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Stabilin-1, a homeostatic scavenger receptor with multiple functions

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Abstract

The multifunctional scavenger receptor stabilin-1 (STAB1, FEEL-1, CLEVER-1, KIAA0246) was originally identified as the MS-1 antigen, expressed by sinusoidal endothelial cells in human spleen. Extensive histological studies revealed that stabilin-1 is also expressed by tissue macrophages and sinusoidal endothelial cells in the healthy organism; its expression on both macrophages and different subtypes of endothelial cells is induced during chronic inflammation and tumorigenesis. *In vitro* induction of stabilin-1 in macrophages requires the presence of glucocorticoids. Stabilin-1 is involved in two intracellular trafficking pathways: receptor mediated endocytosis and recycling; and shuttling between the endosomal compartment and trans-Golgi network (TGN). The latter intracellular pathway of stabilin-1 trafficking is mediated by GGAs, clathrin adaptors that interact with the DDSLL motif in the cytoplasmic tail of stabilin-1. When expressed by alternatively activated macrophages, stabilin-1 mediates the uptake and targeting for degradation of acLDL and SPARC, a regulator of tissue remodeling. Likewise, stabilin-1 in macrophages is involved in intracellular sorting and lysosomal delivery of the novel stabilin-1-interacting chitinase-like protein (SI-CLP). Indirect evidence suggests that stabilin-1 is involved in adhesion and transmigration in various cell types (including tumor cells, leukocytes, and lymphocytes); however, its rapid recycling and scant level of surface expression argue against its universal role in cell adhesion. In summary, stabilin-1 is a homeostatic receptor which links signals from the extracellular environment to intracellular vesicular processes, creating a potential impact on the macrophage secretion profile.

Keywords: scavenger receptor • macrophage • sinusoidal endothelial cells • fasciclin domain • chitinase-like protein • SPARC

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Identification of the MS1 antigen and cloning of the stabilin family

The first member of the stabilin family, human stabilin-1, was originally identified by Goerdt *et al.* as the MS-1 antigen, a selective histological marker for noncontinuous sinusoidal endothelial cells in human spleen with no expression in continuous endothelium of the same organ [1]. The monoclonal antibody (mAb) MS-1 raised against human spleen was selected by immunohistological reactivity with cadherin-negative noncontinuous endothelium. Biochemical analysis revealed the complex structure of the MS-1 antigen. In human spleen lysates, immunoprecipitation with the MS-1 mAb followed by SDS-PAGE with reducing conditions resulted in the identification of three specific bands migrating with approximate molecular weights (MW) of 320, 215 and 120 kDa. Immunoprecipitation from myeloid leukemia KG-1 cells revealed specific bands with approximate MWs of 300, 280, 205 and 120 kDA. Additional experiments using nonreducing conditions, pulse-chase and proteolysis analysis suggested that the MS-1 antigen is first synthesised as a 280 kDa precursor, followed by formation of a 300 kDa mature form. Variable cleavage of the 300 kDa protein results in 205 and 120 kDa products. We anticipate that the final array of stabilin-1 forms will be found to be even more complex and cell-type specific, as various high molecular weight isoforms were detected in later studies performed by us and other research groups [2–4].

Using the MS-1 mAb, we cloned the cDNA for the MS-1 antigen [5]. Applying classical biochemical methodology, the MS-1 protein was purified and analyzed by MALDI-TOF. The MALDI-TOF MS chromatogram allowed partial verification of the cDNA sequence. Using 3' and 5'-RACE, a full length cDNA of about 8 kb was generated, with a corresponding amino acid sequence that represents a predicted 280 kDa protein. Northern blot analysis revealed selective expression of the cDNA in tissues with noncontinuous endothelial cells (spleen, liver, lymph nodes) and in alternatively activated macrophages *in vitro*. These data very well correlated with the results of previous immunochemical findings and supported the identity of the MS-1 cDNA and the MS-1 antigen [1, 6, 7]. Antibody generated against the recombinant GST-tagged cytoplasmic tail of stabilin-1 recognized the same pattern as the MS-1 mAb in double immunofluorescence, finally confirming the identity of the MS-1 antigen and stabilin-1 [5].

Bioinformatic analysis of the cDNA sequence revealed that stabilin-1 is a type I transmembrane protein with clusters of EGF-like and fasciclin domains (cell/matrix interactions and homotypic adhesion, respectively) and with one putative hyaluronan (HA)-binding Link domain (Fig. 1). Stabilin-2, a homologous protein cloned in parallel with the stabilin-1 cDNA, appears to be a true HA receptor of the hepatic sinusoidal endothelial cells [5, 8]. Stabilin-1 and stabilin-2 are 55% identical on the protein level, but show no significant DNA homology. The murine stabilins have also been cloned and analyzed: The homology between human and mouse is 86% for stabilin-1 and 79% for stabilin-2, an indication of the high evolutionary conservation of these two proteins. Interestingly, the major differences between the two stabilins are in the C-termini. Stabilin-2 contains a classical tyrosine-based endosomal sorting signal, while stabilin-1 contains a unique dileucine-based sorting motif [5, 9].

Tissue and cell-type specific expression

Extensive analysis of human tissues using the MS-1 mAb revealed stabilin-1 expression primarily in two cell types: tissue macrophages and noncontinuous endothelial cells (ECs) (see Table 1 for summary). Human sinusoidal ECs in spleen, liver, adrenal cortex, and lymph nodes express high levels of stabilin-1 [1, 3, 10, 11]. Interestingly, in lymph nodes stabilin-1 is expressed not only by sinusoidal ECs, but also by sinusoidal macrophages (Fig. 2). In contrast, stabilin-1 has never been detected in Kupffer cells (liver macrophages), which are located in close proximity to liver sinusoidal ECs [11]. Sinusoidal ECs form noncontinuous endothelium, whereas the endothelium in most blood vessels of a healthy organism is continuous. During angiogenesis rapidly growing blood vessels pass though a temporary noncontinuous state. Studies performed by us and others, using different antibodies, demonstrated the expression of stabilin-1 in blood vessels in various angiogenic conditions,

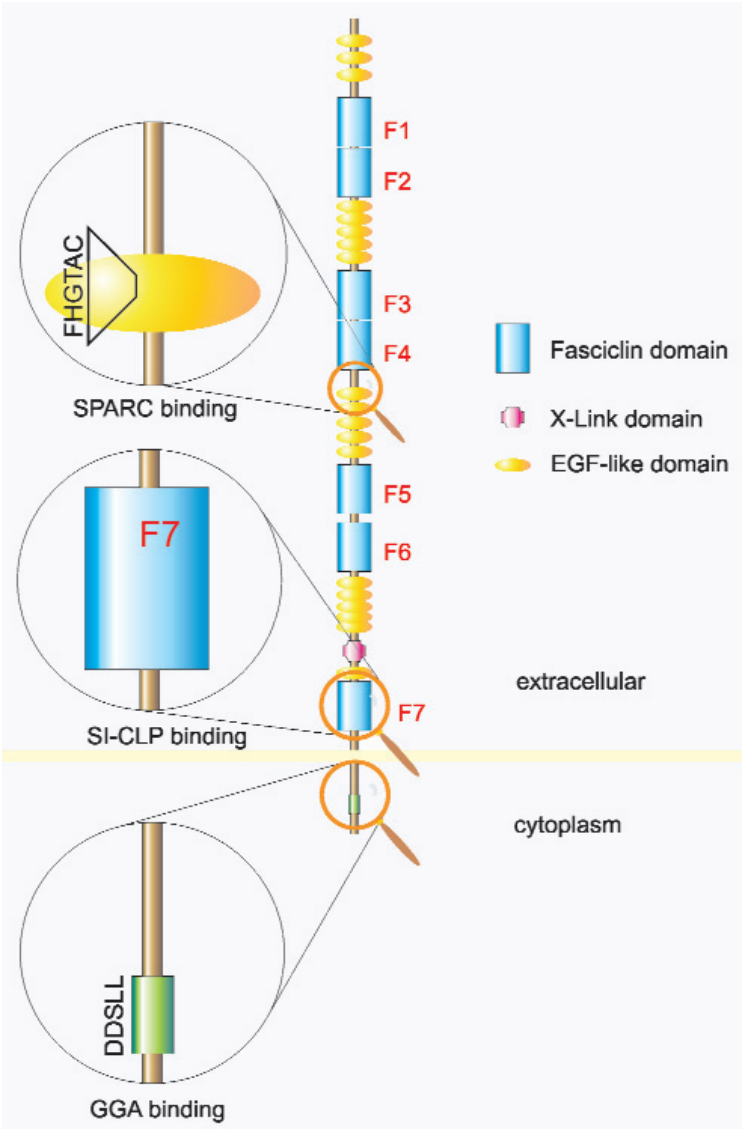

Fig. 1 Stabilin-1 domain organization and binding sites for ligands. The large extracellular fragment of stabilin-1 is composed of seven fasciclin domains (blue cylinders F1-F7) and multiple EGF-like domains (yellow ovals). The Link domain is shown in pink. SPARC binds to the EGF-like domain located between F4 and F5; the FHGTAC sequence was identified as a SPARC-binding peptide by phage display. The chitinase-like protein SI-CLP binds to F7. The short cytoplasmic tail contains the DDSLL motif, which interacts with intracellular sorting adaptors GGA1, GGA2 and GGA3L.

including wound healing, tumor vascularization, and chronic inflammatory conditions of the skin such as psoriasis [4, 6].

There is less agreement concerning the presence of stabilin-1 in lymphatic endothelium. A search with the MS-1 mAb for the expression of stabilin-1 in lymphatic vessels in different organs revealed a very weak signal only in healthy human skin [12]. Prevo *et al.* used a panel of antibodies against various domains of stabilin-1 to carefully examine localization of stabilin-1 in lymphatic vessels [3]. Only the anti-CLEVER-1 3-372 mAb weakly recognized stabilin-1 in lymphatic vessels in frozen sections of human skin; antibodies generated against the last 14 amino acids of the stabilin-1 cytoplasmic tail and against the Link domain failed to recognize stabilin-1 in LYVE-1 positive vessels in various organs, including stomach and colon. In contrast, the Jalkanen group demonstrated that the anti-CLEVER-1 3–372 mAb is able to detect stabilin-1 in lymphatic vessels in various tissues and in various physiological conditions [4, 13, 14] (see Table 1 for details). It is currently not clear whether the discrepancies between different studies are due to the differences in experimental procedures, such as fixation or staining conditions, or whether various antibodies recognize distinct, cell-type specific epitopes in stabilin-1.

Macrophage model systems revealed that stabilin-1 expression *in vitro* requires stimulation by glucocorticoids. In human monocyte-derived macrophages, the stabilin-1 protein can be detected

Table 1 Cell-type specific expression of stabilin-1

Antibody used in the study	Tissue or cell type specificity		Reference
	Endothelial cells (EC)	Macrophages (MΦ)	
MS-1	Sinusoidal EC of spleen, lymph node, liver, adrenal cortex, HEV in tonsils (human)	MΦ in placenta (human)	Goerdt *et al.*, *J Cell Biol.*, 1991 [1]
MS-1	Weak expression in skin LEC (human)	Subpopulation of skin macrophages ("dermal dendrocytes") (human)	Walsh *et al.*, *Lab Invest.*, 1991 [12]
MS-1	EC of continuous origin in wound healing, T-cell lymphoma, psoriasis, melanoma metastasis (human)	MΦ in skin lesion of Kaposi's sarcoma and in juvenile xanthogranuloma; *in vitro* monocyte-derived $MΦ_{Dex}$ and $MΦ_{IL-4/Dex}$ (human)	Goerdt *et al.*, *Am J Pathol.*, 1993 [6]
MS-1		MΦ in non-Langerhans cell histiocytosis	Goerdt *et al.*, *J Pathol.*, 1993 [78]
MS-1	EC in synovial tissue of patients with rheumatoid arthritis and osteoarthritis (human)	MΦ in synovial tissue of patients with rheumatoid arthritis and osteoarthritis	Szekanecz *et al.*, *Arthritis and Rheumatism*, 1994 [79]
MS-1, F4		*In vitro* monocyte-derived M IL-4/Dex	Politz *et al.*, *Bioch J.*, 2002 [5]
3-372	LEC, sinusoids and HEV in lymph nodes (human); HEV-like vessels in inflamed synovium, sinusoids in lymph nodes (rabbit)		Irjala *et al.*, *Eur J Imm.*, 2003 [14]
3-372	Peritumoral LEC in HNSCC and breast carcinoma); HEV-like vessels (human)		Irjala *et al.*, *Cancer Research*, 2003 [13]
MS-1		Decidual MΦ (human)	Cupurdija *et al.*, *Amer J of Reprod Immun.*, 2004 [80]
3-372	LEC in normal and psoriatic skin; blood ECs in psoriatic skin (HEV-like); cultured vascular and lymphatic EC (human)		Salmi *et al.*, *Blood*, 2004 [4]
F4		*In vitro* monocyte-derived M IL-4/Dex	Kzhyshkowska, *J Leuk Biol.*, 2004 [9]
a-stabilin-1 CT, a-stabilin-1 LINK 3-372	HEV and sinusoidal ECs in lymph nodes; weak expression in skin lymphatic EC; primary LSEC; skin-derived LEC; HUVEC (human)	Individual MΦ in colon, stomach, skin (human)	Prevo *et al.*, *JBC* 2004 [3]
F4	Primary LSEC (rabbit, pig)		Hansen *et al.*, *Exp Cell Res.*, 2005 [10]
MS-1, F4	Sinusoidal EC in lymph nodes (human)	Sinusoidal MΦ in lymph nodes (human)	Martens *et al.*, *J Pathol.*, 2006 [11]
1.26		MΦ associated with tumorigenesis and angiogenesis; bone marrow derived $MΦ_{IL-4/Dex}$ (mouse)	Schledzewski *et al.*, *J Path.*, 2006 [76]

Abbreviations used: EC- endothelial cells; **HUVEC** - human umbilical vein endothelial cells; **LEC** - lymphatic endothelial cells; **LSEC** - liver sinusoidal endothelial cells; **MΦ** - macrophages; **HEV** - high endothelial venules; **mAb** - monoclonal antibody; **HNSCC** - head and neck squamous cell carcinoma; **MS-1** - mouse mAb against human spleen; recognise extracellular part of stbailin-1, precise epitope unknown; **F4** - rabbit polyclonal Ab against recombinant cytoplasmic tails of humans stabilin-1; **a-CLEVER-1 3-372** - mouse mAb against isolated efferent lymphatic vessels of human lymph nodes; recognise extracellular part of stbailin-1, precise epitope unknown; **1.26** - mouse mAb against N-terminal fragment of stabilin-1; **a-stabilin-1 CT** - rabbit polyclonal Ab against synthetic peptide (last 14 aa from stabilin-1 cytoplasmic tail); **a-stabilin-1 LINK** - rabbit polyclonal against Link domain of human stabilin-1 (Gly^{2208}-Val^{2305}).

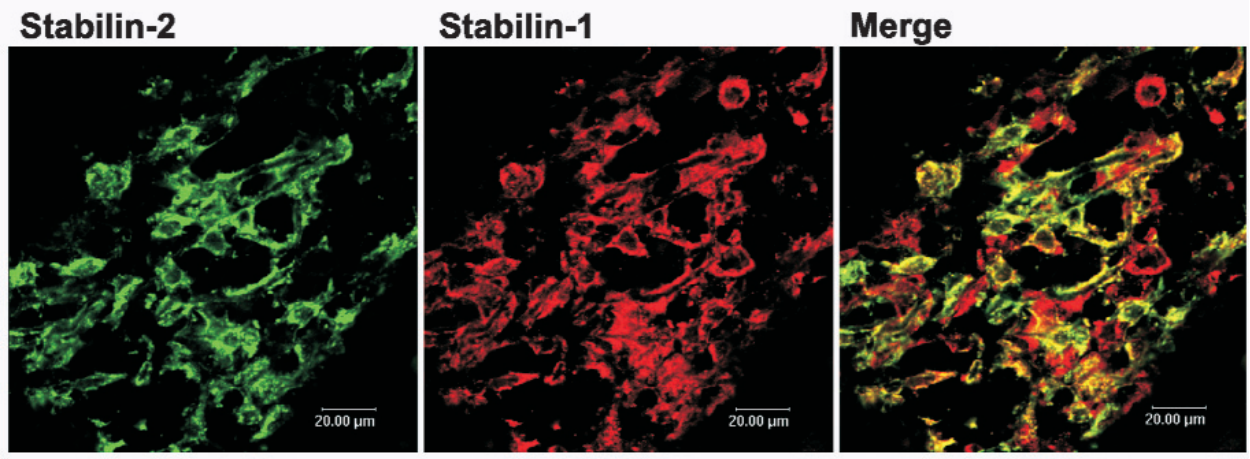

Fig. 2 Stabilin-1 and stabilin-2 distribution in human lymph node sinus. Stabilin-1 (red) is localized in both macrophages and endothelial cells in lymph node sinus. Stabilin-2 is exclusively localized in sinusoidal ECs. Yellow color in merge indicates co-localization of stabilin-1 and -2 in ECs. Red cells in merge are stabilin-1-positive macrophages. Confocal microscopy was performed on acetone-fixed lymph node cryo-sections.

in cultures stimulated by IL-4 in combination with dexamethasone or by dexamethasone alone (specifically, in alternatively activated macrophages), while IFNγ has a negative effect on stabilin-1 expression [5, 6, 9].The apparent upregulation of stabilin-1 mRNA under IL-4 stimulation in early studies was due to the propagation of macrophages in the presence of fetal calf serum which was not glucocorticoid depleted [5, 6]. Stabilin-1 is used as a specific marker for alternatively activated macrophages *in vivo* [15, 16].

Both tissue macrophages and sinusoidal endothelial cells are professional scavenging cells. Their major common function is clearance of non-self and unwanted self components from circulating blood and lymph (sinusoidal cells) and from extracellular space in tissues (*via* tissue macrophages). This barrier activity is requisite for maintaining homeostatic balance in tissues and in the organism. As we recently demonstrated, the scavenging function of macrophages *in vitro* is stimulated by glucocorticoids [17]. The expression of the type I transmembrane protein stabilin-1 in both cell types suggested its potential function as a scavenger receptor. Detailed investigation of the intracellular trafficking pathways of stabilin-1 and ligand studies performed in various systems provided further evidence for the function of stabilin-1 as a specific clearance receptor in sinusoidal ECs and macrophages.

Intracellular distribution and interaction with adaptor machinery

The identity of stabilin-1 as a surface receptor has been controversial for some time, since all previous histological data has indicated its predominant localization in intracellular vesicles in both macrophages and sinusoidal endothelial cells. FACS analysis of enhanced sensitivity provided us our first indication that a small portion of stabilin-1 is present on the surface of alternatively activated macrophages [9]. Detailed investigation of stabilin-1 trafficking in alternatively activated macrophages revealed the complex function of this receptor (Fig. 3). First pathway of stabilin-1-trafficking is classical for an endocytic receptor. Specifically, stabilin-1 was found to be predominantly localized in EEA-1- positive early endosomes; a minor portion was found in late endosomes. (Using fluorescently labelled transferrin, we also demonstrated that a portion of stabilin-1 recycles to the cell surface.)

However, stabilin-1 was unexpectedly found in the second intracellular vesicular pathway. Using double immunofluorescence, we detected stabilin-1 in the TGN; at the same time, its absence in Golgi indicated that its localization in the TGN is not due to accumulation of freshly synthesized stabilin-1 (Fig. 3). Treatment of macrophages with brefeldin A induced accumulation of stabilin-1 in the TGN and its depletion from early endosomes, indicating

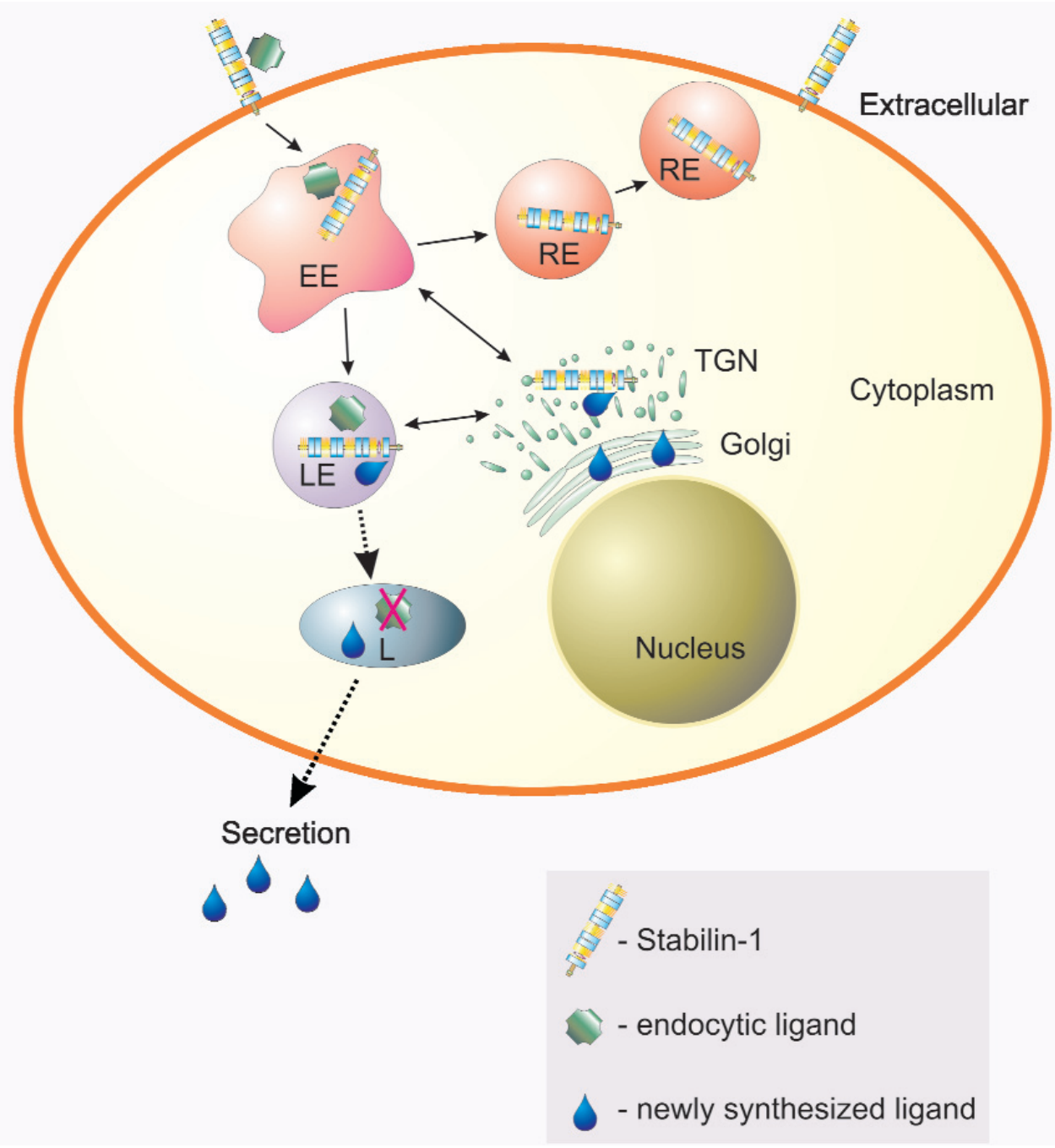

Fig. 3 Schematic representation of stabilin-1 trafficking pathways in macrophages. Upon binding to surface-expressed stabilin-1, ligand is internalized and delivered to early/sorting endosomes (EE). A portion of the ligand-free receptor can recycle back to the cell surface *via* recycling endosomes (RE). Stabilin-1 delivers its ligand to late endosomes (LE). No stabilin-1 had been identified until now in Lamp-1-positive lysosomes, indicating that ligands dissociate from stabilin-1 in late endosomes. Endocytic ligands (green) are further degraded in lysosomes. Stabilin-1 is involved in the second intracellular sorting process; it shuttles between endosomal and biosynthetic compartments *via* the TGN. We propose a model whereby newly synthesized ligand (dark blue) is recognized by stabilin-1 in the late Golgi compartment and delivered to early or late endosomes and so to lysosomes. Macrophages which actively use lysosomal secretion pathways can secrete stabilin-1-interacting ligands into the extracellular space.

that stabilin-1 shuttles between the endosomal system and biosynthetic compartments. Our analysis of stabilin-1 trafficking in rat liver sinusoidal endothelial cells demonstrated that stabilin-1 is involved in the endocytic pathway, but no evidence was found for its shuttling between the endosomal system and the TGN [10]. Searching for the intracellular sorting machinery used by stabilin-1, we found that its cytoplasmic tail interacts with GGAs. GGAs (Golgi-localized, γ-ear-containing, Arf (ADP-ribosylation factor)-binding proteins) function as clathrin adaptors for intracellular sorting of mannose-6 phosphate receptors [18]. GGAs are highly specialized adaptors responsible for TGN/endosomal shuttling of the cation-independent mannose 6-phosphate receptor (CI-MPR) and sortilin, both of which combine endocytic and sorting functions [19–21]. The dual function of CI-MPR includes receptor-mediated endocytosis of insulin-like growth factor-II, and sorting of newly synthesized lysosomal hydrolases from the Golgi compartment to late endosomes followed by delivery to lysosomes [19]. Similarly, shuttling of stabilin-1 between the biosynthetic and endosomal/lysosomal compartments in macrophages is mediated by GGAs, which bind to the DDSLL motif in its cytoplasmic tail [9] (Fig. 3). The similarity between stabilin-1 and CI-MPR in intracellular routing was intriguing. Given that CI-CMR is ubiquitously expressed, and stabilin-1 is highly specific for tissue macrophages, we suggested that stabilin-1 is the first known macrophage-specific receptor that combines endocytic and intracellular sorting functions. Below we present the evidence demonstrating ligand specificity for both the endocytic and intracellular sorting functions of stabilin-1.

Stabilin-1 as a scavenger receptor: endocytosis of acLDL

Many of the classical scavenger receptors (SRs) expressed by sinusoidal ECs and macrophages are characterized by a repertoire of overlapping ligands [22, 23]. Originally, SRs were defined by the ability to endocytose modified lipoproteins (reviewed in [24]. Macrophages express a panel of SRs which can bind, internalize and target for degradation modified forms of LDL, such as oxLDL and its analog acetylated (ac)LDL [24]. Therefore we chose CHO cells lacking the endogenous scavenger receptors for acLDL (including stabilin-1) and used them for stable ectopic expression of stabilin-1 in order to analyze its specific contribution in the uptake of acLDL. FACS analysis and confocal microscopy revealed that stably expressed stabilin-1 functions as an efficient endocytic receptor for acLDL in CHO cells. Similar results were obtained by other groups with CHO [25] and HeLa cells [3] transiently transfected with stabilin-1. Further, we demonstrated that stabilin-1/acLDL trafficking along the lysosomal-targeted endocytic pathway requires PI3K activity [2]. Class III PI3K is responsible for the production of phosphatidylinositol-3-phosphate (PtdIns(3)P) [26, 27], which is found primarily in the membranes of early endosomes and the internal vesicles of multivesicular endosomes in mammalian cells [28, 29]. Membrane-incorporated PtdIns(3)P is recognized by FYVE-domain-containing proteins and sorting nexins. This recognition event is required for Rab-5-dependent endosomal fusion and consequent vesicular trafficking [30, 31]. We found that internalization of acLDL and delivery to early endosomes mediated by stabilin-1 were not affected by the PI3K-specific inhibitor wortmannin. However, the translocation from early endosomes to late endosomes was blocked by wortmannin, resulting in the arrest of stabilin-1/acLDL in swollen EEA-1 positive compartments [2]. Its dependence on PI3-K activity confirmed that stabilin-1 uses the classical endocytic pathway [32] for the trafficking of extracellular ligands.

Stabilin-2

Stabilin-2 (FEEL2; HARE (hyaluronan receptor for endocytosis)) is the closest homolog of stabilin-1; together they comprise the family of fasciclin-like hyaluronan (HA) receptor homologs [5]. Stabilin-2 was originally identified as an endocytic receptor in rat liver sinusoidal endothelial cells (LSECs) by antibody-mediated inhibition of the uptake of HA. The same study reported that stabilin-2 has affinity for the N-terminal propeptide of type I procollagen [8]. Weigel *et al.* demonstrated that HA accumulation by cultured LSECs was inhibited > 98% by an antibody against stabilin-2 (HARE) and unaffected

by antibodies to ICAM-1 or CD44, indicating that virtually all specific HA uptake is mediated by stabilin-2 and not by ICAM-1 or CD44 [33]. Stabilin-2 can exist in two major isoforms: 175/300 kDa and 190/315 kDa for rat and human respectively. Short form of stabilin-2 is sufficient to mediate uptake of HA and its targeting for degradation[34–38]. Stabilin-2 has a very similar domain organization to stabilin-1, but differs from stabilin-1 by its absence in macrophages and its exclusive expression in sinusoidal endothelial cells [5, 39] (Fig. 2). A significant difference is observed in the cytoplasmic tails of stabilin-1 and stabilin-2. Stabilin-2 has the classical motif for binding the μ-subunit of AP-2, which is not present in the stabilin-1 cytoplasmic tail. However, there are no GGA-specific sorting motifs in the cytoplasmic tail of stabilin-2; and an in vitro pull-down assay performed by us confirmed the absence of GGA binding to stabilin-2 [9]. Stabilin-2 is much more abundant on the cell surface than stabilin-1; up to 50% of the total receptor was found to be expressed in plasma membrane [37]. Currently, stabilin-2 is recognized as a classical scavenger receptor responsible for the clearance and degradation of diverse modified, unwanted self products [5, 8, 10, 38, 40]. Stabilin-2 ligands include hyaluronan (HA), AGE-modified BSA, formaldehyde-treated BSA, collagen N-terminal propeptides, and acLDL [10, 25, 38, 40].

Stabilin-1 and ligands of stabilin-2

Of the various stabilin-2 ligands, stabilin-1 expresses weak binding activity only with AGE-BSA; however, the physiological significance of this in vitro interaction remains unclear [40]. Since stabilin-1 carries the lectin-like Link domain module, known to function as an HA-binding site in such receptors as CD44, LYVE-1 and CD44, much attention has been given to the examination of its functional status [3, 4, 10, 41]. However, all attempts to detect the binding of HA to stabilin-1 have failed, despite the use of various *in vitro* systems and various sources of HA. Currently, the Link domain of stabilin-1 is considered to be rudimentary and nonfunctional.

Phagocytosis performed in CHO cells transfected with stabilin-1 and stabilin-2 suggested that both proteins are involved in the internalization of bacteria (*E. coli* and *S. aureus*) [25]. However, the authors did not demonstrate co-localization of internalized bacteria with ectopically expressed stabilin-1 and –2, either in the same cells nor in intracellular vesicles. Moreover, quantification of phagocytosis by FACS showed effective phagocytosis of fluorescent bacteria in the control non-transfected CHO cells. The putative competition effect of the anti-stabilin-1 mAb FE1-1 on stabilin-1-transfected cells was not controlled by the addition of FE1-1 to nontransfected cells nor by incubation of the stabilin-1-transfected cells with an isotype control. In addition increased bacterial uptake in the cells transfected with stabilin-1 and -2 cells can be explained by the increased scavenging capacity of isolated clones, an effect for which there were no parallel control experiments with stabilin-independent ligands. Thus, involvement of stabilin-1 and -2 in recognition and internalization of bacteria remains to be proven. The current state of knowledge indicates that the only ligand shared by stabilin-1 and stabilin-2 is acLDL [25] and our unpublished data).

Endocytosis of SPARC

The homeostatic function of stabilin-1 was initially suggested by the identification of an unusual function: internalization and clearance of SPARC, a universal regulator of tissue remodeling. The matricellular glycoprotein SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin and BM-40) is a soluble, nonstructural component of extracellular matrix (ECM). SPARC is a crucial regulator of developmental processes, tissue remodeling, angiogenesis, and wound healing. Its multiple biological functions include active modulation of ECM organization, binding of growth factors, and induction of an anti-adhesive state in different cell types [42, 43]. SPARC modulates tumor-stromal cell interactions that are dependent on vascularization, immune reactions, and the type of malignancy [44]. Despite the multiple effects of SPARC in development and pathology, the cellular receptor(s) transducing responses to and regulating the activity and extracellular concentration of SPARC have remained unknown for over two

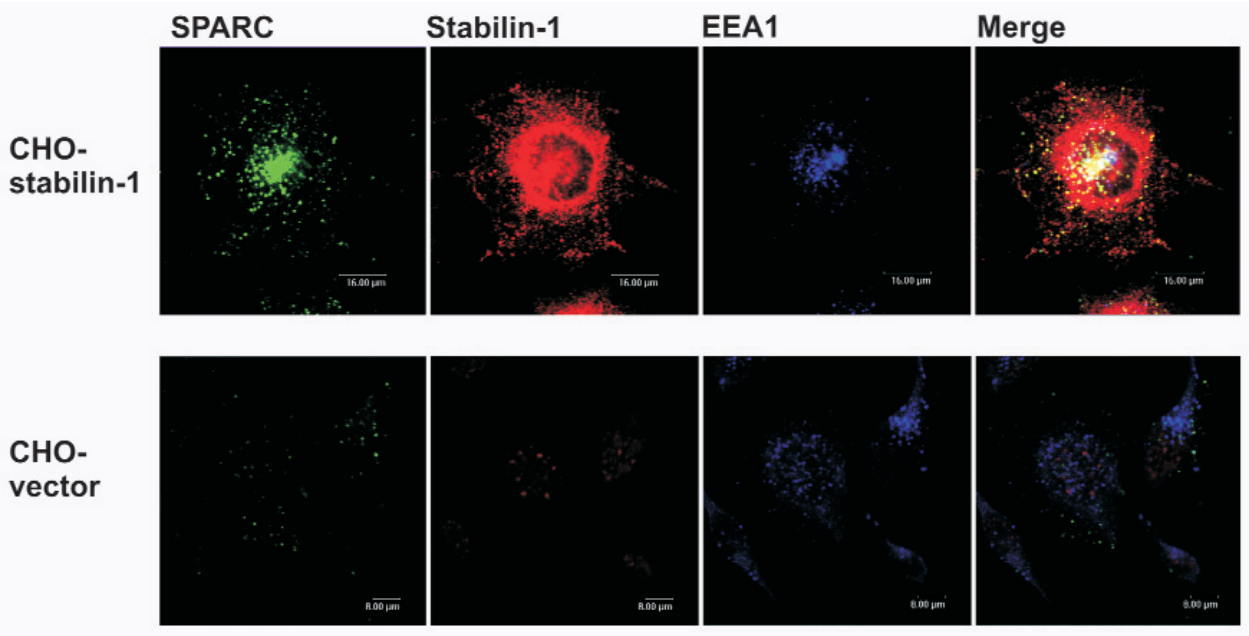

Fig. 4 Stabilin-1 in CHO cells mediates endocytosis of SPARC. Fluorescently labeled SPARC (green) was incubated with CHO cells stably transfected with stabilin-1 (red). At 30 minutes, most of the SPARC was delivered by stabilin-1 to EEA-1 positive early/sorting endosomes (blue). Sites of triple co-localization are shown in white. SPARC and stabilin-1 also co-localized in endocytic vesicles (shown in yellow) during their migration to EEA-1-positive compartments. In control CHO cells stably transfected with empty vector, very low levels of SPARC were observed, none of which was delivered to early endosomes.

decades. In our recent collaborative work, we identified stabilin-1 as a cellular receptor for SPARC, starting with phage display technology [45]. By *in vitro* binding assays we demonstrated that stabilin-1 interacts with SPARC through the extracellular EGF-like domain containing the sequence FHGTAC. *In vivo* endocytosis assay showed that stabilin-1 mediates the internalization and delivery of SPARC to the endocytic pathway in stably transfected CHO cells (Fig. 4), an observation further supported by the concentration-dependent competition of acLDL with SPARC internalization. Stabilin-1-expressing alternatively-activated macrophages, when stimulated by IL-4 in combination with dexamethasone, endocytose SPARC efficiently. In contrast, stabilin-1-negative macrophages stimulated by IL-4 alone or by IFNγ were unable to perform receptor-mediated uptake of SPARC. In stabilin-1-positive macrophages, fluorescently labeled SPARC was delivered into EEA-1 positive early endosomes, trafficked into late endosomes, and then partially degraded (43, and our unpublished observations). Endocytosis experiments performed in parallel time-courses with fluorescently labeled SPARC and acLDL demonstrated that the kinetics of trafficking and degradation along the endocytic pathway and were similar for both stabilin-1 ligands. The SPARC and the acLDL were rapidly (within 5 minutes) endocytosed, and their degradation commenced at approximately 30 minutes. The major portion of the fluorescent signal disappeared after two hours of uptake. Stabilin-1 appears to be the major macrophage receptor mediating the uptake and clearance of SPARC.

This study not only described SPARC/stabilin-1 as a ligand/receptor pair, but also identified a novel function of alternatively activated macrophages and a novel mechanism for the regulation of SPARC activity. Through the uptake and partial degradation of SPARC, alternatively activated macrophages can actively regulate extracellular concentrations of SPARC and adjust them to the changing physiological needs of the organism. In pathological situations, SPARC activity can be controlled by macrophages, which have a flexible phenotype and constitute the major regulatory immunological

component of a tissue. An intriguing question is the physiological role of stabilin-1-mediated SPARC endocytosis in endothelial cells. Notably, blocking experiments with the anti-stabilin-1 mAb FE1-1 on HUVEC cells have revealed that stabilin-1 is involved in the regulation of angiogenesis *in vitro*. The questions of whether this effect is related to the endocytic function of stabilin-1, and whether FE1-1 can interfere with stabilin-1-mediated SPARC uptake, remain to be investigated.

Intracellular sorting of the novel chitinase-like protein SI-CLP

The shuttling of stabilin-1 between endosomal and biosynthetic compartments and its interaction with GGA adaptors suggest its functional homology with CI-MPR, a ubiquitously expressed sorting receptor for lysosomal hydrolases [19]. The highly selective expression of stabilin-1 in alternatively activated macrophages and sinusoidal endothelial cells suggest that its sorting function is also cell-type specific. Since the most abundant stabilin-1 expression was found in placental macrophages, we performed a yeast two-hybrid screen of a human placental cDNA library by which we were able to identify an intracellular ligand for stabilin-1: the novel chitinase-like protein SI-CLP (***S***tabilin-***I***nteracting ***C***hitinase-***L***ike ***P***rotein [46]).

SI-CLP is a newly discovered member of the mammalian family of Glyco_18 domain-containing proteins, comprising true enzymes as well as secreted chitinase-like proteins lacking enzymatic activity. Enzymatic activity of human chitinases is linked to host defenses against chitin-containing pathogens and is associated with the progression of asthma [47–53]. At the same time, enzymatically inactive Glyco_18 domain-containing chitinase-like proteins do not bind chitin but rather act as soluble mediators and are involved primarily in tumor progression [54–59] and disorders characterized by changes in the vascular system or ECM remodeling such as rheumatoid arthritis [60], inflammatory bowel disease [61], hepatic fibrosis and cirrhosis [62, 63], and systemic sclerosis [64]. Numerous biological functions have been assigned to mammalian chitinase-like proteins, including mediation of cell differentiation and the activation of proliferation, migration, and adhesion [65–69]. However no receptors for these proteins have been found until now. SI-CLP is classified as a chitinase-like cytokine, due to its domain organization, lack of a catalytic site, and presence in macrophage conditioned medium. Thus, identification of stabilin-1/SI-CLP as a ligand-receptor couple is the first known example of a protein-protein interaction between the mammalian chitinase-like protein and a transmembrane receptor.

The initial indication that an interaction between stabilin-1 and SI-CLP could occur intracellularly was obtained by expression analysis of SI-CLP. Using real-time PCR and the novel rat mAb 1C11, we found that SI-CLP gene expression is strongly upregulated by a combination of IL-4 and dexamethasone in human primary monocyte-derived macrophages ($M\phi_{IL\text{-}4/Dex}$); the same type of stimulation is necessary for induction of stabilin-1 expression [5, 9]. Analysis of markers for various intracellular vesicular compartments by confocal microscopy revealed that SI-CLP is sorted into late endosomes and consequently into Lamp1-positive and secretion-committed CD63-positive lysosomes. Other mammalian Glyco_18 domain-containing proteins are also sorted to lysosomes; however, the lack of N-glycosylation typical of lysosomal enzymes indicates that their lysosomal routing differs from the classical MPR-mediated sorting [70]. Several lines of evidence support the point of view that stabilin-1 acts as a sorting receptor for SI-CLP, at least in $M_{\phi IL\text{-}4/Dex}$. Thus, interaction with SI-CLP appears to be mediated by the extracellular fasciclin 7 (F7) domain of stabilin-1. The extracellular domain of the type-1 transmembrane receptor becomes intravesicular after formation of the endocytic vesicle. Extracellular domain of sorting type 1 transmembrane receptor is also exposed to late Golgi compartments for binding with its newly synthesized cargo protein [18]. Stabilin-1 co-localization with SI-CLP in the dynamic TGN is transient; SI-CLP is probably detached from stabilin-1 due to the low pH in late endosomes, as has been established for many other classical receptor-ligand interactions. When it is transiently overexpressed, stabilin-1 mediates re-localization of recombinant SI-CLP-FLAG in H1299 cells, which lack the endogenous lysosomal sorting machinery for this protein. Moreover, siRNA knock-down of stabilin-1 in $M\phi_{IL\text{-}4/Dex}$ leads to impaired lysosomal sorting

of SI-CLP and its accumulation in perinuclear areas [46]. However, we were able to detect SI-CLP in lysosomes in stabilin-1 negative monocyte-related MonoMac6 cells (Kzhyshkowska, unpublished observations). This fact suggests that stabilin-1 is not the only receptor to mediate trafficking of newly synthesized SI-CLP. Intriguing questions remain: How many other receptors for SI-CLP exist, are they expressed in stabilin-1-positive cells, and do these receptors cooperate with stabilin-1 or antagonize its sorting activity?

Selective receptor-mediated delivery of newly synthesized product to macrophage lysosomes is of great significance for our understanding of the regulatory functions of macrophages. Besides the classical, constitutively operating ER/Golgi secretory pathway, macrophages use nonclassical [71] and lysosomal secretory pathways [72–74]. Constitutive sorting of soluble cargo proteins from the Golgi to the endosomal/lysosomal system is mediated by the ubiquitously expressed mannose 6-phosphate receptors CD-MPR and CI-MPR [19, 75]. The identification of stabilin-1 as a sorting receptor for SI-CLP is the first known example of a lysosomal sorting process which occurs specifically in alternatively activated macrophages. This finding creates the impetus for investigation of a novel parameter of macrophage polarization: activation-dependent specificity of the lysosomal and consequently secretory repertoire.

Stabilin-1 and cell-cell adhesion

The involvement of stabilin-1 in establishing cell-cell contacts is currently a very contentious issue. Irjala *et al.* [14] reported that the administration of the anti-CLEVER-1 (anti-stabilin-1) mAb 3-372 reduces lymphocyte infiltration in rabbits immunized with keyhole limpet hemocyanin. The authors suggest that the 3-372 mAb interferes with the entrance of lymphocytes into both high endothelial venules (HEVs) and lymphatic sinusoids. The same 3-372 mAb blocked approximately 50% of lymphocyte binding to lymph node sections in both static and dynamic conditions. The next study from this group [13] presented evidence for the involvement of stabilin-1 in the adhesion of malignant cells to lymphatic endothelium. The same methodological approach (blocking of binding with the 3-372 mAb) resulted in a 30–50% inhibition of adhesion of malignant cells to lymphatic endothelium and HEVs. In a subsequent study from the same group, Salmi *et al.* claimed that stabilin-1 is responsible for the transmigration of peripheral blood monocytes through lymphatic endothelium [4]. A mixture of anti-CLEVER-1 mAbs 3-266 and 3-372 was found to have a 40% inhibiting effect on the transmigration of PBMCs through a monolayer of human umbilical vein endothelial cells (HUVECs), used as a model cell system. An intriguing issue is which surface determinants (of those assumed to be common to lymphocytes, PBMCs, and various tumor cell lines) mediate binding to stabilin-1 on the surface of various endothelial cells. Identification of stabilin-1 ligands on the surface of interacting cells would clarify the question about the specificity of the blocking effect of the 3-372 mAb.

The controversy regarding the involvement of stabilin-1 in cell-cell contacts is exacerbated by the extremely low level of stabilin-1 exposure on the cell surface, observed in numerous studies of endogenous and recombinant stabilin-1. Salmi *et al.* [4] failed to detect a significant level of stabilin-1 at the surface of lymphatic endothelium, and its in vitro propagation was necessary to produce a stabilin-1-positive signal by FACS. It is not clear at the moment whether such *in vitro* conditions reflect the physiological situation *in vivo*. At the same time, the intracellular localization of stabilin-1 and its function as an endocytic receptor argue against a role for stabilin-1 in cell-cell adhesion [3, 9–11, 76, 77]. Prevo *et al.* [3] applied high resolution confocal microscopy and used two different rabbit polyclonal antibodies recognizing the stabilin-1 cytoplasmic tail (anti-stabilin-1 CT) and Link domain (anti-stabilin LINK), as well as the 3-372 mAb. All three antibodies revealed intracellular stabilin-1 localization in HEVs in contrast to clear surface localization of L-selectin. The intracellular localization of stabilin-1 was found to be the same in paraffin-embedded sections of human lymph nodes by IHC and in frozen lymph node sections by confocal microscopy [11]; and the vesicular pattern of stabilin-1 was demonstrated in both sinusoidal endothelial cells and macrophages. Prevo *et al.* reported very a low expression level of stabilin-1 in lymphatic vessels of nonlymphoid tissues, including colon, stomach, and skin, in contrast to high

LYVE-1 expression; stabilin-1 was here recognized only by the 3-372 mAb. LYVE-1 is commonly considered to be a highly specific markers for lymphatic vessels. Prevo *et al.* detected only a very minor amount of stabilin-1 on LYVE-1 positive structures by immunohistochemistry. Our recent detailed investigation of the cell-type specificity of LYVE-1 expression revealed that a subpopulation of macrophages in an animal model of angiogenesis and tumorigenesis as well as in human tumors can co-express LYVE-1 and stabilin-1, bringing even more doubt about the degree of expression of stabilin-1 in lymphatic endothelium that would suffice to mediate cell-cell adhesion [76]. In addition, most of our extensive histological studies using the MS-1 mAb, the F4 rabbit polyclonal antibody, and our recently generated mAb against murine stabilin-1 (1.26) failed to detect stabilin-1 in lymphatic endothelium in human and rodent tissue (see Table and the references therein).

Using fluorescently labeled anti-stabilin-1 antibody and acLDL, Prevo *et al.* demonstrated that stabilin-1 functions as an endocytic receptor in transfected HeLa cells [3]. However, both FACS analysis and biotinylation of surface proteins of unpermeabilized cells did not detect stabilin-1 on the surface of cultured HUVEC cells, which were used as a model system by Salmi *et al.* to demonstrate stabilin-1-mediated transmigration of PBMCs [4]. Since the steady-state level of stabilin-1 was below the limits of detection, and the extracellularly added fluorescent ligand was delivered to early endosomes in merely 5 minutes, stabilin-1 was obviously functioning as a rapidly recycling endocytic receptor. Whether such a low surface level of the receptor is sufficient for the numerous cell adhesion activities assigned to stabilin-1 remains a question for debate and further study.

In summary, the low level of stabilin-1 expression on the cell surface, its questionable expression in lymphatic endothelium, and the broad cell-type specificity seen in studies using the 3-372 mAb as a blocking tool argue against the specific and universal functions of a cell adhesion molecule for stabilin-1. Highly speculatively, we can suggest here that a function for stabilin-1 in cell-cell contacts cannot be excluded, but identification of stabilin-1-interacting molecules on the surface of migrating lymphocytes or tumor cells is required as proof.

Conclusion and perspectives

Stabilin-1 is expressed in sinusoidal cells responsible for clearance and selective transport of modified-self and non-self compounds, and in tissue macrophages which regulate homeostasis and the resolution of inflammation. Thus, stabilin-1 with its unusual ligand repertoire is involved in complex physiological clearance processes. In macrophages, stabilin-1 links two different processes: uptake and degradation of unwanted self molecules and intracellular sorting. Hence, stabilin-1 can contribute to the regulation of the secretion repertoire of extracellular mediators. Intriguing questions include: 1) whether endocytosis performed by stabilin-1 can influence its sorting activity and ligand selection in the biosynthetic compartment and 2) how endocytic and intracellular sorting pathways communicate on the molecular level. The question of whether stabilin-1 can function as an intracellular sorting receptor in sinusoidal endothelial cells or other types of endothelial cells has not been addressed in detail and is of particular interest for elucidation of the regulatory role of endothelial cells in inflammatory conditions. Apparently, the large and complex extracellular domain of stabilin-1 is involved in numerous interactions which remain to be discovered, as do the tissue-specific functions of stabilin-1. (For example, abundant expression of stabilin-1 in placenta suggests its active participation in developmental processes.)

Further identification of new stabilin-1 ligands and investigation into the nature of the intracellular interaction between the clearance and secretion functions of stabilin-1 will advance our understanding of homeostatic mechanisms in sinusoidal barrier structures and in tissues.

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