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Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding

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Abstract

Liver sinusoidal endothelial cells (LSECs) mediate clearance of hyaluronan (HA) and scavenger receptor ligands, for example, advanced glycation end product (AGE)-modified proteins and oxidized lipids from the circulation. We recently cloned stabilin-1 and -2, two members of a novel family of transmembrane proteins expressed in LSECs. By using primary LSECs and HEK293 cells separately expressing either stabilin, we have investigated their roles in the early events of endocytosis with respect to localization, ligand-binding properties, and associations with clathrin and adaptor protein (AP)-2. Both stabilins were present at the cell surface, although surface levels of stabilin-1 were limited. In addition, stabilins were present in early endosomal antigen (EEA)-1+ organelles colocalizing with endocytosed AGE-modified bovine serum albumin (BSA). Treating cells with monensin further pronounced this distribution. Recombinant stabilin-2, but not recombinant stabilin-1, bound HA and the scavenger receptor ligands AGE-modified BSA, formaldehyde-treated BSA, and collagen N-terminal propeptides. In LSECs, both stabilins were associated with clathrin and AP-2, but not with each other. These interactions did not change upon addition of exogenous HA, suggesting that stabilins are constitutively internalized. In conclusion, hepatic stabilins are both present in the early endocytic pathway, associating with clathrin/AP-2, but whereas stabilin-2 has a clear scavenging profile, stabilin-1 does not recognize these ligands.

Keywords: Scavenger receptors; Liver; Sinusoidal endothelial cells; Clearance; Internalisation; Hyaluronan; AGE; Collagen propeptides; FSA

Introduction

The sinusoidal endothelial cells (SECs) of the mammalian liver (LSECs) are highly specialized cells that have a major role in the filtration of blood. These cells remove significant amounts of hyaluronan (HA) and other extracellular matrix compounds from the circulation every day [1–3]. LSECs are also important in the clearance of so-called scavenger receptor ligands (SRLs) [3], a heterogeneous group of waste products of the body [4–6], including atherogenic lipids, collagen N-terminal propeptides (PNP), and advanced

Abbreviations: AGE, advanced glycation end product; AP, adaptor protein; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EEA-1, early endosomal antigen-1; FCS, fetal calf serum; FSA, formaldehyde treated bovine serum albumin; GST, glutathione *S*-transferase; HA, hyaluronan; HARE, hyaluronan receptor for endocytosis; immuno-EM, immunoelectron microscopy; LSEC, liver sinusoidal endothelial cell; PBS, phosphate-buffered saline; PNP, N-terminal propeptides of collagen; PINP and PIIINP, PNP of collagen I and III; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC, sinusoidal endothelial cell; SRL, scavenger receptor ligand; TBS, Tris-buffered saline.

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glycation end products (AGEs), to name a few. All of these ligands need to be efficiently cleared from the circulation since elevated levels are potentially harmful.

We recently reported on the cloning of stabilin-1 and stabilin-2, which constitute a novel family of transmembrane proteins [7]. Stabilin-2 was first identified as the endocytic hyaluronan (HA)/scavenger receptor on rat LSECs by antibody-mediated inhibition of HA uptake [8]. Besides its affinity for HA [8,9], results from our previous studies suggested that stabilin-2 from rat LSECs has the ability to bind and endocytose PINP and formaldehyde-treated bovine serum albumin (FSA) [4,8], as well as AGE-modified proteins [10]. Stabilin-1 was originally identified as a surface marker (MS-1) for the SECs of the spleen, lymph nodes, and liver [11]. Upon cloning, it was found that the MS-1 antigen and the HA receptor were members of the same protein family, and they were named stabilin-1 and stabilin-2, respectively [7]. Sequences corresponding to those of the stabilins have been published hereafter, but under different names. These include FEEL1 (stabilin-1), FEEL2 (stabilin-2) [12], hyaluronan receptor for endocytosis (HARE; a truncated form of stabilin-2) [13], and CLEVER-1 (stabilin-1) [14,15].

In addition to LSECs, both stabilins are also expressed in SECs of the spleen, bone marrow, and lymph nodes [7,16]. Stabilin-1 is also expressed in alternatively activated macrophages (Mϕ2) [7,17,18]. Bioinformatic analysis of the extracellular portions of stabilin-1 and -2 shows that they feature one Link domain, as well as seven fasciclin and several epidermal growth factor (EGF) domains [7]. The Link domain has been found to be a key component in binding HA and is present in a number of different HA-binding proteins, such as aggrecan, versican, LYVE-1, and TSG-6 [19–21]. The cytoplasmic domains of stabilin-1 and stabilin-2, however, exhibit no obvious similarities [7].

Receptor-mediated endocytosis via coated pits requires interactions between sorting signals present in the cytoplasmic portion of the receptor and proteins present at the plasma membrane, such as adaptor proteins (APs), various accessory factors, and the structural component, clathrin [22,23]. Adaptor proteins bind clathrin as well as the sorting signals of transmembrane proteins, bridging these two units. Four different APs have been identified, AP-1 to AP-4, which have different roles in cellular vesicle transport and are located at different sites in the cell. They are hetero-tetramers composed of two large subunits (β1–β4 plus γ, α, δ, or ϵ, respectively), and two smaller subunits (μ1-4 and σ1-4, respectively). AP-2 is present at the plasma membrane and is the adaptor specifically involved in endocytosis.

In the present study, we have characterized endogenous stabilins in primary rat and pig LSECs, as well as recombinant stabilins, expressed separately in HEK293 cells. The aim has been to investigate if the stabilins have similar ligand-binding properties and if they both may be involved in the early events of endocytosis. Direct evidence suggesting that stabilin-2 is not only an HA receptor, but also can bind and endocytose SRLs, is presented, together with data suggesting interactions between stabilin-2 and clathrin/AP-2. Stabilin-1 also associated with clathrin and AP-2 and colocalized with stabilin-2 and endocytosed AGE-BSA in the early endocytic pathway. Stabilin-1 did not, however, bind any of the ligands bound by stabilin-2, suggesting that they may have distinct functions in LSECs.

Materials and methods

Chemicals and reagents

Collagenase (Grade V), pepstatin A, *N*-ethylmaleimide, sodium vanadate, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), monensin, and Triton X-100 (molecular biology grade) were obtained from Sigma Co., St. Louis, USA. Complete Mini Protease Inhibitor Cocktail Tablets were obtained from Roche, Norway. Bovine serum albumin (BSA) was obtained from Lee Biosolutions, St. Louis, USA, and hyaluronan was from Pharmacia, Uppsala, Sweden. High-range prestained molecular mass standards were obtained from Bio-Rad Laboratories, Hercules, USA. Protein A Hi Trap columns, Sephadex G-25 (PD-10), gelatin Sepharose 4B, $Na^{125}I$, and Percoll were obtained from Amersham Biosciences, UK, and protein A-gold from the University of Utrecht, The Netherlands. Fibronectin was purified from human plasma on a column of gelatin Sepharose 4B as described by the manufacturer. Culture medium RPMI 1640 (FG1215) and Dulbecco's MEM (FG0435), both supplemented with 100 μg/ml penicillin/streptomycin, were purchased from Biochrom Ag, Berlin, Germany. Fluoromount was purchased from Southern Biotechnology Associates, Birmingham, USA. Commercially available antibodies were monoclonal antibodies against clathrin, the α-adaptin subunit of AP-2 and early endosomal antigen (EEA)-1 (BD Transduction Laboratories, Pharmingen), and goat antirabbit/mouse HRP (Amersham Biosciences, UK). Fluorescence-labeled secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, USA.

Animals

All animals received humane care according to the guidelines of the National Institutes of Health, USA. All animal experiments were performed and approved by animal ethics committees in Tromsø, Norway, and Baden-Württemberg (Regierungspräsidium Karlsruhe AZ: 35-9185.82/A-30/02), Germany.

Cells

HEK293 cells stably expressing stabilin-1 or stabilin-2 were established by lipofection with either Not-1 linearized full-length human stabilin-1 in pEF6V5His-TOPO vector or