Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues

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MS-1, a high-molecular-mass protein expressed by non-continuous and angiogenic endothelial cells and by alternatively activated macrophages (Mϕ2), and the hepatic sinusoidal endothelial hyaluronan clearance receptor are similar with respect to tissue distribution and biochemical characteristics. In the present study we purified these proteins by immuno- and hyaluronan-affinity chromatography respectively, sequenced tryptic peptides and generated full-length cDNA sequences in both mouse and human. The novel genes, i.e. *stabilin-1* and *stabilin-2*, code for homologous transmembrane proteins featuring seven fasciclin-like adhesion domains, 18–20 epidermal-growth-factor domains, one X-link domain and three to six B-(X_7)-B hyaluronan-binding motifs. Northern-blotting experiments revealed the presence of both stabilins in organs with predominant endothelial sinuses such as liver, spleen and lymph node: stabilin-1 mRNA was also detected in organs with predominant Mϕ2 cells, such as placenta, and in interleukin-4/glucocorticoid-stimulated Mϕ2 cells *in vitro*. A polyclonal antibody made against human recombinant stabilin-1 confirmed the expression of stabilin-1 protein in splenic sinus endothelial cells *in vivo* and in Mϕ2 *in vitro*. On the basis of high similarity at the protein level and the unique domain composition, which differs from that of all other known fasciclin-like proteins and hyaluronan receptors, stabilin-1 and stabilin-2 define a novel family of fasciclin-like hyaluronan receptor homologues that might play a role in cell–cell and cell–matrix interactions in vascular function and inflammatory processes.

Key words: endothelial cells, macrophage, MALDI-TOF MS, X-link domain.

INTRODUCTION

Vascular heterogeneity is the basis for the diverse functions exerted by different sections of the vascular tree, in different organs and during angiogenesis. Among the few molecules known to date to be differentially expressed by endothelial cells, the monoclonal antibody (mAb) MS-1 uniquely identifies a high-molecular-mass protein that, in the adult healthy organism, is expressed by all non-continuous endothelia such as the sinusoidal endothelial cells of the liver, spleen, and lymph nodes, but not by continuous endothelia [1]. Ultrastructurally, MS-1 antigen is found on those parts of the plasma membrane of splenic sinusoidal endothelial cells with close intercellular contacts. Endothelial expression of MS-1 antigen is also found in endothelial cells of continuous origin that undergo angiogenesis, e.g. during wound healing, in chronic inflammation and in malignant tumours [2,3].

Besides its involvement in endothelial differentiation and angiogenesis, the MS-1 antigen is differentially expressed by Mϕ2 [alternatively activated macrophages (Mϕ)], i.e. polarized antigen-presenting cells induced by Th2 cytokines [4]. MS1$^+$ Mϕ2 are found in immunologically privileged normal tissues such as placenta; they are also identified during the healing phase of acute inflammatory reactions, in chronic inflammatory diseases such as rheumatoid arthritis and psoriasis, and in wound-healing tissue [2,5,6]. MS-1$^+$ Mϕ2 act as suppressor Mϕ [7] and are associated with a high degree of vascularization *in vivo*. Pro-angiogenic activity of MS-1$^+$ Mϕ2 [3,8] is accompanied by interleukin (IL)-4 induced expression of fibronectin and its splice variants and of other matrix molecules, such as the fasciclin-domain-containing adhesive protein βIG-H3 [9]. Ultrastructurally, the MS-1 antigen is found in Mϕ2 in cytoplasmic vesicles and focally on dense plasma-membrane-associated plaques [10] that resemble the plaque-like fibronexus serving structural integration of intracellular actin fibers and extracellular fibronectin [11].

Biochemically the MS-1 antigen occurs as two major species, of 300 and 220 kDa, and a minor form, of 120 kDa; a precursor of 280 kDa is also detected that matures into the 300 kDa species by complex glycosylation. The 220 and 120 kDa forms derive from the 300 kDa species by proteolytic cleavage [1].

Abbreviations used: ECM, extracellular matrix; EGF, epidermal growth factor; EST, expressed sequence tag; FD, fasciclin domain; HA, hyaluronan; HUAEC-p, primary human umbilical artery endothelial cells; hstabilin, recombinant human stabilin; HUVEC-p, primary human umbilical vein endothelial cells; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; MALDI-TOF MS, matrix-assisted laser-desorption–ionization time-of-flight MS; mstabilin, recombinant mouse stabilin; MTN™ (ClonTech), multiple-tissue Northern; Mϕ, macrophage(s); Mϕ2, alternatively activated macrophages; PSD, post-source decay; RHAMM, receptor of HA-mediated motility; RACE, rapid amplification of cDNA ends; TSG-6, tumour-necrosis-factor-stimulated gene-6.

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The final human stabilin-1 (hstabilin-1) cDNA sequence (7870 bp), the final mstabilin-1 cDNA sequence (7935 bp), the final hstabilin-2 cDNA sequence (8266 bp) and the final mouse stabilin-2 (mstabilin-2) cDNA sequence (8147 bp) have been deposited with the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AJ275213, AF290914, AJ295695 and AF364951 respectively.

Similar to the MS-1 antigen, the hepatic hyaluronan (HA) clearance receptor is expressed by liver sinusoidal endothelial cells. It serves to remove the large amounts of HA released into the peripheral blood during steady-state tissue remodelling, and it protects the blood from becoming overly viscous. The hepatic HA clearance receptor occurs as a high-molecular-mass protein with a major species of 270 kDa and a minor species of 180 kDa after HA affinity chromatography; the MS-1 antigen was not co-eluted from these columns. A polyclonal antibody made against this protein shows that it is located on the cell surface of liver sinusoidal endothelial cells; it is retained in early endosomes of these cells when recirculation of endocytic receptors is blocked by monensin. Besides HA, the hepatic HA clearance receptor also has affinity for type I procollagen, indicating a broader scavenging function [12].

Here we report on the purification and molecular characterization of the MS-1 antigen and of the hepatic HA clearance receptor, i.e. stabilin-1 and stabilin-2. The cloning of the cDNA for human stabilin-1 (hstabilin-1) was based on the matrix-assisted laser-desorption–ionization time-of-flight MS (MALDI-TOF MS) analysis of the purified MS-1 antigen. The mouse stabilin-1 (mstabilin-1) cDNA was cloned by homology with the human gene. h- and m-stabilin-2 cDNAs were cloned using peptide sequences of the HA-affinity-purified rat hepatic HA clearance receptor. Stabilin-1 and -2 were expressed at a similar level in placenta, spleen, lymph nodes and liver; stabilin-1, but not stabilin-2 expression was also detected in specialized endothelial cells *in vivo* and *in vitro* as well as in Mϕ2. Bioinformational analysis showed that the stabilins feature HA-binding domains and other adhesive domains such as fasciclin and epidermal-growth-factor (EGF) domains. Owing to their unique common structure, the stabilins thus constitute a novel protein family of fasciclin-like HA receptor homologues.

EXPERIMENTAL

Cell culture, mediators and antibodies

Peripheral-blood monocytes/Mϕ were isolated and cultured as described in [8]. Mediators were used at the following final concentrations: IL-4 (PromoCell G.m.b.H., Heidelberg, Germany), 300 units/ml; interferon (IFN)-γ (Tebu, Le Perray en Yvelines, France), 50 ng/ml; dexamethasone (Sigma), 100 nM. KG-1, 293 and HepG2 cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). KG-1 cells were propagated in RPMI-1640 medium, HepG2 and 293 were cultured in Dulbecco's modified Eagle's medium, both supplemented with 10 % (v/v) foetal-calf serum and appropriate amounts of penicillin/streptomycin (all from Biochrom KG, Berlin, Germany). The human microvascular endothelial-cell line CDC/EU.HMEC-1 [13] was propagated in endothelial-cell growth media (PromoCell). Primary human umbilical-vein endothelial cells (HUVEC-p) and primary human umbilical-artery endothelial cells (HUAEC-p) were obtained from PromoCell and were also cultivated in endothelial-cell growth media as was the permanent HUVE cell line EA.hy926, kindly provided by Dr C.-J. S. Edgell, Department of Pathology and Laboratory Medicine, University of North Carolina Medical School, Chapel Hill, NC, U.S.A. The cell line 293-hStabilin-1 was generated by stable transfection of 293 cells with *Not*I-linearized pEF6V5His-hStab1, followed by blasticidin (Calbiochem) selection.

Mouse mAb MS-1 was used as MS-1 hybridoma supernatant or purified from the supernatant by HiTrap-ProteinG chromatography (Amersham Pharmacia Biotech). For the generation of rabbit antiserum against the stabilin-1, a recombinant hstabilin-1 was emulsified in Freund's adjuvant and used for intramuscular immunization in the rabbit carried out essentially as described in [12]. Secondary antibodies used in the present study were the following: horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech), FITC-conjugated anti-mouse antibody (Dako), Cy2 (Cyanine Cy2™ fluorochrome with emission 505 nm)-conjugated anti-mouse and Cy3 (Cyanine Cy3™ fluorochrome with emission 570 nm)-conjugated anti-rabbit antibodies (Dianova, Hamburg, Germany).

Purification of MS-1 protein peptides from Mϕ2 and MALDI-TOF MS analysis

The MS-1 antigen was isolated with standard immunoprecipitation from 1 litre of culture of primary human Mϕ2 after stimulation with IL-4 and dexamethasone for 6 days. Proteins were eluted from affinity matrix loaded with anti-mouse IgG antibody and separated by SDS/PAGE.

Protein bands cut out of the SDS/PAGE gel were destained in methanol/acetic acid/water (45:10:45, by vol.) overnight with several changes of the solution and dried with acetonitrile (gradient grade; Merck). Proteins were reduced and alkylated by washing the gel pieces in water, drying with acetonitrile, reswelling in 10 mM dithiotreitol/0.1 M NH_4HCO_3 and incubation at 56 °C for 30 min. The gel pieces were shrunk again in acetonitrile, reswollen in 55 mM iodoacetamide/0.1 M NH_4HCO_3 and incubated at room temperature for an additional 20 min in the dark. Finally, iodoacetamide solution was removed, and the gel pieces were washed with 0.1 M NH_4HCO_3 for 15 min and dried with acetonitrile.

For the tryptic digest the gel pieces were reswollen in 50 mM NH_4HCO_3 containing tosylphenylalanylchloromethane ('TPCK')-treated bovine pancreatic trypsin (Sigma) at a ratio of approx. 1:50 to the calculated amount of protein present in the gel pieces. The gel pieces were just covered by the solution and the digest was completed by incubation at 37 °C overnight. After addition of 10–15 μl of 25 mM NH_4HCO_3 and shaking at 37 °C for 15 min, the resulting peptides were first extracted from the gel by addition of 1–2 gel vol. of acetonitrile and shaking at 37 °C for 15 min. The supernatant was transferred to a fresh tube and the gel pieces extracted further by adding 40–50 μl of 5 % formic acid (Merck), shaking at 37 °C for 15 min and finally shrinking again with acetonitrile.

All supernatants were pooled and dried in a vacuum concentrator. The final purification of the peptide mixture was performed with ZipTip™ (Millipore, Eschborn, Germany) according to the manufacturer's instructions. The peptides were directly eluted with a mixture of 50 % acetonitrile in water containing the MALDI-TOF MS matrix and applied to the MALDI-TOF MS target using a fast-evaporation nitrocellulose matrix [14].

Chromatograms were recorded with REFLEX (Bruker Daltronik, Bremen, Germany) in reflector mode and continuous extraction. The post-source decay (PSD) spectra were recorded with the FAST method (Bruker Daltronik) using a FAST-pulser.

Purification and peptide sequencing of the rat liver HA receptor

Purification of the rat liver sinusoidal endothelial-cell HA receptor was performed largely as described by McCourt et al. [12]. Typically, ten snap-frozen rat livers were homogenized and the soluble fraction was purified on a wheatgerm agglutinin–Sepharose column. The eluate was concentrated on an Amicon YM 30 (Millipore) membrane and then applied to an S-300 column (Amersham Pharmacia Biotech). After washing, the proteins were eluted and 10 ml fractions were collected. Those

fractions enriched in proteins of molecular mass greater than 80 kDa (by SDS/PAGE) were pooled and applied to control–ethylenediamine–Sepharose and HA–ethylenediamine–Sepharose columns in series [15]. Bound proteins were eluted with a pulse of HA oligosaccharides. The eluate from six to eight purifications was pooled and applied to a lentil lectin–Sepharose column. Bound proteins were eluted with methyl mannoside; the eluate was concentrated in a centrifugal concentrator and fractionated on a Superose 6 column (Amersham Pharmacia Biotech). Fractions eluted in the void volume were subjected to preparative SDS/6 %-(w/v)-PAGE under non-reducing conditions. The approx. 270 kDa protein band was visualized, excised and digested 'in-gel' according to the method of Rosenfeld et al. [16]. The liberated peptides were then purified by HPLC and sequenced.

Plasmids and sequencing

hstabilin-1 clone D87433 was identified after the evaluation of the MALDI-TOF MS chromatogram with the MS-Fit and PepIdent programs. The total length of the D87433 clone was 6777 bp. The missing 5′ end of hstabilin-1 was determined using 5′-rapid amplification of cDNA ends (RACE) with Marathon Ready cDNA from spleen (ClonTech). The complete cDNA sequence of hstabilin-1 was generated by PCR and cloned into the pEF6/V5His-TOPO-TA vector (Invitrogen), resulting in plasmid pEF6/V5His-hStab1 containing the hstabilin-1 gene driven by the EF-1α promoter.

A hstabilin-2 clone DKFZp434E0321, containing 3.9 kb of the coding sequence, was obtained from the Primädatenbank am Ressourcenzentrum (RZPD), Berlin, Germany. The rest of the coding sequence was obtained by PCR with primers selected according to the predicted exon–intron structure of the corresponding genomic sequence. PCR products were cloned into a pCRII-TOPO vector (Invitrogen).

The DKFZp402F1819Q2 mstabilin-1 cDNA clone, containing 1.8 kb of the coding sequence was obtained from RZPD. The rest of the cDNA was amplified with predicted gene specific primers. PCR products were cloned into a pCRII-TOPO vector (Invitrogen) or sequenced directly.

mstabilin-2 cDNA was amplified as three overlapping fragments of 2.5, 3.2 and 4.0 kb. Fragments were cloned into pCR-XL-TOPO vector (Invitrogen) and sequenced.

hstabilin-2 splicing analysis

hstabilin-2 splicing was analysed by PCR with the following primers: St2F037 (5′-AGT GGA CTA TGG ACC TAG ACC CAA C), positions 7002–7026, and St2R037 (5′-AGT AAG CAG CCA AGG CAA CAG C), positions 7627–7607.

Bioinformatics and phylogenetic analysis

Peptide mass fingerprints obtained by MALDI-TOF MS analysis were evaluated using the internet facilities of the Protein-Prospector (http://prospector.ucsf.edu/). The evaluation of the chromatogram was done with the MS-Fit and PeptIdent programs.

Public databases were searched for homologueies using BLAST programs at the National Center for Biotechnology Information (NCBI, National Library of Medicine, Bethesda, MD, U.S.A.). The DNA sequences were analysed for possible coding regions using the DNATools program (S. W. Rasmussen, Carlsberg Research Center, Valby, Copenhagen, Denmark). The deduced protein sequences were analysed for putative functional domains by comparing with the Prosite and Pfam databases at the ProfileScan server (http://www.isrec.isb-sib.ch/software/PFSCAN-form.html). We used the ExPASy proteomics tools at http://www.expasy.ch/tools/ to further analyse the protein sequence for functionally important features such as membrane topology (TMHMM), sorting signals (PSORT) and glycosylphosphatidylinositol-anchor analysis. Analysis of genomic sequences for possible exon–intron structure was performed using the FEX program at the Computational Genetics Group (CGG, The Sanger Centre, Hinxton, Cambridge, U.K.) internet page (CGG at http://genomic.sanger.ac.uk/).

The accession numbers of the proteins used for phylogenetic analysis are as follows:

(A) Proteins containing fasciclin domains (FDs)

BGH3_human (pir||I52996); RGD-CAP_chicken (dbj|BAA21479.1); OSF-2_human (pir||S36111); OSF-2_mouse (pir||S36109), MPT_70_*Mycobacterium* (sp|Q50769), MPB70 *Synechocystes* (pir||S77329.), M-FAS_Drosophila (gi|AAC09252), FAS1_ *Drosophila* (pir||B29900), EST_*Pinus* (pir||S52995), Algal-CAM_*Volvox* (pir||S51614), BEP_sea_urchin (pir||A60672).

(B) Proteins containing X-link domains (the X-link domain is an HA-binding region found in proteins of vertebrates that are involved in the assembly of extracellular matrix, cell adhesion, and migration).

TSG-6 (tumour-necrosis-factor-stimulated gene-6)_human (ref|NM_007115); LYVE-human (gb|AF118108); KIAA0527_human (dbj|BAA25453) CD44_human (pir||A47195); CD44_chicken (gb|AF153205); link protein_mouse (sp|Q9QUP5); aggrecan_bovine (sp|P13608); brevican_bovine (sp|Q28062); neurocan_mouse (sp|P55066); versican_human (sp|P13611).

The alignment of sequences for similarity identification was made with the CLUSTAL-X windows interface [17] and dendrograms visualized with Treeview [18].

RNA isolation and Northern-blot analysis

Total RNA from cultivated Mϕ was prepared and used for Northern blots as described in [8]. Multiple Tissue Northern (MTN™; ClonTech) blots were also used for investigation of gene expression. Membranes were hybridized with ^{32}P-labelled probes in ExpressHyb™ solution (ClonTech).

Indirect immunofluorescense

The following samples were used for immunofluorescense: Mϕ stimulated with IL-4 for 6 days, 293-hStabilin-1 cells fixed with acetone and spleen sections fixed with xylol. All procedures were performed as described previously by McCourt et al. [12]. Primary antibodies were used as follows: MS-1 mouse hybridoma supernatant, non-diluted; anti-hstabilin-1 rabbit polyclonal serum and rabbit preimmune serum (negative control), 1:250. The secondary antibodies were: Cy3-anti-mouse 1:300 and Cy2-anti-rabbit 1:400 (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.); Cy2-anti-mouse 1:100 and Cy3-anti-rabbit 1:400 (Dianova). Samples were viewed with a Leica DM RB microscope and photographed with a Leitz WILD MPS46/52 camera.

RESULTS

Purification and MALDI-TOF MS analysis of human MS-1 antigen (hstabilin-1) from Mϕ2

Analysis of MS-1 antigen from Mϕ2, spleen or cell lines showed a predominant 300 kDa protein band in immunoprecipitation

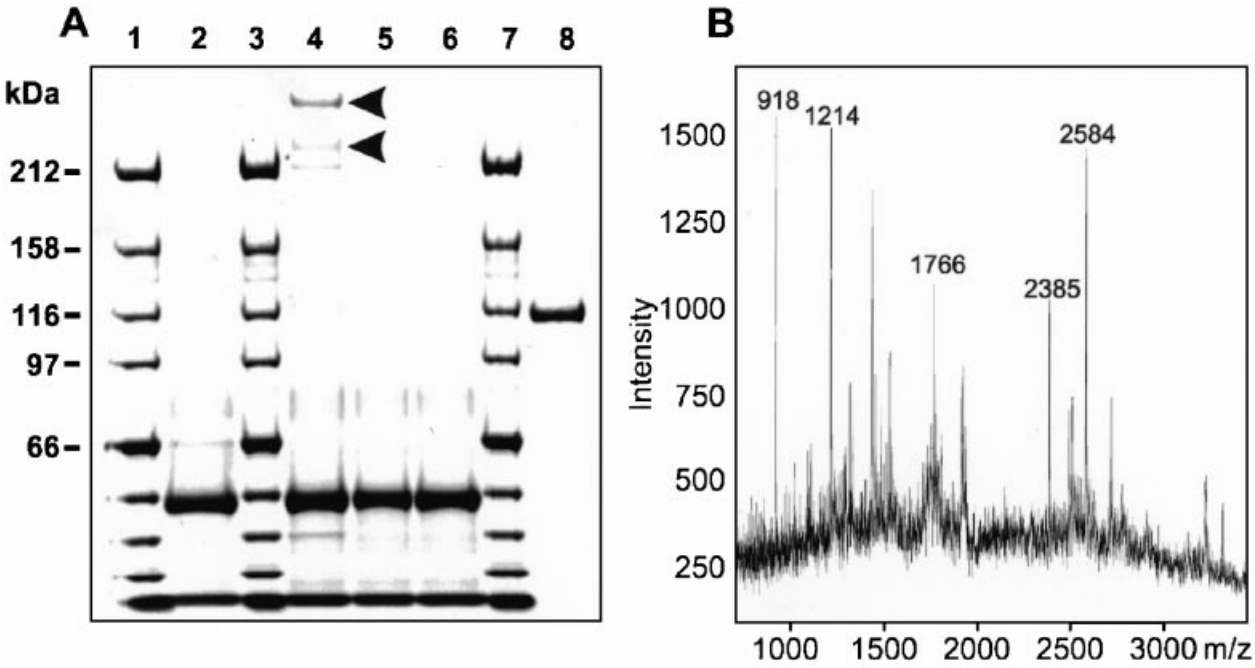

Figure 1 MS-1 antigen isolation and MALDI-TOF MS analysis

(**A**) Coomassie Brilliant Blue-stained SDS/PAGE gels of MS-1 antigen immunoprecipitated from cultivated macrophages. Lanes 1, 3 and 7, molecular-mass markers; lane 2, immunoprecipitation from buffy-coat culture supernatant; lane 4, immunoprecipitation from buffy-coat lysate; lane 5, Sepharose beads only; lane 6, Sepharose beads with mAb MS-1; lane 8, β-galactosidase (1 μg). (**B**) MALDI-TOF MS chromatogram obtained for the 300 kDa band of MS-1 antigen. Masses of the well separated peaks are indicated.

Table 1 MS-1 antigen-derived MALDI peptides identified in hstabilin-1 cDNA (clone D87433)

Peptide molecular mass (Da)	Position in D87433	Modification	Sequence
918	1040–1046		AFWLQP
1213	1021–1032		VTALVPSEAAVR
1766	204–218		CPQNTQCSAEAPSCR
2385	546–568	1 × Cys_CAM	GGCSENAECVPGSLGTHHCTCHK
2584	1642–1665	4 × Cys_CAM	SGFAGTACELCAPGAFGPHCQACR

experiments. As MS-1 antigen expression was strongest in Mϕ2 *in vitro* after stimulation by IL-4 and dexamethasone, MS-1 protein was precipitated with mAb MS-1 from the latter cells. The 300 kDa protein obtained (Figure 1A) was subjected to MALDI-TOF MS analysis.

The peptide patterns obtained (Figure 1B) were then analysed using Protein-Prospector, MS-Fit and PeptIdent programs, resulting in several matches with an uncharacterized cDNA KIAA0246 (D87433) (Table 1). The identity of the MS-1 protein and this cDNA was demonstrated by generating a peptide sequence of peak 918 using PSD. This cDNA was named hstabilin-1.

Cloning of hstabilin-1 and mstabilin-1

A search for hstabilin-1 homology in the NCBI/GenBank® High-Throughput Genomic Sequences ('HTGS') database produced a genomic sequence located on chromosome 3 (AC006208). Exon–intron analysis revealed several potential exons. The actual sequence was then established by reverse-transcription PCR with primers derived from the putative exon sequences using Marathon-Ready™ cDNA from peripheral blood leucocytes and spleen. The extreme 5′-end of the hstabilin-1 mRNA was identified by 5′-RACE.

The mouse homologue of hstabilin-1 was cloned by searching mouse expressed-sequence-tag (EST) databases with the hstabilin-1 sequence; 26 EST sequences were identified. Using these data, we could amplify and clone 5.5 kb of the 3′-end of the mstabilin-1 cDNA. For the identification of the 5′-end of mstabilin-1, we screened a mouse genomic library with a 5′ probe for hstabilin-1. Sequence analysis of one positive clone enabled us to derive the putative exon 1 sequence. These sequence data were used to design primers and amplify the missing 5′ part of the mstabilin-1 cDNA. Alignment of the sequences of hstabilin-1 and mstabilin-1 showed an identity of 83 and 82% at the DNA and protein levels respectively (Table 2).

Purification of the rat hepatic HA clearance receptor and cloning of hstabilin-2 and mstabilin-2

Similar to stabilin-1, the rat hepatic HA clearance receptor runs on SDS/PAGE gels as two bands of 270 and 180 kDa. Large-

Table 2 Homology between mouse and human Stabilin-1 and -2

	Homology (%)			
	hstabilin-1	mstabilin-1	hstabilin-2	mstabilin-2
hstabilin-1	100	81.8	38.5	37.4
mstabilin-1		100	37.7	37.7
hstabilin-2			100	71.0
mstabilin-2				100

scale purification of this HA receptor by HA-affinity chromatography allowed direct sequencing of a total of six peptides. Five peptides out of these six, namely

KEAATIATYNQLSAQK

KALEALPQEQQDFLFQDNK

KVLSDIISTNGVIHVIDK

KNPSTSQYFFQLQEHAVVE

KLIQDSGLLSVIT

showed an identity of only 31–47% with hstabilin-1, but a considerably higher identity of 62.3–92.3% (Table 3) with a partial cDNA sequence (DKFZp434E0321) from the human DKFZ (Das Deutsche Krebsforschungszentrum, Heidelberg, Germany) library. Further searches resulted in identification of the corresponding genomic sequence on human chromosome 12q (AC063946). For the cloning of the full-size cDNA the same strategy as for hstabilin-1 was used. Comparison of the full-length clone with hstabilin-1 gave an identity of 39% on the protein level, allowing designation of the protein as hstabilin-2 (Table 2).

Analysis of the sequences of several hstabilin-2 clones showed the presence of a 107 bp deletion between nucleotides 7067 and 7173 in some of them. This deletion resulted in a frameshift and in the appearance of a stop codon at position 7238. The truncated mRNA codes for a hstabilin-2 protein that lacks the C-terminal FD (see below) and the transmembrane domain, suggesting that this short form must be secreted. Reverse-transcription PCR analysis showed that both forms of the hstabilin-2 mRNA are present in spleen and liver; however, the amount of the truncated form of hstabilin-2 was considerably lower (results not shown).

For the cloning of mstabilin-2, a similar strategy as for cloning of mstabilin-1 was used. Mouse EST databases were screened for homology with the hstabilin-2 sequence. Several ESTs were identified within the EST sequences obtained, PCR primers were selected and the mstabilin-2 sequence was amplified and sequenced, resulting in cDNA of about 8 kb. Comparison of mstabilin-2 and hstabilin-2 nucleotide and protein sequences revealed a 77.2 and 71% identity respectively (Table 2).

Domain structure of stabilin proteins

The analysis of the primary and secondary structure of hstabilin-1 revealed a protein with an apparent molecular mass of 275 kDa deduced from the amino acid sequence. This corresponds to the previously described mass of the immature protein (280 kDa)

Table 3 Peptides of the purified rat hepatic hyaluronan clearance receptor corresponding to hstabilin-2 cDNA

Peptide sequence	Position in hstabilin-2	Identity (%)	Position in mstabilin-2	Identity (%)
KEAATIATYNQLSAQK	2222–2237	68.8	2230–2245	62.5
KALEALPQEQQDFLFQDNK	1785–1803	62.3	1792–1810	73.7
KVLSDIISTNGVIHVIDK	1705–1722	66.7	1712–1729	77.8
KNPSTSQYFFQLQEHAVVE	1605–1623	78.9	1612–1630	94.7
KLIQDSGLLSVIT	1758–1770	92.3	1765–1777	92.3

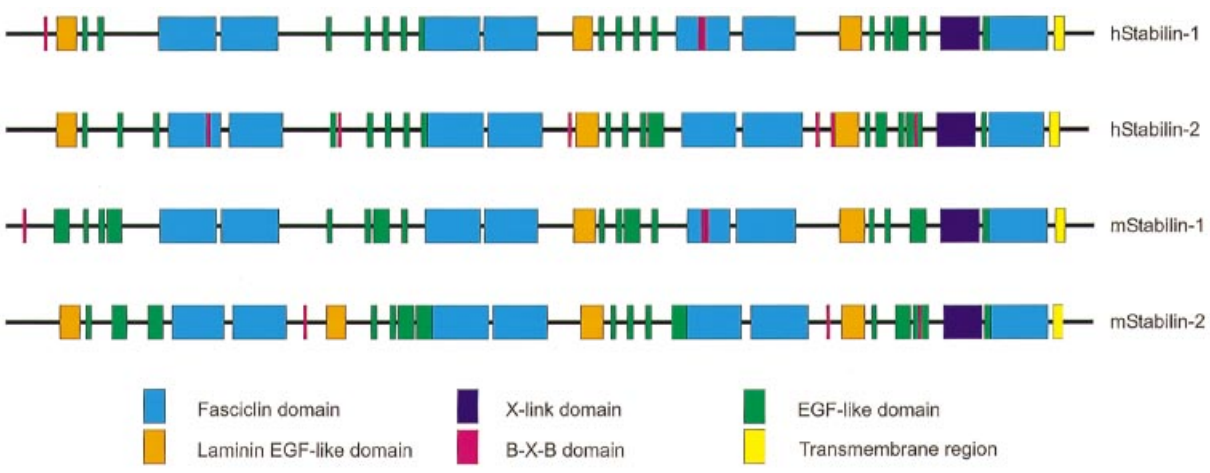

Figure 2 Domain structure of stabilin-1 and stabilin-2

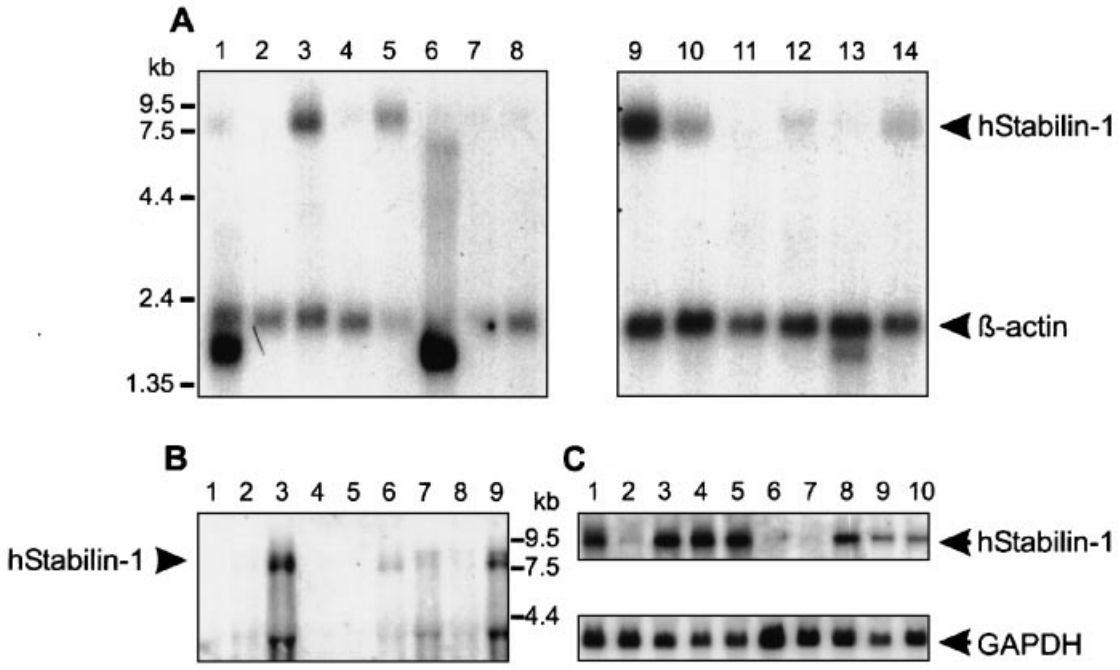

Figure 3 Analysis of stabilin-1 expression

(**A**) Northern-blot analysis of hstabilin-1 expression in different tissues. MTN™ blots were hybridized with a 5′ probe of hstabilin-1 and with a probe for β-actin. Lanes 1–14 contain mRNA from following tissues:

Lane	Tissue	Lane	Tissue
1	Heart	8	Pancreas
2	Brain	9	Spleen
3	Placenta	10	Lymph node
4	Lung	11	Thymus
5	Liver	12	Peripheral blood leucocytes
6	Skeletal muscle	13	Bone marrow
7	Kidney	14	Foetal liver

Bands corresponding to hstabilin-1 and β-actin are indicated by arrows. (**B**) Northern-blot analysis of stabilin-1 expression using a 3′ stabilin-1 probe. Lanes 1–6 contain total RNA from primary HUVEC, HUVEC 926, HUAEC, HepG2, HL 60 and CDC respectively. Lanes 7–9 contain macrophages after 3 days of culture without stimulation (lane 7), with IFNγ stimulation (lane 8) and with dexamethasone/IL-4 stimulation (lane 9). (**C**) Northern-blot analysis of stabilin-1 expression in cultured macrophages. Hybridization with stabilin-1 probe A and with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. Lanes 1–5, day 3 of activation; lanes 6–10, day 6 of activation. Lanes 1 and 6, non-activated cells; lanes 2 and 7, IFNγ treatment; lanes 3 and 8, IL-4 treatment; lanes 4 and 9, dexamethasone treatment; lanes 5 and 10, dexamethasone and IL-4 treatment.

before complex glycosylation, as shown by immunoprecipitation in pulse–chase experiments [1] and to the mass of the recombinant hstabilin-1 protein (results not shown). hstabilin-2 is predicted to have a molecular mass of 273 kDa, which corresponds well to the value of 270 kDa for the purified natural rat protein.

Stabilin-1 and -2 proteins show a mixed content of α-helical and β-sheet secondary structures according to PREDATOR analysis. Scanning the protein sequence using the PROSITE and Pfam databases showed that stabilin-1 and stabilin-2 are composed of three blocks with two FDs each and a single FD at the C-terminal end. Apart from these domain blocks, there are 15–17 EGF-like domains and two to four laminin EGF-like domains distributed between FD blocks and at the N-terminus of the protein (Figure 2). In addition, there is one X-link domain common to HA-binding proteins. Searches for HA binding B-(X_7)-B motifs revealed several motifs in both stabilin-1 and -2 [the HA-binding B-(X_7)-B motif is nine amino acids in length, where B is either arginine or lysine and X_7 contains no acidic residues (such as aspartic acid or glutamic acid) and at least one basic amino acid]. The hydrophobicity analysis of stabilin-1 and -2 revealed a transmembrane region C-terminal from the last FD (Figure 2). Scanning of stabilin-1 for sorting signals using the PSORT program also identified features for protein kinase C/kinase CK2-dependent sorting into the endosomal compartment, a tyrosine-based motif (YLRAR) and the dileucine motif SLLEE, suggesting 33.3 % probability of Golgi localization, 33.3 % probability of plasma-membrane localization and 33.3 % probability of endoplasmic-reticulum localization. This implies a shuttle of the stabilin-1 protein between the plasma membrane and the endosomal compartment [19]. Stabilin-2 analysis did not reveal any endosomal sorting signals, suggesting preferential plasma-membrane localization.

Expression analysis of stabilins

The expression of hstabilin-1 and -2 mRNA was analysed using Northern blots made with total RNA from cultivated Mϕ, endothelial cells, and diverse cell lines, and using MTN™ blots.

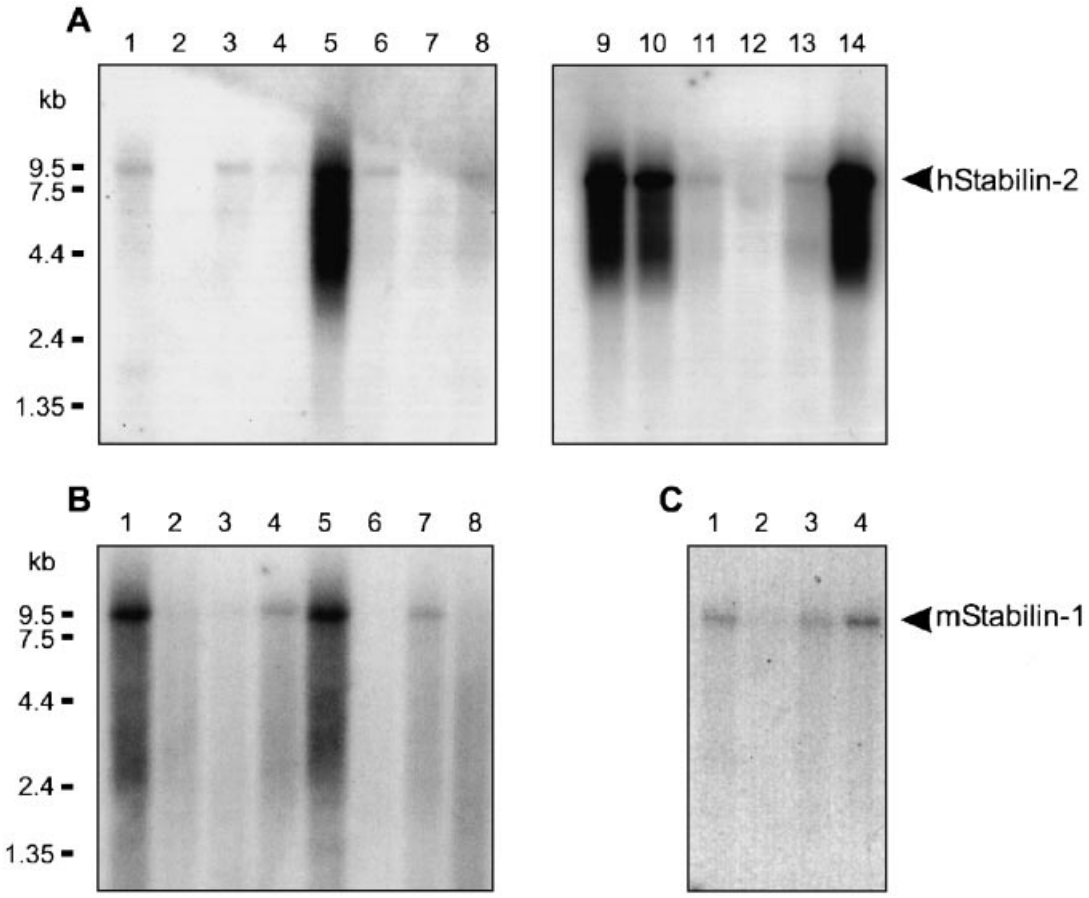

Figure 4 Analysis of hstabilin-2 and mstabilin-1 expression

(**A**) Northern-blot analysis of hstabilin-2 expression in different tissues. MTN™ blots hybridized with a hstabilin-2 probe. Lanes 1–14 contain mRNA from following tissues:

Lane	Tissue	Lane	Tissue
1	Heart	8	Pancreas
2	Brain	9	Spleen
3	Placenta	10	Lymph node
4	Lung	11	Thymus
5	Liver	12	Peripheral blood leucocytes
6	Skeletal muscle	13	Bone marrow
7	Kidney	14	Foetal liver

(**B**) Northern-blot analysis of mstabilin-1 expression in different tissues. Lanes 1–8 contain mRNA from heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis respectively. (**C**) Northern-blot analysis of mstabilin-1 expression in mouse embryo. Lanes 1–4 contain mRNA from 7-, 11-, 15- and 17-day embryos respectively.

MTN™ blot analysis revealed hstabilin-1 expression in organs with prominent endothelial sinuses, such as spleen, liver and lymph node, and also in placenta, an organ rich in Mϕ2 (Figure 3A). Except for human aortic endothelial cells, all other endothelial cells and the liver carcinoma cell line HepG2 were negative (Figure 3B). hstabilin-1 expression was also detected in Mϕ2 *in vitro*. On day 3 of cultivation, a transcript of approx. 9 kb was detected in Mϕ2 stimulated by IL-4, dexamethasone or a combination thereof, as well as in non-stimulated monocytes/Mϕ, albeit to a lesser extent (Figure 3C, lanes 1 and 3–5). In contrast, Th1-associated cytokine IFNγ suppressed the expression of stabilin-1 to a barely detectable level (Figure 3C, lane 2). On day 6 of cultivation, the highest level of hstabilin-1 expression was detected in Mϕ2 stimulated by IL-4 alone (Figure 3C, lane 8), while no expression of hstabilin-1 was observed in IFNγ-stimulated or non-stimulated Mϕ.

The expression of hstabilin-2 in general paralleled the expression of hstabilin-1, with the strongest signals in foetal and adult liver, lymph node and spleen (Figure 4A). By contrast, hstabilin-2 expression was neither detected in endothelial cells or cell lines nor in cultured Mϕ in Northern-blot experiments (results not shown).

Expression of mstabilin-1 in mouse tissues was similar to the results obtained in human tissues, except for the lack of mstabilin-1 in mouse spleen. Interestingly, mouse spleen – in contrast to human spleen – does not contain any endothelial sinuses. Thus comparison of the mRNA expression patterns of hstabilin-1 and mstabilin-1 confirms its association with sinusoidal endothelial

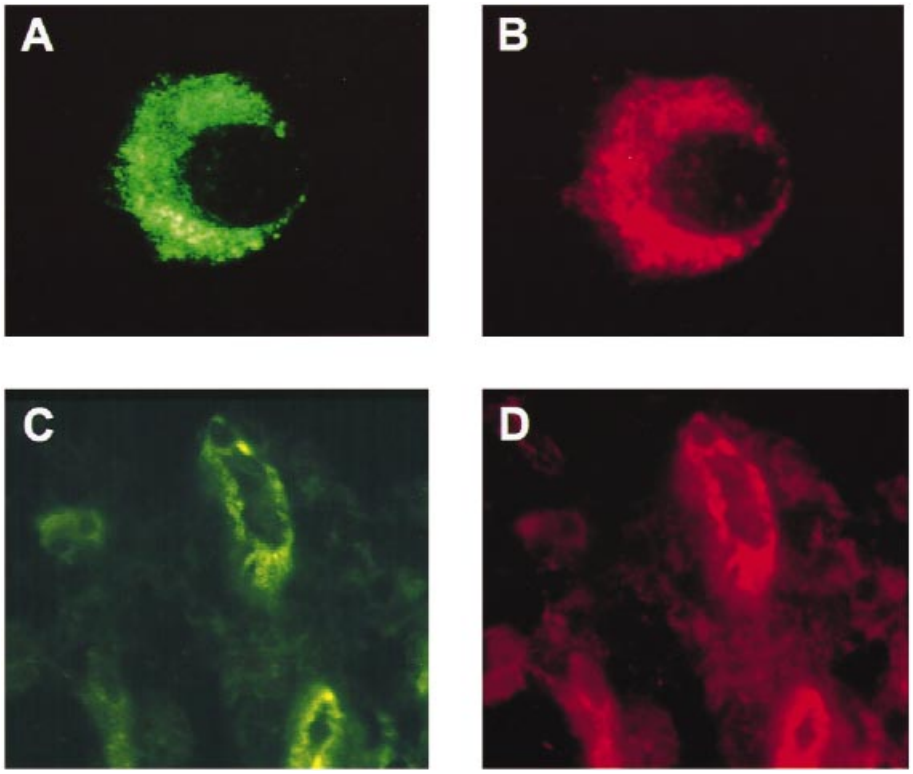

Figure 5 Immunofluorescense analysis of hstabilin-1 protein localization

Acetone-fixed Mϕ2 cells (**A**, **B**) and a xylol-fixed spleen section (**C**, **D**) were stained with a combination of mouse MS-1 mAb and rabbit polyclonal anti-hstabilin-1, followed by incubation with mixture of Cy2-conjugated anti-mouse and Cy3-conjugated anti-rabbit secondary Ab. The green colour corresponds to MS-1 staining (**A**, **C**) and the red colour corresponds to anti-hstabilin-1 staining (**B**, **D**).

cells (Figure 4B). Furthermore, in the mouse embryo, mstabilin-1 expression is a closely regulated early event (Figure 4C).

Analysis of hstabilin-1 protein localization

The localization of natural hstabilin-1 protein was analysed in cultivated Mϕ2 cells on day 6 of stimulation with IL-4 using indirect immunofluorescense. The MS-1 mouse mAb, as well as the rabbit polyclonal anti-hstabilin-1 antibody, recognized identical patterns of hstabilin-1 distribution (Figures 5A and 5B). Stabilin-1 was exclusively localized to cytoplasmatic granular structures. A similar hstabilin-1 subcellular-distribution pattern was observed in 293-hstabilin-1 cells (results not shown).

Spleen sections were stained by the combination of MS-1 mAb and polyclonal anti-hstabilin-1 antibody. An identical staining pattern corresponding to spleen sinusoidal endothelium was detected with both antibodies (Figures 5C and 5D). These data confirm the identity of hstabilin-1 and MS-1 antigen.

Phylogenetic analysis

The FD is used in many different organisms, including bacteria, plants, and lower and higher animals. A phylogentic analysis of representative members of this superfamily resulted in a phylogenetic tree that shows formation of four major clusters:

1. Bacterial fasciclins
2. Stabilins and the *Drosophila melanogaster* (fruitfly) fasciclins
3. Extracellular-matrix protein βIG-H3/osteoblast specific factor-2 and the butanol-extracted proteins (1 and 4) from sea urchins ('beps')
4. Plant fasciclins

A closer analysis of the FD-containing proteins found in higher animals shows that the osteoblast specific factor-2/βIG-H3 group and the stabilins define clear-cut protein families.

The X-link-domain-containing proteins are organized into three subgroups:

1. Link-protein homologues
2. CD44 subgroup
3. Proteins sharing a higher homology with TSG-6 [20]

A phylogenetic analysis based on a multiple sequence alignment of representative X-link domains (roughly 90 amino acids) reveals a clustering of the stabilin proteins within the TSG-6 subgroup.

DISCUSSION

In the present study we have purified and cloned four new high-molecular-mass transmembrane proteins, namely hstabilin-1, hstabilin-2, mstabilinin-1 and mstabilin-2. hstabilin-1 is identical with MS-1 antigen, as shown by the use of a polyclonal antiserum against the recombinant protein and is confirmed here to be associated with non-continuous endothelial cell and Mϕ2 differentiation [3,4,21]. hstabilin-1 is selectively expressed by Mϕ2,

but not by Mϕ1; hstabilin-1 expression in Mϕ2 is induced by Th2 cytokines such as IL-4 and by anti-inflammatory agents such as glucocorticoids, and is inhibited by Th1 cytokines such as IFNγ. Owing to similarities in biochemistry and tissue distribution to those of hstabilin-1, rat stabilin-2 was purified by HA-affinity chromatography and the human and mouse homologues of this liver sinusoidal endothelial-cell HA receptor were cloned by using peptide sequences.

Both stabilin-1 and stabilin-2 are multidomain proteins with seven FDs, multiple EGF-like domains and a single X-link domain. Among these domains, the X-link domain is the best functionally characterized domain and is often responsible for protein binding to HA [22].

HA is a very large polysaccharide present in the extracellular matrix (ECM), at the cell surface and inside the cell. HA functions range from ECM formation to intracellular signalling (reviewed in [23]). There is considerable steady-state turnover of HA that amounts to approx. 5 g daily. Most of it is degraded by sinusoidal macrophages and endothelial cells in the lymph nodes [24], organs that strongly express stabilin-1 and stabilin-2. The remainder of the HA not disposed of in the lymph nodes enters the bloodstream. Removal of HA from the bloodstream is crucial for maintaining the blood at an acceptable viscosity, and this is most efficiently performed by liver sinusoidal endothelial cells [25]. Stabilin-2 may well play this role, as antibodies generated against the purified natural protein were able to block HA endocytosis [12]. This is also in accordance with the bioinformational finding that stabilin-2 preferentially localizes to the plasma membrane.

In contrast with stabilin-2, stabilin-1 did not bind to HA under the conditions used for HA-affinity chromatography (results not shown). Even when conditions were used as described for HA binding of the X-link protein TSG-6, recombinant hstabilin-1 did not bind. This may be either due to the primary sequence of stabilin-1, to non-complete processing of the recombinant hstabilin-1 in 293 cells or to discrepancies between *in vitro* conditions and *in vivo* requirements for HA–stabilin-1 complex formation. With respect to the latter possibility, it has been described that HA – in addition to steady-state turnover – is also involved in inflammation and in wound healing. During these processes, oligomers of depolymerized HA of 4–14 oligosaccharides in length are generated and induce activation of antigen-presenting cells, whereas high-molecular-mass HA (1000–200 kDa) is ineffective [26]. As far as polarization of antigen-presenting cells is concerned, HA fragments induce Mϕ1 and synergize with the Th1 cytokine IFNγ in the process of pro-inflammatory cytokine induction in Mϕ1 [27]. In Mϕ2, however, expression of pro-inflammatory chemokines is not induced upon HA stimulation. In conjunction with the selective expression of stabilin-1 in Mϕ2, but not in Mϕ1, the question arises as to whether HA oligomers may induce anti-inflammatory pathways in these cells [28] and whether stabilin-1 might mediate this reaction.

Similar to the preferentially intracellular receptor of HA mediated motility (RHAMM) that serves cellular locomotion along HA fibrils [29], stabilin-1 was not shown to occur on the plasma membrane, but to preferentially localize to cytoplasmic granular structures in permeabilized cells transfected with recombinant stabilin-1 as well as in natural Mϕ2. This is supported by the bioinformational finding of strong endosomal sorting signals in stabilin-1. Nevertheless, localization to sites of cell contact has been shown by immunoelectron microscopy for stabilin-1 in splenic sinusoidal endothelial cells [1]; furthermore, stabilin-1 is known to occur in Mϕ2 in close proximity to the plaque-like fibronexus integrating intracytoplasmic (intermediate filament) and extracellular (ECM) requirements for spatial stabilization [10]. In order to function as a receptor, stabilin-1 – similarly to RHAMM – might shuttle to the plasma membrane under certain, yet undefined, conditions. As has been described for furin, the shuttle between the endosomal compartment/*trans*-Golgi network and the plasma membrane may depend on the phosphorylation state of the sorting signals [30].

Among the other domains of the stabilins, the FDs stand out, as they are present in proteins involved in adhesion and cell sorting in several distantly related organisms such as algae (*Volvox*) [31], sea-urchins (*Paracentrotus lividus* [32] and *Anthocidaris crassispina* [33]), the fruitfly (*D. melanogaster*) [34] and mammalian species (human [35] and mouse [36]). The prototype molecule of the FD-containing superfamily is the *Drosophila* fasciclin I neural-cell adhesion protein, which is implicated in a variety of functions, such as axon guidance, formation of embryonic axon commissures and synaptic plasticity [34]. The functional unit in proteins mediating adhesion was supposed to be the FD, since the fasciclin I protein is composed almost entirely by four FDs. Recently, the mechanism of FD-dependent adhesion was directly demonstrated for ECM protein βIG-H3. Two out of four FDs were involved in interaction with integrin expressed on human corneal epithelial cells [37]. However, the relationship between structural diversity, the number of FDs and protein function is unclear, since it ranges from one FD in mycobacterial MPB70 protein to seven FDs in stabilin-1 and stabilin-2 and comprises a set of highly conservative motifs spaced by diverse non-conservative fragments [31,38].

In addition to seven FDs with a putative cell-adhesion function, both stabilins contain multiple EGF-like domains also supposed to mediate cellular adhesion properties [39]. The presence of multiple different domain modules responsible for cell adhesion and interaction with ECM is indicative of concomitant or sequential interactions with multiple partners leading to the integration of extracellular events with intracellular signal transduction. These features are well known from other cell adhesion and attachment proteins, including the immunoglobulin and cadherin superfamilies. Owing to their unique common domain structure, the high level of protein sequence similarity and the phylogenetic relationship, the stabilins constitute a novel protein family. The molecular mechanisms of stabilin function and their physiological role in vascular function and angiogenesis as well as in antigen presentation and immune reactions remain to be elucidated.

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