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Identification of protein-protein interactions of human HtrA1

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1. ABSTRACT

The human heat shock protein HtrA1, a member of the HtrA family of serine proteases, is a evolutionarily highly conserved factor which displays a widespread pattern of expression. The yeast two-hybrid technique was employed to identify new cellular proteins physically interacting with HtrA1, and thus potential targets of this serine protease. An enzymatically inactive HtrA1 point mutant, HtrA1-S328A, was generated and used as bait in a yeast two-hybrid system. Fifty-two plasmids were isolated from primary positive yeast clones. Subsequent sequencing and BLAST analysis revealed cDNAs encoding for 13 different proteins. These putative binding partners of HtrA1 appeared to be a) components of extracellular matrix; b) factors related to signal pathways, and c) unknown proteins. Among the 13 positive clones identified and reported here, it is worth of note that the interaction of HtrA1 with tubulin and collagen (extracellular matrix proteins) and with tuberin (cytoplasmic protein) is confirmed by other studies, and this further supports previous findings in which HtrA1 can be found active as an intracytoplasmic protein or as secreted protein as well.

2. INTRODUCTION

The heat shock protein HtrA1 belongs to the evolutionarily conserved HtrA family of serine proteases that were initially identified in *E.coli* and subsequently described in a variety of species, including Gram-negative and -positive bacteria, plants and mammals (1,2). Until now, four human homologues of *E.coli* HtrA have been identified: HtrA1 (L56 or PRSS11) (3,4), HtrA2 (Omi) (5,6), HtrA3 (PRSP) (7) and HtrA4 (8). All the proteins belonging to this family share a highly conserved trypsin-like serine protease domain and one or two PDZ domain at the C-terminus (9), plus specific distinct regulation domains at the N-terminus, in relation with their physiological functions,.

HtrA1 has been the first member of the HtrA family to be cloned and sequenced. The amino acid sequence of the human HtrA1 contains an open reading frame of 480 amino acids that shares 58% similarity with the bacterial protein, and this homology is particularly well pronounced in the regions surrounding the active serine and histidine residues. Structurally, the N-terminus of HtrA1

shares a putative signal sequence for secretion (codons 1-22) followed by an insulin-like growth factor binding protein (IGFBP) domain (codons 33-100) and a Kazal-type inhibitor motif (codons 101-155); this first part of HtrA1 is homologous to mac25, a gene product related to IGFBP and to follistatin, an activin-binding protein, and this specific domain probably makes HtrA1 able to regulate many biological processes, perhaps modulating several growth factor systems as well as IGF and the activin-inhibin system (4). Moreover, the protease inhibitor motif suggests that the human HtrA1 may be a self-regulating enzyme or that it may regulate other serine proteases (4). The C-terminus HtrA1 moiety is more than 40% identical to the bacterial HtrA serine protease, and shows a highly conserved trypsin-like serine protease domain (codons 204-364) and a PDZ domain (codons 365-467). This region allows HtrA1 to perform its endo-proteolytic activity, including autocatalytic cleavage. It is also known that conversion by site-directed mutagenesis of the putative conserved active site serine 328 to alanine (S328A) disrupts completely such enzymatic activity (4).

HtrA1 has a widespread pattern of expression, and its level in human tissues is modulated both in normal tissues with different physiological activities and in pathological condition (10). Indeed, increase of HtrA1 expression has been detected during the progress of human pregnancy (8,10,11) and in osteoarthritic cartilage (4,12), whereas the expression of this protease is decreased in progression and invasion of melanomas, breast cancer, ovarian and endometrial cancers, lung cancer, and mesotheliomas (13-19). Moreover, it is known that stable over-expression of HtrA1 inhibits proliferation in a metastatic melanoma cell line (13) and that induces cell death in ovarian cancer cells (15), suggesting the possibility that HtrA1 acts as a tumor suppressor. Recently, the pivotal role of HtrA1 down-regulation in cancer progression and in the acquisition of aggressive tumor traits in tumors of diverse origins has been highlighted (20,21).

Until now, several targets of HtrA1 have been identified. Most of them are components of the extracellular matrix, such as type III collagen (22) and fibronectin (12) or components of cartilage, such as aggrecan, decorin and fibromodulin (23,24). Moreover, the ability of purified HtrA1 to degrade various Amyloid Precursor Protein fragments involved in the mechanism of plaque formation in the brain of Alzheimer´s disease patients has been described (25). Recently, several studies reveal an involvement of HtrA1 also in age-related macular degeneration (AMD) (26-29).

With the aim to better understand the role of HtrA1 and to identify new binding partners of this protein, we used the enzymatically inactive form of HtrA1, called HtrA1-S328A, as a bait in the yeast two-hybrid screening methodology.

3. MATERIALS AND METHODS

3.1. Reagents and plasmids

Human wild-type HtrA1 cDNA was kindly gift by Dr. Trueb (University of Bern, Switzerland). The HtrA1-S328A variant expression cDNA was obtained by site-directed mutagenesis of the original pcDNA3T7Tag-HtrA1 expression construct using the Stratagene "QuikChange site-directed mutagenesis kit" as previously described (Hu *et al.*, 1998). The reaction of mutagenesis was made using the following primers:

5'-CATCAACTATGGAAACGCGGGAGGCCCGTTAG-3'
5'-CTAACGGGCCTCCCGCGTTTCCATAGTTGATG-3'

The HtrA1-S328A cDNA was cloned into the EcoRI/BamHI sites of vector pGBKT7 (Clontech, Mountain View, CA) in frame with the GAL4 binding domain.

The human fetal brain cDNA library encoding GAL4-transactivation domain fusion proteins into pACT2 vector and the MATCHMAKER GAL4 Two-Hybrid System 3 (containing pGBKT7 series vectors) were from Clontech. Agar, peptone and the yeast nitrogen base without amino acids were purchased from BD Clontech. All amino acids, 3-amino-1,2,4-triazole (3-AT), glucose, x-gal and glass beads (425-600 µm) were from SIGMA (St. Louis, USA).

3.2. Cell line and proliferation assay

LM cell line derived from a supraclavicular lymph node metastasis of cutaneous melanoma and was cultured in RPMI 1640 plus 10% FCS in a 5% CO2 atmosphere. LM cells display high proliferation rate and clonogenic ability (30). To perform proliferation assay, cells were seeded in a 96-well plate (5 x 10^3 per well) 12 h before the experiment. Cell growth was evaluated using the Cell Proliferation Kit II (Roche Molecular Biochemicals) following Manufacturer's instructions. This method employs a colorimetric procedure based on the tetrazolium salt sodium, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt, which is transformed in a formazan dye only in metabolically active cells. The formazan dye was measured, after 4 h, at a 492 nm wavelength. Values ± SD are the average of three experiments done in duplicate.

3.3. Yeast transformation

Transformation was carried out using the lithium acetate method. The yeast strain AH109, carrying UAS-His3, UAS-LacZ and UAS-ADE2 reporter genes, was co-transformed with the pGBKT7-HtrA1-S328A bait and with the human fetal brain cDNA library (Clontech) fused to the GAL4 activation domain in the pACT2 vector (Clontech). Cells were plated on minimal synthetic defined (SD) medium (Clontech) supplemented with the required bases and amino acids, and lacking tryptophan (Trp), leucine (Leu), histidine (His) and adenine (Ade). Plates were incubated for 7 days at 30°C, then His^+ Ade^+ transformants were isolated. Further confirmation of initial positive clones was performed via detection of the β-galactosidase activity resulting from the third reporter gene.

3.4. Identification, sequencing and BLAST analysis of putative positive clones

The positive clones isolated from primary positive yeast clones were transformed into E. Coli XL1

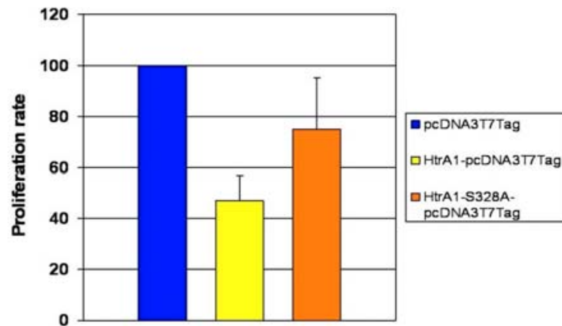

Figure 1. Effect of Htra1-S328A on cellular growth. LM cells were transfected with HtrA1-pcDNA3T7Tag, HtrA1-S328A-pcDNA3T7Tag or with the empty vector. Proliferation rate appeares significantly reduced by HtrA1 over-expression , as compared to parental cells. In contrast, HtrA1-S328A transfectants displayed lower reduction in cell proliferation.

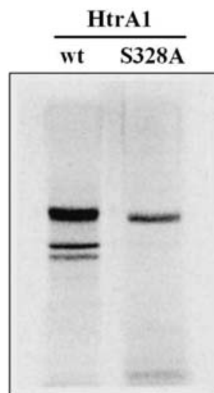

Figure 2. HtrA1-S328A does not exhibit autoproteolysis. Full-length wild-type HtrA1t and HtrA1-S328A were translated *in vitro* in the presence of [^{35}S]methionine and resolved on 10% SDS-PAGE to verify the inactivation of enzymatic activity in the point mutant form. Indeed, HtrA1-S328A did not exhibit autoproteolysis.

blue cells (Stratagene, Santa Clara, CA) by heat-shock method. The plasmids were amplified and the products of DNA extractions were characterized by restriction digest using EcoRI/BamHI. The sizes of fragments were analyzed by agarose gel electrophoresis to check for repetitive inserts. Recovered library-derived plasmids were analyzed by DNA sequencing and the sequences were employed to perform the BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4. RESULTS

4.1. Effect of Htra1 and Htra1-S328A on human melanoma cell growth

In accordance with previous observations describing HtrA1 as a tumor suppressor in several types of cancers (13-15,17), we investigated the effect of the functionally inactive mutant HtrA1-S328A on cellular proliferation in a cutaneous melanoma cell line. The HtrA1-S328A variant expression cDNA was obtained by site-directed mutagenesis of the putative conserved active site serine 328 to alanine using specific primers (see Materials and Methods section). As shown in Figure 1, HtrA1 over-expression significantly reduced the proliferation rate of LM cells, as compared to parental cells (p = 0.0001). In contrast, HtrA1-S328A over-expression appeared appreciably less effective than the native form in inhibiting cell proliferation.

4.2. Identification of Expression Bait Plasmid

The human HtrA1 protein is a secreted serine protease which exhibits proteolytic activity, including autocatalytic cleavage (4). In order to avoid the digestion of possible targets or auto-digestion of this protease during the screening in yeast, we performed the two-hybrid screening using as a bait the point-mutant HtrA1-S328A form, which lacks proteolytic activity (4). Both full-length wild-type HtrA1 and HtrA1-S328A point mutants were translated *in vitro* in the presence of [^{35}S]methionine and the resulting products resolved by means of SDS-PAGE, in order to verify the inactivation of enzymatic activity in the mutant protein (Figure 2).

4.3. Screening of the Human Fetal Brain cDNA Library

The HtrA1-S328A bait used in the yeast two-hybrid system contained the full-length cDNA fused in-frame with the GAL4 DNA-binding domain of pGBKT7. pGBKT7-HtrA1-S328A was first transformed into AH109 by the lithium acetate-mediated method. No auto-activation was detected. Subsequently, the human fetal brain cDNA library, which contained the GAL4 activation domain fused into random cDNAs, was transformed into AH109/pGBKT7-HtrA1-S328A to find the primary positive clones. From over 3.5 million primary colonies screened, 77 clones were grown in SD/Trp-/Leu-/His-/Ade-. Then, approximately 52 positive colonies were identified by a colony-lift filter assay to detect β-galactosidase activity.

4.4. Identification of Putative Positive Clones and BLAST Analysis

The 52 plasmids isolated from primary positive yeast clones were transformed into E.coli and controlled for the presence of the insert by enzymatic digestion. Sequencing and subsequent BLAST analysis of the inserts from the 25 plasmids positive to the digestion, revealed cDNAs encoding for 13 different proteins (Table 1). All sequences were in-frame with the GAL4AD, and most cDNAs were represented two or three times. Among the HtrA1 putative binding partners, we found several extracellular matrix components (Group 2), proteins related to cellular signal pathways (Group 3), and some unknown proteins (Group 4).

5. DISCUSSION

It is known that HtrA1 expression is highly regulated both during development in normal human tissues (8,10,11) and during progression and invasion of

several human cancer. In particular, HtrA1 down-regulation plays important roles in the malignant progression of human melanoma (13), ovarian cancer (15,16), endometrial cancer (17), lung cancer (18) and mesothelioma (19).

Table 1. Putative binding partners of HtrA1. BLAST results for the positive clones obtained from the screening of the human fetal brain cDNA library using HtrA1-S328A as a bait

Group	Clone N°	Gene Name	Ref seq ID	Description
1	04	HtrA1	NM_002775	Homo sapiens HtrA serine peptidase 1
2	06	ADAMTS7	NM_014272	Homo sapiens ADAM metallopeptidase with thrombospondin type 1 motif, 7
	11	CTNND2	NM_001332	Homo sapiens catenin (cadherin-associated protein), delta 2
	12	TUBB2A	NM_001069	Homo sapiens tubulin, beta 2A
	61	COL1A1	NM_000088	Homo sapiens collagen, type I, alpha 1
	67	FBLN1A	NM_006487	Homo sapiens fibulin 1, transcript variant A
3	10	MTCO2	NM_173705	Homo sapiens cytochrome c oxidase II
	13	TCTEX1L	NM_006520	Homo sapiens dynein, light chain, T-complex-associated-testis-expressed
	23	WAC	NM_016628	Homo sapiens WW domain containing adaptor with coiled-coil, transcript variant 1
	46	EIF3S5	NM_003754	Homo sapiens eukaryotic translation initiation factor 3, subunit 5 epsilon
	51	TSC2	NM_021056	Homo sapiens tuberous sclerosis 2, transcript variant 3
4	05	FJ25045	AK057774	Homo sapiens cDNA FLJ25045 fis, clone CBL03591
	09	KIAA1813	XM_046743	Homo sapiens KIAA1813 protein

It has been demonstrated the presence of HtrA1 either in the extracellular space, as a secreted protein, or in the cytoplasm of the cells. Moreover, it has been shown the ability of HtrA1 to promote degeneration of extracellular matrix components, including fibronectin (12,31), collagen (23) and amyloid precursor protein fragments (APP) involved in Alzheimer´s disease (25).

Here we demonstrate that over-expression of the point-mutant form of this serine protease apparently interferes with the growth-suppressive function of the native molecule, since LM human melanoma cells overexpressing HtrA1-S328A showed lower reduction in cell proliferation, when compared to those overexpressing the wild-type protease. This finding suggests that the role of HtrA1 as a suppressor of cellular proliferation is, at least in part, sustained by its protease activity. Therefore, identification of cellular factors target of HtrA1 could be instrumental in defining its role as a tumor suppressor.

In order to better define the role of HtrA1 in human cells, a yeast two-hybrid system was employed to identify new possible targets of this protease.

Among the 13 positive clones identified in our screening, we found both extracellular and cytoplasmic proteins (Table 1, Group 2 and 3, respectively), supporting the findings of previous reports in which HtrA1 is found either as a secreted protein or within the cytoplasmic compartment.

Interestingly, clone 04 (Table 1) was HtrA1 itself. Since the functional unit of the HtrA factors appears to be a omotrimer (32), self association of protease monomers could be possible under specific conditions.

The positive clones in Table 1, Group 2 were matrix proteins, most of them involved in a complex network of filaments and tubules that transmit mechanical and chemical stimuli within and between cells. It is already known the role of HtrA1 in cartilage degradation (4,23), matrix mineralization (33) and proteolytic cleavage of extracellular matrix components, such as aggrecan, decorin, fibromodulin, soluble type II collagen (23), fibronectin (12) and type III collagen (22). Therefore, the identification in our screening of several components of extracellular matrix could be considered as a confirmation of the reliability our yeast two-hybrid screening procedure.

A recent study which identifies novel extracellular substrates for HtrA1 (34), investigates the expression level of HtrA1 and its potential substrates in the context of primary RPE cell extracellular milieu, to define the role of the serine protease in AMD. Among the 196 extracellular proteins identified in the RPE secretome, 8 were found to be selectively cleaved by human recombinant HtrA1. These include fibromodulin, clusterin ADAM9, vitronectin, and alpha2-macroglobulin. Interestingly, one of the genes identified in our screening is *ADAMTS7*, a members of the ADAMTS (A-disintegrin and metalloproteinase with thrombospondin motifs) family. The 'A-disintegrin and metalloproteinase' (ADAM) and 'A-disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS) genes make up two similar, yet distinct, gene families. ADAM and ADAMTS proteins participate in a wide range of cellular processes, including cell adhesion and migration, ectodomain shedding, proteolysis, development, ovulation and angiogenesis (35). Recent studies have demonstrated the importance of cartilage oligomeric matrix protein degradation by ADAMTS7 (36). Moreover, this protein has been found significantly over-expressed in the cartilage and synovium of patients with rheumatoid arthritis (37). HtrA1 is able to promote degeneration of extracellular matrix components (12,31), and its expression is increased 3-7 fold in the synovial fluids from both osteoarthritis and rheumatoid arthritis patients (12). Therefore, an interaction between ADAMTS7 and HtrA1 appears conceivable in this patho-physiological context.

Among the matrix proteins (Table 1, Group 2), the sequence of *COL1A1*, a gene encoding the pro-alpha1 chains of type I collagen is listed. This protein is a fibril-forming collagen found in most connective tissues, abundant in bone, cornea, dermis and tendon (38). Recently, Hadfield and collaborators reported that over-expression of HtrA1 in the 2T3 osteoblasts cell line down-regulats type I collagen mRNA, even if recombinant HtrA1 appears not able to cleave this protein (31). These data further support the possibility of the physical interaction between the pro-alpha1 chains of type I collagen and HtrA1.

Catenin delta 2 (CTNND2) is an adhesive junction-associated protein of the delta-catenin superfamily (39). It was initially identified as a neural-specific protein in the brain, interacting with Alzheimer's disease protein presenilin (39-41). Moreover, Catenin delta 2 protein expression appears up-regulated in over 80% of prostatic adenocarcinomas (42). These data shown a strong correlation between catenin delta 2 and Htra1, which appear both involved in Alzheimer's disease and in prostatic cancer (43,44) as well. Once again, a direct interaction between these two proteins appears reasonable.

Concerning Tubulin beta 2A (TUBB2A), it has been recently demonstrated that HtrA1 associates with alpha- and beta- tubulins (45). Chien and collaborators show that during microtubule assembly, intracellular HtrA1 associates with centrosomes and modulates microtubule stability and cell motility.

Finally, Fibulin-1 is listed among matrix proteins in Table 1, Group 2. Fibulins are a family of seven calcium-binding secreted glycoproteins associated with the basement membrane, cell adhesive structures, and elastic fibers (46). Fibulin-1 is also found in association with extracellular matrix structures including fibronectin (47).

Among the genes listed in Table1, Group 2, it is interesting to focus the attention on TSC2 (tuberous sclerosis 2), a tumor suppressor gene responsible, together with TSC1, for Tuberous Sclerosis Complex (TSC), a rare inheritable disorder characterized by the development of benign tumors (48-50). Recently, our group demonstrated the identification of TSC2, but not TSC1, as a substrate of HtrA1 (51). We showed that HtrA1 cleaves TSC2 both *in vitro* and *in vivo*. Moreover, we found that both HtrA1 and TSC2 are able to physically interact and co-localize in the cytoplasmic compartment.

The other clones identified, listed in Group 3 and 4 of Table 1, code for proteins that act as transcriptional factors (EIF3S5, DYNLT3) or play a role in intracellular pathways. We can speculate that HtrA1 could be able to interact with them to modulate the expression of genes related to their pathways.

In conclusion, this study confirms that HtrA1 plays a key role in several fundamental cellular processes in physiologic and pathologic contexts, ranging from proliferation to cell adhesion and invasion. Indeed, the data presented here represent a good starting point for new investigations regarding the complex role played by this intriguing and multifaceted protease.

6. ACKNOWLEDGEMENTS

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