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Intracellular Localization of the Tumor Suppressor HtrA1/Prss11 and its Association With HPV16 E6 and E7 Proteins

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ABSTRACT

We have a long-standing interest in a nuclear protease which appears to be involved in carcinogenesis. We recently identified the protease as high temperature requirement factor A 1 (HtrA1), also known as Prss11, which is member of an oxidative stress-response family of proteases. HtrA1 has been classified as a secreted protease involved in TGFβ signaling, but recent work has shown HtrA1 to be a tumor suppressor. Here we show that processed forms of HtrA1 are found intracellularly and intranuclearly, and the active intranuclear form of HtrA1 shows an ~Mr 29,000. Further, expression of HPV E6/E7 proteins is associated with a post-transcriptional up-regulation of HtrA1 (most notably the nuclear form), and HtrA1 is found associated with both HPV E6 and E7 proteins. J. Cell. Biochem. 105: 81–88, 2008.

KEY WORDS: HtrA1; Prss11; HPV; CANCER

We have had a long-standing interest in a nuclear protease, which was originally characterized as a calcium-regulated serine protease with chymotrypsin-like substrate preference [Tokes and Clawson, 1989; Madsen et al., 1990; Clawson et al., 1992, 1995a]. A relatively selective chloromethylketone inhibitor, $AAPF_{cmk}$, was identified which efficiently inhibited the nuclear protease activity, both in vitro and in cell culture models [Clawson et al., 1993, 1995b]. More recently, however, we have observed inhibition of spontaneous transformation of a hepatocyte-derived cell line by $AAPF_{cmk}$ [Drubin and Clawson, 2004], which appeared to occur without significant change in the nuclear protease activity. $AAPF_{cmk}$ also produced major growth inhibitory effects in HPV-infected raft cell cultures [Drubin et al., 2006]. Nuclear protease activity could not be assessed in treated raft cultures, since the growth inhibition was so dramatic (there were not enough cells left for analysis), so it is not clear whether the growth inhibitory effects in these raft cultures were mediated by inhibition of proteolytic activity (growth inhibition was not observed in standard monolayer cultures).

Given the potential for non-protease effects of AAPFcmk, we also characterized molecular nuclear targets for $AAPF_{cmk}$ using a biotinylated version and rat liver nuclear extracts. We identified a relatively restricted spectrum of non-protease targets [Dhamne et al., 2007], which included many helicases, SAP-domain containing proteins, and a few other targets. This work also identified one serine protease, which is known as HtrA1 (Gene IDs 65164 for rat, 5654 for human, and 56213 for mouse). The sequence identified in nuclear extracts began at L_{23}, so that the signal sequence was not present [Dhamne et al., 2007].

HtrA1, also referred to as Prss11, is a member of the high temperature requirement factor A (HtrA, and its homologues DegP, Q, and S in bacteria) family of oxidative stress-response proteases. This represents a group of inducible serine proteases, which are involved in protein quality control (they are often called ATP-independent chaperone proteases). The HtrA family of proteases share a number of features, prominently including a conserved protease domain followed by a PDZ domain. The PDZ domain acts as a switch for activating protease activity of bacterial HtrA family members for a cellular stress response [Schlieker et al., 2004; Wilken et al., 2004], as is also the case for HtrA1, where binding of the PDZ domain activates its protease activity [Murwantoko et al., 2004]. HtrA1 is also known as IGF binding protein-5 (IGFBP5) due to the

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existence of an IGF binding domain with homology to mac25 [Hu et al., 1998]. HtrA1 is expressed as an Mr 51,000 precursor; this begins with a 22 aa signal sequence which presumably targets some HtrA1 for secretion. After synthesis in vitro, the precursor undergoes autocatalytic processing to two smaller, proteolytically active forms around Mr 35,000 [Chien et al., 2006]; these active forms begin at or around aa 169, although the difference between the forms is not clear.

In general, HtrA1 has been classified as a secreted protease, where it has been implicated in TGFβ signaling [Oka et al., 2004]. More recently, HtrA1 has been classified as a tumor suppressor in a variety of cancers [Baldi et al., 2002; Allinen et al., 2004; Chien et al., 2004; Bowden et al., 2006], and has been implicated in diverse other diseases [Strauss et al., 2005; Yang et al., 2006]. HtrA1 has also been implicated in resistance to chemotherapy [Chien et al., 2006], and in fact it is one of a cohort of only three genes which could distinguish chemosensitivity of breast cancers [Folgueira et al., 2005].

Here we confirm the presence of the processed forms of HtrA1 in various cell types, including 293 kidney epithelial cells, 293 cells expressing HPV16 E6/E7, and CaSki cells. Immunoblot analyses showed a prominent doublet near Mr 36,000 in CaSki cells, which corresponds to previously reported proteolytically active forms of HtrA1, as well as an Mr 29,000 form, which was the predominant active form in nuclear extracts. The lower band of the doublet (Mr $36{,}000_L$) and the Mr 29,000 form were also observed in 293 T cells. Expression of HPV16 E6/E7 in 293 cells resulted in an up-regulation of HtrA1 expression, particularly the nuclear form, as well as up-regulation of the protooncogene DEK. QPCR results indicated that this HtrA1 up-regulation was post-transcriptional in nature, since HtrA1 mRNA levels remained unchanged. Immunoprecipitation/immunoblot analyses with CaSki cells indicated that HtrA1 was present in E6 and E7 immunoprecipitates. These results show that: (1) HtrA1 is found intracellularly in CaSki (and 293) cells; (2) HPV16 E6/E7 expression is associated with up-regulation of HtrA1 expression; and (3) both the E6 and E7 proteins associate with particular forms of HtrA1.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

AAPFcmk and biotin-AAPFcmk were obtained from MP Biochemicals, and stored lyophilized or in DMSO stocks. Antibodies to HtrA1 were obtained from R&D (Minneapolis, MN) and Imgenex (San Diego, CA). The Imgenex Ab is a polyclonal rabbit Ig targeted to the 234–249 aa region of human (and mouse) HtrA1. The R&D antibody is a monoclonal mouse anti-human antibody which was raised against the full-length HtrA1 protein (23–480 aa; its epitope specificity is not known). Antibodies to HPV16 E6 protein were an affinity purified goat polyclonal preparation, and antibodies to HPV16 E7 protein were a mouse monoclonal preparation; both were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). In some experiments, antibodies to HPV16 E6 (ab70) were a mouse monoclonal preparation obtained from Abcam (Cambridge, MA), and antibodies to HPV16 E7 (ab30731) were a mouse monoclonal preparation also from Abcam. To monitor nuclear fractionation protocols, we also used antibodies to DEK (from Aviva Systems Biology, SanDiego, CA), and to document loading/transfer for immunoblots, we used antibodies to β-actin (from Cell Signaling and Neuroscience, a division of Sigma–Aldrich, St. Louis, MO).

TISSUES AND CELL LINES

CaSki cells, derived from a human cervical cancer, were obtained from ATCC. CaSki cells were grown on ATCC Media (RPMI 1640 + with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, with 10% heat-inactivated FBS), and the cells used were high passage cultures. 293 T cells (a human embryonic kidney line) were obtained from ATCC. $293_{FLIP\text{-}In}$ cells were obtained from Invitrogen (Carlsbad, CA). This system allows stable integration of a single copy of a given gene expression sequence into a predefined site in the genome. $293_{FLIP\text{-}In}$ cells (Invitrogen) were cultured in DMEM, 10% fetal calf serum, 1% glutamine, 400 μg/ml Zeocin (also Invitrogen).

GENERATION OF STABLY TRANSFECTED CELL LINES EXPRESSING HPV16 E6/E7 ($293_{HPVE6/E7}$)

The $293_{FLIP\text{-}In}$ cells were then used to produce stable transfectants expressing high levels of HPV 16 E6/E7. The HPV16E6/E7 sequence (nt 1–776) was isolated by reverse transcription/PCR, using total RNA obtained from CaSki cells. Cells were removed from the plates by trypsinization, and total RNA was isolated with TRIZOL Reagent (Invitrogen). Total RNA preparations were then treated with TURBO DNA-Free kit (Ambion) to remove any trace amounts of DNA. Reverse transcription was performed on 50 ng total RNA using Sensiscript RT Kits (QIAGEN) and an HPV16-specific primer, Claw 428 (5′-TTATGGTTTCTGAGAACAGA). PCR was then performed on the RT product with HotStar Taq (QIAGEN) and Claw 427 (5′-ATGTACCAAAAGAGAACTGC) with Claw 428. The PCR product was cloned into TOPO TA vector (Invitrogen). The HPV 16 E6/E7 construct was verified by sequencing (in the Core Facility at Hershey Medical Center). Restriction endonucleases *Hin*dIII and *Xba*I were used to remove the HPV16E6/E7 fragment, which was then cloned into *Hin*dIII/*Xba*I digested pVAX1 vector (Invitrogen). Next, digests with the restriction endonucleases *Sna*BI and *Apa*I were carried out on both the HPV16 E6/E7-pVAX1 vector (to obtain HPV16E6/E7 insert) and the pCDNA5/Frt vector, to allow for directional subcloning, and the HPV16 E6/E7 insert was cloned into pCDNA5/Frt. The final HPV16 E6/E7-pcDNA5/FRT sequence was confirmed again by sequencing.

An HPV16 E6/E7 stable cell line was generated by co-transfecting HPV16 E6/E7-pcDNA5/FRT and pOG44 (Invitrogen) using Lipofectamine Reagent (Invitrogen) in growth medium without Zeocin, according to manufacturer recommendations. Positive controls were run concurrently, using co-transfections with pcDNA5 containing enhanced green fluorescent protein and pOG44. Negative controls were run using pOG44 only. Twenty-four hours after transfection, hygromycin B (Invitrogen) was added to the medium to a final concentration of 30 μg/ml, and the media were changed every 3–4 days until all the cells on the negative control plate died, and all the cells on the positive control plates showed green fluorescence. A number of cell lines expressing HPV16 E6/E7 were obtained, and designated $293_{HPVE6/E7}$. They were pooled and

tested for HPV16 E6/E7 expression levels using real-time PCR as previously described. The HPV16 E6/E7 expression level in the $293_{HPVE6/E7}$ cell line that we generated is equivalent to E6/E7 expression levels in high-passage CaSki cells.

NUCLEAR AND CYTOPLASMIC FRACTIONATION

In most cases, nuclear and cytoplasmic fractions were obtained using NE-PER extraction reagents (from Pierce Biotechnology, Rockford, IL). As an additional method, we also prepared nuclear and cytoplasmic fractions using manual homogenization as previously described [Dhamne et al., 2007], and obtained similar results.

IMMUNO-ANALYSES

For immunoblot analyses, separation of proteins was performed using 10% SDS–PAGE gels (Invitrogen), using MOPS buffer, with electrophoresis at 200 V at 50 min. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA) for 2 h at room temperature using a mini-trans-blot cell (BioRad, Hercules, CA). Blots were then stained with the various primary antibodies at pretested concentrations (HtrA1 polyclonal Ab at 1 μg/ml, HtrA1 monoclonal Ab at 10 μg/ml, DEK monoclonal Ab at 1 μg/ml, and β-actin monoclonal Ab at 1:2,000 dilution). Secondary antibodies were from Cell Signaling, with horse radish peroxidase detection performed according to manufacturer's instructions. Magic Marker molecular weight standards (Invitrogen) were used to determine approximate Mr's. We also obtained medium from the cell cultures, concentrated them 30× using Centricon columns, and performed immunoblot analyses on 100 μg aliquots using both antibodies to HtrA1.

For analysis of immunoprecipitates, nuclear and cytoplasmic fractions were prepared from C33a, 293 T, and CaSki cells. Five hundred micrograms aliquots were mixed with 5 μg of either the E6 or E7 antibodies (experiments were performed with both the Santa Cruz and Abcam antibodies, for E6 and for E7) and incubated overnight for at 4°C. Following this, the complexes were mixed with Protein A/G Plus Agarose, incubated for 3 h (or overnight, when indicated) at 4°C, rinsed extensively (4×) with PBS, and bound complexes were eluted using electrophoresis buffer (Santa Cruz Biotechnology). Immunoblots were then prepared as described above.

For immunocytochemical staining, cells were plated on 8-chamber slides (10,000 cells/chamber) 1 day before ICC procedures. ICC was performed with Super Picture Polymer Detection kits (Zymed, in South San Francisco, CA), which uses horse radish peroxidase detection with DAB substrate, according to manufacturer's instructions. Polyclonal HtrA1 antibody was used at 2 μg/ml, and DEK antibody was used at 2 μg/ml.

Protease activity for HtrA1 was tested as previously described [Drubin and Clawson, 2004], using $AAPF_{AMC}$ or GR_{AMC} as substrates.

For Protease Activity gels, we used Novex Zymogram gels (Invitrogen). Four to 16% zymogram blue casesin gels were used for PAGE separation. The gel sensitivity is 1.5×10^{-3} U trypsin. Nuclear fractions were prepared as described [Drubin and Clawson, 2004], and the protease activity was found in the high-salt extract as previously reported. Samples included nuclear preparations, as well as the high-salt extracts, which were concentrated using Centricon columns. The high-salt fractions were incubated 48 h at 37°C, and then 60 μg protein (15 μl) was mixed with 15 μl of 2× Novex Tris–glycine–SDS sample buffer and loaded onto the Zymogram gels without heating. Gels were run in 1× Tris–glycine–SDS running buffer according to manufacturer's instructions, at constant 125 V for 90 min, using the blue dye reaches the bottom of the gel. After PAGE, the gel was incubated in 1× Zymogram Renaturing Buffer (Invitrogen) for 30 min at room temperature with gentle shaking. The renaturing buffer was then removed, and the gel was incubated for 30 min at room temperature in Zymogram Developing buffer. The Developing Buffer was then removed, fresh Developing Buffer was added, and the gel was incubated overnight at 37°C. The areas containing protease activity appears clear bands on the blue background. Molecular weight standards were the Seeblue markers (also from Invitrogen).

Expression levels of HtrA1 mRNA were determined by QPCR using TaqMan methodologies as previously described [Pan et al., 2004]. Tata-Box Binding Protein was used as the internal control. The sense primer for HtrA1 represented nt 674–693 (5′-TTGTT-TCGCAAGCTTCCGTT), the reverse primer represented nt 774–755 (5′-ACGTGGGCATTTGTCACGAT), and the FAM-labeled probe represented nt 695–721 (5′-FAM-TCTAAACGAGAGGTGCCGG-TGGCTAGT-BHQ = black hole quencher).

RESULTS AND DISCUSSION

We examined a number of cells types for HtrA1 expression using immunocytochemistry (Fig. 1). In all cell lines examined, we observed cytoplasmic and nuclear staining for HtrA1. While all cell types stained positively for HtrA1 expression, there was some variation in the patterns of the staining. CaSki cells generally showed darker staining, often with a notable perinuclear staining. Staining for HtrA1/Prss11 appeared darker in the $293_{HPVE6/E7}$ cell line compared with the parental $293_{Flip\text{-}In}$ cells (Fig. 1), particularly the nuclear staining. We previously reported a similar staining pattern in an hepatocyte cell line, when we used a biotinylated version of AAPFcmk, which is relatively selective for the nuclear protease [Clawson et al., 1995b].

The various cell lines were further analyzed by immunoblot analysis after cellular fractionation, using two different HtrA1 antibodies. A proteolytically active doublet around Mr 35,000 has been previously reported for HtrA1 in vitro [Chien et al., 2006], where it was produced autocatalytically from the expressed protein. In CaSki cells, the active doublet form of HtrA1 (near Mr 36,000) was prominently present in the cytoplasmic fraction (Fig. 2), as was a smaller previously unidentified ~Mr 29,000 form. In nuclear fractions from CaSki cells, the Mr 36,000 doublet was generally present at considerably lower levels. In some preparations both bands were observed, although in other preparations only the upper or lower bands were evident. We speculate that these forms represent processing intermediates (see below). The smaller Mr 29,000 form was again prominently observed. Intracellular and especially intranuclear HtrA1 has not been previously described. The precursor

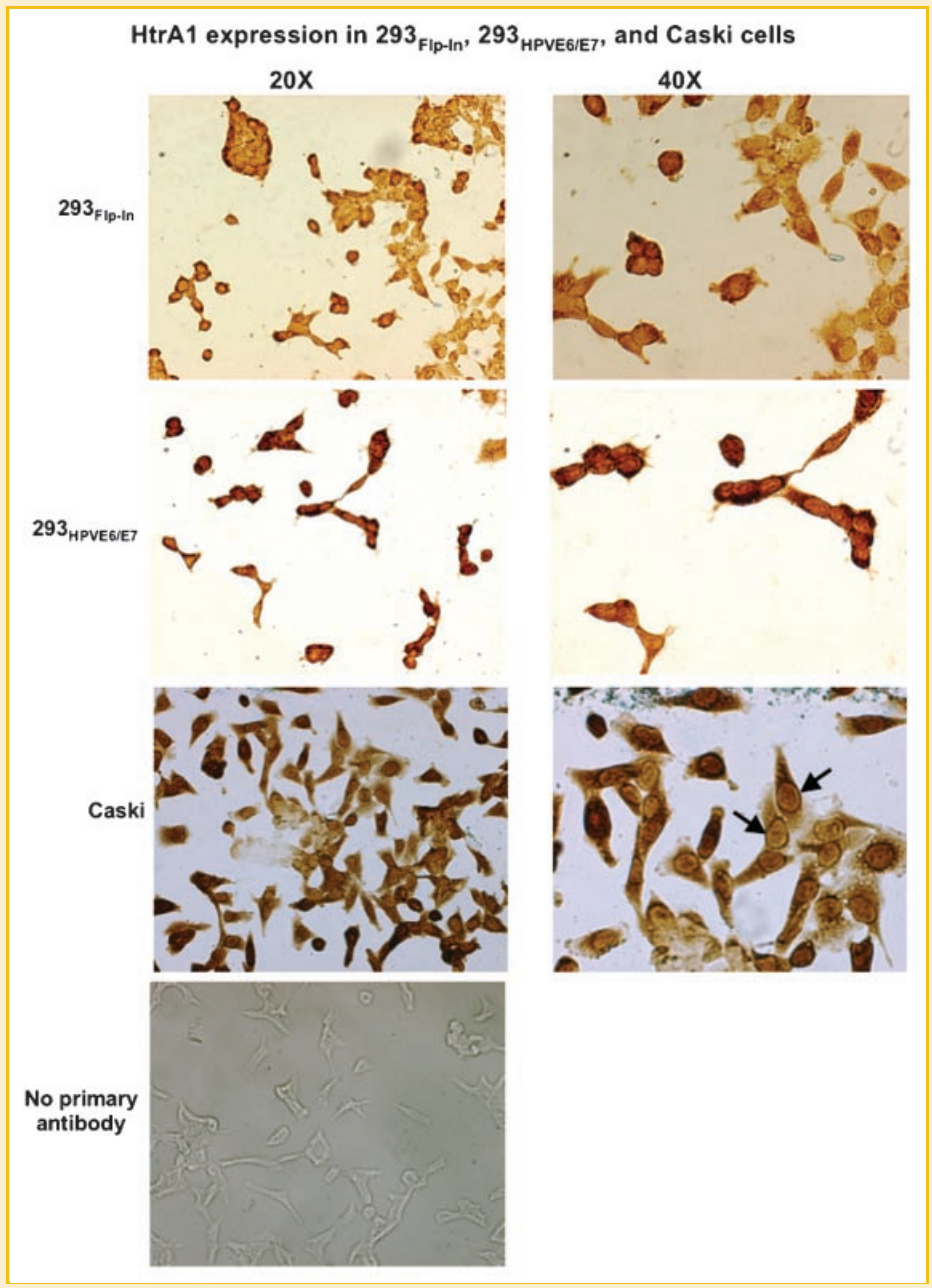

Fig. 1. Immunocytochemical staining for HtrA1 in CaSki, $293_{Flip-In}$, and $293_{HPVE6/E7}$ Cells. CaSki, $293_{Flip-In}$, and $293_{HPVE6/E7}$ cells were grown as described, and subjected to ICC staining with HtrA1 polyclonal and/or monoclonal antibodies, using Vector Impress kits with Vector VP as chromagen. Panels show 20× and 40× magnifications of the various cell lines as indicated, as well as no primary antibody (negative) controls. Arrows in the 40× panels of CaSki cells point to the perinuclear staining often observed.

form of HtrA1 (Mr 51,000) was not observed in cytoplasmic or nuclear fractions, although we have observed it in other cell types (manuscript in preparation). With the $293_{FLIP-In}$ cells, only the lower form of the doublet (Mr $36{,}000_L$) was consistently observed (Fig. 2), as well as the Mr 29,000 form. In the $293_{HPVE6/E7}$ cells, we observed increased expression of the Mr $36{,}000_L$ form and the Mr 29,000 form, with a minor, slightly smaller form often evident near ~Mr 34,000. Interestingly, we observed a notable increase in the Mr $36{,}000_L$ form in the nuclear fraction in the $293_{HPVE6/E7}$ cells (Fig. 2), which was consistent with the immunohistochemical staining observed (Fig. 1).

We also harvested medium from cell cultures, concentrated it using Centricon columns, and performed immunoblot analyses to determine if any HtrA1 was secreted during the culturing. No HtrA1 was detectable in medium from any of the cultures, including a number of additional cell types (data not shown), making it unlikely that HtrA1 functions by regulating IGF bioavailability (at least in epithelial cells).

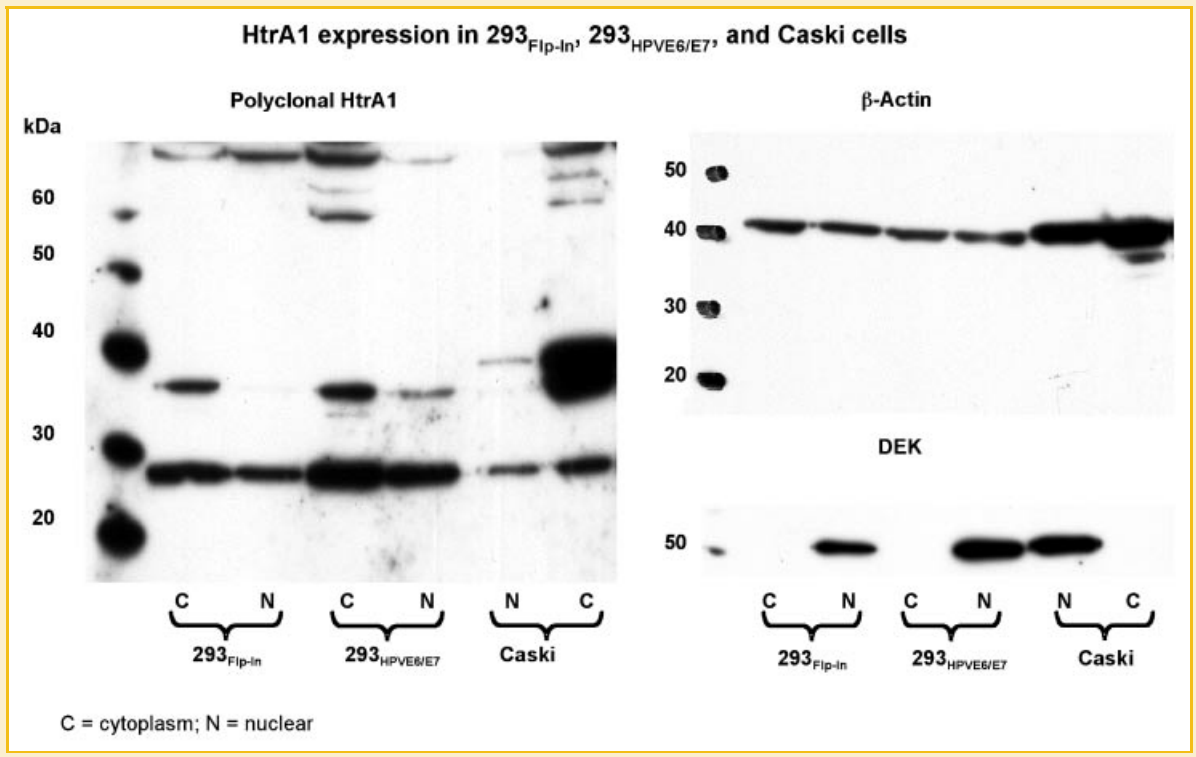

Fig. 2. Immunoblot Analyses of HtrA1 in Cytoplasmic and Nuclear fractions from CaSki, $293_{Flip-In}$, and $293_{HPVE6/E7}$ Cells. CaSki, $293_{Flip-In}$, and $293_{HPVE6/E7}$ cells were grown, and cytoplasmic and nuclear fractions were prepared as described. Fractions were subjected to PAGE in 4–12% gradient gels, proteins were transferred to PVDF membranes. Blots were then stained with primary polyclonal antibodies to HtrA1 (monoclonal antibodies gave analogous staining), DEK, or β-actin, followed by secondary antibodies with peroxidase detection. Non-specific staining of a band at ~Mr 85,000 was noted in all preparations (not shown).

We also stained nuclear and cytoplasmic preparations for DEK. DEK is a nuclear protooncogene which has been shown to be up-regulated with expression of high-risk HPV E7 [Wise-Draper et al., 2005]. As expected DEK staining was only observed in the nuclear fractions (Fig. 2). Of interest, however, was the ~5× up-regulation of DEK in the $293_{HPVE6/E7}$ cells compared with the parental $293_{Flip-In}$ cells, and equivalent loading of these 2 cell lines was documented using β-actin staining (Fig. 2).

We then prepared nuclear and cytoplasmic fractions from CaSki cells, and performed immunoprecipitations with antibodies to HPV16 E6 or E7. The immunoprecipitates were then separated by PAGE, transferred to blots, and probed with antibodies to HtrA1 (both the polyclonal and monoclonal antibodies were used; Fig. 3). Both the E6 and E7 immunoprecipitates contained immunoreactive forms of HtrA1. Surprisingly, a portion of the immunoprecipitates HtrA1 present in the E6 and E7 was found in the cytoplasmic fractions. In the E6 immunoprecipitates with affinity purified polyclonal antibodies (from Santa Cruz), the HtrA1 ~Mr 36,000 doublet was observed (often with the lower form, Mr $36{,}000_L$, predominating), along with the prominent Mr 29,000 form. The Mr 29,000 HtrA1 form was generally not observed in E7 immunoprecipitates; we suggest that E7 may associate with different regions of HtrA1 than E6, and that these regions may be removed during autocatalytic processing of the Mr 36,000 species to the prominent Mr 29,000 form. Also of note is that a significant fraction of E7 in particular appeared to be sequestered in the cytoplasm. In both E6 and E7 immunoprecipitates using the mouse monoclonal antibodies (from Abcam), both bands of the Mr 36,000 doublet were present when the incubations with the primary HtrA1 monoclonal antibodies and A/G Agarose were conducted for 2 h at 4°C, whereas only the lower form (Mr $36{,}000_L$) was found when the incubations were extended to overnight (not shown). This most likely indicates autocatalytic processing of Mr $36{,}000_U$ to Mr $36{,}000_L$ during the extended incubation period, even at 4°C. The Mr 29,000 form was also routinely present, and in some cases, most of the immunoreactive HtrA1 appeared to be converted to the Mr 29,000 form. Parallel immunoprecipitations with fractions obtained from C33a and 293T cell lines (which are HPV-negative) did not contain HtrA1. On protease activity gels, a single protease band was observed at Mr 29,000 (Fig. 3D) in CaSki cell nuclear extracts, suggesting that this prominent form of HtrA1 retains its proteolytic activity. This Mr 29,000 form was also observed in unfractionated nuclear preparations (Fig. 3D). We have subsequently expressed HtrA1 in vitro, and have found that the expressed purified preparations undergo autocatalytic processing to produce the proteolytically active Mr 29,000 form (not shown).

Finally, we examined relative expression levels of HtrA1 mRNA in the various cell lines using QPCR. CaSki cells (used at relatively high passage levels here) expressed HtrA1 mRNA at substantially higher levels than with the $293_{Flip-In}$ or $293_{HPVE6/E7}$ cell lines (Fig. 4), which mirrors the observed HtrA1 protein expression levels. However, $293_{HPVE6/E7}$ cells expressed HtrA1 mRNA at levels which

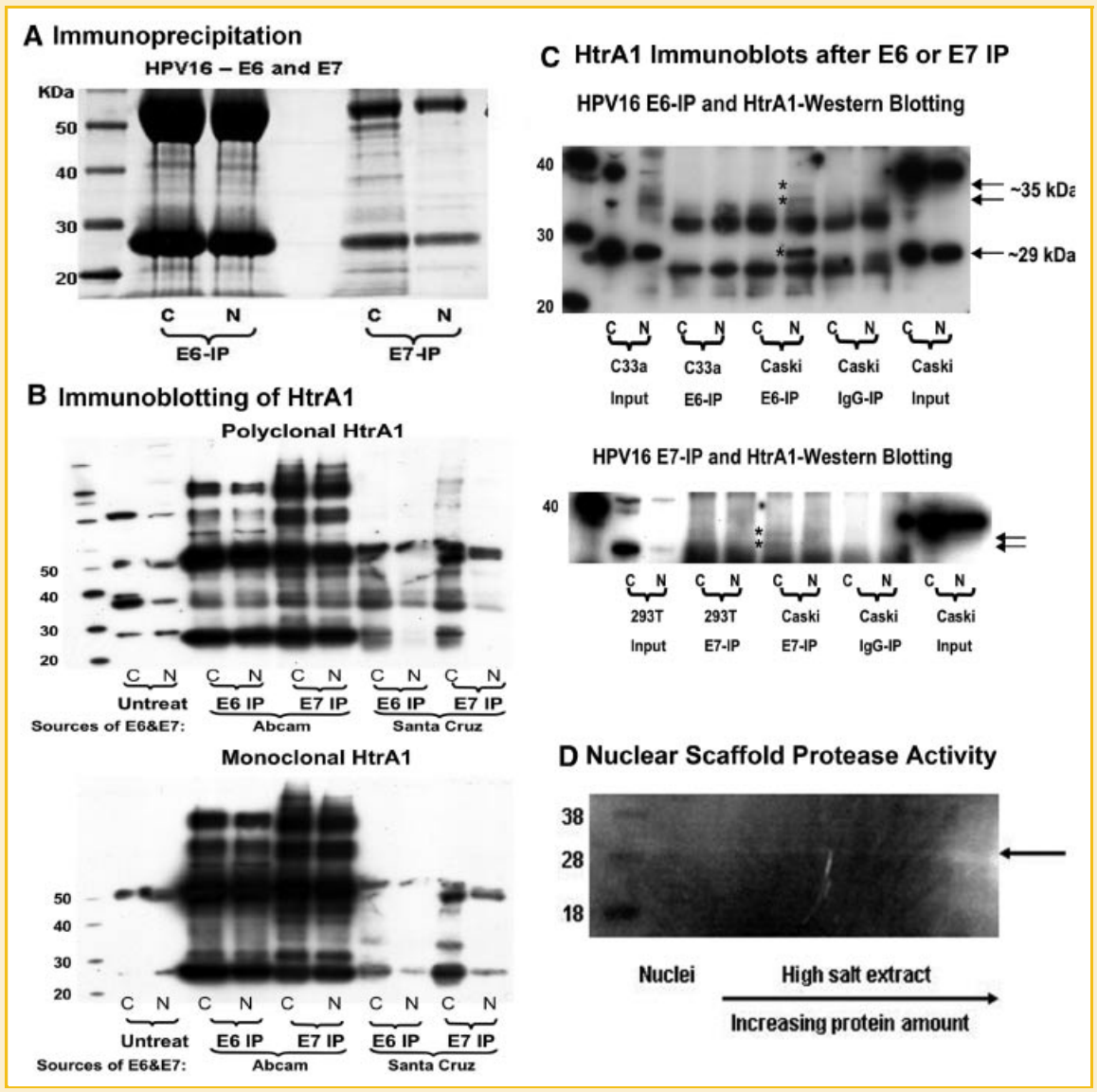

Fig. 3. Immunoprecipitation/Immunoblot Analysis of HtrA1, E6, and E7. CaSki cells were grown, harvested, separated into nuclear and cytoplasmic fractions, and subjected to immunoprecipitation using polyclonal antibodies to HPV16 E6 protein (Santa Cruz) and monoclonal antibodies to HPV E7 (both from Santa Cruz). Immunoprecipitates were harvested and subjected to SDS–PAGE on 10% gels or 4–12% gradient gels. A: A representative stained gel of the immunoprecipitates. B: In a separate set of experiments, HPVE6 and E7 immunoprecipitates (obtained using the antibodies from Abcam and Santa Cruz, as noted) were transferred to PVDF membranes, and probed with primary polyclonal HtrA1 antibodies (from Imgenex, upper portion). The blot was then stripped of the primary antibody (staining was completely removed), and reprobed with the HtrA1 monoclonal antibody (from R&D). C: C33a, 293T, and CaSki cells were harvested, and nuclear and cytoplasmic fractions were prepared. The fractions were immunoprecipitated with antibodies to HPV 16 E6 (Abcam) or HPV E7 (Abcam). Immunoprecipitates were separated by SDS–PAGE, and blots were prepared and probed with polyclonal HtrA1 antibodies. HtrA1 was clearly observed in CaSki cell nuclear fractions, including the ~Mr 36,000 doublet and the Mr 29,000 forms (denoted with asterisks). In contrast, HtrA1 was not detected in the immunoprecipitates from the HPV-negative C33a cells, or in control immunoprecipitates obtained using control mouse IgG antibodies. In the HPV16 E7 immunoprecipitates, HtrA1 is observed as the ~Mr 36,000 doublet (the Mr 29,000 form was not observed), and it was also present in the E7 immunoprecipitates from the cytoplasmic fractions from CaSki cells (no HtrA1 bands were observed in the C33a or normal mouse IgG controls). In other experiments, HPV E6 and E7 were immunoprecipitated with the monoclonal antibodies (Abcam), and HtrA1 was detected with the monoclonal antibodies to HtrA1 (from R&D); however, immunoprecipitations in the A/G Agarose were performed either for 2 h at 4°C, or incubated overnight at 4°C. We noted a clear conversion of the upper Mr $36{,}000_U$ doublet band to the lower form of the doublet (Mr $36{,}000_L$), comparing the 2 h incubations to the overnight incubations. In most cases, processing to the Mr 29,000 form was extensive. D: A casein zymogram gel used for detection of proteolytic activity. A single band at Mr 29,000 was observed in the high-salt extract (which contained essentially all of the nuclear protease activity), indicating that this Mr 29,000 form retained proteolytic activity. The right-most lane shows a 2–3× concentrated high salt extract. This same Mr 29,000 band was also observed in unfractionated nuclear preparations (Nuclei), and this same Mr 29,000 form was also observed with HtrA1 expressed in vitro (not shown). Trypsin was run in the gel as a standard for comparison, and it migrated at ~Mr 26,000 (not shown).

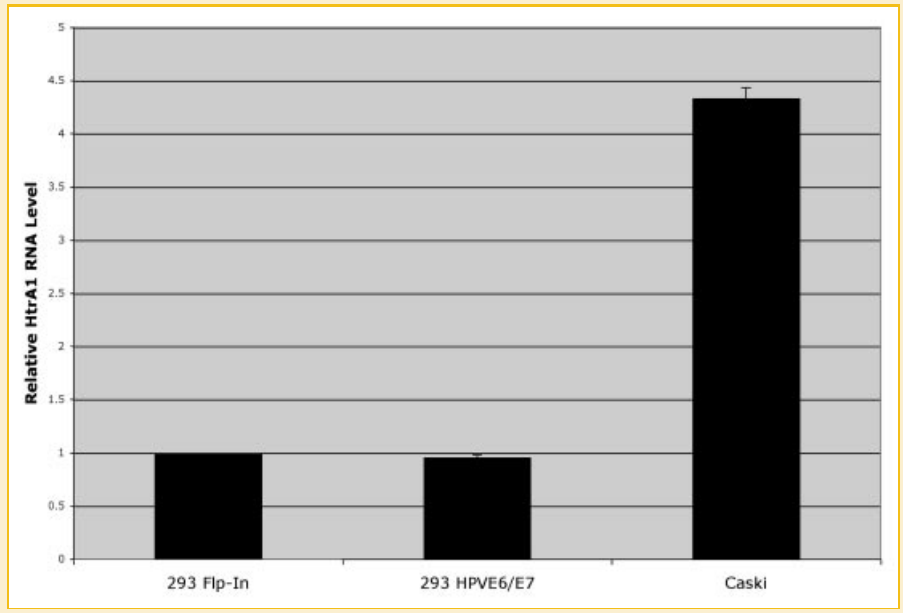

Fig. 4. Quantitative PCR analysis for HtrA1 mRNA levels. RNA was isolated from $293_{Flip-In}$, $293_{HPVE6/E7}$, and CaSki cells and QPCR was performed as described. Results indicate that although HtrA1 expression was increased in $293_{HPVE6/E7}$ cells vs. the parental $293_{Flip-In}$ cells, there was no corresponding change in HtrA1 mRNA levels. Bars show standard errors of the measurements.

were equivalent to levels observed in the parental $293_{Flip-In}$ cells (Fig. 4), showing that the altered levels of HtrA1 protein expression between these cell lines reflects a post-transcriptional change. In a previous report, Eichten et al. 2004 reported cell-associated HtrA1 (therein referred to by the alternative name IGFBP-5). In that study, they showed that expression of HPV E7 protein resulted in a transcriptional up-regulation of HtrA1, which was mediated by NF-κB. In contrast, here we have observed an up-regulation of HtrA1 which is post-transcriptional in nature (Fig. 4), and which appears to preferentially involve nuclear HtrA1.

These results provide straightforward evidence for the presence of HtrA1 in both cytoplasm and nucleus of the various cell types examined here. With CaSki cells, our working hypothesis was that HtrA1 might associate with HPV16 E6, since both have PDZ (and/or PDZ ligand binding) domains. In this regard, E6 has been found to contribute to carcinogenesis in both PDZ-dependent and independent mechanisms [Simonson et al., 2005]. However, the apparent association of HtrA1 with HPV16 E7 was unexpected, and the site of interaction is not known.

It seems reasonable to propose that HtrA1 may have important roles in carcinogenesis. For example, HtrA1 has been shown to function as a tumor–suppressor [Baldi et al., 2002; Allinen et al., 2004; Chien et al., 2004; Bowden et al., 2006], and in fact HtrA1 was originally isolated as a gene whose expression was down-regulated in a human fibroblast cell line after transformation with SV40 [Zumbrunn and Trueb, 1996]. HtrA1 is also involved in apoptosis [Chien et al., 2006], and it has been identified as a pivotal mediator of response to chemotherapy [Folgueira et al., 2005; Chien et al., 2006]. Our studies with AAPFcmk, an inhibitor of chymotrypsin-like proteases (including HtrA1) have suggested a potential role for HtrA1 in transformation [Clawson et al., 1993; Drubin and Clawson, 2004], although it is recognized that AAPFcmk also targets a number of non-protease proteins [Dhamne et al., 2007]. Our studies describing a profound decrease in chemical transformation of fibroblasts by pM to fM concentrations of AAPFcmk could indicate a role for HtrA1 in induction of a mutagenic response to molecular insults, similar to the mutagenic responses observed in other eukaryotic systems [Wintersberger, 1984; Suzuki et al., 2003]. In addition, its association with HPV16 E6 and E7 proteins, as documented here, may have important ramifications in mediating the myriad functions of these important oncogenic proteins.

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