The Serine Protease HtrA1 Specifically Interacts and Degrades the Tuberous Sclerosis Complex 2 Protein

Mara Campioni, Anna Severino, Lucrezia Manente, Ioana L. Tuduce, Stefano Toldo, Michele Caraglia, Stefania Crispi, Michael Ehrmann, Xiaoping He, Jacie Maguire, Maria De Falco, Antonio De Luca, Viji Shridhar, and Alfonso Baldi

Abstract

Hamartin and tuberin are products of the tumor suppressor genes TSC1 and TSC2, respectively. Mutations affecting either gene result in the tuberous sclerosis syndrome, a neurologic genetic disorder characterized by the formation of multiple benign tumors or hamartomas. In this study, we report the identification of TSC2, but not TSC1, as a substrate of HtrA1, a member of the human HtrA family proteins of serine proteases. We show the direct interaction and colocalization in the cytoplasm of HtrA1 and TSC2 and that HtrA1 cleaves TSC2 both in vitro and in vivo. Finally, we show that alterations in HtrA1 expression cause modifications in phosphorylation status of two downstream targets of TSC2: 4E-BP1 and S6K. Our data suggest that, under particular physiologic or pathologic conditions, HtrA1 degrades TSC2 and activates the downstream targets. Considering that HtrA1 levels are significantly increased during embryogenesis, we speculate that one of the targets of HtrA1 activity during fetal development is the TSC2-TSC1 pathway. Mol Cancer Res; 8(9); OF1–13. ©2010 AACR.

Introduction

HtrA1 is the first identified member of the human HtrA family proteins, a group of heat shock–induced serine proteases involved in several aspects of protein quality control and cell fate. This family consists of four members (HtrA1, HtrA2, HtrA3, and HtrA4), which share a highly conserved trypsin-like serine protease domain and one PDZ domain at the COOH terminus (1), and specific distinct regulation domains, in relation with their physiologic functions, at the NH2 terminus.

Structurally, the NH2 terminus of HtrA1 shares a signal sequence for secretion and two homology domains: the first region of the protein is homologous to mac25, a gene sequence for secretion and two homology domains: the first domain at the COOH terminus (1), and specific distinct regulation domains, in relation with their physiologic functions, at the NH2 terminus.

Among these two regions, there is a Kazal-type inhibitor motif. Therefore, the human HtrA1 protein is a secreted serine protease that exhibits endoproteolytic activity, including autocatalytic cleavage. It is also known that conversion by site-directed mutagenesis of the putative conserved active site Ser328 to alanine eliminates this enzymatic activity (4).

Until now, several targets of HtrA1 have been identified, most of which are extracellular proteins. These include components of extracellular matrix, such as type III collagen (5) and fibronectin (6), or components of cartilage, such as aggrecan, decorin, and fibromodulin (7). Moreover, it has been described that purified HtrA1 degrades various amyloid precursor protein fragments, including amyloid-β and its precursor, the C99 peptide, both involved in a metabolic balance able to prevent plaque formation in the brain of Alzheimer’s disease patients (8).

In accordance with its proteolytic activity and extracellular localization, HtrA1 was initially described as a gene downregulated in SV40-transformed human fibroblasts (9) and overexpressed in osteoarthritic cartilage (4). Subsequently, it has been reported that the expression of HtrA1 is also decreased in progression and invasion of melanomas, ovarian cancers, lung cancer, and mesotheliomas (10-13). It has been shown that its overexpression inhibits cell growth and proliferation in vitro and in vivo, thus suggesting a possible role as a tumor suppressor (10). Moreover, it seems that molecular alterations associated with the expression of this HtrA1 serine protease may play an important role in tumor behavior and response to chemotherapy (14). Recently, several studies revealed the involvement of HtrA1 also in age-related macular degeneration. Genome-wide linkage analyses showed a strong association of a variant of HtrA1 gene with increased susceptibility to age-related macular degeneration in different populations (15-19).
To identify new HtrA1-binding partners and thereby obtain new clues toward the functions of this protein, we have used the enzymatically inactive form of HtrA1, called HtrA1-S328A, as a bait in a yeast two-hybrid screening experiment. Among the proteins identified as possible partners of HtrA1, we found one of the two proteins involved in the pathogenesis of tuberous sclerosis, a 1,807–amino acid/220-kDa protein called TSC2.

Tuberous sclerosis complex (TSC) is a rare inheritable disorder characterized by the development of hamartomas in the brain and in other vital organs such as kidneys, heart, eyes, lungs, and skin. The growth of benign tumors is frequently associated with skin rashes, seizures, mental retardation, and renal dysfunction (20-22).

Two tumor suppressor genes responsible for TSC have been identified: TSC1 located on chromosome 9q34 and TSC2 on 16p13, encoding hamartin and tuberin, respectively (23, 24).

It has been elucidated that TSC1 and TSC2 physically interact to form an intracellular heterodimer (TSC1/TSC2 complex) participating in the control of cell growth and division (25, 26). This TSC complex acts upstream of the mammalian target of rapamycin (mTOR), a serine-threonine kinase that increases cell growth and proliferation. Activation of mTOR leads to increased protein synthesis through phosphorylation of two effector molecules: p70S6K and 4E-BP1. p70S6K, the ribosomal protein S6 kinase 1, can phosphorylate translation regulators, such as eukaryotic initiation factor 4E (4E-I), to enhance the translational efficiency of several mRNAs (27, 28). 4E-BP1, the eukaryotic initiation factor eIF4E-binding protein 1, is able to interact with the eukaryotic translation initiation factor eIF4E. Binding of the 4E-BPs to eIF4E is regulated by phosphorylation: Hypophosphorylated 4E-BP1 isoform releases eIF4E and translation initiation ensues (29). When the TSC complex is active, usually under conditions that are adverse for proliferation, it is able to inhibit mTOR through the inhibition of Rheb, a member of the Ras superfamily of GTPases, important for the progression from G0 to G1 phase. Therefore, inhibition of mTOR activity leads to prolonged G1 phase or arrest in G0 (27). On insulin or growth factor stimulation, TSC2 is phosphorylated by several kinases, including Akt/PKB (a serine-threonine kinase involved in cellular survival pathways) and RSK1 (p90 ribosomal S6 kinase 1), thereby suppressing its GTPase-activating protein activity (30). As a consequence, the TSC complex is no more functional, and downstream targets of mTOR act together to increase protein synthesis and cell growth. Mutations in TSC1 or TSC2 genes lead to constitutive activation of this pathway; as a consequence, mTOR uncontrolled activity triggers cell cycle progression and tumor formation (27).

To the best of our knowledge, TSC2 is the first cytoplasmic target of HtrA1 identified until now. In this article, we have characterized in vitro and in vivo the interaction between these two proteins.

Materials and Methods

Plasmids and DNA constructs

Human wild-type HtrA1 cDNA was a kind gift of Dr. Trueb (University of Bern, Bern, Switzerland). The truncated expression constructs encoding the mac25 domain (amino acids 22-155), the KI domain (amino acids 97-155), and the COOH-terminal HtrA (amino acids 150-480) were generated by PCR.

The HtrA1-S328A variant expression cDNA was obtained by site-directed mutagenesis of the original HtrA1 expression construct using the Stratagene QuickChange kit as previously described (4).

For transfection experiments, the full-length HtrA1 and its variant HtrA1-S328A cDNAs were cloned as EcoRI/BamHI fragments into pcDNA3T7Tag mammalian expression vector (Invitrogen).

For production of the glutathione S-transferase (GST)–HtrA1 recombinant fusion protein, the full-length HtrA1 (HtrA1-S328A) and the COOH-terminal truncated fragments (HtrA, mac25, and KI) were cloned as EcoRI/BamHI fragments into the pGEX2TK vector (Invitrogen).

The full-length TSC1-pcDNA3.1 and TSC2-pcDNA3.1 expression constructs were kindly provided by Dr. Halley (Erasmus Medisch Centrum, Rotterdam, the Netherlands). Truncated TSC2 cDNAs were obtained by PCR using TSC2-pcDNA3.1 as template and specific primers: TSC2 (1-607), 5′-GATCGGATCCATGGCGAG-TSC2 (607-1099), 5′-GATCGGATCCATGGCGAG-TSC2 (150-480) were generated by PCR.

Antibodies

The TSC1 and TSC2 polyclonal antibodies used in this study were purchased from Santa Cruz Biotechnology and used at the manufacturer’s recommended dilutions. The anti-T7Tag monoclonal antibody was from Novagen. The anti-4E-BP1 antibody was from Cell Signaling. Anti-phospho-ribosomal S6 kinase (p-S6K) and anti-total S6K antibodies were from Santa Cruz Biotechnology. The rabbit polyclonal antiserum raised against HtrA1 has been previously described (10). To normalize each Western blot experiment, we used the anti-β-actin antibody from Santa Cruz Biotechnology.

Yeast two-hybrid selection

The HtrA1-S328A cDNA was cloned into the EcoRI/BamHI sites of vector pGBK7 (BD Clontech) in frame with the GAL4-binding domain. The yeast strain AH109,
carrying UAS-His3, UAS-LacZ, and UAS-ADE2 reporter genes, was cotransformed with the pGBKTT7-HtrA1-S328A bait and with a human fetal brain cDNA library (BD Clontech) fused to the GAL4 activation domain in the pACT2 vector (BD Clontech). Transformation was carried out using the lithium acetate method. Cells were plated on minimal synthetic defined medium (BD 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, and then His− Ade− transformants were isolated. The His− Ade− colonies, replica plated on SD-Leu-Trp-His-Ade medium and LacZ−, were identified by a filter-lifting assay for β-galactosidase activity. Plasmid DNA was prepared from candidate clones and transformed into Escherichia coli XL1-Blue cells (Stratagene). Recovered library-derived plasmids were analyzed by DNA sequencing.

**In vitro binding assay, GST pull-down assay, and mapping experiment**

GST fusion protein expression was induced for 3 hours in a 500-mL culture of *E. coli* BL21 by the addition of 1 mmol/L isopropyl-β-D-thiogalactopyranoside. The bacterial pellet was resuspended in 20 mL of NENT buffer [20 mmol/L Tris-Cl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin] or NENT buffer added with 2% Sarkosyl (for insoluble protein) and sonicated thoroughly (10 × 30 seconds). After gentle mixing for 30 minutes at 4°C, the lysate was spun at 12,000 × g for 10 minutes at 4°C. The induced GST fusion protein in the supernatant fraction was bound to 250 mL of pre-washed glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 60 minutes at 4°C. The beads were washed extensively with NENT buffer to remove nonspecifically bound bacterial proteins, and the quantity and purity of the GST fusion protein bound to the glutathione-Sepharose beads were analyzed by SDS-PAGE.

For the in vitro binding assay, 1 µg of plasmid encoding TSC2 was used to program a TnT rabbit reticulocyte lysate (Promega) under the control of the T7 polymerase in the presence of [35S]methionine (Amersham Pharmacia Biotech). Aliquots of the reaction mixture were added to glutathione-Sepharose beads (Amersham Pharmacia Biotech) coupled with 5 µg of GST-HtrA1wt GST-mac25, GST-KI, and GST-HtrA. Incubation was carried out in NENT buffer for 60 minutes at 4°C with gentle rocking. Beads were washed three times in NENT buffer, and electrophoresis was done on 6% acrylamide SDS-PAGE. Gels were dried and exposed at −70°C using Kodak Biomax MS films.

For the mapping experiment, 1 µg of plasmid encoding for the different truncated expression constructs of TSC2 [TSC2 (1-607), TSC2 (607-1099), and TSC2 (1099-1807)] was used for in vitro translation as described above. The same amount of each in vitro–translated constructs was incubated with GST-HtrA1wt (5 µg) or with GST alone as control for 60 minutes at 4°C with gentle rocking. After washing, the beads were analyzed on 6% acrylamide SDS-PAGE.

To do a GST pull-down assay with endogenous TSC2 and TSC1 from cell extracts, an equal amount of GST or GST-HtrA1wt protein (5 µg) and 500 µg of Phoenix cell lysate were used for the assay. The mixture of GST-HtrA1wt beads and cell lysate was incubated with rotation at 4°C for 3 hours. Then, the beads were washed three times with lysis buffer. The complexes were separated on 6% SDS-PAGE. Western blot was done with specific anti-TSC2 or anti-TSC1 antibodies.

**Cell culture, transfection, and Western blot analysis**

Phoenix cells were kept in DMEM supplemented with l-glutamine, 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin. A375P and A375M cell lines were cultured in RPMI 1640 supplemented with l-glutamine, 10% heat-inactivated fetal bovine serum, and penicillin-streptomycin. LM cell line was cultured in RPMI 1640 as described elsewhere (31).

For transfection experiments, Phoenix cells were seeded into 10-cm culture dishes and grown overnight to a confluence of 60% to 80%. Expression constructs were transfected into the cells using standard calcium phosphate protocols. The LM, A375P, and A375M cells were seeded into 10-cm culture dishes and grown overnight to a confluence of 80% to 90%. Expression constructs were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) following the protocol instructions.

Twenty-four hours after transfection, the cells were harvested and lysed in lysis buffer [50 mmol/L Tris-Cl (pH 7.4), 5 mmol/L EDTA, 250 mmol/L NaCl, 50 mmol/L NaF, 0.1% Triton X-100, 0.1 mmol/L Na3VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin] for 30 minutes on ice. Lysates were centrifuged at 14,000 × g for 10 minutes at 4°C. Protein concentration was determined by the Bradford assay (Bio-Rad) at 595 nm. Proteins were denatured by boiling in Laemmli sample buffer (Bio-Rad) and resolved in SDS-PAGE. Gels were transferred to Immobilon P membrane (polyvinylidene difluoride; Millipore) and probed with the indicated antibodies.

A2780 ovarian cancer cell line was used to produce stable clones expressing HtrA1 as described elsewhere (32); SKO3 and TOV21G ovarian cancer cell lines were used to produce HtrA1 short hairpin RNA (shRNA) stable clones as described elsewhere (33).

**Coimmunoprecipitation assay**

For the immunoprecipitation experiment, 800 µg of Phoenix cell lysate transfected with pcDNA3T7Tag-HtrA1 were incubated with 1 µg of anti-T7Tag monoclonal antibody (to pull down HtrA1) or 1 µg of anti-TSC2 polyclonal antibody (to pull down TSC2) on ice for 15 hours before the addition of 30 µL of 50% protein G–Sepharose suspension (Amersham Pharmacia Biotech). As a control, 800 µg of lysate were incubated with a monoclonal antibody against p21 protein (BD Pharmingen) or a polyclonal...
Subcellular localization of HtrA1 with immunoelectron microscopy

Full-term placentas ($n = 15$) were collected, cut into pieces of $<1 \text{ mm}^3$, and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.3) for 2 hours. Tissues were postfixed in osmium tetroxide (4%), dehydrated through graded concentrations of ethanol and propylene oxide, and subsequently embedded in Epon 812. For the immunogold technique, tissues were fixed only in 2.5% glutaraldehyde in phosphate buffer (pH 7.3). Ultrathin sections were cut from blocks and mounted on copper grids for the inspection of morphology or on nickel grids for immunogold. All sections were etched on drops of 3% hydrogen peroxide for 10 minutes to permeabilize the resin. Tissue sections were washed in microfiltered distilled water and floated for 30 minutes on a drop of background blocking solution [0.05 mol/L Tris-HCl (pH 7.4), 0.1% bovine serum albumin (BSA)]. Grids were then incubated at 4°C overnight with a rabbit polyclonal antiserum raised against HtrA1 (10) at 1:10 dilution. After several rinses to remove excess antibody, the grids were incubated with an anti-rabbit antibody conjugated with 10-nm gold particles used as a secondary antibody at 1:100 dilution in diluent for gold-absorbed antibody [0.05 mol/L Tris-HCl buffer (pH 8.2), 1% BSA] for 1 hour. The grids were contrasted with lead citrate and uranyl acetate as for conventional electron microscopy. A preimmune serum was used as a negative control. The sections were observed by means of a Siemens Elmiskop IA electron microscope.

Expression analysis of different factors involved in TSC2 pathway

Two different types of melanoma cell lines, A375 (primary and metastatic of the same cell type) and LM, were transfected with pcDNA3T7Tag-HtrA1, pcDNA3T7Tag-HtrA1-S328A, and empty vector as a control as described above. Lysate of transfected cells was run on gel to make Western blot using different antibodies against proteins involved in TSC pathway. Each experiment has been done at least three times and individually normalized using the antibody against β-actin.

Expression levels of HtrA1, TSC2, p-S6K, and total S6K were analyzed by immunoblot analysis in stable clones overexpressing HtrA1 (A2780 cells) or in stable clone with HtrA1 shRNA (SKOV3 and TOV21G cells). Empty
vector–transfected cells and nontargeted control shRNA–transduced cells served as controls. The protein in the insoluble fraction was used for the analysis of the proteins. SKOV3 or TOV21G batch clonal cells with HtrA1 shRNA were seeded at 5 × 10^5 per well into six-well plates and cultured until cells became confluent. Following washing with 1.5 mmol/L Na_3VO_4 in PBS twice, the cells were placed on ice and the protein lysate was extracted with 0.5 mL of lysis buffer [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.2% NP40, 1.5 mmol/L Na_2VO_4], and cocktail protein inhibitor was added into each well. Two minutes later, soluble fraction was removed by centrifugation and the insoluble fraction was subjected to Western blot analysis.

**Results**

**Identification of TSC2 as a binding partner of HtrA1**

To identify new proteins able to interact with HtrA1, we did a yeast two-hybrid screening experiment on ~5 × 10^5 clones from the human fetal brain cDNA library (Clontech). As a bait, we used the construct encoding full-length HtrA1-S328A, which lacks proteolytic activity and does not exhibit autoproteolysis (4), to avoid the digestion of possible targets of our protease during the screening in yeast. From the 77 colonies that grew on selective medium, 52 were positive in a β-galactosidase selection assay, and 25 clones were recovered from yeast and then transformed into XL1-Blue–competent cells. Individual clones were screened and sequenced using the primers from vector. Among rescued plasmids, we found two clones with the cDNA of the tumor suppressor gene TSC2. The region obtained within the positive plasmids was the NH_2-terminal part of TSC2; this finding suggested that the interaction between TSC2 and HtrA1 might take place in this region.

**Confirmation of HtrA1/TSC2 binding in vitro**

To verify the interaction between HtrA1 and TSC2 and to determine whether this association was specific, we did an in vitro binding assay. The activity of TSC2 in the cell is strictly dependent by its interaction and binding with TSC1, the other member of the TSC complex; the dimer acts as a tumor suppressor only when both the proteins are functional and are able to interact with each other (34). Therefore, we decided to investigate whether HtrA1 is able to interact also with TSC1. Both TSC1 and TSC2 were translated in vitro in the presence of [35S]methionine and incubated with GST-HtrA1wt construct and with the truncated constructs encoding three different domains of HtrA1: GST-mac25, GST-KI, and GST-HtrA (Fig. 1A). We used GST alone as a control. Washed beads were used to detect the presence of TSC2 or TSC1 on acrylamide SDS-PAGE; gels were dried and exposed at -70°C.

As shown in Fig. 1B, TSC2 coprecipitated with GST-HtrA1wt and GST-mac25, but not with GST-HtrA; moreover, there was a weak interaction also between TSC2 and GST-KI, the small domain included inside mac25. This indicates that HtrA1 interacts with TSC2, and the site of the binding of TSC2 on HtrA1 is into the mac25 domain of the serine protease. Finally, no TSC1 was retained by GST-HtrA1wt in this experiment (Fig. 1C), thus excluding the hypothesis of a possible interaction of HtrA1 with the other member of the TSC complex.

To investigate the association between HtrA1 and TSC2, a GST pull-down assay to precipitate TSC2 from cell extracts was carried out. To this end, a cleared lysate from Phoenix cells, expressing endogenous levels of TSC2, was applied to glutathione-Sepharose beads alone or to glutathione-Sepharose beads bound with the GST-HtrA1wt chimeric construct. As shown in Fig. 2A, endogenous TSC2 retained by the GST-HtrA1wt beads was detected by immunoblot analysis with anti-TSC2 polyclonal antibody. Tuberin bound to the GST-HtrA1wt beads, but not to glutathione-Sepharose beads containing only the GST protein, thereby confirming that there is a specific interaction between HtrA1 and TSC2. We repeated the same experiment using a specific anti-TSC1 polyclonal antibody, but no TSC1 was detectable by immunoblot analysis, so it was confirmed that there is not direct interaction between HtrA1 and TSC1 (Fig. 2B).

**HtrA1 interacts with the NH_2-terminal region of TSC2**

To better characterize the binding between HtrA1 and TSC2, we next decided to do a mapping experiment to determine the exact regions of TSC2 involved in the interaction with HtrA1.

Figure 3A shows the structure of full-length TSC2 and of the deletion constructs used for mapping experiment. Each fragment was in vitro translated, [35S]methionine labeled, and used for in vitro pull-down assay using GST-HtrA1wt fusion protein. As shown in Fig. 3B, the only fragment precipitated by the GST-HtrA1wt recombinant protein was the TSC2 (1–607) domain, which is the first part of the NH_2-terminal region of the protein.

**In vivo interaction between HtrA1 and TSC2**

To investigate whether the interaction between HtrA1 and TSC2 occurs also in vivo, communoprecipitation experiments were done using lysate from Phoenix cells transfected with pcDNA3T7Tag-HtrA1. As shown in Fig. 4A, a consistent amount of endogenous TSC2 was communoprecipitated by the antibody against the epitope tag of HtrA1-tag overexpressed protein. The same result is obtained using anti-TSC2 to communoprecipitate endogenous TSC2- and HtrA1-interacted protein (Fig. 4B). This result indicates that TSC2 and HtrA1 associate in vivo, and the data are consistent with the results of the GST-HtrA1wt–binding assay.

**HtrA1 localizes both in the cytoplasm and in the extracellular space of the cell**

The subcellular localization of HtrA1 has been investigated by the use of immunoelectron microscopy on human placental tissue. As shown in Fig. 5, we detected a diffuse immunopositivity for HtrA1 scattered in the cytoplasm. Interestingly, HtrA1 immunogold particles were
not associated to organelles or vesicles inside the cytoplasm. Moreover, as already shown in a previous article (35), it was clear that HtrA1 immunopositivity was present in the extracellular spaces of the same human tissues.

HtrA1 and TSC2 colocalize in the cytoplasm of Phoenix cells

To further examine whether HtrA1 and TSC2 colocalize in the cells, Phoenix cells overexpressing HtrA1 were stained using DAPI, mouse anti-T7Tag, or rabbit anti-TSC2. As shown in Fig. 6A, TSC2 localizes in the cytoplasm as well as in HtrA1. Thus, in transfected Phoenix cells, colocalization of HtrA1 and TSC2 was evident in the cytoplasm. In Fig. 6B, the same cells were transfected with the empty vector as a control. No positive staining was detectable in this case.

TSC2 phosphorylation is not necessary for the interaction with HtrA1

To investigate whether phosphorylation of TSC2 is necessary for the HtrA1/TSC2 interaction, the effect of phosphatase activity on the GST-HtrA1–binding assay was analyzed. Phoenix cells lysates were treated with PP1 before incubation with GST or GST-HtrA1wt beads. After incubation of 60 minutes at 30°C either with or without the addition of PP1, TSC2 was retained by the GST-HtrA1wt beads but not by GST alone (Fig. 7). These data clearly show that the binding between HtrA1 and TSC2 is independent from the phosphorylation status of TSC2.

Delta-ss-mac25-HtrA1 digests TSC2 in vitro and in vivo

To explore whether TSC2 is a substrate of HtrA1, we did a digestion assay using purified recombinant human HtrA1 and human TSC2 translated in vitro and labeled with l-[35S]methionine. Further, we used fibronectin (as a positive control) to test the protease activity in our condition and BSA (as a negative control) to check the specificity of HtrA1 substrate recognition (Fig. 8B). After an overnight incubation, HtrA1 completely digests TSC2 (Fig. 8A). As TSC2 disappeared, a lot of bands of lower mass in the second lane become visible, corresponding to TSC2 digestion products. Probably, the formation of defined proteolytic bands is due to HtrA1 recognition of specific TSC2 sequences. As expected, BSA was not digested by HtrA1.

**FIGURE 1.** TSC2 binds specifically to the GST-HtrA1 fusion protein. A, biochemical structure of full-length HtrA1 and different construct domains used for expression of GST fusion proteins. B, the in vitro–translated TSC2 was retained by GST-HtrA1wt and by the truncated fusion proteins GST-mac25 and GST-KI, but not by the COOH-terminal region of HtrA1wt (GST-HtrA). C, the in vitro–translated TSC1 was not retained by GST-HtrA1. Input was 20% of the sample used for pull-down assay. Equal amount of GST or fusion proteins used in the experiments is confirmed by Coomassie staining.
To investigate if the degradation of TSC2 is physiologically relevant, we decided to analyze the protein level of endogenous tuberin in A2780 ovarian cancer cell line overexpressing HtrA1 and in SKOV3 or TOV21G ovarian cancer cells with HtrA1 shRNA. As shown in Fig. 9B, overexpression of HtrA1 is able to induce degradation of endogenous TSC2 in A2780 cell line; otherwise, downregulation of HtrA1 by shRNA in SKOV3 and TOV21G
cell lines leads to an increase in the endogenous levels of TSC2 as expected. Collectively, these results indicate that TSC2 is a substrate of HtrA1.

4E-BP1 is hyperphosphorylated in cells overexpressing HtrA1

We decided to overexpress both the full-length HtrA1 and the point mutant of our protein (HtrA1-S328A) into two different types of melanoma cell lines: LM (metastatic melanoma cell line) and A375 (both primary melanoma cell line, A375P, and metastatic melanoma cell line of the same cellular type, A375M).

As shown in Fig. 9A, the eukaryotic initiation factor 4E-BP1 seems to be hyperphosphorylated in all three types of cells overexpressing HtrA1 wild-type, whereas, in the cells transfected with the functional mutant of HtrA1, 4E-BP1 is clearly dephosphorylated if compared with the cells overexpressing HtrA1. Moreover, in the LM cells, 4E-BP1 seems to be hypophosphorylated also compared with the cells used as a control, which express the physiologic level of HtrA1.

S6K is hyperphosphorylated in stable clones overexpressing HtrA1 and hypophosphorylated in stable clone with downregulation of HtrA1

Immunoblot analysis, shown in Fig. 9Ba, shows that enhanced expression of HtrA1 in A2780 ovarian cancer cell line leads to diminished levels of TSC2. Consistent with this downregulation, there was an increase of p-S6K protein level. Conversely, stable downregulation of HtrA1 in SKOV3 (Fig. 9Bb) and TOV21G (Fig. 9Bc) cells leads to an increase in the endogenous levels of TSC2 with a concomitant decrease of p-S6K form.

Discussion

HtrA1 is a serine protease ubiquitously expressed in normal human adult tissues, which is found either as a secreted protein or in the cytoplasm of the cells (35). The transcription of this gene is highly regulated both during development and in the adult (35, 36), and in particular, it has been shown that HtrA1 is upregulated during the progress of human pregnancy, suggesting an important role of this protein in placental formation and function (37, 38). It is known that HtrA1 expression is decreased in progression and invasion of ovarian and endometrial cancers, melanomas, lung cancer, and mesotheliomas (10-13, 39, 40), and it is also involved in chemotherapy-induced apoptosis (32). Moreover, it has been shown HtrA1 has the ability to promote degeneration of extracellular matrix components, including fibronectin (6, 41), collagen (7), and amyloid precursor protein fragments involved in Alzheimer’s disease (8).

To obtain additional information about the role of HtrA1 in the cell, a yeast two-hybrid system was used to identify new possible targets of our protease. We decided to use the point mutant of full-length HtrA1 as a bait to avoid the degradation of possible targets of our protease.

It is known that the expression of HtrA1 in human embryos is dramatically upregulated from the first to the third trimester of gestation (36). Because of the high expression of HtrA1 in the brain during embryo development, we decided to screen a human fetal brain cDNA library.

One of the proteins detected in the screening was TSC2, a tumor suppressor gene associated with tuberous sclerosis disease. Tuberous sclerosis is characterized by a variety of hamartomatous growths in different organs and tissues. The defects in cell proliferation, migration, and differentiation that these lesions display indicate that the TSC1 and
TSC2 gene products participate in the control of cell growth and division (40), and it is now recognized that the primary function of the TSC1-TSC2 complex is a critical negative regulation of the mTOR activation (27). mTOR plays an evolutionarily conserved role in the control of cell growth (i.e., an increase in cell size), but it has also been found to regulate aspects of cell proliferation, survival, and metabolism in specific settings. In particular, mTOR acts through two well-characterized classes of downstream targets: the ribosomal S6 kinases S6K1 and S6K2, and 4E-BP1 and 4E-BP2 (42, 43). Genetic experiments in mammalian cells showed that mTOR-mediated phosphorylation of both S6K1 and 4E-BP1 is inhibited by TSC1-TSC2 complex overexpression and is activated in cells lacking the TSC1-TSC2 dimer (43-46). As a consequence of TSC gene disruption, the mTOR signaling becomes robustly activated in a growth factor–independent manner; moreover, elevated mTOR activity has also been detected in all rodent and human tumors and tumor-derived cell lines lacking TSC1 or TSC2 (47-50).

The direct interaction between HtrA1 and TSC2 was confirmed by in vitro binding experiments and by GST pull-down assays. These binding experiments indicate that HtrA1 is able to interact with TSC2 but not with the other component of the TSC complex, TSC1, thereby confirming that there is a specific interaction between HtrA1 and TSC2.

![Figure 6](image)

**Figure 6.** Colocalization of TSC2 and HtrA1 in Phoenix cells. A. Phoenix cells transfected with pcDNA3T7Tag-HtrA1. Blue, cells stained with DAPI; red, cytoplasm localization of HtrA1; green, cytoplasm localization of TSC2. Colocalization (yellow) of HtrA1 and TSC2 in the cytoplasm. B. Phoenix cells transfected with pcDNA3T7Tag empty vector. No yellow staining was detectable.

![Figure 7](image)

**Figure 7.** Tuberin phosphorylation is not necessary for binding to HtrA1. A cleared Phoenix lysate was incubated at 30°C for 60 min either with (+) or without (−) PP1 before incubation with GST or GST-HtrA1 beads. The lysate was also run on gel without incubation at 30°C. TSC2 retained by the GST-HtrA1 was detected by immunoblotting. Equal amount of GST or GST-HtrA1wt used in the experiment is confirmed by Coomassie staining.

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<th>Phoenix lysate</th>
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**Table 1.** Effect of PP1 on tuberin phosphorylation.
TSC2. It is well known that the PDZ domain is the prime site of interactions for HtrA1 (41, 51-53). Nevertheless, our study shows the first interaction in which HtrA1 binds to another protein through the mac25 domain. Because this domain sometimes is cleaved off during HtrA1 activity (8), it is possible to suggest that this proteolytic event is important for HtrA1 function, as it could reduce the ability of HtrA1 to interact with other proteins. Interestingly, in our experimental conditions, full-length HtrA1 is able to interact with TSC2, but the activated form of the

FIGURE 8. HtrA1 digests TSC2 in vitro. A, autoradiography of 35S-labeled TSC2 incubated overnight at 37°C alone or in combination with HtrA1. In the presence of HtrA1 are detectable lower mass bands of TSC2. B, Coomassie-stained HtrA1, fibronectin, and BSA. Fibronectin, used as a control substrate of HtrA1, is digested as shown by the presence of a lower band of 30 kDa. BSA was used as a negative control of the protease activity of HtrA1.

FIGURE 9. Effect of HtrA1 on downstream effectors of TSC2. A, 4E-BP1 is hyperphosphorylated in 3 different melanoma cell lines overexpressing HtrA1wt but not in the same cells overexpressing the point mutant of the protein. B, stable clone overexpressing HtrA1 (a) or with downregulation of HtrA1 (b, c) were analyzed to detect protein levels of TSC2 and S6K; moreover, the phosphorylation status of S6K was investigated in relation to the presence of HtrA1 and TSC2. Abbreviation: V1, empty vector; WT20, A2780 overexpressing HtrA1; sh1, clonal cells with HtrA1 shRNA.
protein, lacking ss-signal peptide and mac25 domain, is still able to proteolyse TSC2.

Evidences suggested that HtrA1 is essentially a secreted protein; most of it is located in the extracellular space and only a small percentage of the protein remains into the cells (54). It is also true, however, that several evidences show the presence of this protease in the cytoplasm. Clawson and colleagues (55) have shown that processed forms of HtrA1 are found intracellularly and intranuclearly, and the active intranuclear form of HtrA1 shows an Mr ∼29,000. Chien and colleagues (33) have reported that HtrA1 localized to the cytoplasm and associates with tubulins within the cell. They have shown that HtrA1 colocalizes with intracellular microtubules and degrades tubulins into the cytoplasm. Moreover, they observed coimmunoprecipitation of α-, β-, and γ-tubulins when endogenous HtrA1 is immunoprecipitated. In a different study, He and colleagues (56) showed, with immunoprecipitation and confocal assays, that HtrA1, as well as its 35-kDa active form, can bind to epidermal growth factor receptor on the cell membrane. Interestingly, in a few cells, the colocalization of HtrA1 and epidermal growth factor receptor in the nucleus was also observed.

Therefore, to confirm that the binding between our protease and TSC2 takes place in the cytoplasm of the cells, we decided to show this interaction also in vivo. First, by immunoelectron microscopy, we have shown that HtrA1 is indeed detected in the cytoplasm of the cells and that HtrA1 immunogold particles were not associated to organelles or vesicles inside the cytoplasm. Moreover, endogenous TSC2 associated with HtrA1 and was coimmunoprecipitated in Phoenix cell lysate. Finally, colocalization analysis with immunofluorescence showed that HtrA1 and TSC2 interact into the cytoplasm of the cells.

TSC2 is phosphorylated at multiple amino acid residues, and this phosphorylation is important for the properties of binding of TSC2. Therefore, we investigated if the binding between HtrA1 and TSC2 is independent by the phosphorylation state of TSC2. Indeed, our data indicate that this posttranscriptional regulation of TSC2 does not affect the binding of HtrA1.

The role of HtrA1 as a protease capable to cleave extracellular proteins such as fibronectin, collagen, or components of cartilage is known (5, 7). In the present report, we identified the intracellular protein TSC2 as a substrate of HtrA1. By using the purified activated form of HtrA1, called delta-ss-mac25-HtrA1, we have shown that HtrA1 effectively degraded in vitro–translated TSC2, generating fragments of various sizes with low molecular weight. The same phenomenon was found in vivo using three different cellular systems engineered to overexpress or silence HtrA1.

As previously described, we found that the binding site of HtrA1 on TSC2 is in the same region in which TSC1

![Figure 10. Schematic representation of TSC pathway and the possible role of HtrA1. A, following the activation of the pathway, insulin receptor substrate, activated by insulin-like growth factor, recruits phosphoinositide 3-kinase to the membrane. Phosphoinositide 3-kinase converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate and activates the serine/threonine kinase Akt. Active Akt inhibits TSC2 activity through direct phosphorylation; thus, Akt-driven TSC1/TSC2 complex inactivation allows Rheb to accumulate in a GTP-bound state. Rheb-GTP then activates the protein kinase activity of mTOR. Downstream targets of mTOR include S6K and 4E-BP1. Once phosphorylated by mTOR, 4E-BP1 and S6K can initiate translation. B, hypothetical role of HtrA1.](mcr.aacrjournals.org/article-figures/10.png)
interacts with TSC2 during the dimer formation; furthermore, our protease is able to digest TSC2. The observation that TSC2, when phosphorylated, translocates to the cytoplasm (57) further supports the idea that HtrA1 interacts with TSC2 predominantly within cells due to this mechanism of phosphorylated TSC2 becoming cytosolic. It might be that in particular physiologic conditions in which HtrA1 is present in high level in the cells, TSC2 is degraded, and the downstream factors of the pathway are activated as depicted in the cartoon in Fig. 10.

To confirm our hypothesis, we decided to investigate if upregulation or downregulation of HtrA1 in vitro is able to cause altered expression of the factors involved in the TSC pathway. In the first experiment, we used two different types of melanoma cell lines (A375 and LM). In our experimental conditions, 4E-BP1 was hyperphosphorylated in cells overexpressing HtrA1, whereas the level of the same molecule was lower when we used the point mutant form of HtrA1 (HtrA1-S328A) to transfect the cells, thus confirming that the proteolytically active form of HtrA1 is able to interfere with the expression of downstream targets of TSC2. Therefore, we decided to investigate in a different experimental setting the effects of HtrA1 upregulation or downregulation on S6K phosphorylation status. Indeed, we also found that this downstream effector of TSC2 is hyperphosphorylated when HtrA1 is upregulated and, coherently, hypophosphorylated when HtrA1 is silenced.

In conclusion, in our study, we have shown for the first time an interaction involving TSC2 and HtrA1; moreover, we have shown that HtrA1 is able to digest TSC2 both in vitro and in vivo. The functional role of this interaction is still unclear, but we can speculate that, in particular physiologic or pathologic conditions, HtrA1 degrades TSC2 and activates the downstream targets. Considering that HtrA1 levels are significantly increased during embryogenesis, we speculate that one of the targets of HtrA1 activity during fetal development is the TSC2-TSC1 pathway. Further experiments are indeed required to further define this interaction and to investigate its role during differentiation and tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Serine Protease HtrA1 Specifically Interacts and Degrades the Tuberous Sclerosis Complex 2 Protein

Mara Campioni, Anna Severino, Lucrezia Manente, et al.

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