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Research Article

Molecular cloning and characterisation of DESC4, a new transmembrane serine protease

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Abstract. Type II transmembrane serine proteases (TTSPs) are a growing family of multidomain proteins. Among the TTSPs, a new subfamily of HAT/DESC1-like (human airway trypsin-like protease/differentially expressed in squamous cell carcinoma gene 1) proteases is emerging consisting so far of four members: DESC1–3 and HAT. The cDNA of a new member of this subfamily, named DESC4, was isolated from rat tongue tissue and characterised. Analysis of selected tissues by RT-PCR demonstrated expression of DESC4 in brain, colon, heart, liver, lung and tongue. At the cellular level, DESC4 expression is confined to epithelial cells within the cleft of the circumvallate papillae extending into the ducts of minor salivary glands, the respiratory epithelium of the nasal cavity and tear gland ducts of the eyes as analysed by in situ hybridisation of sensory organ tissues. In transfected mammalian cells, DESC4 is localised to the plasma membrane as shown by immunocytochemistry and subcellular fractionation experiments. Our results suggest that we have identified a protease that is an important constituent of sensory systems and other organs.

Key words. In situ hybridisation; transmembrane serine protease; sensory system; recombinant expression.

Introduction

Secretion of fluids onto epithelia exposed to the environment fulfils a variety of important biological functions. In general, fluid secretion will prevent these epithelia from drying out. More specialised functions of secretions include the protection of the host by participating in innate and acquired immunity [1]. In the case of chemosensorically active epithelia, they provide the perireceptor milieu necessary for the detection of some ligands [2], and they are indispensable for the biological functions of the respiratory system [3]. Different types of glands secrete a plethora of molecules including water, small inorganic ions, proteoglycans and proteins to fulfil this multitude of functions. Among the components frequently isolated from a variety of different secretions are soluble forms of transmembrane serine proteases, such as the type II transmembrane proteases, enteropeptidase from bovine duodenal mucosal fluid [4] and human airway trypsin-like protease (HAT) from sputum of patients with chronic airway disease [5].

Type II transmembrane serine proteases (TTSPs) are a growing class of proteins sharing common structural motifs. They all consist of an N-terminally located small cytoplasmic domain, followed by an extracellular transmembrane domain, a very variable stem region, and a carboxy-terminally located proteolytic domain of the chymotrypsin fold [6]. With the exception of enteropeptidase, most TTSPs have only been found and characterised recently, so their specific physiological roles are

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often unknown. However, existing data point towards a great diversity of functions, such as the participation in proteolytic cascades [7], the processing of pro-hormones [8], cellular signalling events [7], embryonic development [9] and host immune defence [10]. A number of TTSPs have been identified through their association with cancer [11–15], suggesting a possible role in extracellular matrix remodelling events involved in tumour development and progression.

Although the presence of a transmembrane domain suggests predominant or even exclusive cellular localisation, members of the TTSP family such as enteropeptidase and HAT were isolated in soluble form. The soluble forms of both enteropeptidase and HAT exhibit enzymatic activity for their substrates trypsinogen and fibrinogen, respectively [4, 5]. Subsequent cloning and characterisation of the cDNA for HAT [10] revealed that it has the typical multidomain architecture of TTSPs. The short presumably intracellular located N terminus is followed by a SEA domain (sea urchin sperm protein, enterokinase, agrin [16]) and a trypsin-like proteolytic domain at the C terminus. Recently, the adrenal secretory serine protease (AsP) [17] which is involved in the processing of N-POMC-derived peptides was reported to be a secretory isoform of rodent airway trypsin-like proteases arising by alternative splicing. A similar cDNA variant for HAT, however, was not detectable [18].

The isolation of DESC1 [11] (differentially expressed in squamous cell carcinoma) and the recent reports on DESC2 and DESC3 [19, 20], all sharing a high degree of similarity with each other and with HAT, reveals the existence of a subfamily of HAT/DESC1-like proteases among the TTSPs. Currently, not much is known about cellular expression patterns, biochemical characteristics and subcellular localisation of HAT/DESC proteins. Whereas the HAT protein has been detected in cytoplasmic vesicles and at cilia of ciliated cells of the human lung [21], and mouse airway trypsin-like protease/AsP is found on tongue surface epithelium, oesophagus, trachea, gut and adrenal gland [18], the expression of the DESC proteases has been determined only at the level of tissues. Until now, the transmembrane topology leading to their classification as TTSPs has not been determined experimentally. The predictions of a transmembrane-anchoring sequence and of a disulphide bond linking the non-catalytic N-terminal domains with the C-terminal proteolytic domain even after cleavage at a conserved activation motif [19] suggest that HAT/DESC proteases remain membrane bound following activation. The identification of a catalytically active soluble form of HAT [5], however, indicates the necessity to experimentally confirm these predictions for each member of this family individually. Only for HAT and its rodent homologue have natural substrate proteins been identified [5, 10, 17]. The critical residues constituting the cleavage site of substrate proteins for HAT/DESC proteases are unknown. In the present study, we report on the isolation and characterisation of the cDNA for DESC4, a novel TTSP highly similar to the HAT/DESC family. The cDNA was isolated by a differential screening of cDNAs prepared from rat circumvallate papillae. We provide evidence for expression in different tissues and at the cellular level, the transmembrane topology, subcellular localisation of recombinantly expressed DESC4 and the presence of an interchain disulphide bridge.

Materials and methods

Differential screening of rat tongue epithelial cDNA

Tissues from circumvallate papillae and taste bud-free tongue epithelium were obtained from Wistar rats using a collagenase/dispase treatment [22]. Approximately 0.3 µg of total RNA extracted with peqGOLD RNApure (PEQLAB) from both samples was used for SMART-cDNA synthesis (Clontech). Both cDNA pools were amplified by PCR using the following conditions: 1 min 95 °C followed by 18 cycles of: 15 s 95 °C, 30 s 65 °C, 6 min 68 °C. To isolate cDNAs enriched in circumvallate papillae, a suppression subtractive hybridisation [23] was performed using the PCR-Select Differential Screening Kit (Clontech). The resulting cDNA fragments were subcloned to create a plasmid library. The successful enrichment of cDNAs specific for circumvallate papillae was monitored by random analysis of the plasmid library for α-gustducin, a taste receptor cell-specific G protein [24]. About 7% of all cDNAs corresponded to gustducin. The same enrichment was achieved for plasmids corresponding to the 3′-untranslated region of a novel cDNA species we chose for further analysis. Subsequent screening of a cDNA library prepared from rat circumvallate papillae led to the isolation of cDNAs containing the entire coding region of rat DESC4.

Tissue distribution of DESC4 mRNAs

A male rat was euthanised to obtain tissues for total RNA isolation using peqGOLD RNApure (PEQLAB). RT-PCR analysis of total RNA was done as before [25]. The following oligonucleotides/PCR conditions were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward primer: 5′-ACCACAGTCCATGCCATCAC-3′, reverse primer: 5′-TCCCACCACCCTGTTGCTGTA-3′/28 cycles, 1 min 94 °C, 1 min 58 °C, 1 min 72 °C. DESC4; forward primer: 5′-GATGAAGGGTGTTCACTGGAAGGT-3′, reverse primer: 5′-AATATGGGTGGGTGAACCGCTTAA-3′/40 cycles 1 min 94 °C, 1 min 65 °C, 1 min 72 °C; DESC4, splice variants: forward primer: 5′-TGGCTTTGGCAGTGCTCATCGGTC-3′, reverse primer: 5′-CCAGAATCACCCTCACAGGCATCC-3′/40 cycles, 1 min 94 °C, 1 min 68 °C, 1 min 72 °C; Equal amounts of

DNAseI-digested RNA were subjected to PCR as negative controls. To verify the specificity of DESC4 amplification, Southern hybridisation under high-stringency conditions was performed. Briefly, after agarose gel electrophoresis, PCR products were blotted onto a nylon membrane (Hybond-N+; Amersham) by capillary transfer according to standard procedures. The membrane was prehybridised in hybridisation buffer [50% formamide, 5 × SSC, 0.2% SDS, 1% blocking reagent (Roche), 100 µg/ml salmon testes DNA] for 2 h at 42 °C. Hybridisation was done for 1 h at 42 °C in the same buffer supplemented with approximately 10 ng/ml of a digoxigenin-labelled PCR fragment generated from DESC4 cDNA using the same DESC4-specific oligonucleotides described above. The membrane was washed twice for 15 min at room temperature in 0.1 × SSC, 0.1% SDS, followed by an incubation for 30 min at 50 °C in the same buffer. Detection of positive bands was done by colorimetry using an anti-digoxigenin antibody linked to alkaline phosphatase (1:5000; Roche).

In situ hybridisation

For the generation of riboprobes, a full-length cDNA clone covering the entire coding region as well as 5′- and 3′-untranslated sequences of the DESC4 variant including exon 6 (fig. 1) was digested with *Sac*I and *Eco*RV. The resulting fragments of 877, 546, and 926 bp were cloned separately into pBluescript KS(+). Digoxigenin-labelled riboprobes were generated and subsequently used as a mixture combining equal amounts of antisense and sense probes, at a concentration of 1 µg/ml. In situ hybridisation was done as described previously [25]. Briefly, 10-µm cross-sections of circumvallate papillae of rat tongues and embryo heads [embryo day 17 postconception (E17)] were processed. For hybridisation, the sections were postfixated, permeabilised and acetylated. Prehybridisation was done at 50 °C for 5 h, followed by hybridisation overnight at 50 °C. After hybridisation, the slides were washed several times at low stringency, followed by RNAse A treatment and high-stringency washes using 0.4 × SSC buffer at 50 °C. Hybridised riboprobes were detected using an anti-digoxigenin antibody and colorimetry. Photomicrographs were taken with a CCD camera (RT slider; Diagnostic Instruments) mounted onto a Zeiss Axioplan microscope.

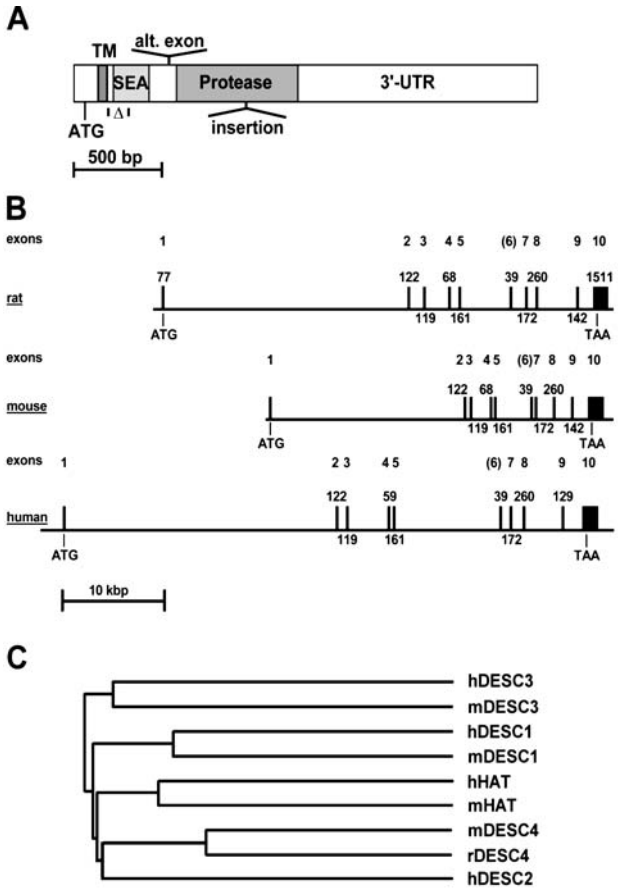

Figure 1. Structures of DESC4 cDNA, the DESC4 gene and phylogenetic tree of the HAT/DESC family. (*A*) Schematic of DESC4 cDNAs: TM, transmembrane domain; SEA; SEA-domain; alt. exon, alternative exon coding for 13 amino acids; Protease, trypsin-like proteolytic domain; 3′-UTR, 3′-untranslated region; Δ, missing exon found in one cDNA clone; Insertion, part of an intron interrupting the proteolytic domain found in one cDNA-clone. The open reading frame of DESC4 mRNA including the alternative exon codes for a protein of 417 amino acids. (*B*) Comparison of the genomic structures of the DESC4 genes of rat, mouse and human. Exons 1–10 are indicated by numbers. Exon (6) refers to the alternative exon. The length of exons is given in base pairs. Numbering starts with the most 5′ located nucleotide found in rat DESC4 cDNA clones. (*C*) Phylogenetic tree of the published members of the HAT/DESC family of transmembrane serine proteases (accession numbers and references: hHAT, AB002134 [10]; mHAT, AF448809 [19]; hDESC1, NM_014058 [11]; mDESC1, XM_144390 [19]; hDESC2, AL833167 [19]; hDESC3, XM_068227 [19]; mDESC3, XM_132179 [19]; rDESC4, AJ617528 (variant including alternative exon); mDESC4, AK036981. The multiple sequence alignment was done using the pileup program of the Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc.

Immunocytochemistry

For the generation of C-terminally HSV-tagged DESC4, the coding region of the cDNA variant including exon 6 was amplified by PCR using the following oligonucleotides: forward primer: 5′-CCACCATGTATCAGCCAGGAATC-3′, reverse primer: 5′-TATATAGCGGCCGCTGATGTTAGTTTTAGATTTAATC-3′. The resulting fragment was cloned into the vector pcDNA3 (Invitrogen) which included a sequence coding for the HSV-tag in frame with the *Not*I restriction site [26]. The resulting HSV-tagged cDNA was subcloned into the vector pcDNA5FRT (Invitrogen). HEK 293 cells (Flp-In-293; Invitrogen) transiently transfected with C-terminally HSV-tagged DESC4 were grown on poly-D-lysine-coated glass coverslips. For detection, cells were processed (at room temperature if not indicated otherwise) in two different

ways. After washing the coverslips with PBS, cells were either fixated for 10 min with 4% paraformaldehyde or with methanol/acetone (1:1 v/v) for 2 min, followed by 4-min incubation in 0.25% Triton X-100 in TBS. After fixation, coverslips were processed mainly as described before [27]. Briefly, after rinsing with TBS and blocking with 3% normal goat serum in TBS, anti-HSV antibody (1:30,000; Novagen) was allowed to react overnight at 4 °C. Cells were then rinsed with TBS and incubated for 1 h with a fluorescent dye-labelled secondary antibody (1:200, anti-mouse Alexa488; Molecular Probes). Excess antibody was removed by washing with TBS followed by a brief rinse with deionised H_2O. Coverslips were mounted onto slides and analysed by confocal microscopy using a Leica TCS SP2 equipped with an argon/krypton laser emitting at 488 nm. Fluorescence was detected in the range from 510 nm to 550 nm.

Enteropeptidase digestion of DESC4-expressing cells

The construct for the expression of internally FLAG-tagged DESC4 was generated by PCR-mediated recombination [28]. Briefly, the N-terminal subfragment of DESC4 was amplified with the following primer pair: forward primer: 5′-CCACCATGTATCAGCCAGGAATC-3′, reverse primer: 5′-CTTATCGTCGTCATCCTTGTAATCTCCCAAGCCACAGTTGCTG-3′. The C-terminal subfragment was amplified with the following oligonucleotides: forward primer: 5′-GATTACAAGGATGACGACGATAAGATCGCTGACGGCAAACCTGC-3′, reverse primer: 5′-TCCTCGAGCTGTTGCATTAGATGTTAG-3′. After fusing both subfragments by PCR, the construct was cloned into the vector pcDNA5FRT and transfected into HEK 293 cells. After 48 h in culture, cells were washed twice with 37 °C warm PBS buffer and harvested using ice-cold calcium-TBS buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 2 mM $CaCl_2$). Cells were pelleted by centrifugation (5 min, 500 g, 4 °C) and resuspended in 50 µl calcium-TBS buffer; 1 µl enteropeptidase (2 U/µl, Novagen) was added and incubated under gentle shaking for 1 h at room temperature. Cells were pelleted again, resuspended in the same buffer including protease inhibitors (2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1 mM PMSF, 2 mM EDTA) and homogenised by 20 strokes with a Potter-Elvehjem. Nuclei were removed by centrifugation (5 min, 500 g, 4 °C). The supernatants were subjected to centrifugation (10 min, 6000 g, 4 °C). The resulting supernatants were stored at –80 °C. The pellets enriched for plasma membranes [29] were extracted for membrane proteins (see cell fractionation below), recentrifuged and stored at –80 °C.

Cell fractionation

HEK 293 cells were transiently transfected using lipofectamine 2000 (Invitrogen) with DESC4 constructs containing an internal FLAG epitope. Fourty-eight hours after transfection dishes were rinsed with 37 °C warm PBS buffer (20 mM NaH_2PO_4, 80 mM Na_2HPO_4, 65 mM NaCl, pH 7.4) and cells were harvested and resuspended in ice-cold homogenisation buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 1 mM EDTA). Homogenisation was performed by 20 strokes using a Potter-Elvehjem. Nuclei were removed by centrifugation (5 min, 500 g, 4 °C). The supernatant was subjected to ultracentrifugation (1 h, 50,000 g, 4 °C). The supernatant containing cytosolic proteins was stored at –80 °C. The pellet enriched in membrane proteins was washed with homogenisation buffer, resuspended in homogenisation buffer including 1% Triton X-100, kept on ice for 30 min, and centrifuged (30 min, 16,000 g, 4 °C). The resulting supernatant containing extracted membrane proteins was stored at –80 °C. Protein concentrations were determined using a Bio-Rad Protein Assay.

Western blotting

Protein extracts were separated on SDS-polyacrylamide gels and transferred onto cellulose nitrate membranes (Optitran BA-S 83; Schleicher&Schuell). For detection of FLAG epitopes, membranes were blocked with 5% non-fat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20). Immunodetection was done by sequential application of rabbit anti-FLAG M2 antibody (1:2000; Sigma) and anti-rabbit antibody coupled to alkaline phosphatase (1:15,000; Sigma). HSV-tagged DESC4 was detected after blocking with 2% BSA in TBST buffer, and subsequent incubation with mouse anti-HSV-Tag antibody (1:5000; Novagen) and anti-mouse antibody coupled to alkaline phosphatase (1:15,000, Sigma). Colorimetric detection was performed with BCIP (0.175 mg/ml) and NBT (0.25 mg/ml).

In vitro transcription/translation

Subcloning the entire cDNA of the DESC4 variant including exon 6 into the vector pSP64 Poly(A) (Promega) generated the construct used for in vitro transcription/translation. Coupled transcription/translation reactions were done in the presence of ^{35}S-methionine (Amersham) and canine microsomal membranes (Promega) using the TNT Quick coupled transcription/translation system (Promega). After incubation for 90 min at 30 °C, reactions were stopped and microsomal membranes were harvested by centrifugation (15 min, 15,000 g, +4 °C). The resulting pellets were washed with PBS buffer and recentrifuged. The final pellet was either dissolved in sample buffer or prior to SDS-PAGE and autoradiography subjected to protein extraction by alkaline treatment [30].

Results

Isolation of cDNA clones coding for a new transmembrane serine protease from vallate papillae

By suppression subtractive hybridisation [23] between rat circumvallate papillae and adjacent lingual epithelium, we identified cDNAs corresponding to a novel TTSP, which we named DESC4. Most of the isolated cDNAs code for an N-terminal membrane-anchoring sequence, followed by a SEA domain, and a C-terminal trypsin-like proteolytic domain. The cDNAs differed by the presence or absence of an alternative exon preceding the proteolytic domain. In addition, two cDNAs containing sequences deleted or inserted within the N-terminal coding region and the C-terminal coding region, respectively, were identified. These interruptions introduced frameshifts rendering the resulting proteins non-functional, and were therefore considered to be the results of aberrant splicing and not analysed further (fig. 1A).

The intron-exon structure of the DESC4 gene is highly conserved among mammals (fig. 1B). The rat and the mouse genes code for proteins that are 91% identical at the deduced amino acid level. Within the rat genome, the DESC4 gene is located on chromosome 14 (14p21). The region of the primary transcript spans about 44 kb of genomic DNA and contains ten exons. Exon 6 is subjected to alternative splicing as it can be found to be present or absent in mRNA/cDNA (see fig. 1A, fig. 2D). The genomic area covered by the mouse DESC4 gene is about 33 kb and located on chromosome 5 (5E1). The human gene spans 53 kb and maps to chromosome 4q13. By RT-PCR using oligonucleotides directed against exons 7–10 of the human gene, we detected DESC4 mRNA in human circumvallate papillae (not shown). The primary transcript appears to be subjected to extensive alternative splicing making a more detailed analysis necessary to deduce amino acid sequences for the resulting proteins. Although the overall lengths of rat, mouse and human genes differ considerably, the arrangement of exons and their relative spacing by the corresponding introns suggests a direct ancestry. This is further supported by the fact that even the nucleotide sequences of non-coding regions share a high degree of similarity. We compared the nucleotide sequences of approximately 400 bp upstream of the start codons and found 100% identity between the rat and mouse genes and 74% between rat/mouse and human genes (not shown), indicating a conservation of gene regulation between species. The phylogenetic tree showing the relationships among the members of the HAT/DESC family demonstrates that the rat and mouse

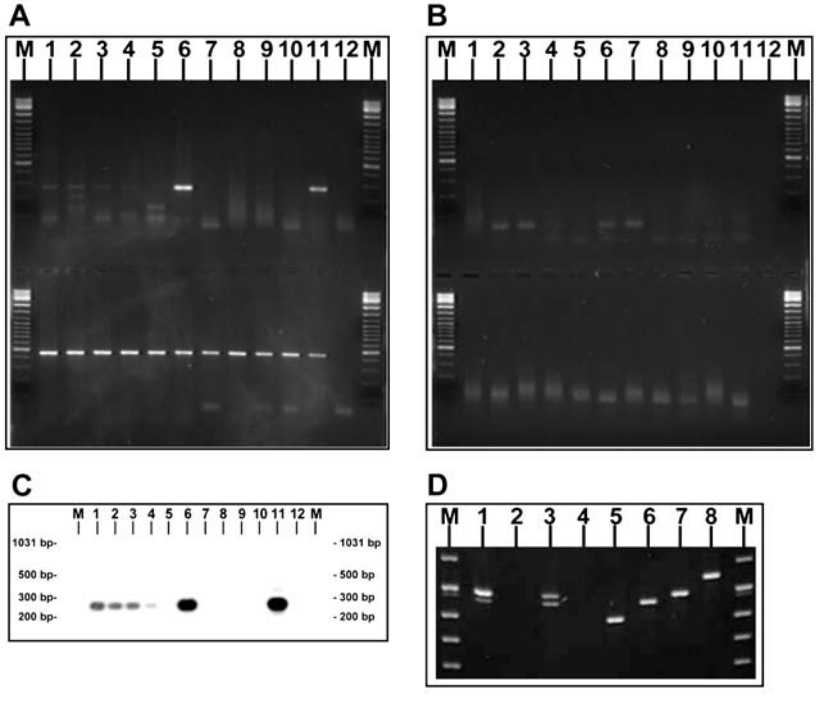

Figure 2. Tissue distribution of rat DESC4 mRNA and detection of splice variants. (*A*) RT-PCR analysis of DESC4 expression within different tissues. The top row shows PCR-products specific for DESC4; the bottom row demonstrates the presence of cDNA as detected by GAPDH-specific oligonucleotides used as positive controls. M, molecular-weight standard; 1, brain; 2, colon; 3, heart; 4, kidney; 5, liver; 6, lung; 7, skeletal muscle; 8, spleen; 9, stomach; 10, testis; 11, tongue; 12, H_2O control. (*B*) Control experiment, same as *A* minus reverse transcriptase. (*C*) Southern hybridisation of PCR-products using a digoxigenin-labelled DESC4-specific probe. Application of PCR-products was done exactly as in *A*, top row. (*D*) RT-PCR analysis using a set of oligonucleotides flanking all splice variants detected by colony screening. M, molecular-weight standard; 1, tongue cDNA; 2, tongue –reverse transcriptase (RT); 3, lung cDNA; 4, lung –RT; 5–8, plasmids corresponding to the different isolated splice variants.

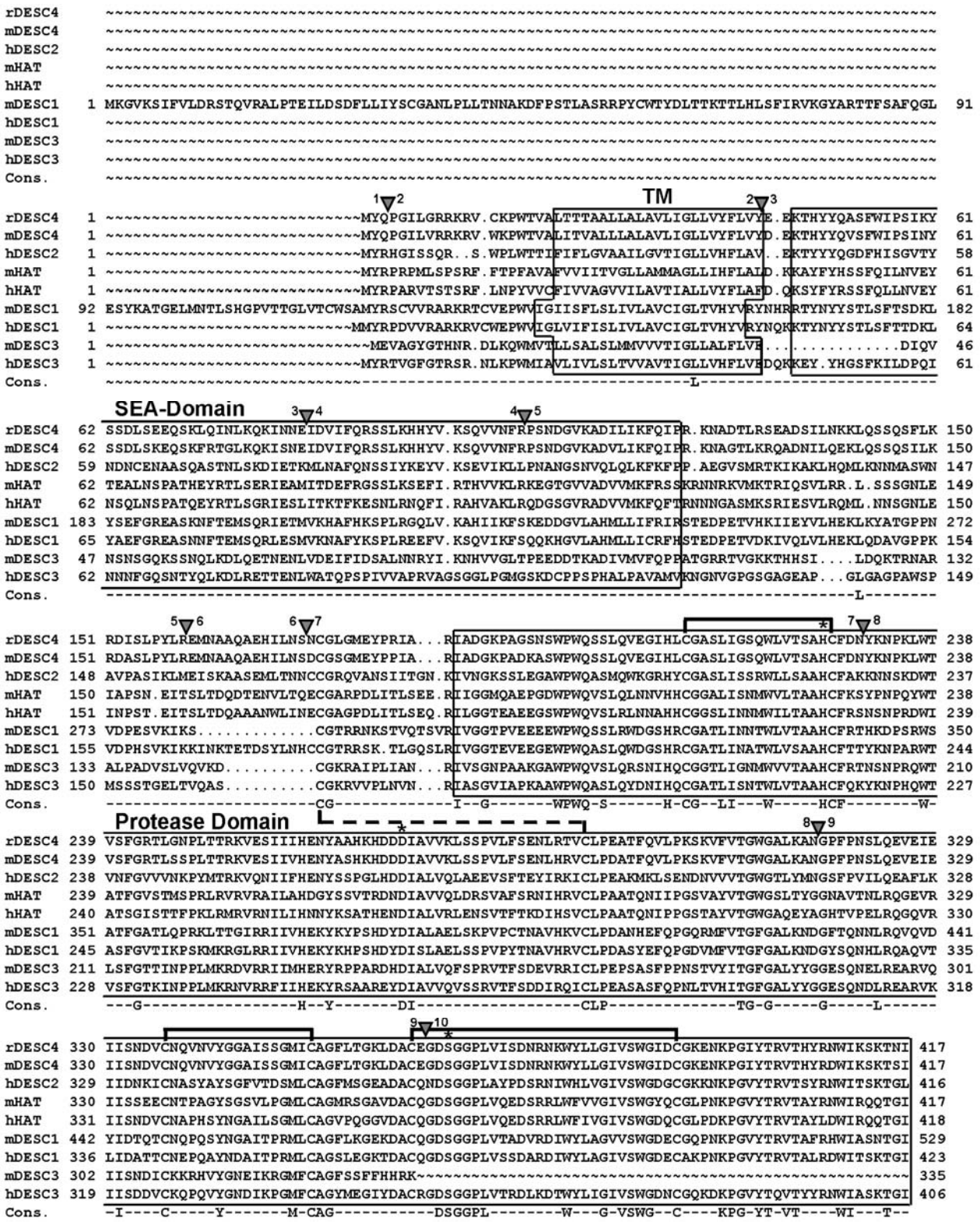

Figure 3. Comparison of the deduced amino acid sequences of the HAT/DESC family. The transmembrane domains (TM), the region showing similarities to SEA domains in some HAT/DESC family members and the proteolytic domains are boxed. Asterisks label the amino acids constituting the charge relay system. Putative disulphide bridges within the proteolytic domain deduced by analogy to the secondary structure of enteropeptidase [41] are indicated by lines, the interchain disulphide bridge by a dotted line. Exon-exon boundaries as determined by genomic analysis of rat DESC4 are indicated and numbered at the top of the rDESC4 amino acid sequence. The multiple sequence alignment was done using the pileup program of the Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, Wisc.

DESC4 genes are grouped together and therefore might represent orthologues (fig. 1C).

DESC4 belongs to a family of structurally related membrane-associated serine proteases. Comparison of the deduced 417-amino-acid peptide sequence of the rat DESC4 variant including exon 6 with its mouse orthologue and all other published members of the HAT/DESC family indicates that they belong to a family of structurally related transmembrane serine proteases (fig. 3). All proteases contain a short cytoplasmic N terminus followed by a single transmembrane domain. The TMHMM program predicted a type II membrane topology for all proteins (http://www.cbs.dtu.dk). A SEA domain is located close to the plasma membrane on the extracellular side. Originally, the SEA domain was described as an extracellular domain associated with O-glycosylation [16], suggesting an interaction of the HAT/DESC family proteases with glycosylated extracellular matrix components.

The highest homology among the proteases lies within their proteolytic domains. Many serine proteases are expressed as enzymatically inactive zymogens depending on proteolytic cleavage at the N-terminal end of their proteolytic domain for activation [31]. Characteristically, the cleavage occurs between a basic residue and a hydrophobic residue. A canonical activation site in agreement with these characteristics can be found in DESC4 and all other members of this gene family. An aspartate residue at the bottom of the S1 specificity pocket (aa 361) indicates that DESC4 will cleave its substrate molecules after a basic residue. Among the common features of this protease family is the conservation of structurally important cysteine residues. By analogy to enteropeptidase, the presence of conserved disulphide bridges can be postulated (fig. 3).

Analysis of the tissue distribution of DESC4 mRNA by RT-PCR

To determine the tissue specificity of DESC4 expression in rat, we analysed brain, colon, heart, kidney, liver, lung, skeletal muscle, spleen, stomach, testis and tongue tissues by RT-PCR (fig. 2A, B). The most prominent PCR products were obtained from lung and tongue tissues. Additionally, DESC4 mRNA was found in brain, colon, heart and liver. The specificity of PCR products was confirmed by Southern hybridisation under high-stringency conditions using a digoxigenin-labelled probe specific for DESC4 (fig. 2C). The tissues that apparently express the highest amounts of DESC4 mRNA, lung and tongue, were further analysed for the presence of splice variants that were observed during the initial cDNA screening. Using oligonucleotides spanning the region that includes all identified sequence variations, we detected the presence of the two variant mRNAs of DESC4 differing in only the presence or absence of the alternative exon 6. The two variants considered to have resulted by aberrant splicing were not detected. Interestingly, the long variant of DESC4 containing exon 6 is the predominant form in tongue tissue, whereas in lung tissue, both variants are expressed in similar amounts (fig. 2D).

Cellular expression pattern of DESC4 mRNA in adult circumvallate papillae and embryonic tissues

Cryostat cross-sections of circumvallate papillae, of adult rats and E17 embryo heads were hybridised with DESC4-specific riboprobes. Within the circumvallate papillae, the DESC4 mRNA is restricted to epithelial cells lining the trench of the papillae on both sides (fig. 4A). The signal is localised in epithelial cells surrounding the taste buds

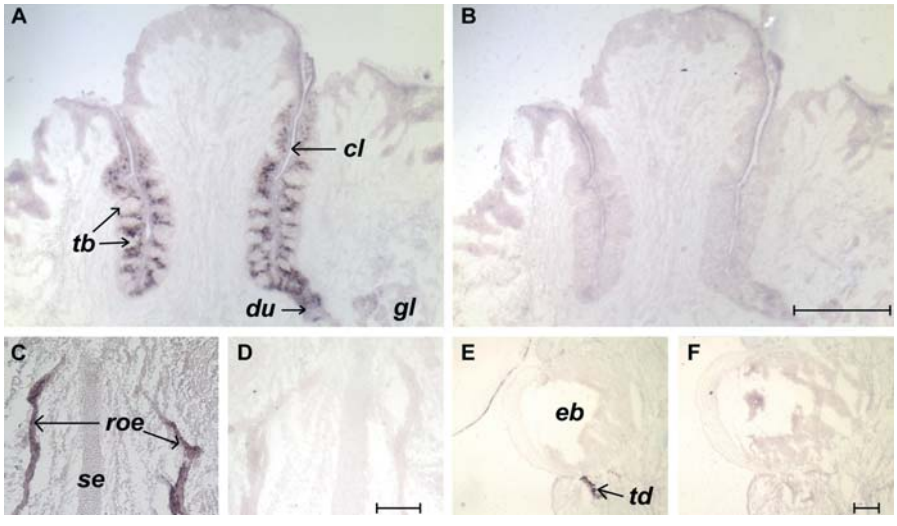

Figure 4. In situ hybridisation of DESC4 mRNA in different sensory organs. Ten micrometre cryostat cross-sections of adult rat circumvallate papillae (*A, B*) embryonic (E17) nasal cavity (*C, D*) and E17 embryonic eye (*E, F*) were hybridised with digoxigenin-labelled antisense (*A, C, E*) or sense (*B, D, F*) riboprobes. cl, cleft of the circumvallate papilla; du, duct of minor salivary gland; eb, eyeball; gl, minor salivary gland; roe, respiratory olfactory epithelium; se, nasal septum; tb, taste buds; td, tear gland duct. Scale bars, 200 µm.

but is absent from the taste receptor cells themselves. The tongue surface shows no signals, whereas the zone of expression extends into the ducts of minor salivary glands. Within head structures of E17 embryos, DESC4 mRNA is detected within two additional sensory systems, the olfactory system and the visual system. Within the nose, signals are present in the respiratory epithelium of the nasal cavity that is located in the anterior part of the nose (fig. 4C). The posterior part of the nasal cavity containing the sensorically active olfactory neuroepithelium is devoid of hybridisation signals (not shown). Within the visual system, signals can be visualised within tear gland ducts and epithelial cells surrounding the eyeball close to tear gland duct openings (fig. 4E).

Subcellular localisation of epitope-tagged DESC4 protein in HEK 293 cells

To determine the membrane association of DESC4 and to experimentally confirm the type II transmembrane topology, which has been predicted but not thoroughly investigated for this TTSP family, we tagged the DESC4 protein with HSV and FLAG epitopes, and transfected HEK 293 cells with these constructs. By immunocytochemistry of non-permeabilised, paraformaldehyde-fixed cells, and permeabilised, methanol/acetone-fixed cells, we observed staining of the plasma membrane using a DESC4 construct, which had been C-terminally tagged with the HSV epitope, in combination with an anti-HSV antibody (fig. 5A, upper and lower panel).

Separation of DESC4-HSV-transfected HEK 293 cells into cytosolic and membrane-associated protein fractions, followed by Western blotting and detection of HSV-tagged DESC4 demonstrates that the DESC4 protein is strictly membrane-associated (fig. 5B). In addition to the major band that corresponds to the predicted size of HSV-tagged DESC4, we observed a slightly smaller fragment specific for DESC4 within membrane protein extracts, suggesting that co- or posttranslational processing occurs in HEK 293 cells.

To further demonstrate the type II transmembrane topology, we placed an internal FLAG epitope/enteropeptidase cleavage site just N terminal of the proteolytic domain of DESC4 (fig. 5C). After transient transfection, we incubated HEK 293 cells with enteropeptidase. Enteropeptidase with access to the cleavage site from the extracellular site would generate an N-terminal fragment carrying the FLAG epitope while the proteolytic domain will be cleaved off. After enteropeptidase digestion, a fraction enriched in plasma membrane proteins was prepared and subjected to SDS-PAGE, Western blotting and immunodetection using an anti-FLAG antibody. The epitope-tagged 19-kDa fragment that remains plasma membrane associated after enteropeptidase cleavage (fig. 5C, lane 1) clearly demonstrates the type II transmembrane topology of DESC4. In contrast to the observation of an additional smaller variant of HSV-tagged DESC4 (fig. 5B, lane 1), a low-molecular-weight form of FLAG-tagged DESC4 was not detected. This difference might result from the different types and localisations of epitope tags affecting the maturation of both protein variants.

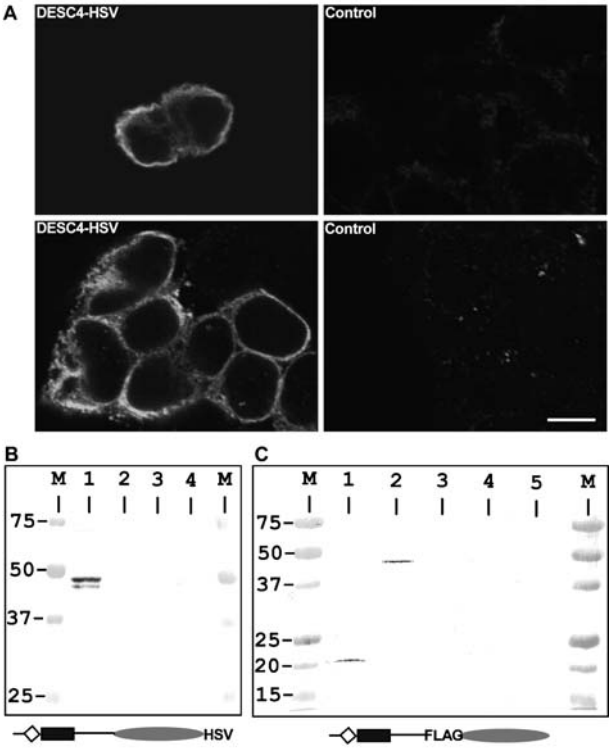

Figure 5. Subcellular localisation and transmembrane topology of DESC4. (*A*) HEK 293 cells transfected with DESC4 C-terminally tagged with the herpes simplex glycoprotein D epitope (HSV tag). Transfected cells were stained using an anti-HSV antibody after fixation with 4% paraformaldehyde (upper panel, left) or acetone/methanol fixed and Triton X-100 permeabilised (lower panel, left). Non-transfected cells were treated identically for a control of specificity (upper and lower panel, right). Scale bar, 8 µm. (*B*) Subcellular fractionation of DESC4 C-terminally tagged with the HSV epitope. M, molecular-weight standard; 1, membrane protein fraction of DESC4 transfected cells; 2, membrane proteins of non-transfected HEK 293 cells; 3, cytosolic fraction of DESC4-transfected cells; 4, cytosolic fraction of non-transfected HEK 293 cells. (*C*) Enteropeptidase digestion of cells transfected with DESC4 epitope tagged with a FLAG peptide/enteropeptidase cleavage site N terminal of the proteolytic domain. M, molecular-weight standard; 1, plasma membrane protein fraction of DESC4-FLAG transfected cells after enteropeptidase treatment; 2, plasma membrane proteins of transfected cells without enteropeptidase treatment; 3, cytosolic protein fraction of transfected cells; 4, plasma membrane proteins of non-transfected cells; 5, cytosolic proteins of non-transfected cells.

Elucidation of the possibility of DESC4 secretion in vitro or in cell culture

So far we have provided evidence for an association of DESC4 protein with the plasma membrane. However, this does not rule out the possibility of secretion either via the conventional secretory pathway or by membrane

shedding. To elucidate the potential for DESC4 secretion in greater detail, we performed in vitro translation experiments in the presence of microsomal membranes. After translation in the presence of ^{35}S-methionine, we isolated microsomal membranes and subjected them to SDS-PAGE analysis and autoradiography (fig. 6A). A band with the apparent molecular weight of 46.6 kDa predicted for the DESC4 variant including exon 6 was generated (fig. 6A, lane 1) indicating co-translational membrane insertion. We next treated the isolated microsomes with a basic sodium carbonate buffer to break up the vesicles, and separated the luminal vesicle contents from the integral membrane components by centrifugation. The majority of DESC4 protein was found in the pellet of the carbonate-treated microsomes (fig. 6A, lane 5) providing further evidence for its being an integral transmembrane component. The release of some DESC4 protein from the membrane (Fig. 6A, lane 3) is most likely due to the harsh extraction conditions inherent to this method [see also ref. 30]. However, at present we cannot rule out the generation of a minor amount of secreted DESC4 during co-translational membrane insertion.

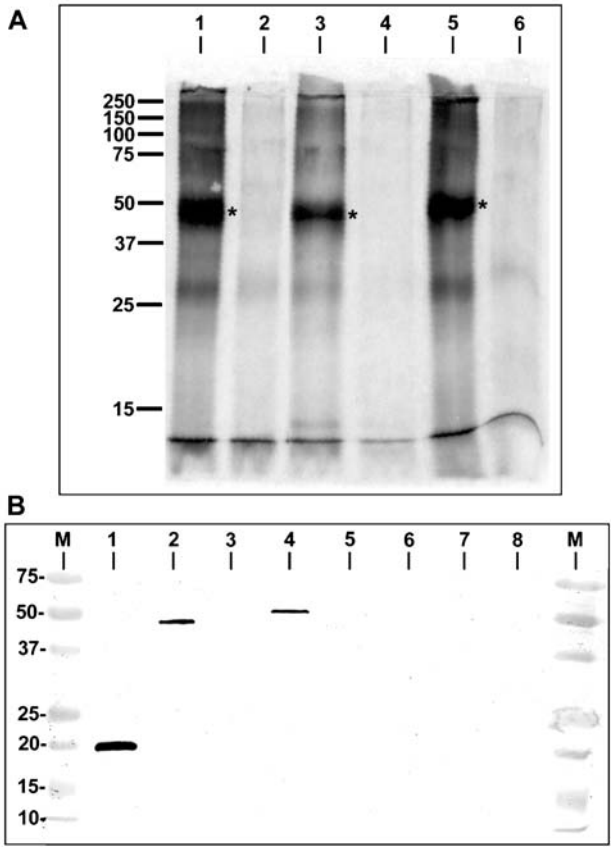

Figure 6. In vitro translation and non-reducing SDS-PAGE. (*A*) Autoradiography of SDS-PAGE-separated DESC4 generated by in vitro translation in the presence of canine pancreatic microsomes and ^{35}S-methionine. Lane 1, isolated microsomes; lane 3, supernatant after alkaline treatment of microsomes; lane 5, pellet of alkaline treated microsomes; lanes 2, 4 and 6, corresponding negative controls obtained by in vitro translation without DESC4 cDNA. Asterisks label bands corresponding to DESC4. (*B*) Subcellular fractionation of HEK 293 cells transfected with internally FLAG-tagged DESC4 followed by enteropeptidase digestion reveals a disulphide bridge linking the proteolytic and non-catalytic domains as shown by SDS-PAGE under reducing and non-reducing conditions prior to Western blotting. M, molecular-weight standard; 1, membrane protein fraction of DESC4-transfected cells subjected to enteropeptidase digestion and reduced with 100 mM DTT; 2, undigested membrane proteins of DESC4-transfected cells (+DTT); 3, bovine serum albumin (–DTT); 4, same as lane1 (–DTT); 5, bovine serum albumin (–DTT); 6, cytosolic fraction of DESC4 (–DTT); 7, membrane proteins of non-transfected cells (–DTT); 8, cytosolic proteins of non-transfected cells (–DTT).

Since many serine proteases are expressed as catalytically inactive zymogens requiring proteolytic activation resulting in a two-chain conformation linked by a disulphide bridge, we investigated if this is also the case for DESC4. We subjected a membrane protein preparation of DESC4 internally tagged with a FLAG tag/enteropeptidase cleavage site to enteropeptidase digestion followed by SDS-PAGE and Western blotting (fig. 6B). The FLAG-tagged N-terminal fragment of enteropeptidase-cleaved DESC4 has an apparent molecular weight of 19 kDa (lane 1), whereas the uncleaved protein is 48 kDa in size (lane 2). Cleaved DESC4 applied to SDS-PAGE analysis under non-reducing conditions shows an apparent molecular weight slightly larger than the uncleaved protein, demonstrating the presence of an interchain disulphide bond linking the N-terminal and proteolytic domain (lane 4).

Discussion

By differential screening of cDNAs derived from taste/non-taste tongue epithelium, we cloned DESC4, a transmembrane-type serine protease. The domain architecture and amino acid sequence identity between DESC4, HAT and DESC1–3 suggest that they all belong to a subfamily within the class of TTSPs. All TTSPs characterised to date exhibit a relatively restricted expression pattern, although some are expressed in several tissues and cell types. Our results obtained by RT-PCR show that DESC4 mRNA is expressed in tongue, lung and colon. By in situ hybridisation, we detected DESC4 expression in structures of the eye and the nasal cavity in addition to the signals seen within the trenches of circumvallate papillae. All of these structures contain exocrine glands producing mucus, and one is therefore tempted to speculate that DESC4 might be involved in the processing of secreted components or modification of the secretion process itself. Both processes have been shown to require limited proteolysis, e.g. the membrane shedding of MUC1 by the matrix metalloprotease TACE/ADAM 17 [32] or the activation of the epithelial sodium channel (ENaC) by epithelial serine proteases [33]. Since all structures expressing DESC4 come into contact with a large variety of micro-organisms

including pathogens, a role in defence against bacterial infections as has been demonstrated for human mast cell tryptase βI [34] appears possible too. To our surprise, DESC4 is also expressed in brain, heart and liver, hampering such speculations about functional implications, although similarly heterogeneous expression patterns have been obtained for several TTSPs [6, 19, 20].

The variety of tissues expressing DESC4 indicates a complex and intricate mode of action for this protease. This assumption is further supported by the observed splice variants (fig. 2D). For tongue and lung, the long variant containing the alternative exon 6 is predominant in tongue whereas both variants are about equally abundant in lung. Although a database search with this 13-amino-acid-long alternative exon did not reveal any functional implications, its location within the stem region immediately N terminal of the proteolytic domain and the conservation of this peptide between rat DESC4 and its mouse orthologue suggest an important function.

To better understand the function of DESC4, we sought to determine its subcellular localisation and transmembrane topology. The localisation of charged amino acids surrounding the hydrophobic transmembrane domain is an important determinant for the transmembrane topology adopted by a nascent polypeptide chain during co-translational insertion into the lipid bilayer. Positively charged amino acids flanking the transmembrane domain are generally believed to be located on the cytoplasmic side whereas negative charges will be found on the luminal side of the endoplasmic reticulum [35]. Consequently, the transmembrane topology prediction for DESC4 suggests a type II topology as the charged amino acids surrounding the transmembrane domain agree perfectly with the classical 'positive-inside rule'. However, since membrane insertion is a dynamic process with multiple determinants [36], we needed to confirm the membrane topology experimentally. This has not been done before for any member of the DESC1/HAT-like family of TTSPs. Immunocytochemistry of HEK 293 cells transfected with DESC4 revealed an extracellular localisation of the HSV-tagged proteolytic domain (fig. 5A). Extraction and Western blotting of membrane proteins of DESC4-transfected cells confirms the strict membrane association (fig. 5B). The extracellular localisation of the proteolytic domain was further demonstrated by in vitro digestion of DESC4-expressing cells with enteropeptidase and immunostaining (fig. 5C).

In contrast to the single bands specific for DESC4 obtained with the internal FLAG tag (fig. 5C), the anti-HSV tag antibody identified a doublet of bands (fig. 5B). Since the HSV tag is located at the extreme C terminus of DESC4, this raises the possibility that co- or posttranslational processing occurs within the N terminus leading to secreted DESC4.

Recent evidence suggests a role for SEA domains aside from extracellular matrix association. The SEA domain of the G protein-coupled receptor Ig-Hepta is cleaved within its SEA domain during passage through the endoplasmic reticulum in HEK 293T cells. Interestingly, this cleavage by an unknown protease did not cause a dissociation of the two fragments. They remained tightly bound to each other by unknown non-covalent interactions [37]. This principle could hold true for all SEA domain-containing membrane resident proteins leading to highly specific ligand-receptor alliances after processing within the SEA domain [38]. Recently, a SEA domain adjacent to the transmembrane domain of matriptase was recognised. The authors demonstrated that intracellular processing within the SEA domain was an important prerequisite for the catalytical activation of matriptase [39].

To determine if the cleavage at the N terminus of DESC4 occurs co-translationally, within the endoplasmic reticulum or during later steps of protein maturation, we performed in vitro translation experiments in the presence of microsomal membranes. The apparent molecular weight of bands we detected by SDS-PAGE of the isolated microsomal membranes corresponds well to the results we obtained by our subcellular fractionation experiments. Treatment of the isolated microsomal membranes with an alkaline buffer extracts minor amounts of DESC4, indicating the possibility of secretion of presumably small quantities of DESC4. Since we failed to extract DESC4 completely by alkaline treatment and we never detected secreted DESC4 in a cell culture experiment, we believe that the formation of alkali-released DESC4 protein is most likely an artefact inherent to the harsh extraction method.

However, since the structurally very similar HAT was isolated from sputum of patients with chronic airway disease as a single-chain molecule with an apparent molecular weight of 27 kDa [5], we cannot exclude that DESC4 is released into the extracellular space in vivo by a release mechanism similar to that of HAT. Recently, AsP [17] was reported to be a short secreted isoform of the rodent but not the human airway trypsin-like protease arising from alternative splicing [18].

DESC4, at least under experimental conditions, is present in a two-chain conformation after being cleaved at its activation site (fig. 6B). The two-chain conformation resembles the activated state of enteropeptidase. The catalytic light chain of enteropeptidase is linked to the non-catalytic heavy chain via a disulphide bridge between two conserved cysteine residues [40]. These cysteines are conserved within the DESC4 polypeptide chain (fig. 3), suggesting conformational similarities between DESC4 and enteropeptidase.

In summary, the characterisation of DESC4 demonstrated several important determinants for the physiological properties of HAT/DESC1-like proteases. Future research will perhaps add even more members to this gene subfamily, should clarify the release mechanism of

secreted isoforms and shed light on the substrate specificity/physiological substrates for members of this interesting gene family.

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1 Tlaskalova-Hogenova H., Tuckova L., Lodinova-Zadnikova R., Stepankova R., Cukrowska B., Funda D. P. et al. (2002) Mucosal immunity: its role in defense and allergy. Int. Arch. Allergy Immunol. **128:** 77–89
2 Matsuo R. (2000) Role of saliva in the maintenance of taste sensitivity. Crit. Rev. Oral Biol. Med. **11:** 216–229
3 Lillehoj E. P. and Kim K. C. (2002) Airway mucus: its components and function. Arch. Pharm. Res. **25:** 770–780
4 Fonseca P. and Light A. (1983) The purification and characterization of bovine enterokinase from membrane fragments in the duodenal mucosal fluid. J. Biol. Chem. **258:** 14516–14520
5 Yasuoka S., Ohnishi T., Kawano S., Tsuchihashi S., Ogawara M., Masuda K. et al. (1997) Purification, characterization, and localization of a novel trypsin-like protease found in the human airway. Am. J. Resp. Cell Mol. Biol. **16:** 300–308
6 Hooper J. D., Clements J. A., Quigley J. P. and Antalis T. M. (2001) Type II transmembrane serine proteases – insights into an emerging class of cell surface proteolytic enzymes. J. Biol. Chem. **276:** 857–860
7 Takeuchi T., Harris J. L., Huang W., Yan K. W., Coughlin S. R. and Craik C. S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. J. Biol. Chem. **275:** 26333–26342
8 Yan W., Wu F. Y., Morser J. and Wu Q. Y. (2000) Corin, a transmembrane cardiac serine protease, acts as a pro-atrial natriuretic peptide-converting enzyme. Proc. Natl. Acad. Sci. USA **97:** 8525–8529
9 Appel L. F., Prout M., Abushumays R., Hammonds A., Garbe J. C., Fristrom D. et al. (1993) The *Drosophila* stubble-stubbloid gene encodes an apparent transmembrane serine-protease required for epithelial morphogenesis. Proc. Natl. Acad. Sci. USA **90:** 4937–4941
10 Yamaoka K., Masuda K., Ogawa H., Takagi K., Umemoto N. and Yasuoka S. (1998) Cloning and characterization of the cDNA for human airway trypsin-like protease. J. Biol. Chem. **273:** 11895–11901
11 Lang J. C. and Schuller D. E. (2001) Differential expression of a novel serine protease homologue in squamous cell carcinoma of the head and neck. Br. J. Cancer **84:** 237–243
12 Lin B. Y., Ferguson C., White J. T., Wang S. Y., Vessella R., True L. D. et al. (1999) Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. Cancer Res. **59:** 4180–4184
13 Shi Y. E., Torri J., Yieh L., Wellstein A., Lippman M. E. and Dickson R. B. (1993) Identification and characterization of a novel matrix-degrading protease from hormone-dependent human breast-cancer cells. Cancer Res. **53:** 1409–1415
14 Takeuchi T., Shuman M. A. and Craik C. S. (1999) Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue. Proc. Natl. Acad. Sci. USA **96:** 11054–11061
15 Wallrapp C., Hahnel S., Muller-Pillasch F., Burghardt B., Iwamura T., Ruthenburger M. et al. (2000) A novel transmembrane serine protease (TMPRSS3) overexpressed in pancreatic cancer. Cancer Res. **60:** 2602–2606
16 Bork P. and Patthy L. (1995) The sea module – a new extracellular domain associated with O-glycosylation. Protein Sci. **4:** 1421–1425
17 Bicknell A. B., Lomthaisong K., Woods R. J., Hutchinson E. G., Bennett H. P. J., Gladwell R. T. et al. (2001) Characterization of a serine protease that cleaves pro-gamma-melanotropin at the adrenal to stimulate growth. Cell **105:** 903–912
18 Hansen I. A., Fassnacht M., Hahner S., Hammer F., Schammann M., Meyer S. R. et al. (2004) The adrenal secretory serine protease AsP is a short secretory isoform of the transmembrane airway trypsin-like protease. Endocrinology **145:** 1898–1905
19 Netzel-Arnett S., Hooper J. D., Szabo R., Madison E. L., Quigley J. P., Bugge T. H. et al. (2003) Membrane anchored serine proteases: a rapidly expanding group of cell surface proteolytic enzymes with potential roles in cancer. Cancer Metastasis Rev. **22:** 237–258
20 Szabo R., Wu Q. Y., Dickson R. B., Netzel-Arnett S., Antalis T. M. and Bugge T. H. (2003) Type II transmembrane serine proteases. Thromb. Haemost. **90:** 185–193
21 Takahashi M., Sano T., Yamaoka K., Kamimura T., Umemoto N., Nishitani H. et al. (2001) Localization of human airway trypsin-like protease in the airway: an immunohistochemical study. Histochem. Cell Biol. **115:** 181–187
22 Striem B. J., Naim M. and Lindemann B. (1991) Generation of cyclic AMP in taste buds of the rat circumvallate papilla in response to sucrose. Cell. Physiol. Biochem. **1:** 46–54
23 Diatchenko L., Lau Y. F. C., Campbell A. P., Chenchik A., Moqadam F., Huang B. et al. (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. USA **93:** 6025–6030
24 Mclaughlin S. K., Mckinnon P. J. and Margolskee R. F. (1992) Gustducin is a taste-cell-specific G-protein closely Related to the transducins. Nature **357:** 563–569
25 Behrens M., Venkatraman G., Gronostajski R. M., Reed R. R. and Margolis F. L. (2000) NFI in the development of the olfactory neuroepithelium and the regulation of olfactory marker protein gene expression. Eur. J. Neurosci. **12:** 1372–1384
26 Roosterman D., Roth A., Kreienkamp H. J., Richter D. and Meyerhof W. (1997) Distinct agonist-mediated endocytosis of cloned rat somatostatin receptor subtypes expressed in insulinoma cells. J. Neuroendocrinol. **9:** 741–751
27 Behrens M., Margolis J. W. and Margolis F. L. (2003) Identification of members of the Bex gene family as olfactory marker protein (OMP) binding partners. J. Neurochem. **86:** 1289–1296
28 Fang G. W., Weiser B., Visosky A., Moran T. and Burger H. (1999) PCR-mediated recombination: a general method applied to construct chimeric infectious molecular clones of plasma-derived HIV-1 RNA. Nat. Med. **5:** 239–242
29 Cabedo H., Luna C., Fernandez A. M., Gallar J. and Ferrer-Montiel A. (2002) Molecular determinants of the sensory and motor neuron-derived factor insertion into plasma membrane. J. Biol. Chem. **277:** 19905–19912
30 Clum S., Ebner K. E. and Padmanabhan R. (1997) Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient in vitro processing and is mediated through the hydrophobic regions of NS2B. J. Biol. Chem. **272:** 30715–30723
31 Khan A. R. and James M. N. G. (1998) Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Sci. **7:** 815–836
32 Thathiah A., Blobel C. P. and Carson D. D. (2003) Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding. J. Biol. Chem. **278:** 3386–3394
33 Vallet V., Chraibi A., Gaeggeler H. P., Horisberger J. D. and Rossier B. C. (1997) An epithelial serine protease activates the amiloride-sensitive sodium channel. Nature **389:** 607–610
34 Huang C. F., De Sanctis G. T., O'Brien P. J., Mizgerd J. P., Friend D. S., Drazen J. M. et al. (2001) Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. J. Biol. Chem. **276:** 26276–26284

35 Krishtalik L. I. and Cramer W. A. (1995) On the physical basis for the cis-positive rule describing protein orientation in biological-membranes. FEBS Lett. **369:** 140–143
36 Goder V. and Spiess M. (2001) Topogenesis of membrane proteins: determinants and dynamics. FEBS Lett. **504:** 87–93
37 Abe J., Fukuzawa T. and Hirose S. (2002) Cleavage of Ig-Hepta at a 'SEA' module and at a conserved G protein-coupled receptor proteolytic site. J. Biol. Chem. **277:** 23391–23398
38 Wreschner D. H., Mcguckin M. A., Williams S. J., Baruch A., Yoeli M., Ziv R. et al. (2002) Generation of ligand-receptor alliances by 'SEA' module-mediated cleavage of membrane-associated mucin proteins. Protein Sci. **11:** 698–706
39 Oberst M. D., Williams C. A., Dickson R. B., Johnson M. D. and Lin C. Y. (2003) The activation of matriptase requires its noncatalytic domains, serine protease domain, and its cognate inhibitor. J. Biol. Chem. **278:** 26773–26779
40 Matsushima M., Ichinose M., Yahagi N., Kakei N., Tsukada S., Miki K. et al. (1994) Structural characterization of porcine enteropeptidase. J. Biol. Chem. **269:** 19976–19982
41 Kitamoto Y., Yuan X., Wu Q. Y., Mccourt D. W. and Sadler J. E. (1994) Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. Proc. Natl. Acad. Sci. USA **91:** 7588–7592

Database submission. The sequences reported in this paper have been deposited in the EMBL Nucleotide Sequence Database under the accession numbers AJ617481 (DESC4, variant without exon 6) and AJ617528 (DESC4, variant including alternative exon 6).