Accumulation of Free Oligosaccharides and Tissue Damage in Cytosolic α-Mannosidase (Man2c1)-deficient Mice

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Background: Cytosolic α-mannosidase (Man2c1) is involved in the catabolism of free oligosaccharides (fOS). Results: Man2c1-deficient mice accumulate fOS in tissues and show biochemical and histological alterations in the CNS, liver, and intestine. Conclusion: Man2c1 plays an essential role in the catabolism of fOS. Significance: Man2c1-deficient mice could be a useful tool to examine the biological significance of Man2c1.

Free Man₉₋₉GlcNAc₂ is released during the biosynthesis pathway of N-linked glycans or from misfolded glycoproteins during the endoplasmic reticulum-associated degradation process and are reduced to Man₅GlcNAc in the cytosol. In this form, free oligosaccharides can be transferred into the lysosomes to be degraded completely. α-Mannosidase (MAN2C1) is the enzyme responsible for the partial demannosylation occurring in the cytosol. It has been demonstrated that the inhibition of MAN2C1 expression induces accumulation of Man₉₋₉GlcNAc oligosaccharides and apoptosis in vitro. We investigated the consequences caused by the lack of cytosolic α-mannosidase activity in vivo by the generation of Man2c1-deficient mice. Increased amounts of Man₉₋₉GlcNAc oligosaccharides were recognized in all analyzed KO tissues. Histological analysis of the CNS revealed neuronal and glial degeneration with formation of multiple vacuoles in deep neocortical layers and major telencephalic white matter tracts. Enterocytes of the small intestine accumulate mannose-containing saccharides and glycogen particles in their apical cytoplasm as well as large clear vacuoles in retronuclear position. Liver tissue is characterized by groups of hepatocytes with increased content of mannosyl compounds and glycogen, some of them undergoing degeneration by hydropic swelling. In addition, lectin screening showed the presence of mannos-containing saccharides in the epithelium of proximal kidney tubules, whereas scattered glomeruli appeared collapsed or featured signs of fibrosis along Bowman’s capsule. Except for a moderate enrichment of invariantly and intestine.

The abbreviations used are: ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; fOS, free oligosaccharide(s); ENGase, β-endo-N-acetylglucosaminidase; Man2c1, α-β-mannosidase; ES, embryonic stem.

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Man$_3$-$_9$GlcNAc oligosaccharides in the cytosol (9, 16). In line with these data, the overexpression of this enzyme caused the decrease of free high mannose-type sugars in COS cells (17). Furthermore, the overexpression of MAN2C1 in HeLa cells led to modifications of the cytosolic pool of free oligomannosides and resulted in the accumulation of small Man$_2$-GlcNAc$_1$ oligosaccharides in the cytosol (18). This accumulation was shown to be correlated with incomplete protein glycosylation.

**FIGURE 1. Neutal free oligosaccharides formation and processing.**

A. generation and trimming of fOS. fOS can be generated either in the lumen of the ER or in the cytosol during the ERAD process. Glc$_x$Man$_y$GlcNAc$_z$ is released from the dolichol pyrophosphate (Dol-PP) anchor in the lumen of the ER by the action of an oligosaccharyltransferase (1). $\alpha$-Glucosidase I and II remove the Glc residues from fOS (2), which are then translocated into the cytosol (3). Misfolded glycoproteins originating in the lumen of the ER move to the cytosol to be degraded (4). An N-glycanase (PNGase) cleaves the N-linked glycans (5), which are reduced to Man$_x$GlcNAc by an ENGase (6). Man$_x$GlcNAc is then trimmed down to Man$_y$GlcNAc by Man2c1 (7) prior to transfer into the lysosome (8). B. Man$_y$GlcNAc cytosolic processing. The removal of mannose residues from Man$_y$GlcNAc is carried out by Man2c1. This enzyme specifically hydrolyzes the $\alpha$-1,2, $\alpha$-1,3, and $\alpha$-1,6 mannose residues linked to the B and C branches of fOS, giving rise to Man$_y$GlcNAc oligosaccharides.
and truncated lipid-linked glycosylation precursors, which yields an increase in N-glycoprotein en route to the ERAD.

Recently, MAN2C1 has been implicated in the growth of tumor cells and malignant transformation processes in cancer cells. Indeed, this protein seems to promote the tumorigenesis in human prostate cancer cells by activating the AKT pathway as a result of PTEN-negative regulation (19). Moreover, the suppression of MAN2C1 expression resulted in mitotic arrest and apoptosis of esophageal carcinoma cells in vitro (20). On the other hand, hMAN2C1 transgenic mice showed an increase in growth, invasion, and metastasis of implanted cancer cells (21).

To deepen the knowledge of the biological significance of Man2c1 and its implication in the cytosolic catabolism of FOS, we generated a Man2c1 knockout mouse model by gene disruption. These mice did not show a visible macroscopic phenotype in growth, invasion, and metastasis of implanted cancer cells and codes a protein of 1039 amino acids (17). To disrupt the transcription initiation site to the RNA cleavage site and truncated lipid-linked glycosylation precursors, which results in a 280-bp fragment for the WT and a 3000-bp fragment for the KO were obtained.

Mice used in this study were housed under standard conditions in a 12-h light-dark cycle with food and water ad libitum. All experiments were carried out in accordance with local and state regulations for research with animals.

**Enzyme Assays**

Kidney, liver, brain, lung, heart, and spleen extracts were prepared from 16-week-old Man2c1+/−, Man2c1−/−, and WT mice. Tissue homogenates were prepared in 10 mM HEPES buffer containing 5 mM MgCl2, 150 mM KAc, and 0.25 M sorbitol (pH 7.4) (1:10 w/v) and centrifuged at 16,000 × g for 10 min. Enzymatic activity was determined using 3 mM fluorogenic substrate 4-methyl-umbelliferyl-α-D-mannopyranoside (Sigma-Aldrich) in 0.1 M citric acid/0.2 M disodium phosphate buffer (pH 6.5 and 4.5, respectively). Lysosomal β-mannosidase activity was also measured using 3 mM 4-methyl-umbelliferyl-β-D-mannopyranoside (Sigma-Aldrich) in the same buffer. The reactions were stopped with 0.2 M glycine/NaOH buffer (pH 10.4). The fluorescence of the liberated 4-methyl-umbelliferone was measured by using a VersaFluor fluorometer (Bio-Rad) (23).

**Western Blot Analysis**

40 μg of proteins from Man2c1+/+, Man2c1+/−, and Man2c1−/− mouse tissue extracts were subjected to reducing sample buffer, boiled for 5 min, and loaded on a 7% SDS-PAGE gel. Proteins were electrophoretically transferred to a nitrocellulose membrane and blocked with 5% nonfat milk in Tris-buffered saline and TWEEN 20 solution (TBS-T). Man2c1 was detected by using a goat anti-Man2c1 antibody (catalog no. sc-66458, Santa Cruz Biotechnology) followed by a secondary antibody. The immunoblot was visualized by chemiluminescence (Immobilon Western, Millipore) on Hyperfilm x-ray film.

**Histology**

Paraffin Histology and Immunohistochemistry/Lectin Histochecmistry—16-week-old mice were killed by an overdose of anesthetic and then perfused sequentially via the left ventricle with 20 ml of phosphate buffer with 1% procaine HCl and then either modified Bouin’s solution (24) or neutral buffered formalin (Sigma-Aldrich). After dissection and embedding of indi-
vidual organs in paraplast, sections were cut at a thickness of 7–10 μm and mounted on aminosilane-coated slides. After deparaffinization in xylene and rehydration in a graded series of ethanolys, sections were either used for a routine H&E stain according to standard protocols or blocked with a mixture of 5% BSA and 5% non-immune serum and then incubated overnight with the primary antibody according to the instructions of the manufacturer. Alternatively, to avoid extraction of cell constituents (especially lipid compounds), fixed organs were encased in agarose and cut on an Leica VT 1200 S high-frequency vibratome prior to staining. After the detection of primary antibody binding via fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 or Alexa Fluor 549), samples were documented with a motorized Nikon 90i microscope equipped with a triple-color eZC1 confocal laser unit.

For lectin histochemistry, sections from samples fixed in modified Bouin’s solution were reacted with concanavalin A, Lens culinaris, and Pisum sativum lectins to detect enrichment of mannose residues in different tissues. To increase lectin staining sensitivity, parallel samples were fixed by immersion rather than perfusion fixation, reducing the loss of fOS from histological material. Additional lectin stains were performed rather than perfusion fixation, reducing the loss of fOS from histological material. Additional lectin stains were performed with Ricinus communis agglutinin I to identify activated microglia as well as with Griffonia simplicifolia agglutinin II, known to detect glycogen with a significantly higher sensitivity than classical periodic acid-Schiff stains (25). Positive lectin staining signals were confirmed by transmission electron microscope as classical periodic acid-Schiff stains (25). Positive lectin staining signals were confirmed by transmission electron microscope as classical periodic acid-Schiff stains (25).

High-resolution Light Microscopy—Mice were killed as described above and perfused transcardially with first 20 ml of Sörensen phosphate buffer with 1% procaine HCl, followed by 6% glutardialdehyde dissolved in the same buffer, again with 1% procaine HCl. Samples were dissected from individual organs, post-fixed in 2% OsO4 for lipid retention, and embedded in Spurr resin in the ERL 4221D variant, according to standard protocols, using propylene oxide as an intermediate. Sections of 1 μm were cut on a Powertome® ultramicrotome, mounted on glass slides, and stained with either toluidine blue/pyronin G or p-phenylene diamine, the latter being employed as a sensitive lipid stain.

Transmission Electron Microscopy—Regions of interest of about 1 mm2 were identified on the semithin sections and prepared as described above. The respective Spurr resin blocks were then trimmed down with a diamond milling cutter and resectioned at 70 nm. Sections were collected on 300 mesh copper grids, contrasted with lead citrate and uranyl acetate, and documented in a Zeiss EM 910 transmission electron microscope equipped with a Tröndle 4 MP digital camera.

Extraction and Analysis of Soluble Oligosaccharides

Extraction of Soluble Oligosaccharides—Tissues of WT and Man2c1 KO mice were disolated in an ice-cold KMH buffer (110 mM potassium acetate, 2 mM magnesium acetate, and 20 mM HEPES pH 7.2) containing a protease inhibitor mixture (Complete, Roche Diagnostics). Cellular homogenization was performed by using a Potter-Elvehjem homogenizer (750 rpm, 5 strokes). The cellular homogenate was then submitted to lipid extraction by addition of methanol and chloroform (MeOH/CHCl3/tissue extract in a ratio of 3/2/1, v/v/v) (26). The aqueous phase containing free oligosaccharides and resulting from the lipid extraction was then lyophilized.

Permethylation of Oligosaccharides—Permethylation of the freeze-dried oligosaccharides and sample cleanup were performed as described previously (27). Internal standard maltoheptaose was permethylated with triply deuterated iododmann than under the same conditions.

MALDI TOF-MS Analysis—For each sample, permethylated oligosaccharides were mixed with a known amount of maltoheptaose standard. MALDI-MS experiments were carried out on a Voyager Elite DE-STR Pro instrument (PerSeptive Biosystem, Framingham, MA) 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, a pulse delay time of 250 ns, and a grid voltage of 72%. All spectra represent accumulated spectra obtained by 500 laser shots. For each sample, three samples were spotted, and the amount of each oligosaccharide was quantified by calculating the ratio between the relative amount of these oligosaccharides and the internal standard in the MALDI-MS spectra. Protein concentration of tissue samples was measured by a micro BCA protein assay reagent kit (Pierce).

RESULTS

Generation of Man2c1-deficient Mice

To generate a Man2c1 KO mouse model, a gene targeting vector was created (Fig. 2A) and used to disrupt the cytosolic α-mannosidase gene by homologous recombination in ES cells. Following drug selection, 6 of 100 ES cell clones screened by PCR analysis indicated homologous recombination. Southern blot analysis of EcoRI-digested genomic DNA resulted in a 12-kb EcoRI fragment of the WT allele and a 5-kb specific fragment of the recombinant allele, confirming the disruption of the Man2c1 gene in all of these clones (Fig. 2B).

Two recombinant ES clones were injected into C57BL/6 blastocysts. Male chimeras were identified by agouti coat color and backcrossed with wild-type C57BL/6 females. Heterozygous mice were identified by PCR and intercrossed to generate homozygous mice, which represented the 25% of the F2 offspring, as expected. Genotyping of these mice was carried out by PCR analysis (Fig. 2C).

Man2c1−/− mice were fertile. They appeared normal and did not show any difference in growth, weight, or lifespan up to 12 months when compared with wild-type mice.

Enzyme Activity

α-Mannosidase activity was measured in the kidney, liver, brain, lung, heart, and spleen of 16-week-old KO, heterozygous, and WT mice at both neutral and acid pH. Almost no neutral activity was found in KO tissues (in Fig. 3A, results obtained with liver and kidney are shown; similar results were achieved with the other tissues), whereas in heterozygous mice, ~50% of the wild-type enzyme activity was detected. The activity at pH 4.5 was similar in all mice and most likely reflects the activity of lysosomal mannosidases with an acidic pH optimum.

To evaluate whether the endosomal-lysosomal system was enhanced as a consequence of oligosaccharides accumulation, β-mannosidase, cathepsin D, and cathepsin E activities were also
measured in the kidney, liver, brain, lung, heart, and spleen. Comparable values between KO and WT animals were observed in all analyzed tissues (data not shown).

**Western Blot Analysis**

The deficiency of Man2c1 in KO mice was confirmed by immunoblotting analysis. The amount of protein in heterozygous tissues was reduced with respect to WT counterparts (in Fig. 3B, the result obtained with liver and kidney is shown).

**Oligosaccharide Analysis**

To obtain functional evidence of Man2c1 knockout, we quantified and compared the structure of free oligomannosides in various tissues of WT and KO male and female mice. Free oligosaccharides were extracted from tissue homogenates and permethylated prior to analysis by MALDI-TOF MS. WT tissues presented a very low-level population of free Man2–5GlcNAc1 (Fig. 4, A and B). These populations represent...
the steady-state level of free oligomannosides originating from the ERAD process. In contrast, a clear accumulation of higher oligomannosides species, particularly Man8–9GlcNAc1, was observed in Man2c1 KO mouse tissues (Fig. 4, A and B). Their abundance is consistent with the function of the Man2c1 because they represent the specific substrates of the KO enzyme. However, the presence of smaller species (Man1GlcNAc1 to Man7GlcNAc1) indicates that another cellular /H9251-mannosidase was able to trim Man2c1 substrates. Interestingly, we observed that the accumulation of Man8–9GlcNAc1 species was tissue-specific. They were more abundant in the liver and spleen and less represented in the brain and heart. Because KO mouse models of lysosomal glycosidases were often accompanied by the presence of oligosaccharides in urines, we checked their presence in Man2c1 KO mice urine, but no trace of oligomannosides was detected (data not shown).

Histological Findings

A screen of α-mannosidase knockout tissues revealed major histopathological changes in several organs (Fig. 5) where the liver, small intestine, kidney, and CNS featured the most impressive alterations. Somewhat in contrast to the tissue distribution of oligosaccharide accumulation, we did not observe major defects in spleen tissue.

CNS—Within the isocortical lamina V, the major CNS white matter tracts (corpus callosum, fimbria hippocampi, internal capsule, and hemispherical subcortical white matter) (Fig. 5, C and D) feature lesions in the form of multiple irregular patches of about 100- to 200-mm diameter, characterized by vacuolated (oligodendro) glial cells. Although here the vacuoles extend into the proximal parts of the glial processes, the myelin sheaths still appear normal, at least in high-resolution light microscopy on semithin sections of epoxy resin-embedded material. Within these patches, activated microglial cells can be revealed by RCA1 staining, and affected cells feature an increased stainability with mannose-binding lectins. Interestingly, such white matter defects were not observed in other regions of the CNS, like the cerebellum (data not shown).

Liver—In the livers of α-mannosidase-deficient mice (Fig. 5, E–H), numerous hepatocytes appear swollen, featuring an abnormally pale cytoplasm studded with mitochondria (Fig. 5, G and H). The presence of large amounts of glycogen and cellular edema could be confirmed by electron microscopy (Fig. 5, I–L). Interestingly, the mitochondria themselves were not affected, and dilated profiles of rough endoplasmic reticulum (RER) were only encountered occasionally, mainly in cells still
FIGURE 5. Key lesions of Man2c1-deficient mice shown in semithin resin sections stained with toluidine blue and pyronine G. In contrast to wild-type isocortex lamina V (A), pyramidal neurons in KO lamina V (B) feature major vacuolation around their basolateral aspect (compare arrows in A and B), typically sparing the dendritic compartment, thus arguing for a specific vulnerability of adaxonal protein synthesis for Man2c1 deficiency. C and D, the subcortical white matter of WT (C) and KO (D) mice. Note again the vacuolation in oligodendroglia in KO mice (C and D, arrows). E–H, morphology of hepatocytes in WT (E), heterozygous (F), and KO (G) mice is compared in semithin sections. Note the accumulation of lipid droplets in heterozygous mice (F) and the occurrence of unusually pale cells in knockout livers (G and H, arrows). Note that, in these pale cells, the cytoplasm is segregated by staining characteristics, featuring spots of dark basophilic material (H, arrow). Transmission electron microscopy on KO livers (I–L) showed a high glycogen content in these cells (I and J), accompanied by cellular edema (K and L), explaining the occurrence of pale cells in light microscopy. Likewise, we compared the morphology of the small intestine in all three genotypes (M–O). Note that, at low magnification detailing the entire intestinal villi, especially WT and heterozygous tissue did not reveal visible differences. Only KO villi of the small intestine featured an irregular basal border of enterocytes, characterized by multiple retronnuclear vacuoles and invading lymphocytes (O and inset). Scale bars = 20 μm (A–D); 15 μm (E, F, and H); 10 μm (G); 5 μm (I–L); 15 μm (M–O).
Cytosolic α-d-Mannosidase (Man2c1)-deficient Mice

appearing normal by other criteria. Both in affected and still normal-appearing cells, droplets filled with material that exhibited staining characteristics compatible with lipids and bile constituents were detected. They were located next to some clear vacuoles and could be indicative of microcholestasis next to a significantly more prevalent lipid accumulation. A more frequent occurrence of lipid droplets was already seen in heterozygous mice. Lectin histochemistry revealed groups of hepatocytes predominantly situated at the periphery of lobuli, featuring increased stainability with both mannose- and glycogen-binding lectins. Similar to the situation in enterocytes, increased lectin staining without overt cell degeneration was, to a smaller degree, also observed in heterozygous mice (not shown).

**Small Intestine**—Within the small intestine and especially within the duodenum (Fig. 5, M–O), numerous large clear vacuoles piling up in a mainly retronuclear (i.e. basal) position in the cells were seen already at the light microscope level, an effect not seen in heterozygous mice. Moreover, lymphocytes were seen to invade the basal region of the gut epithelial layer (Fig. 5O, inset). In contrast, the apical cell region, including the brush border, was not visibly affected by vacuole formation and, besides the glycogen stores (see below), even features a normal ultrastructure.

Enterocytes in knockout animals are characterized by an increased stainability of the supranuclear cytoplasm and, partly, the brush border by mannose-binding lectins as well as lectins detecting glycogen. Interestingly, this effect also occurred in enterocytes of heterozygous mice (Fig. 6, A–F). The presence of significant amounts of glycogen could be verified by transmission electron microscopy (Fig. 6, G and H). Within the villus stroma, these changes are accompanied by dilated intracellular spaces and an increased density of macrophages situated between blood vessels and lymphatics.

**Kidney**—We observed a small number of scattered individual glomeruli being collapsed, i.e. having reduced or lost lumina of both capillaries and Bowman’s space. Some of the still normal-appearing glomeruli featured a moderate fibrosis visible mainly by transmission electron microscopy, with collagen bundles and fibroblasts lined up around the outer leaflet of Bowman’s capsule (Fig. 6, O–Q). Increased lectin stainability, albeit at variable intensity, was mainly observed in the epithelia of proximal tubules, which, however, displayed a normal ultrastructure.

**Oligosaccharide Accumulation**—In general, the lectin data corroborate the accumulation of mannosyl compounds in different tissues and also reveal conspicuously high levels of glycogen in cell types capable of gluconeogenesis. In addition, we sometimes noticed increased lectin binding in the microvascular endothelia of different Man2c1-deficient tissues (Fig. 6, I–N), most notably in the heart and liver. That could be indicative of an increased transendothelial transfer of accumulated saccharides. Interestingly, a limited accumulation was also observed in heterozygous tissues, albeit without the signs of cellular degeneration seen in the respective KO cells.

**DISCUSSION**

Free Man$_{7-9}$GlcNAc$_2$ can be released from dolicholpyrophosphate-linked oligosaccharides during the biosynthesis pathway of N-linked glycans or from misfolded glycoproteins throughout the ERAD process. In the cytosol, fOS are reduced to Man$_n$GlcNAc to enter into the lysosomes and be degraded completely (12).

Until now, the in vivo consequences because of the loss of activity of the enzymes involved in the cytosolic trimming of fOS were unknown. Here we describe the effects caused by the lack of the cytosolic α-mannosidase by the generation of a Man2c1 KO mouse line.

Man2c1-deficient mice were fertile and showed a normal appearance up to the age of 1 year. Notably, no skeletal or gross neurological abnormalities characteristic for deficiency of lysosomal mannosidases were observed. However, a detailed analysis revealed the presence of less obvious biochemical and histological alterations in several tissues.

The accumulation of Man$_{8-9}$GlcNAc oligosaccharides was recognized in the brain, heart, kidney, liver, lung, and spleen of KO mice. A quantitative analysis showed higher levels of fOS in the spleen and liver when compared with the other analyzed KO tissues (Fig. 4, A and B). Their presence can reflect the transport of Man$_{8-9}$GlcNAc from the cytosol into the lysosomes via the ATP-dependent transporter (10, 11). Even if oligosaccharide import was selective toward partially demannosylated fOS, the presence of one GlcNAc residue at the reducing end was described to be of critical importance for its interaction with the ATP-dependent transporter. When transported into the lysosomes, the degrading process of Man$_{8-9}$GlcNAc can be carried out by the acidic α- and β-mannosidases. However, accumulated Man$_{8-9}$GlcNAc could derive from the alternative route followed by some misfolded glycoproteins. These molecules pass from the ER to the Golgi complex, where α1,2mannosidases and/or endo-α-mannosidases act on the N-linked oligosaccharides (16). The resulting glycoproteins are then recycled back to ER and submitted to ERAD targeting, producing fOS (12, 28). Alternatively, Man$_n$GlcNAc$_1$ and Man$_n$GlcNAc$_2$ compounds could be transported by a nonspecific mechanism from the cytosol into the lysosomes, where the degrading process can be carried on by the acidic α-mannosidase. However, the activity of some lysosomal enzymes (α-mannosidase, β-mannosidase, and cathepsin D) and the endosomal enzyme cathepsin E in 16-week-old Man2c1-deficient mice was similar to that in WT littermates. This does not support the hypothesis of an endosomal-lysosomal system activation.

Lectin and transmission electron microscope analysis of glycogen storage, on one hand, confirmed data from biochemical analysis but also revealed two further aspects, i.e. the small-scale accumulation in heterozygous tissues and the presence of conspicuously large amounts of glycogen next to mannosyl compounds, whereby the latter effect seems to be restricted to cells capable of gluconeogenesis (for enterocytes, see Ref. 29). The small-scale accumulation of mannosyl compounds in heterozygous tissues may be seen in accord with the significantly reduced amount of enzyme protein described here, driving cells to the verge of haploinsufficiency. Likewise, we observed degenerative effects on cells only in the knockout material, in line with a loss-of-function effect.
FIGURE 6. Increased lectin binding reveals accumulation of mannose- or GlcNAc-containing saccharides in KO tissues. Comparing WT (A), heterozygous (B), and KO (C) enterocytes, we show that enrichment of these compounds is already visible in the supranuclear portion of heterozygous enterocytes, similar to knockout cells (C). This enrichment is paralleled by an enrichment of glycogen, as shown by both lectin histochemistry (D–F) and electron microscopy (F, G, and H). In the kidney (I–N), lectins recognizing mannosyl compounds featured next to intensely staining glomerular capillaries (Gl_Cap). Multiple, intensely staining dots probably corresponding to lysosomes are visible in KO proximal tubules (PT). Although most of the glomeruli in KO kidneys featured a normal appearance in light microscopy (M), individual units looked collapsed (N), with erythrocytes blocked within capillaries. Transmission electron microscopy featured a normal overall morphology of uncollapsed glomerula with a normal morphology of endothelia and podocytes (P, arrowhead) but featuring a conspicuously thick basal lamina around the outer leaflet of Bowman’s capsule (O and Q, arrowheads), thick bundles of collagen (Q, double asterisk), and the deposition of a multilayered basal lamina-like material around adjacent blood vessels (O and Q, arrowheads). Scale bars = 20 μm (A—E), 15 μm (F), 50 μm (I, J, M, and N); 20 μm (K and L), 20 μm (O), 5 μm (P and Q).
Cytosolic α-d-Mannosidase (Man2c1)-deficient Mice

The relatively high glycogen content of knockout cells, in turn, may be explained by the bidirectional regulation of glucose-neogenesis by ER stress responses (30–32), whereby glycogen formation could simply be a method to reduce intracellular osmotic pressure created by a surplus of glucose. Because accumulation of mannose-containing fos in the cytoplasm, occurring both in Man2c1 knockout as well as overexpressing cells (this work and Ref. 18) has been linked to the activation of an ER stress response, glucose/glycogen accumulation may be a rather straightforward response to mannosyl fOS enrichment or defects of their turnover.

Cellular lesions and/or outright degenerations were only seen in a few instances. Increased retronuclear vacuolization of enterocytes (i.e. affecting the region of the Golgi field of these cells and the nutrient extrusion site toward the blood vessels and lymphatics of the villus stroma) remain unexplained so far, the more so as they do not coincide with glycan storage, which was concentrated in the apical cell pole.

Because ER stress signals (like SEL1L, Ref. 33) have been linked to intestinal epithelial cancer progression and because Man2c1, likewise, has been reported to play a role in the progression toward an increased malignancy of prostate cancer, Man2c1-deficient mice may be a valuable model to further investigate the link between fOS-associated ER stress and epithelial tumorigenesis, e.g. in older mice.

A histological analysis further revealed severe changes in the CNS, kidney, and liver. In the CNS, the predominant occurrence of neuronal lesions in layer V coincides with the presence of predominantly long-axon projection (Golgi type I) neurons, i.e. cells characterized by high protein production from the rough endoplasmic reticulum to sustain their axonal projections. In contrast, other Golgi type I neurons in the cortex, like the small pyramidal cells of layer II/III, have comparatively short axons with associational or commissural trajectories. Therefore, it is of special interest that the vacuolization of neurons is mainly seen in their basal aspect, i.e. close to the axon hillock, which would coincide with an especially important role of Man2c1 with the turnover of sugar moieties from proteins destined for axonal localization, a notion supported by the localization of specific mRNAs at this site (34).

The occurrence of lesions in the subcortical white matter is also intriguing because oligodendroglia appear to be especially prone to ER stress reactions, both during development from its precursors (35) as well as in a variety of neurodegenerative disorders like Parkinson disease or multiple-system atrophy (36), i.e. even in conditions of primarily neuronal origin.

Degeneration of scattered hepatocytes by morphology was always associated with cell swelling, where mitochondria remained conspicuously intact, which, speculatively, may be interpreted as an osmotic origin of water influx, e.g. because of increased cytosolic sugar content. This interpretation would be in line with the high glycogen amounts because hepatocyte swelling (next to ER stress) is a documented strong activator of converting glucose into the osmotically less critical glycogen polymer form (37, 38). Therefore, the high protein turnover characteristic of liver parenchyma may be the ultimate reason for mannosyl compound accumulation by ERAD defects.

Comparison of the phenotype of Man2c1-deficient mice to that of mice lacking lysosomal mannosidases next to numerous differences reveals few surprising similarities. To start with the latter, vacuolation of, specifically, the basal (adaxonal) side of isocortical neurons (mainly long-axon Golgi type I cells) seen in Man2c1 deficiency (Fig. 5B) have been observed in a similar fashion in β-mannosidase knockouts (Fig. 4A in Ref. 39).

A related parallel between Man2c1 deficiency and β-mannosidosis may exist in the case of oligodendrocytes, which are vacuolated in both instances, even if these cells appear to be significantly more affected in β-mannosidosis compared with Man2c1 deficiency (39). Available biochemical data indicate that deficiency for β-mannosidase causes accumulation of disaccharides. That is, their immediate biochemical lesion is clearly distinct from that seen in Man2c1 deficiency. Thus, one might speculate that, in both conditions, the related vacuolation pattern might rather be linked to similarly sorted (e.g. axonal or myelin) glycoproteins, be it from ineffective synthesis and ERAD or ineffective lysosomal degradation (in the case of axons, following retrograde transport). However, despite these similarities, murine Man2c1 deficiency does not reflect the severe phenotype of bovine β-mannosidase. Strikingly, phenotypes differ significantly from lysosomal α-mannosidase deficiency, and even when similar organs are affected, like the kidney or CNS, the distribution pattern of affected cells is clearly different (40). As yet, the few but intriguing similarities between Man2c1 deficiency and β rather than α mannosidosis remain unexplained but may indicate that the identity of misdigested glycoproteins may be more important than the internal structure of the glycan moieties.

The results described in this work demonstrate the essential role of Man2c1 in the fOS degradation process. The tissue damage observed in Man2c1-deficient mice suggests that the genetic lack of this protein could cause a not-yet-described disease in humans with involvement of the CNS.

Furthermore, several reports have confirmed the association between MAN2C1 and malignant transformation processes that occur in cancer cells (19–21). MAN2C1 overexpression promoted tumor growth and metastasis of xenograft tumors in immunocompromised mice. Moreover, up-regulation of MAN2C1 was observed in human prostate cancer cells, where MAN2C1 seemed to attenuate PTEN functions by binding to PTEN (19). Conversely, down-regulation of MAN2C1 induced growth delay or arrest in nasopharyngeal carcinoma cells (41) and led to mitotic arrest and apoptosis in esophageal cancer cells (20). Recently, the antiapoptotic function of MAN2C1 was investigated. MAN2C1 seems to have an enzyme-independent role in the apoptotic signal mechanism. In particular, down-regulation of MAN2C1 caused apoptosis in HeLa cells via a mitochondrion-dependent intrinsic pathway without the occurrence of ER stress (42). In this context, our model could represent a useful tool to better understand the role of Man2c1 in the apoptotic signal pathway and in tumorigenesis of various cancer types in vivo.

Acknowledgments—We thank Drs. Giovanni Ricci and Michela Maserati for contributions to the mouse-related work and Marcello Coli for technical support.
Cytosolic α-D-Manniosidase (Man2c1)-deficient Mice
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