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Mouse liver lysosomes contain enzymatically active processed forms of Hyal-1

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ABSTRACT

It has long been known that liver lysosomes contain an endoglycosidase activity able to degrade the high molecular mass glycosaminoglycan hyaluronic acid (HA). The identification and cloning of a hyaluronidase with an acidic pH optimum, Hyal-1, suggested it might be responsible for this activity. However, we previously reported that this hydrolase could only be detected in pre-lysosomal compartments of the mouse liver using a zymography technique that allows the detection of Hyal-1 activity after SDS–PAGE ("renatured protein zymography"). Present work reveals that the activity highlighted by this technique belongs to a precursor form of Hyal-1 and that the lysosomal HA endoglycosidase activity of the mouse liver is accounted for by a proteolytically processed form of Hyal-1 that can only be detected using "native protein zymography". Indeed, the distribution of this form follows the distribution of β-galactosidase, a well-established lysosomal marker, after fractionation of the mouse liver in a linear sucrose density gradient. In addition, both activities shift toward the lower density region of the gradient when a specific decrease of the lysosomal density is induced by Triton WR-1339 injection. The fact that only native protein zymography but not renatured protein zymography is able to detect Hyal-1 activity in lysosomes points to a non-covalent association of Hyal-1 proteolytic fragments or the existence of closely linked partners supporting Hyal-1 enzymatic activity. The knockdown of Hyal-1 results in an 80% decrease of total acid hyaluronidase activity in the mouse liver, confirming that Hyal-1 is a key actor of HA catabolism in this organ.

1. Introduction

Hyaluronic acid (HA) is a high molecular mass glycosaminoglycan of the extracellular matrix composed of repeating disaccharides of β-D-glucuronyl-(1 → 3)-β-N-acetyl-D-glucosamine connected through (1 → 4) linkages. In 1965, Aronson and Davidson reported that rat liver lysosomes contain a hyaluronidase activity [1]. This conclusion was based on incubation of HA with lysosome-enriched liver subcellular fractions and subsequent detection of the HA reducing ends using the "Reissig assay" [2]. Furthermore, the authors showed that, similar to lysosomal hydrolases, the distribution of this hyaluronidase activity in a linear sucrose density gradient shifted toward the lower density region of the gradient when the animals were injected with Triton WR-1339, a non-ionic detergent that specifically decreases the density of liver lysosomes [1,3,4]. In 1984, Fiszer-Szafarz reported that several hyaluronidase activities, including a rat liver lysosomal hyaluronidase activity, could be detected by an in-gel digestion assay (hereafter called "native protein zymography"), in which samples are resolved by native polyacrylamide gel electrophoresis prior to incubation in conditions that allow the degradation of the HA included in the gel [5]. Using a slightly modified version of this technique (hereafter referred to as "renatured protein zymography") where proteins were resolved by SDS–PAGE and subsequently renatured [6], Stern and colleagues documented that the hyaluronidase purified and cloned from human plasma, named Hyal-1, exhibits a molecular mass of 59 kDa under non reducing conditions as well as an acidic pH optimum [7,8]. High serum levels of HA and accumulation of glycosaminoglycans in lysosome-like structures were reported in patients with Hyal-1 deficiency, who mainly suffer from articular swelling and pains [9–11]. Taken together, these findings suggested that Hyal-1 may be the actor of HA catabolism detected in liver lysosomes.

Abbreviations: DMAB, *p*-dimethylaminobenzaldehyde; ER, endoplasmic reticulum; GPI, glycophosphatidylinositol; HA, hyaluronic acid; Hyal-1, hyaluronidase-1; RSA, relative specific activity.

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However, our recent analysis of the localization of Hyal-1 in the mouse liver using the renatured protein zymography assay only highlighted the presence of Hyal-1 activity in pre-lysosomal compartments that do not undergo a density shift after Triton WR-1339 injection [12]. These results were difficult to reconcile with a lysosomal residence of hepatic Hyal-1. More recently, we discovered that RAW264.7 mouse macrophages contain two forms of Hyal-1: a precursor form that localizes to biosynthetic compartments and endosomes, and a proteolytically processed form that localizes to lysosomes at steady state [13]. The processing of the endocytosed precursor was found to start in endosomes and was completed within 30 min. Interestingly, while the HA-degrading activity of both forms could be detected using native protein zymography, only the precursor forms exhibited activity under renatured protein zymography assay conditions, suggesting that non-covalent associations must be preserved in order to detect the activity of the cleaved form of Hyal-1. These findings lead us to hypothesize that a similar processing of Hyal-1 takes place in mouse liver endo/lysosomal compartments. Using native experimental conditions, we now report that, in addition to full-length pre-lysosomal Hyal-1, the mouse liver contains large amounts of active/processed forms of Hyal-1 that localize to the lysosomes.

2. Material and methods

C57BL/6(N10) Hyal-1$^{-/-}$ mice obtained from the Mutant Mouse Regional Resource Center (MMRRC, University of California, Davis, USA) were raised at the University of Namur animal facilities. All experimental procedures were conducted in accordance with local regulations under the supervision of the Animal Ethics Committee of the University. Male mice were injected intraperitoneally with 17 mg of Triton WR-1339 (Tyloxapol, Sigma–Aldrich, St. Louis, MO, USA) in 0.2 mL of saline solution, 72 h prior to fractionation. The livers of control and Triton WR-1339-injected animals (fasted for the last 16 h) were then fractionated into 5 fractions by differential centrifugation as described by de Duve et al. [14]. The M + L pooled fraction was then centrifuged in a linear sucrose density gradient as described previously [3,12]. The β-galactosidase and alkaline α-glucosidase content of each collected fraction was assayed by fluorometric enzyme assays, while Hyal-1 activity was measured by two methods of zymography (detailed assay protocols can be found in [13]). To measure the total acid hyaluronidase activity, the samples were incubated with 2 mg/mL of rooster comb HA (Sigma–Aldrich, St. Louis, MO, USA) in 0.1 M formate buffer, pH 3.7, containing 0.1 M NaCl overnight at 37 °C. HA fragments were then detected using the Reissig assay [2]. Briefly, after heating in a boiling water bath for 5 min, 30 μL of borate solution (0.8 M boric acid–KOH, pH 10) were added to start the Morgan–Elson reaction. Next, the mixture was heated for 3 min in a boiling water bath prior to the addition of 750 μL DMAB reagent (prepared as described in [2]) and incubated at 37 °C for 20 min. After centrifugation at 14,500*g* for 5 min, the absorbance of the clear supernatant was read at 585 nm.

3. Results

3.1. The mouse liver contains several forms of Hyal-1

The mouse liver was fractionated into N (nuclear), M (heavy mitochondrial), L (light mitochondrial), P (microsomal) and S (soluble) fractions as described by de Duve et al. [14]. As expected, this fractionation scheme allowed some enrichment of lysosomes in the M and L fractions, as indicated by the 2.7 and 3.8 Relative Specific Activity (RSA) of the lysosomal marker β-galactosidase in those fractions compared with the starting homogenate, respectively (Fig. 1A). Altogether, 53% of β-galactosidase was recovered in M + L fractions, while only 4% was detected in the P fraction (Fig. 1A). By contrast, the endoplasmic reticulum (ER), represented by alkaline α-glucosidase, was mainly recovered in L and P fractions (56%) with a similar enrichment in these two fractions (Fig. 1B).

The distribution of Hyal-1 activity was analyzed among the different fractions by zymography, using either renatured or native protein zymography [5,6,13]. Equal amounts of proteins were loaded for each fraction. The results, shown in Fig. 1C, indicate that the activity of Hyal-1 detected by renatured protein zymography is visualized as a 70 kDa doublet and is enriched in L and P fractions. This distribution is reminiscent of the distribution of the ER marker alkaline α-glucosidase (Fig. 1B). Native protein zymography (Fig. 1D) yields a different picture composed of two signals: one is enriched in the L and P fractions (Fig. 1D, open arrow) and thus corresponds to the forms detected by renatured protein zymography, whereas a second hyaluronidase activity is enriched in the M and L fractions (Fig. 1D, closed arrow), similar to β-galactosidase. These results are consistent with the concomitant presence of a precursor form of Hyal-1 (detected by both techniques as a doublet) and a proteolytically processed form (detected only under native conditions) in the mouse liver [13]. As the processed form accounts for the vast majority of the liver hyaluronidase activity, this result indicates that liver Hyal-1 mainly localizes to a compartment that sediments in the M + L fraction. This would be in accordance with a lysosomal localization.

3.2. Most of the active Hyal-1 localizes to the lysosomes

To investigate the putative lysosomal residence of Hyal-1 in mouse liver, the animals were injected with Triton WR-1339 and the subcellular distribution of Hyal-1 was analyzed in N, M, L, P and S fractions (prepared as above), and after fractionation of the M + L liver fraction using isopycnic centrifugation in a linear sucrose density gradient. Triton WR-1339 specifically decreases the buoyant density of lysosomes in this type of gradient, which results in a shift of distribution of lysosomal proteins toward the lower density region of the gradient [3,12]. After differential centrifugation, the distribution of alkaline α-glucosidase remained similar in Triton WR-1339-treated animals (Fig. 1F) compared with controls (Fig. 1B), while the distribution profile of β-galactosidase (Fig. 1E) slightly shifted toward the soluble fraction: the percentage of β-galactosidase recovered in fraction S increased from 20.6 ± 3.6% to 35.2 ± 5.1% (n = 3), most likely as a consequence of the decreased resistance to homogenization of the modified lysosomes. It should be noted that this did not alter the distribution of intact lysosomes within the other fractions. The distribution of the processed form of Hyal-1 in native protein zymography followed the distribution of β-galactosidase, with a clear increase of signal in the S fraction but a similar pattern in the sedimented fractions (N → P) in the Triton WR-1339-treated animals compared with control animals (Fig. 1H vs 1D, closed arrows). By contrast, the precursor form of Hyal-1 (Fig. 1H, open arrow), which was also detected as a doublet by renatured protein zymography (Fig. 1G), exhibited the same distribution after Triton WR-1339 injection.

The density modification of lysosomes induced by the Triton WR-1339 treatment was then visualized by fractionation of the M + L fraction over a linear sucrose density gradient (Fig. 2). The distribution of β-galactosidase shifted from a median density of 1.19 g/mL in control animals to a median density of 1.15 g/mL in Triton WR-1339-injected mice, while the distribution of alkaline α-glucosidase remained centered around 1.20 g/mL in control and treated animals (Fig. 2A and B), confirming the specificity of the density shift. As reported previously by Gasingirwa et al. [12], the Hyal-1 forms detected using renatured protein

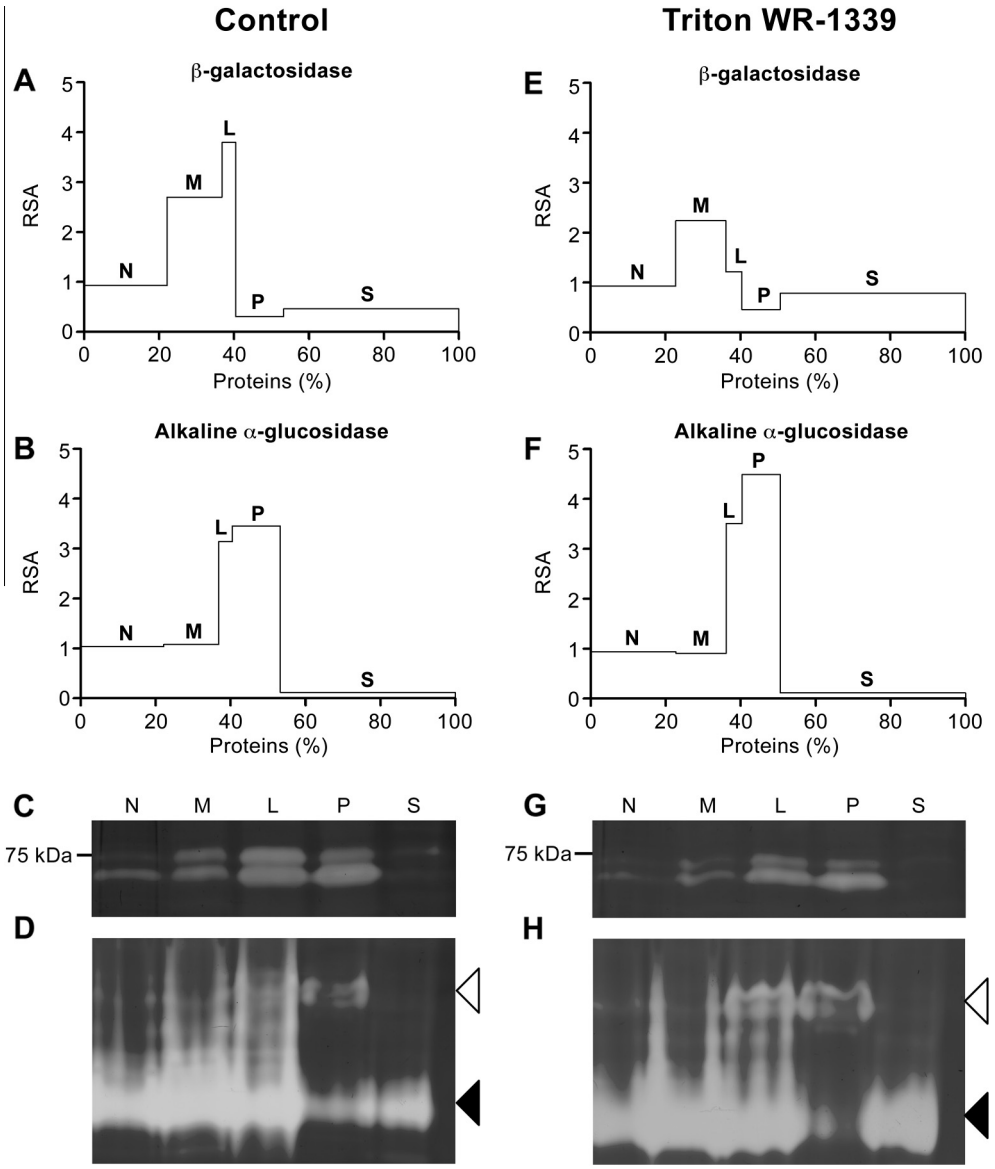

Fig. 1. Analysis of the subcellular distribution of Hyal-1 in the liver of control and Triton WR-1339-injected mice by differential centrifugation. The livers of control (A–D) or Triton WR-1339-injected (E–H) mice were fractionated into 5 fractions (N, M, L, P and S) by differential centrifugation. The activities of the lysosomal hydrolase β-galactosidase (A, E) and of the ER marker alkaline α-glucosidase (B, F) were measured in each fraction by *in vitro* enzyme assays. The graphs show the enrichment factor of the enzyme in the different fractions, i.e., the Relative Specific Activity (RSA: ratio of the percentage of activity of the enzyme in a given fraction to the percentage of proteins in this fraction), plotted against the percentage of proteins in each fraction. The activity of Hyal-1 was analyzed by renatured protein zymography (C, G) and native protein zymography (D, H). The same amounts of proteins were loaded for each fraction. The open and closed arrows indicate the two Hyal-1 forms detected by native protein zymography.

zymography were located in the high density region of the control sucrose gradient, and remained in this region after Triton WR-1339 treatment (Fig. 2C). Comparing their distribution in control and treated animals (established from the quantification of the zymography signals) showed that these forms equilibrate around a median density of 1.20 g/mL in both groups (Fig. 2E), consistent with the presence of Hyal-1 precursor forms in pre-lysosomal compartments. By contrast, the analysis of the gradient fractions by native protein zymography revealed that the Triton WR-1339 treatment strongly shifted the distribution of the processed form of Hyal-1. Indeed, the processed form co-distributed with β-galactosidase in the high density region of the gradient in control animals, and shifted toward the lower density region, similarly to this lysosomal marker, in the treated mice (Fig. 2D, closed arrows, and Fig. 2F). This demonstrates that lysosomes contain large amounts of active processed form of Hyal-1.

To investigate whether Hyal-1 accounts for the liver acid hyaluronidase activity reported by Aronson and Davidson [1], we measured the HA-degrading activity of each gradient fraction by the same *in vitro* assay used in their study, based on the detection of HA reducing ends by the Reissig method [2] (Fig. 2G). The distribution profile of the acid hyaluronidase activity detected by this method was very similar to the distribution of the processed Hyal-1 form, detected using native zymography (Fig. 2F). Most importantly, the two distributions were similarly shifted after Triton WR-1339 injection. Next, we compared the total acid hyaluronidase activity of wild-type and Hyal-1$^{-/-}$ mouse liver homogenates using native protein zymography and the Reissig assay.

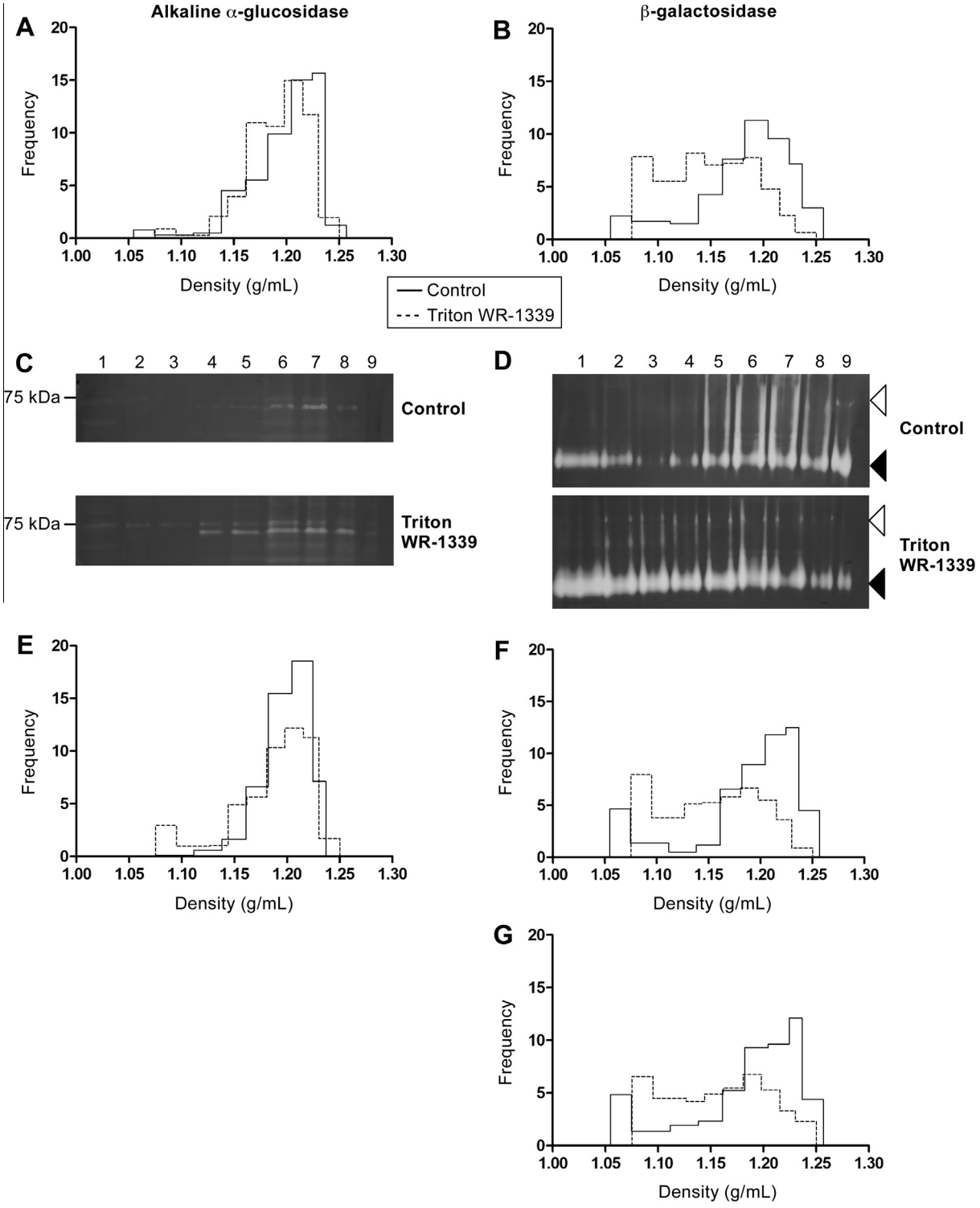

Fig. 2. Analysis of the distribution of the different forms of Hyal-1 in the liver of control and Triton WR-1339-injected mice by isopycnic centrifugation. An ML fraction was prepared from control and Triton WR-1339-injected mouse livers and subjected to isopycnic centrifugation in a linear sucrose density gradient extending from 1.09 to 1.26 g/mL. The activities of alkaline α-glucosidase (A) and β-galactosidase (B) were assayed in the 9 fractions collected from top to bottom in both control (—) and treated (—) animals. The ordinate corresponds to the frequency (Q/SQ.r, where Q represents the activity found in the fraction, SQ, the total activity recovered in the sum of the fractions, and r, the increment of density from top to bottom of the fraction). The fractions were analyzed for Hyal-1 activity by renatured protein zymography (C) or native protein zymography (D). Arrows in (D) point to the two forms of Hyal-1 detected by the latter. The signals obtained in (C) and (D) were quantified and represented as described for the marker enzymes (E–F). Lastly, all fractions were analyzed using the Reissig assay (G).

Zymography showed a complete disappearance of the signal in the Hyal-$1^{-/-}$ mouse livers (as previously reported in [13]) and the Reissig assay detected an 80 ± 6.8% decrease in these mice ($n = 3$), indicating that the vast majority of the liver acid hyaluronidase activity detected by this method is accounted for by Hyal-1.

4. Discussion

Coupling subcellular fractionation of control and Triton WR-1339-treated mouse livers with zymographic techniques, we demonstrate that large amounts of enzymatically active Hyal-1 localize

to the lysosomes of the liver. However, lysosomal Hyal-1 is a proteolytically processed form that does not exhibit enzymatic activity under the assay conditions of renatured protein zymography, suggesting that non-covalent association of Hyal-1 proteolytic fragments or the existence of closely linked partners is required to support Hyal-1 activity in hepatic lysosomes. This conclusion is supported by our recent study of the intracellular trafficking and processing of Hyal-1 in RAW264.7 mouse macrophages, in which we could observe that the proteolytic cleavage of Hyal-1 occurs during endocytic transport of the protein toward lysosomes, i.e., in endosomes [13]. Of note, a processed form of Hyal-1 has also been described in human urine [15].

Our knockout mice observations that Hyal-1-mediated degradation of HA represents 80% of the total acid hyaluronidase activity of the liver confirm that Hyal-1 is a key actor of HA degradation and turnover in that organ, which is acknowledged as the main site of plasmatic HA catabolism [16]. Indeed, the liver exhibits high mRNA levels of Hyal-1, and Hyal-1 deficient mouse livers accumulate HA [17,18]. It has been reported that circulating HA is rapidly endocytosed by the "Hyaluronic Acid Receptor for Endocytosis" (HARE) expressed on endothelial sinusoidal liver cells and that its half-life in the blood is only 3–5 min [19–21]. We recently found evidence that Hyal-1 is targeted to the lysosomes of RAW264.7 macrophages by a secretion/recapture mechanism involving the mannose receptor [13]. Interestingly, this receptor is highly expressed by the non-parenchymal cells of the liver, especially by the sinusoidal endothelial cells, suggesting that Hyal-1 (including plasmatic Hyal-1) could be efficiently captured from the extracellular medium by these cells [22–24]. As other plasma acid hydrolases internalized by this mechanism are known to contribute to the lysosomal degradation of substrates in these mannose receptor expressing cells [25], it is tempting to suggest that the liver sinusoidal cells may be the primary site of HA degradation in the liver. The degradation of HA may start extracellularly with the GPI-anchored cell surface hyaluronidase Hyal-2, which cleaves high molecular mass HA to ~20 kDa fragments [26,27]. The abnormal storage of HA that is observed in the liver sinusoidal cells of mice lacking Hyal-2 supports this hypothesis [28]. It also suggests that Hyal-2 may account, at least partly, for the 20% of acid hyaluronidase activity remaining in the mouse liver in the absence of Hyal-1, albeit its optimum pH is not clear. It was reported that Hyal-2 exhibits a weak hyaluronidase activity at a pH optimum of 3.8 in C6 glioma cell lysates [27], over a broader range of pH when expressed as a soluble form by insect cells [29], and at pH 6.0–7.0 in a membrane fraction of HEK-293 cells when overexpressed together with the HA binding receptor CD44 [30]. The subsequent hydrolysis of the Hyal-2 digestion products by Hyal-1 could begin directly after endocytosis and continue in the lysosomal compartment, as we found that both the precursor form of Hyal-1, which localizes to pre-lysosomal compartments, and the proteolytically processed form of Hyal-1 (i.e., the lysosomal form) are able to degrade HA. The exoglycosidases β-hexosaminidase and β-glucuronidase, two lysosomal acid hydrolases that are also rapidly cleared by the mannose receptor, are also known to contribute to HA degradation [18,31–34]. Of note, albeit a weak contribution of the hyaluronidase Hyal-3 to the liver HA catabolism cannot be excluded, especially since an elevation of its expression has been described in the Hyal-1 deficient mice [35], this hydrolase appears to have no detectable HA-degrading activity in somatic cells [30,36], and its knockdown in mice did not result in any obvious tissue accumulation of HA [37]. The involvement of the other known hyaluronidase-like genes in the liver catabolism of HA seems unlikely, as the expression of Hyalp1, Spam1 and Hyal5 appears restricted to the mouse sperm and Hyal4, found in human placenta and skeletal muscle, may not degrade HA [34,38]. Finally, the recently described HA depolymerizing functions of KIAA1199, a protein markedly overexpressed in several cancers, seems to be indirect and to require potential intracellular partners [39].

In summary, we have shown that 80% of the mouse liver acid hyaluronidase activity belongs to Hyal-1, and that this hydrolase accounts for the liver lysosomal hyaluronidase activity reported by Aronson and Davidson [1]. It will be interesting in the future to elucidate the molecular mechanisms/associations that control the processing and activity of Hyal-1.

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