Cohen syndrome is associated with major glycosylation defects

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Cohen syndrome (CS) is a rare autosomal recessive disorder with multisystemic clinical features due to mutations in the VPS13B gene, which has recently been described encoding a mandatory membrane protein involved in Golgi integrity. As the Golgi complex is the place where glycosylation of newly synthesized proteins occurs, we hypothesized that VPS13B deficiency, responsible of Golgi apparatus disturbance, could lead to glycosylation defects and/or dysfunction of this organelle, and thus be a cause of the main clinical manifestations of CS. The glycosylation status of CS serum proteins showed a very unusual pattern of glycosylation characterized by a significant accumulation of agalactosylated fucosylated structures as well as asialylated fucosylated structures demonstrating a major defect of glycan maturation in CS. However, CS transferrin and α1-AT profiles, two liver-derived proteins, were normal. We also showed that intercellular cell adhesion molecule 1 and LAMP-2, two highly glycosylated cellular proteins, presented an altered migration profile on SDS–PAGE in peripheral blood mononuclear cells from CS patients. RNA interference against VPS13B confirmed these glycosylation defects. Experiments with Brefeldin A demonstrated that intracellular retrograde cell trafficking was normal in CS fibroblasts. Furthermore, early endosomes were almost absent in these cells and lysosomes were abnormally enlarged, suggesting a crucial role of VPS13B in endosomal–lysosomal trafficking. Our work provides evidence that CS is associated to a tissue-specific major defect of glycosylation and endosomal–lysosomal trafficking defect, suggesting that this could be a new key element to decipher the mechanisms of CS physiopathology.

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INTRODUCTION

Cohen syndrome (CS) (OMIM 216550) is a rare autosomal disorder, first described in 1973 (1), and characterized by many clinical features including facial dysmorphism, microcephaly, truncal obesity, intellectual deficiency, progressive retinopathy and intermittent congenital neutropenia (2,3). Both of the latter symptoms have recently been described as the best indicators for the diagnosis of CS (2). CS is due to mutations in the VPS13B gene also called COHI. More than 100 distinct VPS13B mutations, predominantly truncating mutations and genomic deletions/duplications, have already been described in CS (2). The VPS13B gene is located on chromosome 8q22–q23 and composed of 62 exons encoding a putative transmembrane protein of 4022 amino acids (4). VPS13B shows strong homology to Saccharomyces cerevisiae vacuolar protein sorting-associated protein 13 (Vps13p), suggesting that VPS13B plays a role in cellular trafficking (5). Recently, Seifert et al. identified VPS13B as a peripheral membrane protein localized to the Golgi complex (6). Thanks to its C-terminus amino acids, VPS13B colocalizes with a specific marker of the cis-Golgi apparatus, GM130. Furthermore, VPS13B depletion using RNAi technology leads to disruption of the Golgi structure which corresponds to the disrupted Golgi morphology observed in fibroblasts from CS patients (6).

The main cellular functions of the Golgi apparatus include protein sorting and packaging and the posttranslational modification, such as glycosylation, of newly synthesized proteins and lipids. The fundamental involvement of glycosylation in the intricate design of life is dramatically illustrated by a group of inherited human disorders named congenital disorders of glycosylation. The fundamental involvement of glycosylation in protein sorting and packaging and the posttranslational modification of newly synthesized proteins, such as glycosylation, of newly synthesized proteins and genomic deletions/duplications, have already been described in CS (2). The VPS13B gene is located on chromosome 8q22–q23 and composed of 62 exons encoding a putative transmembrane protein of 4022 amino acids (4). VPS13B shows strong homology to Saccharomyces cerevisiae vacuolar protein sorting-associated protein 13 (Vps13p), suggesting that VPS13B plays a role in cellular trafficking (5). Recently, Seifert et al. identified VPS13B as a peripheral membrane protein localized to the Golgi complex (6). Thanks to its C-terminus amino acids, VPS13B colocalizes with a specific marker of the cis-Golgi apparatus, GM130. Furthermore, VPS13B depletion using RNAi technology leads to disruption of the Golgi structure which corresponds to the disrupted Golgi morphology observed in fibroblasts from CS patients (6).

The main cellular functions of the Golgi apparatus include protein sorting and packaging and the posttranslational modification, such as glycosylation, of newly synthesized proteins and lipids. The fundamental involvement of glycosylation in the intricate design of life is dramatically illustrated by a group of inherited human disorders named congenital disorders of glycosylation (CDG) (7). To date, ~150 different types of CDG have been defined genetically and most of the time, the defects impair the biosynthesis (CDG-I) and remodeling of N-glycans (CDG-II). Given the number of biochemical pathways in which glycans are intricately involved, these diseases have a wide range of clinical phenotypes in which nearly every organ could be affected. The initial screening test for CDG is the study of the highly glycosylated iron-binding transferrin glycoprotein and its isoforms, although this screening test has shown its limits (8,9). Recently, the intercellular cell adhesion molecule 1 (ICAM-1) has been described as a new marker for hypoglycosylation for CDG (10). A few years ago, the identification of CDG subtypes caused by altered vesicular Golgi trafficking and Golgi pH homeostasis has marked a new era in CDG pathogenesis (11,12). It is now widely accepted that the incorrect oligosaccharide structures obtained an 80% decrease in mRNA levels (Fig.3B). After 48 h, we observed a shift in LAMP-2 migration indicating a lower molecular weight of this protein due to abnormal glycosylation (Fig. 3C). Furthermore, we observed a dramatic decrease in ICAM-1 protein expression (Fig. 3C). This was also visualized by flow cytometry and confirmed at the mRNA level (data not shown).

RESULTS

Cohort of patients with CS

Ten of the 11 patients fulfilled the CS diagnosis criteria (15) but patient P100–F88 had an atypical clinical presentation with neither obesity nor mental retardation (16). Clinical features included chorioretinal dystrophy with myopia (11/11 cases), congenital neutropenia (11/11 cases), slender extremities (11/11 cases), microcephaly (10/11 cases), intellectual deficiency (10/11 cases), joint hyperlaxity (10/11 cases) and truncal obesity (9/11 cases). The 18 different mutations include six frameshift four nonsense, four splice and one missense mutations, as well as one truncating large duplication and two in frame deletions.

Structural analysis of N-linked glycans and cell glycosylation defects

In all CS patients, mass spectrum of serum N-glycans showed a significant accumulation of agalactosylated fucosylated structures Hex3HexNAc4dHex1 (m/z 1836) and asialylated fucosylated structures Hex4HexNAc4dHex1 (m/z 2040). Monosialylated structures (NeuAc1Hex5HexNAc4, m/z 2431) and monogalactosylated structures (Hex4HexNAc4, m/z 1855) with the corresponding fucosylated ones (NeuAc1Hex5HexNAc4dHex1, m/z 2606; Hex4HexNAc4dHex1, m/z 2040) have also been found increased (Fig. 1). Interestingly, all the aforementioned structures are present in all CS patients but in rather different proportions (see Supplementary Material, Table S1 and Fig. 1 for the relative distribution of the glycoforms). This result then points to a strong disturbance of the Golgi N-glycosylation process in CS patients.

We analysed the pattern of molecular weights of serum transferrin, and interestingly, transferrin glycosylation appeared normal in all of the patients compared with CDG-I and CDG-II serum (Fig. 2A). Furthermore, we also studied the α1-antitrypsin (α1-AT) glycosylation pattern, another liver-derived protein and showed that the migration profile of this protein was normal in CS patients (Fig. 2B).

We thus turned to the analysis of ICAM-1, which has been described as new marker of hypoglycosylation for CDG (10). Peripheral blood mononuclear cells (PBMCs) of four CS patients and healthy donors were then tested for the expression of ICAM-1, as well as the highly glycosylated lysosomal protein LAMP-2. Both proteins were normally expressed in PBMCs but with lower molecular weights in CS patients (differences of 9 kDa for ICAM-1 and 8 kDa for LAMP-2), likely resulting from Golgi glycosylation defects (Fig. 3A).

In order to determine whether the VPS13B deficiency was involved in the observed glycosylation defects in CS patients, we knocked down VPS13B mRNA expression in retinal pigment epithelium (RPE) cell lines using RNAi delivery and obtained an 80% decrease in mRNA levels (Fig. 3B). After 48 h, we observed a shift in LAMP-2 migration indicating a lower molecular weight of this protein due to abnormal glycosylation (Fig. 3C). Furthermore, we observed a dramatic decrease in ICAM-1 protein expression (Fig. 3C). This was also visualized by flow cytometry and confirmed at the mRNA level (data not shown).
Link between VPS13B and the Golgi–lysosomal–endosomal pathway

As previously reported with the cis-Golgi matrix protein GM130 (6), staining with the Golgi markers TGN46 confirmed slight alterations in Golgi morphology in all of our investigated CS fibroblasts, with marked dilation and fragmentation for P68–F58 and P72–F62 (white arrows, Fig. 4A and B). Fragmented and dilated Golgi were quantified, and CS fibroblasts showed ~44–48% of abnormal Golgi. Because the aberrant glycosylation and the defects in Golgi morphology may result from abnormal retrograde trafficking, its integrity was investigated using Brefeldin A (BFA). The loss of Golgi mannosidase-II distribution was rapid and similar in both control and patient cells (data not shown). This underlies that VPS13B deficiency had no impact on retrograde trafficking.
The ortholog of VPS13B in *S. cerevisiae* (Vps13p) is known to be involved in vacuolar protein sorting. In order to get insights into this pathway, the lysosomal and endosomal compartments were investigated by confocal microscopy using the intracellular markers LAMP-2 (lysosome-associated membrane protein 2) and EEA1 (early endosome antigen). As shown in Figure 4A and B, LAMP-2 staining was mainly perinuclear while EEA1 appeared as punctuate staining throughout the cells. Compared with control cells, EEA1 staining was much less marked and almost absent in CS fibroblasts. Furthermore, LAMP-2 staining showed enlarged lysosomes. These results strongly suggest that VPS13B is involved in the endosomal–lysosomal pathway.

DISCUSSION

Our results showed that *VPS13B* mutations, responsible for Cohen syndrome, are associated with strong protein glycosylation defects highlighting an important role for VPS13B in Golgi glycosylation and morphology as well as in lysosomal–endosomal pathway maintenance.

As we confirmed that the Golgi apparatus morphology was altered in CS fibroblasts (previously described by Seifert et al. (6)), and as part of the glycosylation process takes place in this organelle and strong Golgi glycosylation deficiencies have been linked to Golgi alterations, we hypothesized that CS could be linked to a glycosylation disorder, i.e. CDG syndrome. Indeed, some of the CS characteristics belong to the CDG multisystemic clinical features spectrum. Considering the two hallmarks of the syndrome, pigmented retinopathy has been described in CDG-Ia and CDG-II and neutropenia in CDG-IIf, as well as microcephaly in CDG-Ij, -Ik, -IIl, -Ilm, -Ilc and -Ilh (7). Furthermore, intellectual deficiency is present in most CDG syndromes (7). While many clinical characteristics of CDG-II patients are observed in CS patients, the usual CDG diagnosis test based on serum transferrin isoforms analysis appeared normal in CS patients. Moreover, normal western blotting pattern of α1-AT suggests that the defect of glycosylation observed in CS is tissue specific and absent in the liver. This could be similar to the glycosylation abnormality also observed in the G6PC3 syndrome. Indeed, in G6PC3 syndrome, a congenital neutropenia disorder, glycosylation defects were detected in neutrophils, even though serum transferrin isoforms remained normal, suggesting that this disorder could be classified as a CDG syndrome (17). In CS, routine transferrin glycosylation test cannot be used as a diagnosis tool, but our study indicates that western blotting experiments of ICAM-1 associated with LAMP-2 could become an easy and cheap pre-screening test of CS diagnosis, before *VPS13B* sequencing which remains an expensive analysis, in particular in atypical CS, when all phenotypic criteria are not fully met. A mass spectrometry approach could also be considered as a pre-screening test for CS diagnosis. Indeed, the appearance of the abnormal glycan structures at *m/z* 1836 is quite specific from this defect. Although present in *ATP6V0A2* deficient CDG patients’ sera, these structures are rarely seen in other CDG-II patients (18). However, the rapid advent of next generation sequencing in the diagnostic work-up of polysomal-informative syndrome may considerably decrease the cost of *VPS13B* sequencing. Moreover, these results strongly confirm that the use of transferrin isoforms analysis as a routine test for CDG and glycosylation deficiency can present some limits, i.e. normal results do not exclude glycosylation defects.

The glycosylation deficiency was mainly identified in serum proteins by mass spectrometry, but also in primary cells of CS patients such as PBMCs, which exhibited glycosylation defects in two highly glycosylated proteins, ICAM-I and LAMP-2. ICAM-1 has recently been considered as a new biomarker in CDG because of its reduced expression at the cell surface in CDG-I and -II (10). In CS patients, ICAM-1 was well expressed at the PBMCs cell surface but showed a lower molecular weight, suggesting a lower glycosylation status, while RPE cells displayed reduced ICAM-1 expression after siRNA delivery against *VPS13B*. This discrepancy could be explained by the alteration of glycosylation level or the Golgi disorganization resulting from various *VPS13B* mutations, and thus different impacts on VPS13B protein and its function. Nevertheless, these results strongly suggested that the glycosylation deficiency observed in CS patients could be linked to dramatically decreased VPS13B function.

Despite its location in the Golgi apparatus, the precise role of VPS13B is unknown and needs to be determined more clearly. So far, no studies have explored the potential link between VPS13B and Golgi glycosylation, but VPS13B has been identified as a potential transmembrane protein that may function in vesicle-mediated transport and sorting of proteins within the cell (11). The link between *VPS13B* defect in CS patients, the alteration of Golgi glycosylation and all the clinical spectrum of CS is however not yet elucidated. Indeed, the disturbance of the Golgi apparatus is probably the main impact of *VPS13B* deficiency; the key question now is to understand while abnormal protein glycosylation or disturbance of the Golgi function are responsible for neutropenia, truncal obesity, retinopathy, intellectual deficiency, etc. Indeed, two hypotheses could emerge from this new CS element: (i) the defect of glycosylation could affect any protein playing a major role in the cellular mechanisms of neutrophils, adipogenesis, etc., and thus disturb cell...
function, leading to neutropenia, truncal obesity, etc. and (ii) it could also be suggested that the observed hypoglycosylation in CS patients is secondary to the disruption of the Golgi apparatus, like for TMEM165 defects (19), being just another CS symptoms, and resides outside the glycosylation machinery. Furthermore, Golgi apparatus is the place where N- but also O-glycans, GAG and glycolipids synthesis occur. This is highly possible that other types of glycosylation can also be altered, inducing some clinical features.

The disturbance of Golgi trafficking could also been implicated in the appearance of some symptoms in CS patients. One can expect that VPS13B like the COG complex machinery would regulate the vesicle-mediated transport of various Golgi glycosyltransferases and/or substrates. Interestingly, the impacts of VPS13B deficiency on lysosomes and early endosome compartments highly suggest that VPS13B plays a role in the maintenance of these organelles. The lack of VPS13B clearly gives rise to enlarged lysosomes and a lack of early endosomal structures. As lysosome size is regulated by a balance of vesicle fusion and fission, any dysfunction in this process would increase its size. According to our results, we may hypothesize that VPS13B regulates endosome fission. The enlarged lysosomes observed correspond also to a hallmark of both Chediak-Higashi syndrome (CHS) (20), a rare autosomal recessive disorder characterized by deficiencies in CHS (LYST), and lysosomal storage diseases. The link between the endo-lysosomal and biosynthetic pathways becomes obvious as endo-lysosomal deficiencies affect the Golgi glycoprotein biosynthesis. The intricate design of the catabolic pathway in the biosynthetic process needs to be further investigated.

Golgi apparatus disruptions have also been reported in diseases with clinical features shared with CS. In retinitis pigmentosa (RP) resulting from mutations in the \(RP2\), \(Alr3\) or \(Kif3a\) genes, the progressive photoreceptor cell degeneration was linked to the fragmentation of the Golgi network, which leads to the dispersal of cargo vesicles from the Golgi apparatus to the cillum, thus affecting the location and trafficking of cilia proteins (21).

In summary, our results provide evidence that VPS13B plays a major role in the function of the Golgi apparatus associated with major alterations in protein glycosylation in CS patients with \(VPS13B\) mutations. Further studies in a larger cohort of CS patients would be necessary to determine whether the glycosylation status, measured by western blotting experiments of...
ICAM-1 associated to LAMP-2, associated or not to a mass spectrometry analysis could be used as a pre-screening test before \textit{VPS13B} sequencing analysis.

\section*{MATERIALS AND METHODS}

\subsection*{Patient cohort}

In a cohort of 30 patients with two \textit{VPS13B} mutations, blood samples and/or skin biopsies, from which serum, PBMCs and fibroblasts were isolated, could be ascertained in 11 patients (five females and six males) from nine sibships (Table 1). The patients have previously been described (2,3). Written informed consents were obtained according to the French regulatory requirement. The study has been approved by the ethical committee of Dijon University Hospital.

\subsection*{Mass spectrometry: release of the N-glycans and PNGase treatment}

Aliquots of 20 \(\mu\)l of sera from healthy individuals or CS patients were dried in a vacuum centrifuge. The dried samples were
dissolved in 200 μl of 50 mM ammonium bicarbonate containing 0.25% SDS (w/v) and 0.25% β-mercaptoethanol (v/v) and subsequently heated for 20 min at 100°C. After adding 175 μl of 50 mM ammonium bicarbonate and 25 μl Nonidet P40, 1.5 U of PNGase were added and the deglycosylation was incubated at 37°C for 18 h. Released N-glycans were desalted on a column of 150 mg of non-porous graphitized carbon (Alltech, Deerfield, IL, USA). The column was subsequently washed with 5 ml of methanol and 2 × 5 ml of 0.1% (v/v) TFA. Free N-glycans were dissolved in 1 ml of 0.1% (v/v) TFA, applied to the column and washed with 3 × 5 ml of 0.1% (v/v) TFA. The elution of N-glycans was conducted with the application of 4 ml of 25% (v/v) ACN in water containing 0.1% (v/v) TFA. The fractions were freeze-dried.

**Permethylation and MALDI TOF-MS analysis**

Permethylation of the freeze-dried N-glycans and sample clean-up were performed as previously described (22). Samples were cocrystallized with 2,5-dihydroxybenzoic acid as matrix (10 mg/ml) in methanol/water solution (50/50) and freeze-dried. MALDI-MS experiments were carried out on Voyager Elite DE-STR Pro instrument (PerSeptive Biosystem, Framingham, MA, USA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode by delayed extraction with an accelerating voltage of 20 kV and a pulse delay time of 250 ns and a grid voltage of 72%. All the spectra represent accumulated spectra obtained by 500 laser shots.

**Cell culture**

Fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (FCS) (both from Thermofisher Scientific, Illkirch, France) and 1% penicillin/streptomycin (P/S) (Sigma, Saint-Quentin-Fallavier, France). RPE cells (kind gift from Sophie Thomas, Inserm UMR781, Paris, France) were cultured in DMEM:Ham’s F12 (1:1) medium supplemented with 10%
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Nucleotide change and/or CNVs</th>
<th>Intron/exon</th>
<th>Protein mutation</th>
<th>Facial dysmorphism</th>
<th>Clinical features</th>
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<tr>
<td>P5–F13</td>
<td>F</td>
<td>15</td>
<td>IVS30+2T</td>
<td>EX 30</td>
<td>Typical</td>
<td>Typical CRD, NTP, ID, MC, TO, SE, JH, M</td>
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<tr>
<td>P2–F2</td>
<td>F</td>
<td>44</td>
<td>c.10139_10143dupCGCCAarr</td>
<td>8q22.2</td>
<td>Truncating</td>
<td>Typical CRD, NTP, ID, MC, TO, SE, JH, M</td>
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<tr>
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<td>F</td>
<td>47</td>
<td>c.10139_10143dupCGCCAarr</td>
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<td>Truncating</td>
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<tr>
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<td>14</td>
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<td>EX 13EX 61</td>
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<td>Typical CRD, NTP, ID, MC, TO, SE, JH, M</td>
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<tr>
<td>P15–F11</td>
<td>M</td>
<td>11</td>
<td>c.10880insTTdelCTGCGAGG</td>
<td>EX 23EX 56</td>
<td>Typical</td>
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<tr>
<td>P100–F88</td>
<td>F</td>
<td>37</td>
<td>IVS30 T_3AinsTIVS57+2T</td>
<td>8q23</td>
<td>Typical</td>
<td>Typical CRD, NTP, ID, MC, SE, JH, M</td>
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<tr>
<td>P68–F58</td>
<td>F</td>
<td>10</td>
<td>arr 8q23 (100817911–100828887)x1</td>
<td>INT 43–44</td>
<td>In frame large</td>
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<tr>
<td>P72–F62</td>
<td>M</td>
<td>27</td>
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<td>P10–F8</td>
<td>M</td>
<td>23</td>
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<td>EX 15EX 34</td>
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<td>Typical</td>
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CNV, copy number variations; CRD, chorioretinal dystrophy; CS, Cohen syndrome; EX, exon; ID, intellectual deficiency; IN, intron; JH, joint hyperlaxity; M, myopia; MC, microcephaly; NTP, neutropenia; SE, slender extremities; TO, truncal obesity.

Immunofluorescence

For human cells immunofluorescence experiments, cells were grown on glass coverslips for 12–24 h, washed once with PBS and fixed by incubation for 25 min at RT in 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2. The coverslips were then rinsed twice with 0.1 M glycine in PBS for 15 min at RT, then incubated for 1 h at RT with primary antibodies diluted in blocking solution [0.1% Triton X-100 (Sigma), 1% BSA (Roche, Meylan, France) and 5% normal goat serum (Invitrogen, Courtaboeuf, France)]. After washing with PBS, Alexa Fluor 488- or Alexa Fluor 568-conjugated anti-rabbit IgG antibodies (Invitrogen) diluted in blocking solution were applied for 1 h at RT. Immunostaining was detected using an inverted Leica TCS-SP5 confocal microscope. Data were collected and processed in Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

Golgi to ER trafficking assay

Fibroblasts were grown overnight on glass cover slips to ~70% confluence. For the experiment, the medium was changed for pre-warmed medium containing 5 μg/ml of BFA (St. Louis, MO, USA). The assay was stopped at different time points by fixing the cells with 4% paraformaldehyde. After fixation and permeabilization, the coverslips were stained for GM130 and Golgi-mannosidase-II, as described above.

RNA interference

Small interference RNAs (siRNA) against VPS13B and negative control siRNA (both siGENOME SMARTpool, sequences available on request) were purchased from ABgene Ltd (Thermo scientific). Transfection of siRNA was obtained using Lipofectamine™ RNAiMAX reagent (Invitrogen) according to the manufacturer’s protocol. Briefly, 0.1 x 10^6 RPE were seeded in 2 ml of DMEM containing 1% P/S and 10% FCS. After 24 h, siRNA (100 pmol/lipofectamine™ RNAiMAX (5 μl) complexes (in 500 μl Opti-MEM® Medium) were added to the cells. After 48 h, the cells were prepared for subsequent analysis.

RNA extraction and real-time RT–qPCR

Total RNA was extracted using TRI reagent® (Sigma), according to the manufacturer’s protocol. The first-strand cDNA was synthesized from 0.5 μg total RNA using Maxima First Strand cDNA Synthesis Kit for RT–qPCR from Fermentas (Thermo Scientific). The real-time qPCR contained, in a final volume of 20 μl, 2 ng of reverse transcribed total RNA, 300 nM of the forward and reverse primers, and SYBR green buffer (Fermentas, Thermofisher). PCRs were performed in triplicate in 96-well plates, using the LightCycler 480 (Roche). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an invariant control. Sequences of primers used for real-time PCR were VPS13B-forward: TGGAACCATCAAACAAAAGGCTGCA, VPS13B-reverse: TCCTGACACACTGTAGCGA.
GAPDH-forward: TGCACCAACACTGCTTAGC, GAPDH-reverse: GGCATGGACTGGTGTCATGAG.

Western blot analysis

Cells were lysed for 20 min in RIPA buffer containing 50 mM Tris, 150 mM NaCl, 1 mM NaF, 0.1% NP40, 0.25% DOC, 1 mM Na3VO4, 1 mM PMSF and protein inhibitor cocktail (Sigma). Cell lysates were centrifuged at 13,000 g at 4°C for 20 min and protein concentrations were determined with the BCA protein assay (Sigma). Twenty micrograms of proteins from cell lysates or from sera were run on 8% SDS–PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was blotted with antibodies from cell lysates or from sera were run on 8% SDS–PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was blotted with antibodies to α1-AT (clone 1C2, Sigma), ICAM-1 (Ozyme, St Quentin, France), LAMP-2, tubulin (both from Santa Cruz Biotechnology, Inc) and vinculin (Sigma) in PBS, 0.05% Tween 20, 5% non-fat dried milk, washed and probed with the appropriate secondary antibody coupled to horseradish peroxidase. The labeled proteins were detected using ECL reagent (Pierce, Thermo scientific) according to the manufacturer’s recommendations. Bands on western blots were visualized using ChemiDoc™ Imaging System (Bio-Rad). Molecular weights were determined using Image Lab software (Bio-Rad).

Analysis of serum transferrin

Isoelectric focusing was done as previously described (23).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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