EXPERIMENTAL STUDY

Evidence against a role of human airway trypsin-like protease – the human analogue of the growth-promoting rat adrenal secretory protease – in adrenal tumourigenesis

Stefanie Hahner, Martin Fassnacht, Fabian Hammer, Markus Schammann, Dirk Weismann, Immo Alex Hansen and Bruno Allolio

Endocrinology and Diabetes Unit, Department of Medicine, University of Wuerzburg, Josef-Schneider-Strasse 2, D-97080 Wuerzburg, Germany

(Correspondence should be addressed to S Hahner; Email: hahner_s@medizin.uni-wuerzburg.de)

Abstract

Objective: A serine protease from rat adrenal cortex was recently characterized and named adrenal secretory protease (AsP). AsP is expressed in the adrenal cortex and is capable of cleaving pro-γ-melanocyte-stimulating hormone (1-76 N-terminus of pro-opiomelanocortin) into fragments that act as adrenal mitogens. AsP may therefore play a crucial role in adrenal growth and tumourigenesis. The aim of this study was to further characterize the human homologue of AsP and its possible role in adrenal tumourigenesis.

Methods and results: Starting with the rat cDNA sequence of AsP we detected high homology to the catalytic C-terminus of the human airway trypsin-like protease (HAT). Further analysis revealed that the HAT gene is the human homologue of a long splice variant of AsP, which we recently described as rat airway trypsin-like serine protease 1. In contrast to rodents, no short isoform of HAT was found in humans due to a stop codon in exon 6 which prevents the expression of a short isoform. While high expression of HAT mRNA was found in the trachea and in the gastrointestinal tract, expression in the adrenal was only very weak. RT-PCR and real-time PCR analysis revealed a complex tissue expression pattern of HAT, indicating a role for this protease in multiple tissues. We further investigated HAT expression in five normal adrenal glands, 15 adrenocortical adenomas (five hormonally inactive adenomas, five aldosterone-producing adenomas and five cortisol-producing adenomas), nine adrenocortical carcinomas, five phaeochromocytomas and two adrenal hyperplasias. Weak HAT expression was detectable in only two out of five normal adrenal glands, in one out of twenty-four adrenocortical tumours and four out of five phaeochromocytomas. However, the expression in the adrenal tissue was several orders of magnitude lower than in the trachea. In addition, we could not detect any HAT transcripts in a sample of fetal adrenal.

Conclusion: Gene structure and tissue distribution of HAT, the human homologue of the rat adrenal secretory protease AsP, reveal major interspecies differences. The observation of very low expression levels in normal adrenal tissue and adrenocortical tumours casts doubt about a role for HAT in the physiological and pathological growth of adrenocortical cells.

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Introduction

The adrenal gland is a dynamic organ that requires constant stimuli from the pituitary gland to maintain its size, as either hypophysectomy (1) or dexamethasone treatment (2, 3) result in rapid adrenal atrophy. There is substantial evidence that adrenal growth is dependent on pro-opiomelanocortin (POMC)-derived peptides secreted from pituitary corticotrophic cells: it has been demonstrated that peptides derived from the N-terminus of POMC (without the γ-melanocyte-stimulating hormone sequence) are potent adrenal mitogens in rodents, both in vitro and in vivo (4–7). However, as these shorter peptides were not found in the circulation (8), it has been suggested that they are generated locally by a specific protease in the adrenal cortex. In keeping with this concept, a 28 kDa serine protease in the rat adrenal gland was recently described and named adrenal secretory protease (AsP) (9). It has been proposed that AsP plays a key role in the control of adrenal proliferation in rats by specifically cleaving the N-terminal fragment of rat 1-74 N-POMC to generate smaller peptides which act as adrenal mitogens. AsP is expressed in the outer adrenal cortex in rats...
and is upregulated in the contralateral adrenal after unilateral adrenalectomy (8–10). It has been suggested that AsP may also play a critical role in neoplastic growth, as expression of AsP antisense mRNA in mouse adrenocortical cancer cells (Y1 cells) led to a significant reduction in growth rate (9). Is is therefore of considerable interest to elucidate the role of this system for physiological and neoplastic adrenal growth in humans.

We recently demonstrated two alternatively spliced isoforms of the adrenal protease AsP in both rat and mouse (11). The longer form has a type II transmembrane protein structure showing high overall homology with the human airway trypsin-like protease (HAT) and was therefore designated rat airway trypsin-like protease 1 and mouse airway trypsin-like protease 1. The shorter isoform rat airway trypsin-like protease 2 is identical with the formerly described AsP, which does not contain a transmembrane domain but an N-terminal signal peptide to direct the enzyme to the secretory pathway. The C-terminus covering the catalytic triad is identical in both isoforms.

So far there have been no reports of a short isoform of human HAT. In previous studies, HAT expression was detectable only in trachea, but not in 16 other different types of tissues examined, such as heart, brain, pancreas, liver and thymus (12). HAT expression in adrenal tissue has never been investigated.

The aim of the present study was to further characterize the human homologue of AsP and to study the possible role of HAT in adrenal tumourigenesis in man. It was hypothesized that constitutive expression of this protease by neoplastic adrenocortical cells would provide a growth stimulus generated from circulating human 1-76 N-POMC, as N-terminal peptides derived from 1-76 N-POMC possess mitogenic activity in the human adrenocortical cancer cell line NCI-h295 (13).

### Materials and methods

#### Human tissue

We analyzed 36 adrenal surgical specimens from patients who had undergone adrenalectomy for primary adrenal tumours or as part of nephrectomy for renal cell carcinoma. The patients’ data are given in Table 1. Tissues were collected after written informed consent was obtained from the patients and with the approval of the ethics committee of the University of Wuerzburg. After resection, the specimens were frozen and stored at −80°C until RNA extraction. The tumour specimens included fifteen adrenocortical adenomas (five aldosterone-secreting adenomas, five cortisol-secreting adenomas and five hormonally inactive adenomas), two adrenocortical hyperplasias (one from a patient with Cushing’s disease and one from a patient with ectopic adrenocorticotrophin (ACTH) syndrome), five benign phaeochromocytomas and nine adrenocortical carcinomas. In all cases the clinical diagnosis was confirmed by histopathology.

#### Nucleotide and amino acid sequence analysis

Genome searches were performed using basic local alignment (BLAST) (14) at the National Center for Biotechnology Information databases (NCBI databases, http://www.ncbi.nlm.nih.gov/), sequence similarities were studied using ClustalW (15) at the European Molecular Biology Laboratory (EMBL; http://www2.ebi.ac.uk/clustalw/) and analysis of the modular domain architecture of proteins was performed with SMART (16) at EMBL (http://smart.embl-heidelberg.de).

#### Isolation of RNA from solid tissues and cDNA synthesis

Total RNA from human adrenals and adrenocortical tumours was isolated by means of a commercially available modification (TRizol; Invitrogen, Karlsruhe.
Germany) of the one-step phenol/guanidinium thiocyanate method. PolyA⁺ RNA was isolated using the Oligo-tex mRNA mini kit (Qiagen, Hilden, Germany).

Total RNA from human whole adrenal gland, trachea, lung, kidney, heart, placenta, spinal cord, fetal brain, brain, cerebellum, fetal liver, liver, testis, prostate, stomach, small intestine, colon and bladder were purchased from BD Clontech (Heidelberg, Germany), total RNA from human fetal adrenal cortex and oesophagus from Ambion (Huntingdon, Cambs, UK), human tongue total RNA from Stratagene (Amsterdam, The Netherlands) and total RNA from human fetal adrenal gland from BioCat (Heidelberg, Germany). Additionally, total RNA from the human adrenocortical cancer cell line NCI-h295 was extracted. For analysis of adrenal tumour tissue, the commercially available sample from Clontech served as reference.

Three micrograms of total RNA were used to synthesize single-stranded cDNA using the commercially available superscript first-strand synthesis system (Invitrogen, Paisley, Strathclyde, UK). All steps were carried out according to the manufacturer’s instructions.

**DNA extraction and PCR**

Leukocyte DNA was isolated from whole blood lysates using Qiagen DNA blood isolation Kit (Qiagen). To confirm the findings made by the data base search we further amplified a 177 bp fragment from human DNA containing the region of exon 6. PCR was performed using the Qiagen PCR Core Kit (Qiagen); the primers are listed in Table 2. The resulting PCR fragments were introduced into the PCR-TOPO vector (Invitrogen, Karlsruhe, Germany) and sequencing was performed by TOPLAB (Martinsried, Germany).

**Rapid amplification of 5'-cDNA ends (RACE)**

Rapid amplification of the 5'-cDNA end was performed using the SMART RACE cDNA Amplification Kit (Becton Dickinson, Heidelberg, Germany) following the manufacturer’s instructions. PolyA⁺ RNA (0.5 μg) was used as starting material. The sequences of the oligonucleotides used as primers are given in Table 2. RACE products were analysed on 1.5% agarose gels. Isolated bands were cut out of the gel; agarose was removed using Ultrafree-DNA spin columns (Millipore, Eschborn, Germany). The cDNAs were introduced in PCR-TOPO vector (Invitrogen, Karlsruhe, Germany) following the manufacturer’s instructions. Sequencing was performed by TOPLAB.

**RT-PCR**

The reactions were carried out using total RNA (500 ng) as template and Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer’s instruction. For sequences of the primers see Table 2. RT-PCR was

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**Table 2** Primers and their location within the sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Symbol</th>
<th>Sequence</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAT-a-forward</td>
<td>●</td>
<td>5' GGC TAG GAA TCT GCA TTT TAG AGG ACC ACC TCC C 3' TAG CAG TAA GTG C 3'</td>
<td>PCR-Exon6</td>
</tr>
<tr>
<td>HAT-a-reverse</td>
<td>●</td>
<td>5' GCC AAT TTG CTG CAG CCT GGT CAG TAA GTG C 3'</td>
<td>PCR-Exon6</td>
</tr>
<tr>
<td>HAT-b</td>
<td>▲</td>
<td>5' TGA GTG TCC TTG TAC TAG TGG GCC ACC AGA AGT TGA AGG GTT TAT TCC CAG GTT CCG AGA GTT ATT 3'</td>
<td>5' RACE</td>
</tr>
<tr>
<td>HAT-c</td>
<td>□</td>
<td>5' ATA CAG ACT TTG CAA CAG GGA TGC ACT TGT T 3'</td>
<td>5' RACE</td>
</tr>
<tr>
<td>HAT-d</td>
<td>○</td>
<td>5' AGT TGA AGG GTT TAT TCC CAG GTT TCC AGA GTT ATT 3'</td>
<td>5' RACE</td>
</tr>
<tr>
<td>HAT-e forward</td>
<td>●</td>
<td>5' ATG TAT AGG CCA GCA GGT GTA ACT TCG ACT TC 3'</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>HAT-e reverse</td>
<td>●</td>
<td>5' CAC TCA AAG TCC GTG ATT CCT GTG TAG CTG GTG 3'</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>β-actin-forward</td>
<td>●</td>
<td>5' AAC TGG GAC GAC ATG GAG AAA ATC TGG CAC 3'</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>β-actin-reverse</td>
<td>●</td>
<td>5' GTA CAG GGA TAG CAC AGC CTG GAT AGC AAC GTA 3'</td>
<td>RT-PCR</td>
</tr>
</tbody>
</table>
performed as follows, using a Mastercycler gradient (Eppendorf, Hamburg, Germany): 30 min of 50 °C reverse transcription, 15 min of 94 °C inactivation of the reverse transcriptase and activation of Taq-polymerase, followed by 37 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. The PCR products were analysed on 2% agarose gels stained with ethidium bromide.

**Figure 1** Derived amino acid alignment of HAT and the rat airway trypsin-like protease (RAT with the long isoform RAT1 and the short isoform AsP). Completely conserved amino acids are marked in black and amino acids with strong similarity are marked in grey. HAT shows an overall amino acid identity of 67% with rat airway trypsin-like protease 1. Amino acids that are part of the catalytic triad: His (227), Asp (272) and Ser (368) are indicated by arrows and boxes. HAT and rat airway trypsin-like protease 1 possess four structural regions: an N-terminal cytoplasmic domain (amino acids 1–20), a transmembrane domain (amino acids 21–41), an SEA domain (amino acids 44–164) and a trypsin-like serine protease domain (amino acids 187–417); AsP possesses three structural regions: a signal peptide which is supposed to direct the protein to the secretory pathway (amino acids 1–24), a 23 amino acid linker peptide and the 232 amino acid catalytic domain.
**Real-time PCR**

Relative quantification was performed using commercially available HAT- and 18s-specific FAM dye-labelled TaqMan MGB probes (Assays on Demand Gene Expression Products; Applied Biosystems, Foster City, CA, USA) and TaqMan universal Master Mix (Applied Biosystems). Each reaction was run in a total volume of 25 μl in triplicates using 250 ng total RNA for the tissue distribution analysis and up to 500 ng for the analysis of the different adrenal tissues. The universal thermal cycling parameters were used as recommended (2 min 50°C, 10 min 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C). The experiments were run in an iCycler thermocycler (BioRad, Munich, Germany) and results were calculated with the iCycler iQ real-time detection system software. Expression levels of each variant were determined by comparison with standard curves generated using a fourfold serial dilution series of cDNA from human trachea (Becton Dickinson). As an endogenous control, quantification of 18s was performed in separate tubes. All results were normalized to expression of 18s.

**Results**

**Humans lack the short isoform of HAT corresponding to the rat AsP**

We used the rat cDNA sequence of AsP to perform a BLAST sequence analysis and found only one similar sequence in the human databases which has been described recently as HAT consisting of 418 amino acids (12, 17). HAT shows strong homology to AsP only in the C-terminus, whereas the N-terminus differs considerably (Fig. 1). In contrast, using the cDNA encoding the long isoform of rat AsP (rat airway trypsin-like protease 1), which we recently cloned from both rat trachea and adrenal gland (11), an identical genomic and protein structure was detected indicating that HAT is the human homologue of the longer splice variant of AsP (rat airway trypsin-like protease 1) (Fig. 1). Both genes contain 10 exons (Fig. 2) and the corresponding proteins have a simple type II transmembrane protein structure, consisting of a short cytoplasmic domain, a ‘SEA’ module named after the first proteins described with such a domain (sea urchin sperm protein, enteropeptidase, agrin), and the serine protease domain containing the functionally essential catalytic triad His, Asp and Ser. The C-terminus, covering the catalytic triad, is identical in both the long and the short isoform of AsP. The overall identity of the aligned amino acid sequence demonstrated a high homology of HAT with rat airway trypsin-like protease 1 (67%) and to a minor degree with AsP.

To identify a short isoform in humans, 5′-RACE-PCR experiments were performed. However, no shorter HAT cDNA was detected in a number of human tissues (adrenal, trachea, oesophagus, tongue). In rodents, exon 6 contains an internal splicing site, which is used when the long isoform is transcribed (Fig. 3), whereas the complete exon 6 is used as the first exon for the short isoform. In humans, the genomic sequence is similar. There is also a potential start ATG corresponding to the initiation codon for exon 6 of rat airway trypsin-like protease 2 (AsP); however, shortly after this start ATG a stop codon prevents the translation of this isoform (Fig. 3). Thus in humans the genomic structure of the HAT gene precludes the generation of a shorter isoform corresponding to rat AsP. We confirmed this finding by amplification of a short fragment from human DNA containing the critical region of exon 6. Gene sequencing verified the existence of the stop codon.

**HAT expression in different tissues**

In contrast to the recently published Northern blot data (12), real-time PCR analysis revealed a complex expression pattern of HAT. High expression of HAT mRNA was found in human trachea and in tissues...
of the upper gastrointestinal tract (Fig. 4a), with the highest expression in oesophagus and tongue. In addition, HAT mRNA was clearly detectable in lung, bladder, prostate, testes and the spinal cord. Using RT-PCR, a similar expression pattern was observed (Fig. 4b).

**HAT expression in the normal adrenal gland and in adrenal tumours**

HAT expression was either not detectable or in very low abundance in all investigated adrenal tissues as assessed by both RT-PCR and quantitative PCR. Quantitative analysis demonstrated that expression in human trachea was several orders of magnitude higher than the expression in the reference adrenal gland (Clontech). Assessing HAT expression in adrenal tissues by real-time PCR, weak expression was detectable in only two out of five normal adrenal glands, one out of fifteen adrenocortical adenomas, none out of nine adrenocortical carcinomas and four out of five phaeochromocytomas (Fig. 5), whereas in three tumours a faint band was visible using RT-PCR analysis (Fig. 6). In addition, we could not detect any HAT expression in a sample of fetal adrenal gland and also no HAT expression was detectable in NCI-h295 cells. It is important to note that the observed HAT expression in all adrenal tissues was close to the detection limit of both RT-PCR and quantitative PCR (see Materials and methods).

**Discussion**

In this paper we have demonstrated that no direct human homologue of the rodent AsP exists. The homologous gene in humans encodes the previously described HAT. In contrast to AsP, HAT is a transmembrane protein and has a completely different N-terminus. However, we have demonstrated that human HAT corresponds to the long splice variant of the AsP gene recently described as rat airway trypsin-like protease 1 and to the mouse airway trypsin-like protease 1 (11). Comparison of the amino acid sequence demonstrated high homology of HAT with both rat airway trypsin-like protease 1 and to the mouse airway trypsin-like protease 1 (66%). Moreover, like rat airway trypsin-like protease 1 and mouse airway trypsin-like protease 1, HAT has a transmembrane structure consisting of a short cytoplasmic domain, a transmembrane domain, a ‘SEA’ module and the serine protease domain, whereas the short isofrom AsP (= rat airway trypsin-like protease 2) contains an alternative N-terminus including a signal peptide to direct the protease to the secretory pathway. This shorter cDNA is derived from the rat airway trypsin-like protease gene by alternative transcription of exon 6, while in the long isofrom rat
airway trypsin-like protease 1 only the second part of exon 6 is transcribed, the complete exon 6 is utilized as first exon of the short isoform (11). Usage of alternative initial exons is a well-described phenomenon (18). In contrast to rodents, in humans no shorter isoform is present. All our attempts to identify shorter human HAT cDNAs in various human tissues by 5'-RACE failed. Interestingly, genomic sequence analysis revealed that the human HAT gene has an equivalent to the described alternative initial exon with a potential start codon at

Figure 4 Expression of HAT mRNA in various human tissues. (a) Quantitative PCR analysis of relative levels of HAT mRNA. Relative quantification was performed using FAM dye-labelled TaqMan probes. As template, 250 ng total RNA was used. Normalization was performed with 18s. (b) RT-PCR analysis of tissue distribution of HAT mRNA expression. For RT-PCR, 500 ng total RNA was used as template. All probes were positive for β-actin. tr, trachea; lg, lung; ht, heart; pl, placenta; sc, spinal cord; fb, fetal brain; br, brain; cb, cerebellum; fl, fetal liver; lv, liver; tt, testis; pr, prostate; st, stomach; fag, fetal adrenal gland; si, small intestine; co, colon; bl, urinary bladder; ac, adrenal cortex; oe, oesophagus; tg, tongue; −, negative control (no RNA). For the results of total adrenal gland see Fig. 6.
the same position. However, in humans a stop codon at the position of the potential amino acid 4 prevents the translation of the short splice variant. Transcripts of the short splice variant may be degraded via nonsense mediated mRNA decay, a mechanism that detects and rapidly destroys aberrant transcripts containing premature termination codons (19).

This unexpected difference between humans and rodents raises the question as to whether the short isoform was lost during human evolution or represents a new development in rodents. However, both isoforms share exons 7–10 and have, therefore, identical catalytic domains. Thus it seems possible that both isoforms have similar substrates and that in humans
Figure 6 RT-PCR analysis of HAT mRNA expression in adrenal tissues. Expression was analyzed in five normal adrenal glands (N1-5), five hormonally inactive adenomas (HI1-5), five aldosterone-producing adenomas (A1-5), five cortisol-producing adenomas (C1-5), five adrenocortical carcinomas (CA1-5), five phaeochromocytomas (P1-5) and two ACTH-dependent hyperplasias (H1-2). For patient data see Table 1. As controls, mRNA from total adrenal gland (ag), trachea (tr) and liver (lv) were used; −, negative control (no RNA).
HAT serves the same physiological function as AsP in rodents.

The tissue distribution of HAT described in this paper clearly extends the initial observations (12) as significant expression was observed not only in trachea but also in the upper gastrointestinal tract (particularly in oesophagus and tongue) as well as in other organs like bladder, testes and spinal cord. The expression pattern of HAT resembles the expression of rat airway trypsin-like protease 1 and mouse airway trypsin-like protease 1 in rodents (11).

The expression level in the adrenal gland was several orders of magnitude lower than in tracheal tissue. This observation is in keeping with low expression of rat airway trypsin-like protease 1, the corresponding long isoform of AsP in the rat adrenal gland, whereas expression of the short isoform AsP itself is considerably higher (11). Thus a growth-promoting role of HAT in the human adrenal cortex is not supported by our findings. The differences in adrenal expression of HAT in humans and AsP in rodents are also evident in fetal tissue: while high expression of AsP has recently been described in the fetal adrenal of rodents (20), we were unable to detect HAT expression in human fetal adrenal tissue.

Overexpression of an adrenal protease cleaving circulating human 1-76 N-POMC to locally generate adrenal mitogens would provide adrenal tumour cells with a growth advantage, indicating that such a protease has the potential of an oncogene. Accordingly, a role for AsP in the growth of adrenocortical tumour cells derived from rodents has been demonstrated (9). However, in addition to the low or absent expression in normal adrenals, we could detect HAT transcripts in only one out of 24 adrenocortical tumours, whereas the other 23 were negative in the real-time PCR analysis. These findings suggest that HAT plays no role in adrenocortical tumorigenesis and casts further doubts on a role for HAT in adrenocortical growth. Intriguingly, we found weak HAT expression in four out of five phaeochromocytomas. As expression of POMC peptides is a typical feature of adrenomedullary chromaffin cells (21), HAT may be involved in the cleavage of locally generated POMC peptides. Accordingly, expression of HAT was detectable in extracts of total adrenal tissue consisting of both cortex and medulla, whereas it was negative in a sample consisting only of adrenal cortex, further supporting the view that chromaffin cells may be a physiological source of HAT. Shorter POMC peptides may be generated in the adrenal medulla and act through paracrine mechanisms on both adrenomedullary cells and the adjacent adrenal cortex.

The function of HAT remains to be elucidated. Initially HAT was isolated from the sputum of patients with chronic airway disease (17). It has been reported to degrade fibrinogen and also to activate protease-activated receptor 2 (PAR 2) (22, 23). It has further been suggested that HAT has a dual action on airway mucin regulation both increasing mucin production via an amphiregulin–epidermal growth factor receptor pathway and enhancing mucin secretion through a PAR 2-mediated pathway (24).

In keratinocytes, HAT induced PAR-2-mediated interleukin-8 release and it was shown to be upregulated in psoriatic epidermis (25). The function of HAT in the upper gastrointestinal tract is still unclear and remains to be elucidated.

The widespread expression of HAT in many tissues questions a role for HAT in specifically cleaving N-POMC to generate adrenal mitogens, as this cleavage would take place in multiple sites seemingly unrelated to adrenal physiology. Thus it seems possible that the physiological function of rat AsP for adrenal growth has been taken over by a protease different from the human homologue HAT. Possible candidates may be the closely related differentially expressed in squamous cell carcinoma (DESC) proteases. DESC proteases have a similar structure to that of HAT and exhibit the same exon/intron organization probably as a result of gene duplication. Like HAT, human DESC is located on the long arm of chromosome 4 (26). Expression of DESC in the adrenal gland has, however, never been studied.

In summary, our findings do not support a role of HAT, the human homologue of the recently described adrenocortical protease AsP, in the regulation of human adrenal growth or adrenal tumorigenesis.

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