

Changes in expression of serine proteases HtrA1 and HtrA2 during estrogen-induced oxidative stress and nephrocarcinogenesis in male Syrian hamster

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Serine proteases HtrA1 and HtrA2 are involved in cellular stress response and development of several diseases, including cancer. Our aim was to examine the involvement of the HtrA proteins in acute oxidative stress response induced in hamster kidney by estrogen treatment, and in nephrocarcinogenesis caused by prolonged estrogenization of male Syrian hamster. We used semi-quantitative RT-PCR to estimate the *HtrA1* and *HtrA2* mRNA levels in kidney tissues, and Western blotting to monitor the amount of the HtrA proteins. Within the first five hours following estrogen administration both *HtrA1* mRNA and the protein levels were increased significantly. No changes in the expression of *HtrA2* were observed. This indicates that HtrA1 may be involved in the response against oxidative stress induced by estrogen treatment in hamster kidney. During prolonged estrogenization, a significant reduction of the *HtrA1* mRNA and protein levels was observed after 6 months of estradiol treatment, while the expression of *HtrA2* was significantly elevated starting from the third month. This suggests an involvement of the HtrA proteins in estrogen-induced nephrocarcinogenesis in hamster. Using fluorescence *in situ* hybridization we localized the *HtrA1* gene at the qb3-4 region of Syrian hamster chromosome 2, the region known to undergo a nonrandom deletion upon prolonged estrogenization. It is possible that the reduced level of *HtrA1* expression is due to this chromosomal aberration. A full-length cDNA sequence of the hamster *HtrA1* gene was obtained. It codes for a 50 kDa protein which has 98 and 96% identity with mouse and human counterparts, respectively.

Keywords: HtrA proteases, oxidative stress, estrogen-induced carcinogenesis, 17- β -estradiol

INTRODUCTION

The HtrA family of proteins consists of evolutionarily conserved serine proteases, homologues of the model HtrA protein of the bacterium *Escherichia coli*. Members of the family are characterized by the presence of a trypsin-like protease domain and at least one PDZ domain at the C-terminal end, and are widely distributed among organisms, prokaryotic as well as eukaryotic, including humans. Prokary-

otic HtrAs are involved in the defense against cellular stresses, including the heat and oxidative shocks, and act by degradation of aggregated or misfolded proteins (Clausen *et al.*, 2002).

To date at least four human HtrA homologues have been identified (Clausen *et al.*, 2002) and the best characterized are the HtrA1 and HtrA2 (Omi) proteins.

The *HtrA1* gene was initially identified as being repressed in SV40-transformed fibroblasts (Zum-

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Abbreviations: IAP, inhibitor of apoptosis proteins.

brunn & Trueb, 1996). Its 50 kDa protein product contains an N-terminal secretory sequence, a domain with homology to follistatin, and a Kazal-type inhibitor motif in addition to the evolutionarily conserved serine protease and PDZ domains at the C-terminal end (Zumbrunn & Trueb, 1996; Hu *et al.*, 1998). There are several lines of evidence suggesting that *HtrA1* may function as a tumor suppressor gene. Recent studies reported that *HtrA1* mRNA was either absent or significantly reduced in ovarian cancers and some brain, liver and breast cancer cell lines (Shridhar *et al.*, 2002; Chien *et al.*, 2004). Primary ovarian cancers showed high frequencies of loss of an allele of microsatellite markers at the *HtrA1* locus on 10q26 (Chien *et al.*, 2004). Down-regulation of *HtrA1* by antisense transfection promoted anchorage-independent growth, while heterologous overexpression of *HtrA1* in an ovarian cell line induced cell death (Chien *et al.*, 2004). Overexpression of *HtrA1* in a highly invasive metastatic melanoma cell line inhibited proliferation *in vitro* and tumor growth *in vivo* (Baldi *et al.*, 2002). The molecular basis of the *HtrA1* function in carcinogenesis is poorly understood.

The HtrA2 (Omi) protein is a tissue-specifically expressed protein whose involvement in apoptosis has been well established. HtrA2 is synthesized as a precursor protein that is autocatalytically processed in mitochondria to produce a mature protein. This processing results in exposure of a tetrapeptide motif, AVPS, allowing interaction with the inhibitor of apoptosis proteins (IAPs). Upon induction of apoptosis the mature HtrA2 protein is released to the cytoplasm, where it interacts with IAPs, neutralizing the IAP-mediated inhibition of caspases, and thus enhancing apoptotic cell death (Hedge *et al.*, 2002; Martins *et al.*, 2002; Verhagen *et al.*, 2002; Yang *et al.*, 2003; Srinivasula *et al.*, 2003). HtrA2 can also induce apoptosis in a caspase-independent manner *via* its proteolytic activity, but the mechanism of this process is still not well understood (Cilenti *et al.*, 2003). Recent studies show that HtrA2 can activate apoptosis without being released from mitochondria. This occurs at the early stages of apoptosis by cleaving the mitochondrial anti-apoptotic HAX-1 protein (Cilenti *et al.*, 2004).

There is a strong epidemiological and clinical evidence that estrogens have a role in induction, promotion and progression of a variety of cancers in numerous target organs, including breast, endometrium, ovary, prostate, liver, brain and lymphoid tissues. Chronic administration of estrogens induces a high frequency of mammary cancers in rat strains and malignant kidney tumors in Syrian hamster (*Mesocricetus auratus*) (Li & Li, 1990; Liehr, 2000). The Syrian hamster estrogen-induced and -dependent renal cancer is a well-established animal model used to elucidate the cellular and molecular mechanisms

involved in estrogen-induced carcinogenesis. Prolonged exposure to natural or synthetic estrogens, administered to intact or castrated male hamsters, causes microscopic changes (dysplasia, carcinoma *in situ*) at approx. 4–5 months of treatment, and macroscopically visible kidney tumors after 6 months of estrogenization, with an incidence approaching 100% (Li & Li, 1987; Liehr *et al.*, 1988; Li *et al.*, 1995). Although the pathogenesis of the tumor is not clear, oxygen free radicals were proved to play a crucial role in the carcinogenic process (Bhat *et al.*, 2003). 4-Hydroxylation of estradiol seems to be an initial point of oxidoreductive cycling of hydroxylated derivatives of estradiol, its semiquinone and quinone metabolic intermediates, which may cause, apart from non-nuclear lesions, genetic instability, including chromosomal aberrations, gene mutations and amplification (Hodgson *et al.*, 1998; Liehr, 1998; 2001; Cavalieri *et al.*, 2000; Li *et al.*, 2001). In our recent studies we found that the carbonyl group level, a marker of protein oxidation, increased significantly in hamster kidney as soon as after 1 h exposition to the hormone and progressed further after 3 h (at that time the increase was at least fourfold) (Kobiela *et al.*, 2007), and was elevated in response to prolonged estradiol treatment (Stefaniak *et al.*, 2002).

Taking into account the facts that: (1) the HtrA1 and HtrA2 proteins belong to the family of proteases the members of which participate in defense against oxidative stress; (2) protein oxidation increases upon estradiol treatment in hamster kidneys; (3) prolonged estradiol treatment causes nephrocarcinogenesis in hamster; (4) HtrA1 is a potential tumor suppressor; (5) HtrA2 is involved in apoptosis, a process which is tightly connected to oncogenesis, we decided to investigate whether HtrA1 and HtrA2 proteins could be involved in estrogen-induced oxidative stress and nephrocarcinogenesis in male Syrian hamster. Our initial goal, presented in this work, was to find out whether oxidative stress and tumorigenesis induced by estrogen treatment cause any changes in the expression of *HtrA1* and *HtrA2* genes in hamster kidney tissues. We used RT-PCR to estimate the *HtrA1* and *HtrA2* mRNA levels, and Western blotting to monitor the HtrA proteins. Our results, demonstrating significant changes of the expression of the *HtrA* genes, suggest an involvement of HtrA1 and HtrA2 in oxidative stress and nephrocarcinogenesis, and indicate that the hamster model can be used to clarify the mechanism of the HtrA proteins' action in carcinogenesis.

MATERIALS AND METHODS

Chemicals. The chemicals were purchased from the following sources: 17- β -estradiol from

Sigma (Poznań, Poland); restriction enzymes and molecular mass markers from Fermentas (Vilnius, Lithuania); oligonucleotides used in PCR from Interactiva Biotechnologie GmbH (Ulm, Germany); nitrocellulose type BA 83 0.2 µm for immunoblotting from Schleicher & Schuell (Dassel, Germany). Other chemicals were purchased from Sigma or Fluka (Poznań, Poland) and were of the highest purity.

Animal treatments. Male Syrian hamsters (n = 168), all 6–8 weeks old, were divided into two groups consisting of 48 and 120 animals. Thirty-six animals of the first group received intraperitoneal injections of 75 mg of 17-β-estradiol suspended in 1 ml of 0.5% (w/v) carboxymethylcellulose saline solution and 12 animals served as an untreated control group. The animals were killed after 1, 3 or 5 h (at each point n = 12) after the injection of estradiol. The second group was divided into two subgroups: subgroup 1 (n = 60) received subcutaneous implants of 25 mg pellets of 17-β-estradiol every 3 months; subgroup 2 (n = 60) served as a sham-operated control group. The animals from the subgroups were killed after 1, 3, 6, 9 or 12 months (at each point n = 12) after initial implantation of estradiol. Kidneys of control and estradiol-treated animals were excised as described previously (Stefaniak *et al.*, 2002). All the procedures concerning animals were approved by the Local Ethical Committee (LEC) and performed according to instructions authorized by the LEC.

Reverse transcriptase (RT). Total RNA was extracted from kidney of male Syrian hamster using RNeasy Isolation kit (Qiagen, Wrocław, Poland). All RNA samples were DNaseI-treated. To obtain cDNA, RNA was reverse-transcribed using MuLV Reverse Transcriptase and oligo(dT)₁₅ primer, according to the supplier's protocol (Fermentas).

Amplification of *HtrA1* and *HtrA2* cDNA fragments. Fragments of cDNA encoding hamster *HtrA1* and *HtrA2* were amplified in polymerase chain reaction using *pfu* DNA Polymerase (Fermentas). The following pairs of primers were used: *HtrA1* forward1, 5'-ATG GAG CGA CTT ATG AAG CC-3' and *HtrA1* reverse1, 5'-CTC CTG CCT CTG CCT AGG G-3'; *HtrA1* forward2, 5'-ATT TTC GGC TGT GGT CCA G-3' and *HtrA1* reverse2, 5'-CCG ATG GCT ACC ACA AAC TC-3'; *HtrA1* forward3, 5'-TGA AAG TGA CAG CAG GCA TC-3' and *HtrA1* reverse3, 5'-CTT TGA GGG AAA CAT GAA GTC-3'; *HtrA2* forward1, 5'-TGG GCT CAT CGT AAC CAA CG-3' and *HtrA2* reverse1, 5'-CTA GAA GCA TTT GCG GTG GA-3'. The following hybridization temperatures for each pair of the primers were applied: 57, 60, 57, and 58°C, respectively. Primers *HtrA1* forward1, *HtrA1* reverse1, *HtrA1* forward2 and *HtrA1* reverse3 were based on the nucleotide sequence of the rat *HtrA1* gene (GenBank Accession No. AF179370), and *HtrA1* reverse2 and *HtrA1* for-

ward3 were based on the hamster *HtrA1* sequence (obtained during the course of this study, GenBank Accession No. EF185310). Primers *HtrA2* forward and *HtrA2* reverse were based on the nucleotide sequence of the rat *HtrA2* gene (GenBank Accession No. XM_001066414). The amplified fragments were then sequenced.

Semi-quantitative RT-PCR. Total reverse-transcribed cDNA was used in a multiplex reaction with the following primers: *HtrA1* forward 5'-AGC CAA AAT CAA GGA TGT GG-3', *HtrA1* reverse 5'-ACA TCT GGG AAG TCT CGG TG-3', *HtrA2* forward 5'-AAG GAG CCT CTT CCC ACA CTG-3', *HtrA2* reverse 5'-TAC CGT CTT CTC TCC GAT GG-3', *β-actin* forward 5'-GTC ACC CAC ACT GTG CCC AT-3', *β-actin* reverse 5'-GAA ATC GTG CGT GAC ATT AAG -3', *α-MHC* (*α*-myosin heavy chain) forward 5' -CGG TGC AGG AGT GTA GGA AT-3', *α-MHC* reverse 5' -CGG AAC TTG GAC AGA TTG GT-3'. The RT-PCR conditions were optimized with respect to: (1) primers' concentration, (2) temperature of primer hybridization, (3) Mg²⁺ concentration, (4) number of cycles, and (5) control for competition between primers. The PCR conditions were always such that the PCR products were in the exponential phase of amplification and amplification did not reach a plateau. The slopes and efficiencies of PCR were similar in multiplex PCRs. This permitted quantitative interpretation of the results. The standard RT-PCR reaction contained a pair of primers: 0.15 µM each *HtrA1* primer and 0.08 µM primers for *β-actin*, or 0.15 µM each *HtrA2* primer and 0.08 µM primers for *α-MHC*, 200 µM dNTPs, 2 mM Mg²⁺. To prepare 12 µl of PCR mixture 0.6 U of REDTaq Polymerase (Sigma, Poznań, Poland) was used. The conditions for standard amplification were: 95°C for 2 min, then 28 cycles of 92°C for 1 min (to amplify *HtrA1* and *β-actin*) or 30 cycles of 92°C for 1 min (to amplify *HtrA2* and *α-MHC*), next 60°C for 1 min, 72°C for 2 min, and final extension at 72°C for 5 min. Products of the reaction were resolved in 2% (w/v) agarose gel, stained with ethidium bromide and analyzed densitometrically using Quantity One software (BioRad, Warszawa, Poland).

Fluorescence *in situ* hybridization. *Preparation and labeling of hamster HtrA1 probe.* Two fragments of hamster *HtrA1* gene, of 17 000 bp and 5 500 bp, were amplified by PCR using genomic DNA extracted from hamster kidney as a template. The following pairs of primers, based on the nucleotide sequence of the hamster *HtrA1* gene, were used: FISH forward1, 5'-ATT GTC AGC ACT ACC CAA CGA GG-3' and FISH reverse1, 5'-ACT GAC TGT CCA TTG ATG CTG ATG-3'; FISH forward2, 5'-CTC GTT GGG TAG TGC TGA CAA TC-3' and FISH reverse2, 5'-AAC TTT ATT GCT GAC GTG GTG GA-3'. AccuTaq LA Polymerase Mix was used

(Sigma, Poznań, Poland). PCR reactions were carried out according to manufacturer's protocols, applying 0.4 μ M concentration of each primer. The conditions for amplification were as follows: 98°C for 30 s, then 32 cycles of 94°C for 10 s, 63°C for 30 s, 68°C for 6 min and 17 min for amplification of 5 500 bp and 17 000 bp, respectively, and final extension at 72°C for 20 min. Amplified genomic DNA fragments were labeled with digoxigenin (DIG) by nick translation (DIG-Nick Translation Mix, Roche, Warszawa, Poland), purified on Nick Translation Columns (Amersham, UK) and next, precipitated. Briefly, to 1 μ g of DIG-labeled DNA in 0.1 ml of column buffer (0.1% (w/v) SDS, 1 \times SSC), 0.1 μ g of herring sperm DNA, 0.1 volume of 3 M sodium acetate pH 5.5 and 2 volumes of cold 96% (w/w) ethanol were added. Probe was incubated at -70°C for 30 min and centrifuged (15 000 \times g, 30 min) at 4°C. Next, DIG-labeled DNA was washed with 1 ml of cold 70% (v/v) ethanol, centrifuged again and dissolved in 50 μ l of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0).

Chromosome preparation and in situ hybridization. Metaphase chromosomes were obtained by use of standard procedures after short-term culture of hamster skin fibroblasts in RPMI-1640 medium (Gibco BRL, Paisley, UK) with antibiotics and 10% (v/v) fetal calf serum (Gibco BRL, Paisley, UK). The hamster *HtrA1* genomic probe, 600 ng, was used for FISH experiments. Both probe and metaphase chromosomes on the slide were co-denatured at 75°C on a hot plate. Hybridization was performed at 37°C in 10 μ l of hybridization solution (50% (v/v) formamide, 2 \times SSC, 10% (w/v) dextran sulfate, 0.05% (v/v) Tween 20). Hybridization took place overnight at 37°C and was followed by posthybridization washing in 0.4 \times SSC/0.3% (v/v) NP-40 at 73°C and then 2 \times SSC/0.1% NP-40 (v/v) at room temperature. Digoxigenin-labeled DNA was detected using TRITC-SheepAntiDigoxigenin primary antibody (Roche, Warszawa, Poland) and TexasRed-DonkeyAntiSheep secondary antibody (JacksonImmunoResearch, Suffolk, UK). Chromosomes were counterstained with DAPI/Antifade (Vector, Burlingame, CA, USA) and hybridization signals were analyzed by using an Axioscope fluorescence microscope (Zeiss, Poznań, Poland) equipped with a cooled CCD camera and run by the CytoVision software for capture and analysis (Applied Imaging, UK).

Construction of plasmids for expression of HtrA proteins. The pET Protein Expression System (Novagen, San Diego, CA, USA) was chosen for expression of the recombinant proteins, fragments of rat HtrA1 and human HtrA2 with His₆-tags at their N-terminal end. Fragments of rat *HtrA1* and human *HtrA2* were amplified by PCR using as a template rat and human cDNA, respectively. A 1047 bp fragment of the rat *HtrA1* cDNA, corresponding to amino ac-

ids 133 to 480, was amplified using the following primers, based on the rat *HtrA1* gene sequence (GenBank Accession No. AF179370): rat*HtrA1* forward 5'-CGG AAT TCA TAT GAA TGG AGC GAC TTA TGA AGC C-3' and rat*HtrA1* reverse 5'-GGG ATC CGT CTC CGT CCT CCT AGG AC-3'. The *EcoRI*/*NdeI* and *BamHI* restriction endonuclease sites were incorporated into the primer sequences (underlined). The obtained *HtrA1* cDNA fragment was firstly subcloned into the *EcoRI* and *BamHI* restriction sites of the pUC18 vector and subsequently excised, using *NdeI* and *BamHI* restriction endonucleases, for cloning into the expression plasmid, pET28b (Novagen, San Diego, CA, USA). The obtained construct was named pDZ1. The following primers, based on the human *HtrA2* nucleotide sequence (GenBank Accession No. AF141305), were used for amplification of a 975 bp fragment of the human *HtrA2* cDNA (amino acids 133 to 458): hum*HtrA2* forward 5'-CCG GCC GTC CAT ATG GCC GTC CCT AGC C-3' and hum-*HtrA2* reverse 5'-GGT GCT CGA GTC ATT CTG TGA CCT CAG GG-3'. The *NdeI* and *XhoI* restriction sites were incorporated into the primer sequences (underlined) so that the resultant *HtrA2* cDNA fragment was cloned into the *NdeI* and *XhoI* restriction sites of the expression plasmid pET28b. The construct was named pJN1.

Protein expression and purification. *Escherichia coli* strain BL21(DE3) was transformed with the pDZ1 or pJN1 plasmids and protein expression was induced by a standard procedure (Novagen, San Diego, CA, USA). The recombinant His₆-rHtrA1 (133-480 aa) and His₆-humHtrA2 (133-458 aa) proteins were purified by affinity chromatography on Ni-NTA columns according to the manufacturer's instructions (Qiagen, Wrocław, Poland).

Antibody preparation. Polyclonal antibodies against the rat HtrA1 and human HtrA2 recombinant proteins (obtained as described above) were produced in rabbits (Pound, 1998) and then purified by affinity chromatography on Sepharose 4B (Pharmacia GmbH, Freiburg, Germany) conjugated with the rat HtrA1 or human HtrA2 recombinant proteins.

Preparation of kidney tissue homogenates. Each sample, approx. 30 mg of kidney tissue, was homogenized with 0.2 ml of cold 10 mM Tris, pH 7.4, containing 150 mM KCl, and centrifuged (15 000 \times g, 30 min) at 4°C. Supernatant was collected, stored at 70°C and subjected to protein analysis.

Protein electrophoresis and assay. Proteins were quantitated by Amido Black staining (Lipinska *et al.*, 1990). SDS/PAGE electrophoresis was carried out according to Laemmli (1970).

Immunoblotting (Western blotting). The immunoblotting was performed essentially as described by Pound (1998). Samples of renal tissues,

containing equal amounts of total protein, originating from estrogen treated hamsters and an appropriate control group were resolved on the same gel by SDS/PAGE and then subjected to electrotransfer on Immobilon. After that, proteins bound to the membrane surface were stained by a standard procedure with the Ponceau S dye. This step was introduced into the procedure in order to confirm proper protein resolution by SDS/PAGE and electrotransfer, and also to check if protein amounts in the samples were comparable. Polyclonal anti-rat HtrA1 and anti-human HtrA2 immunoglobulins were used as primary antibodies. As the secondary antibody, anti-rabbit polyclonal immunoglobulins conjugated with horseradish peroxidase were used at a dilution of 1/10 000 (Sigma, Poznań, Poland). To visualize the HtrA1 and HtrA2 proteins chemiluminescence detection was performed by using Lumi-Light Western Blotting Substrate from Roche (Warszawa, Poland) and following the manufacturer's protocol. Each assay was repeated 3 times and the differences did not exceed 10%.

Statistical analysis. The RT-PCR products were analyzed densitometrically using Quantity One software, and band intensity ratios were calculated. The RT-PCR signals were averaged from at least three independent experiments using cDNA from kidneys of estrogenized hamsters and control samples. Western blotting data were generated from replicate runs. Student's *t*-tests with Bonferroni's correction were used to compare the groups. Statistica 7.1 PL was used for statistical analysis. Statistical significance was assumed at $P < 0.05$.

RESULTS

Amplification and sequencing of hamster *HtrA1* and *HtrA2* cDNA fragments

Since the sequences of hamster *HtrA1* and *HtrA2* genes were not available, we set out to amplify fragments of *HtrA1* and *HtrA2* cDNA in order to obtain sequence data necessary for designing primers for the RT-PCR assays of the mRNA levels. Additionally, the *HtrA1* sequence was necessary to design primers used in probe preparation for the hamster *HtrA1* gene mapping by fluorescent *in situ* hybridization (FISH).

To amplify cDNA fragments of hamster *HtrA1*, PCR was performed using cDNA obtained in reverse transcription of total RNA isolated from hamster kidney. First, specific primers complementary to the rat *HtrA1* gene coding sequence were designed to amplify the 759 bp fragment of hamster *HtrA1* encoding the proteolytic domain and part of the PDZ domain. Subsequently, two cDNA fragments of hamster *HtrA1* were obtained by PCR and primers based on rat *HtrA1* and hamster *HtrA1* sequences: the first fragment, of 888 bp, encoding a signal peptide, the IGF-binding domain and the Kazal-type inhibitor motif from the N-terminus of the HtrA1 protein, and the second fragment, of 447 bp, encoding C-terminal PDZ domain of HtrA1 (Fig. 1). The obtained full-length cDNA sequence was deposited in the GenBank (Accession No. EF185310).

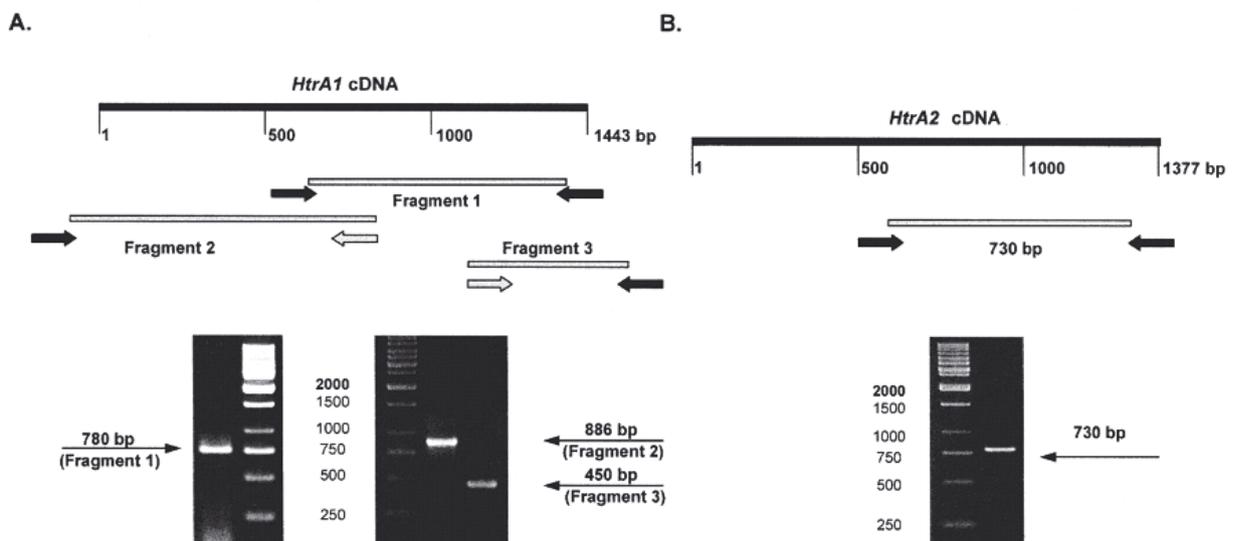


Figure 1. Amplification of hamster *HtrA1* and *HtrA2* cDNA fragments.

Each panel represents schematic localization of amplified cDNA fragments in coding sequence of *HtrA1* (A) and *HtrA2* (B), and results of agarose electrophoresis of obtained PCR products. Black arrows represent primers complementary to sequences of rat *HtrA1* and *HtrA2*, designed to amplify cDNA fragments of hamster *HtrA1* and *HtrA2*. Grey arrows represent primers based on hamster *HtrA1* cDNA.

The complete hamster *HtrA1* coding sequence of 1443 bp, had the highest homology with cDNA of mouse and rat *HtrA1*, 91 and 90%, respectively, and showed 88% homology with human *HtrA1* cDNA. At the deduced amino acid level the identity of the hamster HtrA1 with the rodent HtrA1 proteins and human HtrA1 was 98% and 96%, respectively.

RT-PCR was also used to amplify a fragment of the hamster *HtrA2* coding sequence using primers based on the rat *HtrA2* sequence (Fig. 1). The obtained cDNA fragment of the hamster *HtrA2*, of 730 bp, encoding the proteolytic domain and a fragment of the PDZ domain from the C-terminus, was 93% identical with mouse *HtrA2* cDNA sequence and 90% identical with human *HtrA1* cDNA. At the deduced amino acid level the identity was 97% and 96%, respectively. The sequence was deposited in the GenBank (Accession No. EF185311).

HtrA1 gene expression, unlike that of *HtrA2*, is up-regulated in response to oxidative stress induced by estradiol in male Syrian hamster kidney

To find out whether oxidative stress induced by estrogen treatment in hamster kidney causes any changes in the expression of *HtrA1* and *HtrA2* genes, we injected the animals with 17- β -estradiol and then used a semi-quantitative RT-PCR method to estimate the *HtrA1* and *HtrA2* mRNA levels, and Western blotting to monitor the amount of HtrA proteins in kidney tissues.

Based on the obtained hamster *HtrA1* and *HtrA2* coding sequences, two pairs of primers were designed for RT-PCR. The first pair of primers was used for amplification of a 507 bp *HtrA1* cDNA fragment, in a multiplex semi-quantitative RT-PCR, with a 654 bp fragment of β -actin gene as a reference. The method allowed us to determine the relative level of the *HtrA1* gene transcript by calculating the ratio of *HtrA1* mRNA to β -actin mRNA. The second pair of primers was used to amplify a 555 bp fragment of *HtrA2* cDNA in a multiplex RT-PCR with a 460 bp fragment of the gene encoding α -myosin heavy chain (α -MHC) as a reference. This allowed us to calculate the relative level of the *HtrA2* mRNA over α -MHC mRNA. We estimated the *HtrA1* and *HtrA2* transcript levels in kidney samples derived from hamsters treated with estrogen for 1, 3 or 5 h, and from untreated control animals. The kidney samples were parallelly subjected to immunoblotting analysis to estimate the levels of the HtrA proteins. The results are presented in Fig. 2.

Although no changes in the expression of the *HtrA2* gene were observed, the *HtrA1* mRNA level was increased, starting from the first hour of estrogenization. After 1, 3 and 5 h from initial

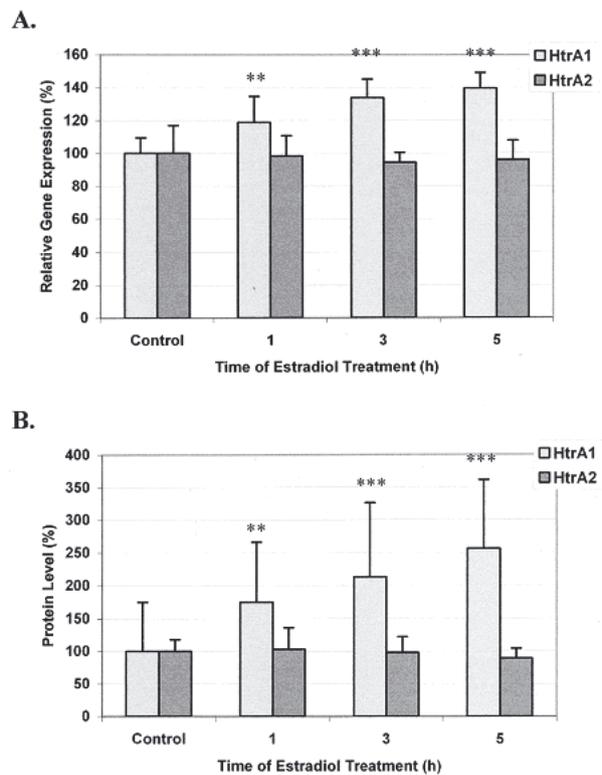


Figure 2. Influence of oxidation stress induced by estradiol treatment on *HtrA1* and *HtrA2* expression in male Syrian hamster kidney.

(A) Semi-quantitative RT-PCR analysis of *HtrA1* and *HtrA2* transcript levels in kidneys after 1, 3 and 5 h of estradiol treatment. The relative transcript level of *HtrA1* was calculated as ratio of *HtrA1* mRNA to β -actin mRNA. The relative transcript level of *HtrA2* was calculated over mRNA of gene coding for α -myosin heavy chain (α -MHC). (B) HtrA protein levels in kidneys of estrogenized male Syrian hamsters. Equal amounts of protein from kidney homogenates were resolved by SDS/PAGE and analyzed by immunoblotting using anti-HtrA1 and anti-HtrA2 antibodies and chemiluminescence. Immunoblots were analyzed densitometrically. Each bar represents protein level (HtrA1 or HtrA2) quantitated using Quantity One software and expressed as percentage of control values (from untreated animals). Standard deviation did not exceed 15%. Statistical significance between the control and estradiol-treated groups was determined by Student's *t*-test, data significantly different at $P < 0.01$ and $P < 0.001$ are marked as a ** and *** sign, respectively.

estradiol implantation the *HtrA1* mRNA level increased significantly by approx. 19, 34 and 40%, respectively (Fig. 2A), compared to the non-treated controls. These observations were supported by Western blotting analysis, which showed increased contents of the HtrA1 protein in the kidney tissues of estradiol-treated animals. After 1, 3 and 5 h of estrogenization the HtrA1 level was approx. 1.8, 2.1 and 2.6-fold higher comparing to the control group (Fig. 2B). The results were statistically significant.

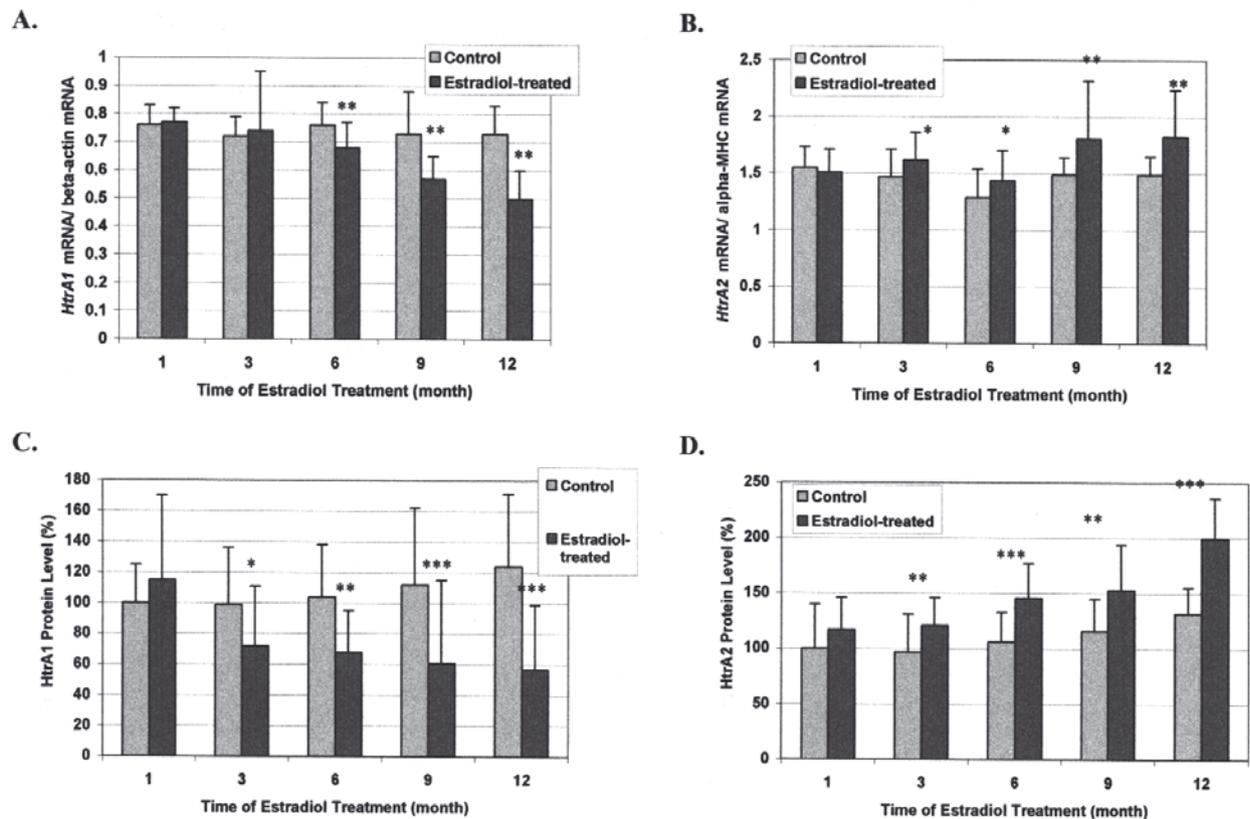


Figure 3. Effects of prolonged estradiol treatment on *HtrA1* and *HtrA2* gene expression in male Syrian hamster kidneys.

(A, B) Semi-quantitative RT-PCR analysis of *HtrA1* and *HtrA2* transcript levels in kidneys after 1, 3, 6, 9 and 12 months of estrogenization. The relative transcript level of *HtrA1* gene was calculated as the ratio of *HtrA1* mRNA to β -actin mRNA (A). The relative transcript level of *HtrA2* gene was calculated over mRNA of the gene encoding α -myosin heavy chain (α -MHC) (B). (C, D) HtrA protein levels in kidneys of estrogenized male Syrian hamsters. Equal amounts of protein from kidney homogenates were resolved by SDS/PAGE and analyzed by immunoblotting using anti-HtrA1 (C) and anti-HtrA2 (D) antibodies and chemiluminescence. Immunoblots were analyzed densitometrically. Each bar represents protein level (HtrA1 or HtrA2) quantitated using Quantity One software and expressed as percentage of control values (from untreated animals). Statistical significance between the control and estradiol-treated groups was determined by Student's *t*-test, data significantly different at $P < 0.05$, $P < 0.01$ and $P < 0.001$ are marked as a *, ** and ***sign, respectively.

Down-regulation of *HtrA1* gene expression and increasing expression of the *HtrA2* gene accompany nephrocarcinogenesis of male Syrian hamster

To find out if any significant changes in the transcription of the *HtrA1* and *HtrA2* genes accompany nephrocarcinogenesis of male Syrian hamster caused by prolonged estrogenization, the animals received subcutaneous implants of 17- β -estradiol and then semi-quantitative RT-PCR was used to estimate relative expression of the genes, and Western blotting to monitor the HtrA protein levels in the kidney tissues. Development of the oncogenic process was monitored by histopathological analysis of kidney tissues performed at consecutive stages of carcinogenesis. Significant, characteristic proliferative changes (interstitial or tubular) were observed starting from the third month of estro-

genization and showed an increase in frequency throughout the experiment. Macroscopically visible foci were observed starting from the ninth month (not shown), which is in agreement with other authors' reports and our experience (Oberlay *et al.*, 1991; Hacker *et al.*, 1991; Stefaniak *et al.*, 2002; Kobiela *et al.*, 2003).

The results of the RT-PCR analysis showed that the *HtrA1* mRNA level decreased significantly starting from the 6th until 12th month of estradiol treatment (Fig. 3A). These observations were supported by the results of Western blotting analysis which showed a reduced level of the HtrA1 protein in kidney tissues after 6–12 months of estrogenization (Fig. 3C).

In contrast, expression of the *HtrA2* gene in the kidney was increased by the estradiol treatment. We observed a significantly increased transcript lev-

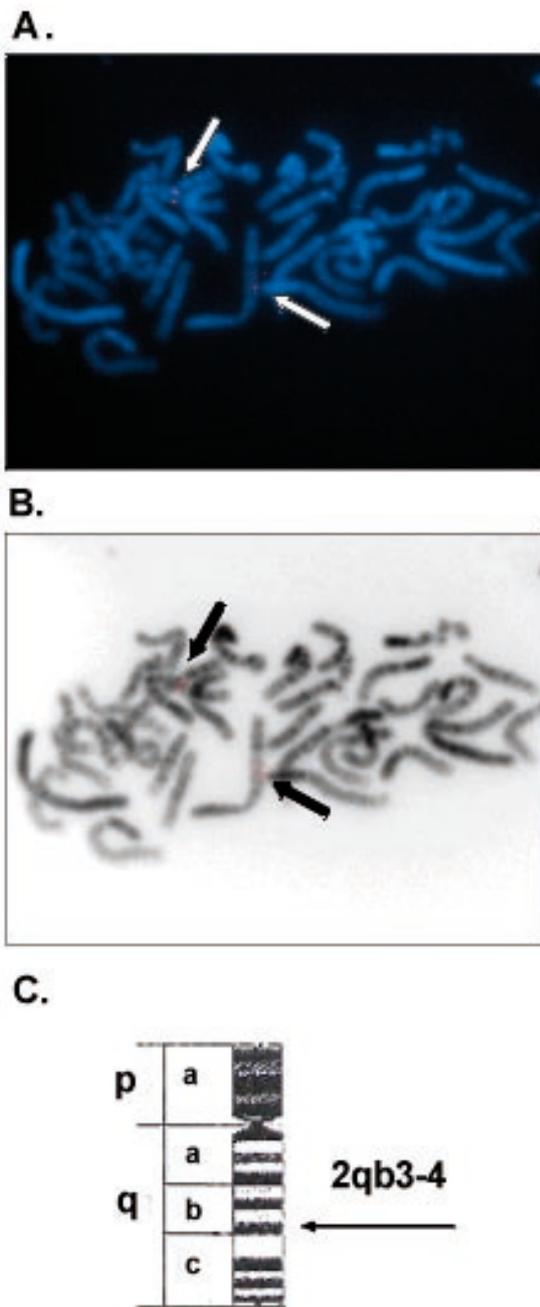


Figure 4. Localization of *HtrA1* gene to chromosome 2 by fluorescence *in situ* hybridization (FISH).

Hybridization signals on homologous chromosomes corresponding to the *HtrA1* locus are indicated by arrows. (A) Image of metaphase chromosomes (DAPI stained) after FISH with hamster *HtrA1* genomic probe. (B) Reversed image of the same DAPI-stained metaphase chromosomes. (C) Ideogram of Syrian hamster chromosome 2 (Mamaeva, 2002).

el of the *HtrA2* gene (Fig. 3B) and a higher *HtrA2* protein content after 3–12 months from the initial estradiol implantation (Fig. 3D).

Localization of *HtrA1* gene on Syrian hamster chromosome by fluorescent *in situ* hybridization (FISH)

To localize the *HtrA1* gene on hamster chromosomes, we performed a FISH study using as a probe a mixture of two fragments of *HtrA1* genomic sequence. The fragments, of 17 kb and 5.5 kb, were amplified using PCR and labeled with digoxigenin. The probe was hybridized to metaphase spreads obtained from cultured normal hamster fibroblasts. Staining of the same chromosome spreads with DAPI gave a G-banded pattern, which permitted the *HtrA1* gene to be localized to chromosome region 2qb3-4 (Fig. 4), based on a published map of Syrian hamster chromosomes (Mamaeva, 2002).

DISCUSSION

The *HtrA1* and *HtrA2* proteins belong to the *HtrA* family of serine proteases that participate in defense against cellular stresses causing aberrations in protein structure, for example heat and oxidative stress. Human *HtrA* proteins are involved in cell growth and differentiation, apoptosis, and disturbances in their function may induce several diseases, including cancer (Clausen *et al.*, 2002). There is accumulating evidence suggesting that *HtrA1* may function as a tumor suppressor, though the molecular basis for the suppression is poorly understood. The role of *HtrA2* protein in apoptosis is well established but so far there are no data available indicating its involvement in carcinogenesis. In this study we set out to investigate the involvement of the *HtrA1* and *HtrA2* proteases in estrogen-induced oxidative stress and carcinogenesis. Our aim was to find out whether oxidative stress and tumorigenesis, both induced by estrogen treatment, cause any changes in the expression of the *HtrA1* and *HtrA2* genes. To achieve this goal we used an animal model in which carcinogenesis in the kidney of male Syrian hamster was induced by 17- β -estradiol (natural female sex hormone). This experimental model is a reliable rodent model of human estrogen-dependent breast carcinoma (Roy & Liehr, 1999; Liehr, 2000).

We demonstrated both at the mRNA and protein levels that expression of the *HtrA1* gene was significantly increased in hamster kidney in response to acute estrogen treatment while there were no statistically significant changes in the *HtrA2* gene expression (Fig. 2). Our previous studies assessing the dynamics of oxidative damage in kidney of estradiol-treated male Syrian hamster showed that among the cellular macromolecules, proteins appear to be the primary target of oxidative damage. Protein oxidation increased as soon as 1 h after exposition to the hormone while DNA oxidative damage

was unchanged at that time and increased only after 5 h of estradiol treatment (Kobiela *et al.*, 2007). Taking these facts into account it is possible that up-regulation of the *HtrA1* gene, observed as early as after one hour of estrogenization, may be a result of induction of pathways involved in the response to the appearance of oxidatively damaged proteins, and the HtrA1 protease could be an important element of the oxidative stress response. Although our present study concerned the influence of acute estradiol treatment on *HtrA1* expression in hamster kidney tissue, the response mechanism in which HtrA1 participates may be more general. Up-regulation of *HtrA1* expression and autocatalytic activation of the protease were demonstrated in ovarian cell lines under treatment with agents causing protein and DNA damage, such as cisplatin. It has been suggested that HtrA1 may function as a stress-induced protease whose main function is removal of unfolded proteins (Chien *et al.*, 2006). Our results are in agreement with this hypothesis; moreover, they suggest that HtrA1 could participate in the response to estradiol-induced oxidative stress that triggers early stages of estrogen-dependent carcinogenesis in hamster kidney.

We showed both at the mRNA and protein levels that prolonged estrogenization influenced significantly expression of both the *HtrA1* and *HtrA2* genes. After 6–12 months of estrogen treatment, leading to nephrocarcinogenesis, we observed a significant down-regulation of the *HtrA1* gene (Fig. 3A and C). Numerous studies have been published supporting the hypothesis that HtrA1 may function as a tumor suppressor involved in promoting cell death and that down-regulation of HtrA1 is correlated with malignant progression and metastasis, and also with poor response to chemotherapy. Down-regulation of the *HtrA1* gene expression was observed in several types of cancers, including ovarian ones, some breast cancer cell lines, and also metastatic melanomas (Shridhar *et al.*, 2002; Baldi *et al.*, 2002; Chien *et al.*, 2004; 2006). It has been shown that in primary ovarian cancers a decrease of *HtrA1* expression is correlated with the loss of heterozygosity of the *HtrA1* gene and its epigenetic inactivation by hypermethylation (Chien *et al.*, 2004). In the present study, using fluorescence *in situ* hybridization, we localized the Syrian hamster *HtrA1* gene to chromosome 2, region qb3-4 (Fig. 4). This region has been shown by Papa *et al.* (2003) as a region that undergoes a nonrandom deletion upon prolonged estradiol treatment. It is possible that the observed reduced level of *HtrA1* gene expression could be due, at least partially, to the chromosomal aberration. If that is the case, then our results would suggest that the loss of the chromosomal region encoding HtrA1 is a general mechanism occurring during oncogenesis.

Our results showed that during the acute oxidative stress HtrA1 level went up (after 1–5 h) but after a prolonged and milder stress (6 months) it decreased, in spite of the fact that after 6 months of estrogenization the level of protein oxidation was elevated (Stefaniak *et al.*, 2002) as well as the level of 8-iso-prostaglandin $F_{2\alpha'}$, a known marker of oxidant stress (Bhat *et al.*, 2003). We assume that *HtrA1* is induced by the oxidant stress — and this happens in hours — but after several months of the continuous exposure to reactive oxygen species *HtrA1* regulation changes. One of the reasons of this change may be a deletion of the fragment of chromosome where the *HtrA1* gene is located.

We found that prolonged estrogenization caused an increase of *HtrA2* expression starting from the 3rd month of the estradiol treatment (Fig. 3B and D). Among the human homologues, HtrA2 was found to be up-regulated in response to heat shock or tunicamycin treatment, both of which cause aberrations in protein structure (Gray *et al.*, 2000). As a mitochondrial protein, HtrA2 provides protein quality control in the mitochondrion (Jones *et al.*, 2003), an important target of estrogen action and a predominant source of reactive oxygen species, also of those arising under estrogen action (Felty & Roy, 2005). It is possible that the observed increase of HtrA2 level is caused, at least partially, by accumulation of aberrant proteins in the mitochondrion. It is known that mature HtrA2 is released from the mitochondrion to the cytoplasm, degrades inhibitors of apoptosis proteins and activates a cascade of caspases (Yang *et al.*, 2003; Srinivasula *et al.*, 2003; Seong *et al.*, 2004). It has been shown by Jin *et al.* (2002), that HtrA2 is implicated in a p53-dependent apoptosis. Under treatment with etoposide — a DNA damaging agent that induces apoptosis in a p53-dependent manner — the *HtrA2* gene expression is upregulated in HeLa cells and primary mouse thymocytes. Taking into account the fact that genetic instability is a common feature of estrogen-induced carcinogenesis in Syrian hamster kidney (Banerjee *et al.*, 1992; 1994; Hodgson *et al.*, 1998; Li *et al.*, 1999), it is possible that the observed increase of *HtrA2* expression could be an element of the cellular response to DNA damage. This is, to our knowledge, the first time that changes in *HtrA2* gene expression during carcinogenesis were described.

Summarizing, the observed changes in the *HtrA1* and *HtrA2* genes' expression suggest that both HtrA proteins could be involved in estrogen-induced nephrocarcinogenesis of male Syrian hamster, though acting by different mechanisms. Additionally, the HtrA1 protease could participate at a very early stage of estrogen treatment, most probably as a response to estrogen-induced oxidative stress.

While achieving the main goals of this work, we have sequenced the Syrian hamster *HtrA1* cDNA and found that it codes for a protein of a predicted molecular mass of 50 kDa, with a high identity (96%) to human HtrA1.

Our findings open the way for further investigation of the molecular basis of the HtrA protein action in carcinogenesis, using the Syrian hamster as a model. One of the possible pathways of investigation is to study the interplay between the HtrA proteases and TGF β proteins, since recent studies by Oka *et al.* (2003) have shown that HtrA1 participates in the regulation of signaling by various proteins of the TGF β family, including TGF β 1 and TGF β 2, which are regulators of cell growth and differentiation. HtrA1 inhibits signaling by TGF β presumably by preventing receptor activation (Oka *et al.*, 2003).

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