Endocytosis of hyaluronidase-1 by the liver

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It has been suggested that intracellular Hyal-1 (hyaluronidase-1), which is considered a lysosomal enzyme, originates via endocytosis of the serum enzyme. To test this proposal we have investigated the uptake and intracellular distribution of rhHyal-1 (recombinant human Hyal-1) by mouse liver, making use of centrifugation methods. Experiments were performed on wild-type mice injected with 125I-labelled rhHyal-1 and on Hyal-1−/− mice injected with the unlabelled enzyme, which were killed at various times after injection. Activity of the unlabelled enzyme was determined by zymography. Intracellular distribution of Hyal-1 was investigated by differential and isopycnic centrifugation. The results of the study indicated that rhHyal-1 is endocytosed by the liver, mainly by sinusoidal cells, and follows the intracellular pathway described for many endocytosed proteins that are eventually located in lysosomes. However, Hyal-1 endocytosis has some particular features. First, endocytosed rhHyal-1 is quickly degraded. Secondly, its distribution, as analysed by differential centrifugation, differs from the distribution of β-galactosidase, taken as the reference lysosomal enzyme. Further analysis by isopycnic centrifugation in a sucrose gradient shows endocytosed rhHyal-1 behaves like β-galactosidase shortly after injection. However the Hyal-1 distribution is markedly less affected than β-galactosidase, following a prior injection of Triton WR-1339, which is a specific density perturbant of lysosomes. The behaviour in centrifugation of endogenous liver Hyal-1, identified by hyaluronan zymography, exhibits some similarity with the behaviour of the endocytosed enzyme, suggesting that it could originate from endocytosis of the serum enzyme. Overall, these results can be explained by supposing that active endocytosed Hyal-1 is mainly present in early lysosomes. Although its degradation half-time is short, Hyal-1 could exert its activity due to a constant supply of active molecules from the blood.

Key words: endocytosis, hyaluronan, hyaluronidase-1, liver, lysosome.

INTRODUCTION

HA (hyaluronan) catabolism depends mainly on two somatic hyaluronidases: Hyal-1 (hyaluronidase-1) and Hyal-2 (hyaluronidase-2) [1–3]. Hyal-1 exhibits a maximal activity at acidic pH and is considered a lysosomal enzyme [2,3], whereas the location and activity of Hyal-2 is somewhat more controversial [4,5]. Hyal-1 is the major hyaluronidase present in the serum and is frequently referred to as serum hyaluronidase [2]. It is also present in various tissues; the liver, the main site of circulating HA catabolism [6], shows by far the largest Hyal-1 expression, followed by the kidney, heart and lungs [7]. Hyal-1 is also expressed in chondrocytes [8]. Considering the high blood concentrations of Hyal-1, it has been proposed that intracellular Hyal-1, especially in the liver, could originate from plasma enzyme that is recovered by endocytosis after its secretion. Previous in vitro findings from Harada and Takahashi [9] favour such a hypothesis; these authors have shown that Hyal-1 added to the medium of cultured cells is internalized and becomes functional intracellularly. It is to be noted that, in contrast with most lysosomal enzymes, the plasma levels of Hyal-1 are not elevated in patients with mucolipidoses II and III [10]. This suggests that liver Hyal-1 has to follow another pathway to reach lysosomes, rather than the classical intracellular phosphomannosyl recognition pathway. Endocytosis of the serum enzyme is a possibility.

To resolve this issue, we have injected rhHyal-1 (recombinant human Hyal-1) into mice. Centrifugation results indicate that rhHyal-1 injected into mice is rapidly endocytosed by the liver, probably by sinusoidal cells, but is degraded relatively quickly in the endosomal/lysosomal compartment. On the other hand, we can show, via differential and isopycnic centrifugation experiments, that the endogenous liver Hyal-1 exhibits a distribution profile distinct from that of reference lysosomal enzymes, but with some similarity to the distribution of injected rhHyal-1. We suggest that the liver enzyme originates from a continuous endocytosis of Hyal-1 secreted into the serum.

EXPERIMENTAL

Tissue fractionation

Experiments were performed with male NMRI mice and C57BL/6 wild-type (Hyal-1+/+) or Hyal-1−/− mice. The latter were a gift from Professor Barbara Triggs-Raine (University of Manitoba, Winnipeg, Canada). Animals were cared for using protocols approved by the FUNDP (University of Namur) Commission d’Éthique en Expérimentation Animale. Mice were injected intravenously via the tail vein with rhHyal-1, a gift from Dr Greg Frost (Halozyme, San Diego, CA, U.S.A.). In some experiments, rhHyal-1 was labelled with 125I prior to injection using the chloramine T method [11]. In some experiments, mice were also

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Abbreviations used: HA, hyaluronan; HARE, HA receptor for endocytosis; Hyal-1, hyaluronidase-1; Hyal-2, hyaluronidase-2; L fraction, light mitochondrial fraction; M fraction, heavy mitochondrial fraction; N fraction, nuclear fraction; P fraction, microsomal fraction; rhHyal-1, recombinant human Hyal-1, S fraction, soluble fraction.

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injected with Triton WR-1339 (17 mg in 0.2 ml of saline) 4 days before rhHyal-1 injections. A Hyal-1−/− mouse was injected with reconstituted lyophilized wild-type murine serum. All mice were killed at the indicated times after injections.

The liver was perfused with 0.15 M NaCl through the posterior vena cava for 5 min. After perfusion, it was removed and homogenized in ice-cold 0.25 M sucrose with a Potter–Elvehjem-type homogenizer consisting of a smooth-walled glass tube fitted with a Teflon pestle (Arthur Thomas). Fractionation of the homogenate, by differential centrifugation, was performed as described by de Duve et al. [12]; an N fraction (nuclear fraction), M fraction (heavy mitochondrial fraction), L fraction (light mitochondrial fraction), a P fraction (microsomal fraction) and an S fraction (soluble fraction) were successively isolated. For isopycnic centrifugation, sucrose density gradients extending from 1.09 g/cm³ to 1.26 g/cm³ were used. The granule preparations were layered at the top of the gradient. Centrifugations were performed at 39000 rev./min for 150 min with the L fraction and overnight with the P fraction, in the Beckman rotor SW 55Ti. After centrifugation fractions were collected by slicing the tube with a device similar to that described by Beaufay et al. [13].

Enzyme measurements
The acid-soluble β-galactosidase activity was assayed by incubating the granules at 37 °C in 0.2 ml of buffer (50 mM sodium acetate buffer, pH 5, containing 0.5 mM 4-methylumbelliferyl-β-D-galactopyranoside, 50 mM NaCl and 0.05 % Triton X-100). β-N-acetylglucosaminidase activity was measured by incubating the granules at 37 °C in 0.2 ml of buffer (50 mM sodium acetate buffer, pH 5, containing 0.5 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosamine and 0.05 % Triton X-100). In each case, the reaction was stopped by adding 1.3 ml of 50 mM glycine buffer, pH 10.5, containing 5 mM EDTA and 0.5 % Triton X-100. The level of the released fluorogenic group (4-methyl umbelliferone) was determined with a Versa Fluor fluorimeter (Bio-Rad Laboratories) using excitation filter 170–2420 and emission filter 170–2421. For the assay of alkaline phosphodiesterase activity, the granules were incubated at 25 °C in a volume of 0.25 ml of buffer (100 mM glycine buffer, pH 9.6, containing 1.5 mM p-nitrophenyl thymidine-5′-phosphate and 2 mM zinc acetate). The reaction was stopped by adding 1 ml of 0.1 M NaOH. The level of the released p-nitrophenol was determined by measuring the absorbance at 400 nm. Hyaluronidase activity was measured using HA zymography as recently described by Jadin et al. [14]. Briefly, granule samples were added to a loading buffer [250 mM Tris/HCl, pH 6.8, containing 4 % (w/w) SDS, 20 % (v/v) glycerol and 0.01 % Bromophenol Blue]. Proteins were separated by SDS/PAGE [10 % polyacrylamide gels to which 0.17 mg/ml of human umbilical cord HA (Sigma–Aldrich) was added] on ice at 200 mA under a constant voltage of 80 V. After electrophoresis, the gels were rinsed with distilled water and incubated for 2 h in a 3 % (v/v) Triton X-100 solution on a rocking table. The gels were subsequently equilibrated with the assay buffer (100 mM sodium formate, pH 3.7, containing 150 mM NaCl) and incubated at 37 °C for 20 h. After incubation, the gel was washed with water and incubated for 2 h at 39 °C in a medium containing 20 mM Tris/HCl and 0.1 mg/ml Pronase solution (Sigma–Aldrich) to remove interfering protein bands. Subsequently, the gel was rinsed in water and equilibrated in 50 % aqueous formamide for 30 min with gentle agitation. Finally, the gel was stained with 0.05 % Stains-All (Sigma–Aldrich) dissolved in 50 % (v/v) formamide for at least 3 days in the dark. After that, gels were washed in water and photographed with a digital camera on a transilluminator.

Isolation of liver cells
Separation of hepatocytes and sinusoidal cells was performed after collagenase perfusion according to the method described by Seglen [16], with slight modifications as described by Lecoq et al. [17]. Cells were used immediately after their isolation, without subsequent culture.

Purification of lysosomes
Purified lysosomes were obtained from livers of rats injected with Triton WR-1339 [18] according to the method of Trouet [19].

RESULTS
Endocytosis of Hyal-1 by the liver was investigated by following the uptake of rhHyal-1 and, in a limited number of experiments, of serum Hyal-1. The experiments were performed with 125I-labelled rhHyal-1 injected into NMRI (wild-type) mice, or unlabelled rhHyal-1 injected into C57BL/6 Hyal-1−/− mice. The intracellular trafficking of endocytosed Hyal-1 was followed by analytical centrifugation techniques. Liver homogenates were prepared at various times after injection and fractionated by differential and isopycnic centrifugation. The distribution of endocytosed Hyal-1 in the fractions was established by determining the radioactivity or hyaluronidase activity, and compared with that of the marker enzymes β-galactosidase and N-acetylglucosaminidase for lysosomes, and alkaline phosphodiesterase for plasma membrane and endocytic vesicles [20]. Centrifugation experiments were also performed to determine the intracellular localization of endogenous liver Hyal-1.

Uptake of 125I-rhHyal-1 by the liver
The total radioactivity recovered in liver and in blood plasma at various times after 125I-rhHyal-1 injection, is shown in Figure 1(A); 125I-rhHyal-1 was quickly endocytosed, and reached a maximum level within a few minutes after injection, which was maintained for at least 30 min. After that, liver radioactivity was markedly decreased and corresponded to only a small percentage of the injected dose after 60 min. The proportion of acid-soluble radioactivity, resulting from proteolysis of 125I-rhHyal-1 increased almost linearly with time, suggesting that the labelled enzyme begins to be degraded soon after it is endocytosed (Figure 1B).

Differential centrifugation
Livers isolated at 2, 10 and 30 min after injection were fractionated by differential centrifugation according to the method described by de Duve et al. [12]. An N fraction, an M fraction, an L fraction, a P fraction and an S fraction were isolated. The mean proportion of the radioactivity and of the β-galactosidase activity, which is used as a reference enzyme for lysosomes, recovered in the total sedimentable fractions for the three fractions, was 80.5 ± 6.4 % and 86.4 ± 6.0 % respectively; the distribution of this activity in the different sedimentable fractions is shown in Figure 2(A). A relatively high amount of radioactivity was constantly associated with the N fraction. This fraction is very heterogeneous and contains nuclei as the main components along with large sheets of plasma membrane fragments, collagen fibres
and undistrupted cells. It is possible that radioactivity found in the fraction results from a binding of $^{125}$I-rHyal-1 to the large plasma membrane fragments present in this fraction. A significant proportion of radioactivity was also recovered in the P fraction, but this decreased with time. In a similar manner to β-galactosidase activity (a lysosomal marker), radioactivity present in the post-nuclear sedimentable structures was mainly found in the mitochondrial fractions (the M and L fractions), but the partitioning of β-galactosidase and radioactivity between these fractions differed; the lysosomal enzyme sedimented mainly in the M fraction, whereas the radioactivity was mainly recovered in the L fraction. These results illustrate that, like several proteins that are endocytosed by the liver [21,22], $^{125}$I-rHyal-1 is first associated with endocytic vesicles (the P fraction) but is very quickly transferred to the endosomes (the P and L fractions) and lysosomes (the L and M fractions). The rapidity of the transfer suggests that the uptake of $^{125}$I-rHyal-1 is carried out by sinusoidal cells [22].

### Isopycnic centrifugation

To identify the localization of $^{125}$I-rHyal-1 more precisely, fractions recovered by differential centrifugation at early (2 min) and later (30 min) time points were analysed using isopycnic centrifugation in a sucrose gradient. As shown in Figure 2(A), 2 min after injection, most of the radioactivity was almost equally distributed between the P and L fractions. Figure 2(B) illustrates the distribution, after isopycnic centrifugation in a sucrose gradient, of the radioactivity recovered in these fractions 2 min after injection of $^{125}$I-rHyal-1. The distribution curves for the radioactivity are the same in the two fractions and coincide with the distribution of alkaline phosphodiesterase (present in plasma membrane and endocytic vesicles). In the L fraction, there is a correlation between the β-galactosidase (lysosomes) and the radioactivity distribution curves, but it is not exact. The median equilibrium density computed from the density distribution histogram of the fractions was approx. 1.16 g/cm$^3$ for the radioactivity and for alkaline phosphodiesterase, whereas it was 1.18 g/cm$^3$ for β-galactosidase.

At longer time intervals after injection, radioactivity was mostly associated with the mitochondrial M and L fractions with it predominating in the L fraction (Figure 2A). Figure 2(C) illustrates the distributions of radioactivity present in these fractions 30 min after injection of $^{125}$I-rHyal-1 as analysed by isopycnic centrifugation. The similarity between the distribution curves of the β-galactosidase and radioactivity is clearly apparent. The median equilibrium density of both components was approx. 1.19 g/cm$^3$.

To locate the bulk of lysosomes more precisely in the gradient, the same experiments were performed with livers obtained from mice where an injection of Triton WR-1339, a non-ionic detergent, had been administered. This compound selectively decreases the density of liver lysosomes, as a result of its accumulation in these organelles [18]. It can be used to purify lysosomes [19,23] and to detect compounds, for example endocytosed molecules, in these organelles [24,25]. As seen in Figure 2(C), after Triton WR-1339 injection, a large proportion of β-galactosidase recovered in the M and L fractions had shifted towards the lower density end of the gradient, with the median equilibrium density being close to 1.10 g/cm$^3$. The distribution of radioactivity recovered in L and M fractions was markedly less affected by the detergent injection, with median equilibrium densities of 1.1 g/cm$^3$ and 1.17 g/cm$^3$ respectively.

### Endocytosis of unlabelled rHyal-1

Studying liver incorporation of $^{125}$I-rHyal-1 in wild-type mice is straightforward and yields quantitative information on the uptake and intracellular fate of the protein. However, under these conditions, it is not possible to trace the enzyme activity after internalization due to the presence of the endogenous enzyme. Moreover, iodination of the enzyme can affect its activity. This is the reason why, in a second set of experiments, we made use of Hyal-1$^{-/-}$ mice, to which unlabelled rhHyal-1 or serum of wild-type mice was injected. The fate of the internalized enzyme was followed by determining its activity using HA zymography at three time points (4, 30 and 45 min) after injection. Such a method does not yield strictly quantitative results as it is obtained with radioactive enzyme; however, it is accurate enough to illustrate the distribution of the enzyme between the fractions isolated by centrifugation. At the baseline condition, hyaluronidase activity is readily detectable in the liver, kidney and blood plasma of wild-type mice but is totally absent from the same tissues of Hyal-1$^{-/-}$ mice (results not shown).

Liver fractions were isolated by differential centrifugation. At 45 min after injection, Hyal-1 activity was barely detectable (results not shown), indicating that by that time rhHyal-1 taken up by the liver had probably been degraded to a large extent, as was seen with $^{125}$I-rHyal-1 (see Figure 1). Figure 3(A) illustrates the activity of rhHyal-1 recovered in the fractions at 4 min (panel a) and 30 min (panel b) after injection. In these experiments,
Figure 2  Distribution of radioactivity after differential and isopycnic centrifugation

The distributions were obtained with livers originating from mice killed at various times after $^{125}$I-rhHyal-1 injection. (A) Distribution of radioactivity after differential centrifugation of liver homogenates from mice killed at the indicated time after injection. The values are given as percentages of the amount present in the total sedimentable components of the homogenate ($N + M + L + P$ fractions). For the sake of comparison, the mean distribution of $\beta$-galactosidase (a lysosomal marker) in the three fractionation experiments is presented (means ± S.D.). (B) Distribution, after isopycnic centrifugation, of the radioactivity recovered in the $P$ and $L$ fractions from mice killed 2 min after $^{125}$I-rhHyal-1 injection. For the sake of comparison, distributions of alkaline (Alk) phosphodiesterase (a marker for plasma membrane and endocytic vesicles) sedimenting in the $P$ fraction and $\beta$-galactosidase (a lysosomal marker) sedimenting in the $L$ fraction are presented. (C) Distribution, after isopycnic centrifugation, of radioactivity and $\beta$-galactosidase recovered in the $L$ and $M$ fractions from mice killed 30 min after a $^{125}$I-rhHyal-1 injection to a control mouse or to a mouse injected with Triton WR-1339 (17 mg in 0.2 ml of saline) 4 days before receiving a $^{125}$I-rhHyal-1 injection. y-axis, average frequency of the component over the density range in each fraction, obtained by dividing the fractional amount present in the fraction ($Q/\Sigma Q$) by $\Delta \rho$ (the increment of density from the top to the bottom of the fraction); x-axis, fractions are numbered from the top of the gradient.

enzymatic activity was measured with an equal amount of protein for each fraction. To allow a comparison with the results obtained with the radioactive enzyme the results illustrated in Figure 2(A) are shown as histograms in Figure 3(A), but are now expressed as the relative specific radioactivity, i.e. the percentage of radioactivity found in the fractions divided by the percentage of protein recovered in the fraction [12]. The similarity between the distributions of radioactivity and of enzymatic activity is evident, both at 4 and 30 min post-injection. After 30 min, except in the $P$ fraction, a second weak band was observed in the gels, characterizing a compound of lower molecular mass.

This band could result from a deglycosylation of the enzyme after its internalization, as glycosylation of rhHyal-1 corresponds to approx. 10% of the molecular mass of the molecule [26]. To summarize, the fractional distributions after differential centrifugation observed after injection of unlabelled rhHyal-1, as identified by the activity of the enzyme, are comparable with the distributions obtained with the labelled enzyme. We also injected reconstituted lyophilized mouse serum, containing endogenous Hyal-1, into a Hyal-1$^{-/-}$ mouse that was killed 30 min after injection. The reaction product was recovered in the four sedimentable fractions, particularly in the $L$ fraction. These results
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Figure 3 HA zymography of liver fractions isolated by differential and isopycnic centrifugation after injection of rhHyal-1 into Hyal-1−/− mice

(A) Analysis of mice liver fractions by differential centrifugation. The distributions were obtained with livers originating from mice killed 4 min (a) and 30 min (b) after injection of rhHyal-1 or 30 min after injection of reconstituted lyophilized mouse serum (c). In these experiments, HA zymography has been performed with an equal amount of protein for each fraction. To allow a comparison with the results obtained with the radioactive enzyme presented in Figure 2, the percentages of radioactivity recovered in each fraction (as shown in Figure 2) and presented as the percentage of protein found in the fraction (relative specific radioactivity). (B) HA zymography of fractions obtained by isopycnic centrifugation of the P and L fractions isolated 4 min after injection of rhHyal-1. For the sake of comparison, distributions of alkaline (Alk) phosphodiesterase sedimenting in the P fraction and of β-galactosidase in the L fraction are shown. (C) HA zymography of fractions obtained by isopycnic centrifugation of the L fraction isolated 30 min after injection of rhHyal-1 into a Hyal-1−/− mouse or to a Hyal-1−/− mouse injected with Triton WR-1339 (17 mg in 0.2 ml of saline) 4 days before receiving the rhHyal-1 injection. Centrifugation conditions are as described in the legend to Figure 2. For the sake of comparison, distributions of the two lysosomal enzymes β-galactosidase and N-acetylglucosaminidase recovered in the L fraction are presented. The approx. molecular mass in kDa (kD) is indicated to the right of the gels.

indicate that the mouse serum Hyal-1 can be endocytosed by the liver in a similar manner to the human recombinant enzyme.

An analysis of the P and L fractions by isopycnic centrifugation was also performed at 4 min after rhHyal-1 injection. The results illustrated that the enzyme present in the P fraction distributes in the same manner as alkaline phosphodiesterase, whereas the enzyme recovered in the L fraction distributes in the same manner as β-galactosidase (Figure 3B). This indicates a rapid transfer of the endocytosed protein from endocytic vesicles to the lysosomal compartment. After 30 min, when rhHyal-1 activity was mainly present in the L fraction, its distribution in the isopycnic centrifugation fractions was similar to that of the two lysosomal enzymes β-galactosidase and N-acetylglucosaminidase (Figure 3C). However, the distribution shift caused by a Triton WR-1339 injection was markedly less pronounced for internalized rhHyal-1 compared with the bona fide lysosomal enzymes, as it was seen for the radioactive enzyme. In conclusion, centrifugation experiments concerning the subcellular localization of endocytosed rhHyal-1 give similar results irrespective of whether 125I-rhHyal-1 or unlabelled active rhHyal-1 is injected into the mice.

Cell specificity of rhHyal-1 endocytosis

Numerous purified lysosomal enzymes, when injected into animals, are preferentially endocytosed by hepatic sinusoidal cells [27,28]. To determine whether this was the case for Hyal-1, its activity was measured in preparations of purified hepatocytes and purified sinusoidal cells obtained 15 min after injection of rhHyal-1 into Hyal-1−/− mice. As shown in Figure 4, the concentration of rhHyal-1 in sinusoidal cells largely exceeds its concentration in hepatocytes.
Preparations were obtained from the liver of a Hyal-1−/− mouse killed 15 min after an injection of rhHyal-1. HA zymography was performed with the same amount of protein from the homogenates of each preparation. Assuming that the concentration of proteins is approximately the same in the two kinds of cells, HA zymography illustrates the concentration of rhHyal-1 in each preparation. The approx. molecular mass in kDa (kD) is indicated to the right of the gel.

Intracellular distribution of endogenous liver Hyal-1

Having determined that exogenous Hyal-1 is incorporated into the liver, probably through the endosomal/lysosomal system, it was relevant to examine the intracellular distribution of endogenous mouse liver Hyal-1 relative to bona fide lysosomal enzymes.

As illustrated in Figure 5(A), mouse liver Hyal-1 was almost exclusively present in the L fraction and the P fraction. Such a distribution differs from the distribution of the β-galactosidase marker of lysosomes, which was predominantly in the L fraction and hardly present in the P fraction. It exhibits some similarity with the distributions of alkaline phosphodiesterase (a marker for plasma membrane and endocytic vesicles), except for the virtual absence of Hyal-1 in the N fraction. The liver enzyme exhibits a molecular mass of approx. 72 kDa, which is higher than the molecular mass of the recombinant enzyme (approx. 52 kDa), but equal to the molecular mass of the serum enzyme. Differences in molecular mass of purified Hyal-1 from different origins (e.g. recombinant, plasma, serum and liver) have been described previously [29] and may be due to different degrees of sialylation [30].

Fractions P and L were then examined in more detail. First, the distribution of Hyal-1 recovered in the P fraction was analysed using isopycnic centrifugation in a sucrose gradient. As shown in Figure 5(B), the distributions of Hyal-1, alkaline phosphodiesterase and β-galactosidase (the latter was only present at a low amount in the P fraction) were broadly similar. The L fraction, which contains a high amount of lysosomes, was examined likewise. Hyal-1 was recovered in the same fractions of the gradient as the reference lysosomal enzymes, β-galactosidase and N-acetylglucosaminidase, and the plasma membrane marker alkaline phosphodiesterase (Figure 5C). However, when the experiment was repeated with an L fraction obtained from the liver of a mouse injected with Triton WR-1339, the distribution curves of the lysosomal enzymes, but not of Hyal-1 or alkaline phosphodiesterase, were strongly shifted towards the lower density fractions of the gradient (Figure 5C).

Effect of lysosomal enzymes on rhHyal-1 and liver Hyal-1

The results from the present study show that endocytosed rhHyal-1 retains its activity for a certain amount of time, but is apparently degraded relatively quickly when it reaches the endosomal/lysosomal system. One possibility is that it is particularly sensitive to the action of hydrodases present in the lysosomes. In favour of this hypothesis is the fact that, when rhHyal-1 is incubated at pH 5 with purified lysosomes (lysed with 0.1% Triton X-100) as illustrated in Figure 6, the activity of the enzyme decreased faster than the activity of the lysosomal hydrolases β-galactosidase and cathepsin C (originating from lysosomes present in the incubation mixture). Moreover, the same situation is observed for endogenous liver Hyal-1.

DISCUSSION

Endocytosis of Hyal-1

Our results obtained in vivo, are in agreement with the ex vivo results of Harada and Takahashi [9] indicating that Hyal-1 present in the culture medium is taken up by the cells and is intracellularly functional. We demonstrate that rhHyal-1 and serum Hyal-1 can be endocytosed by the liver and remains active once inside the cells. It is likely that this endocytosis is carried out mainly by sinusoidal cells. Centrifugation experiments indicate that Hyal-1 follows the intracellular pathway described for many endocytosed proteins, which leads them to lysosomes. However, Hyal-1 does not seem to end up in the lysosomes that contain the bulk of the typical markers β-galactosidase and N-acetylglucosaminidase.

First, Hyal-1 is recovered mainly in the L fraction after differential centrifugation, whereas β-galactosidase predominates in the M fraction. Secondly, the density of the lysosomes, or lysosome-like vesicles, that contain endocytosed Hyal-1 is barely affected by prior injection of Triton WR-1339 into the mice, whereas this procedure induces a large shift in the density of the lysosomes harbouring β-galactosidase and N-acetylglucosaminidase. A third discriminating feature of endocytosed Hyal-1 is its relatively quick degradation as ascertained by the rapid appearance of acid-soluble radioactivity after 35S-rHyal-1 injection into wild-type mice and by the fast decrease of the liver activity after rhHyal-1 injection into Hyal-1−/− mice.

Several authors have shown that there are at least two classes of lysosomes, defined as organelles containing acid hydrolases, through which an endocytosed protein has to travel at the end of its intracellular journey: early (or transfer) lysosomes and late (or accumulation) lysosomes (for a review, see [31]). In the liver, degradation of endocytosed protein takes place sequentially in these two compartments [21, 22, 25]. Those degradation products that cannot diffuse through the organelle membrane and the undegraded protein remain in the late lysosomes where they can accumulate. After differential centrifugation according to the de Duve scheme [12], the lysosomes are recovered in the total mitochondrial fractions (the M and L fraction), with early lysosomes being found in the L fraction and late lysosomes, which contain the bulk of lysosomal enzymes, in the M fraction [22, 25]. Since endocytosed rhHyal-1 is rapidly degraded, the largest proportion of intact and active molecule coming from endosomes would be expected to be present in the early lysosomes, i.e. in the L fraction. On the other hand, endocytosed Triton WR-1339, owing to its structure, cannot be digested by the acid hydrolases present in early and late lysosomes and is unable to cross the lysosomal membrane. As a consequence, it mainly accumulates in late lysosomes, at the end of its intracellular journey, and causes a strong decrease in their density; the density of early lysosomes is only transiently affected by the detergent. If endocytosed Hyal-1 is mostly present in an active form in early lysosomes, it seems logical that its distribution after isopycnic centrifugation is hardly affected by prior injection of Triton WR-1339.

Our results do not permit us to conclude whether endocytosis of Hyal-1 results from fluid-phase, adsorptive or receptor-mediated endocytosis. However, the fast rate of removal of rhHyal-1 from
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Figure 5  HA zymography of liver fractions isolated by differential and isopycnic centrifugation

(A) HA zymography of liver fractions isolated with differential centrifugation. Distributions of reference enzymes alkaline (Alk) phosphodiesterase (a marker for the plasma membrane and endocytic vesicles), β-galactosidase and N-acetylglucosaminidase (lysosomal makers) are indicated after (B) isopycnic centrifugation of the recovered P fraction and (C) isopycnic centrifugation of the L fraction isolated from the liver of a control mouse or from the liver of a mouse injected with Triton WR-1339 (17 mg in 0.2 ml of saline). Centrifugation conditions are as described in the legend to Figure 2. The approx. molecular mass in kDa (kD) is indicated to the right of the gels.

Liver Hyal-1

After differential centrifugation, endogenous liver Hyal-1 recovered from the mitochondrial fractions was found to sediment almost totally in the L fraction. After isopycnic centrifugation of the L fraction, the enzyme was present in the density zone where lysosomes equilibrate, but its distribution profile was not affected by a prior Triton WR-1339 injection. This behaviour suggests that a proportion of liver Hyal-1 is located in early lysosomes, as with endocytosed Hyal-1. A large proportion of endogenous Hyal-1 was also found in the P fraction: this could result from a localization of the enzyme in the intermediate endocytic vesicles/endosomes it has to travel through before reaching the lysosomal system. The almost equal distribution of Hyal-1 between the L and P fractions may translate to a steady-state situation created by constant uptake of the serum enzyme and rapid inactivation during or at the end of its intracellular journey. This could lead to a quasi absence of active Hyal-1 in late lysosomes. It is worthwhile mentioning that previous observations suggest that liver Hyal-1 follows an alternative trafficking pathway to reach lysosomes, rather than via the classical intracellular phosphomannosyl recognition pathway [32]. Endocytosis of the serum enzyme is another possibility. Hyal-1 could be synthesized by hepatocytes like many other plasma proteins. After its secretion into the blood its re-uptake could be carried out by sinusoidal cells, the main site of circulating hyaluronan catabolism. As described above, previous observations have suggested that liver Hyal-1 had to follow a different pathway to reach lysosomes, compared with the classical intracellular phosphomannosyl recognition pathway [32]. Interestingly, Nielsen et al. [33] have shown that kidney proximal tubular cells can incorporate the lysosomal enzyme cathepsin B, which is devoid of mannose-6-phosphate, through a system involving megalin, a multi-ligand receptor. This system allows cathepsin B to be efficiently endocytosed and intracellularly active. If Hyal-1 behaves similarly, the potential
receptor, possibly a mannose receptor, for its endocytosis remains to be identified.

It has long been recognized that the liver is the main site for circulating HA catabolism [6]. The first step in this catabolism is HA uptake into liver sinusoidal endothelial cells through the specific scavenger receptor called HARE (HA receptor for endocytosis)/stabilin-2 [34]. Liver Hyal-1 is also thought to be an essential player in this process [3]. According to the results from the present study, serum Hyal-1 could be continuously endocytosed by liver sinusoidal cells and end up in early lysosomes. Its relative instability in the lysosomal system could be countered, under steady-state conditions, by a high input of active endocytosed Hyal-1 into this compartment. It follows that intracellular hydrolysis of HA by Hyal-1 would mainly take place in early lysosomes. The resulting oligosaccharides would be sent to late lysosomes, where the terminal hydrolases (β-glucuronidase and N-acetylglucosaminidase) are mostly present (as shown by the effect of Triton WR-1339 on the distribution of these hydrolases after isopycnic centrifugation in the experiments in the present study and by Tsunga et al. [35]). However, the exact role of liver Hyal-1 in circulating HA catabolism remains undetermined. The only human being with congenital Hyal-1 deficiency described so far did not seem to suffer from hepatic HA accumulation, but had grossly elevated plasma HA concentrations together with HA-filled vesicles in peri-articular fibroblasts and macrophages [32]. He developed severe joint damage. Hyal-1−/− mice have an even more benign phenotype with some long-term chondrocyte damage, but no hepatic HA accumulation, and only mildly elevated plasma HA levels [8]. Hyal-1 deficiency may thus be accompanied by compensatory mechanisms, such as a decrease in HARE-mediated HA uptake into the liver or an up-regulation of other HA-degrading mechanisms, such as those mediated by the cell-surface-anchored enzyme Hyal-2. Jadin et al. [14] have shown recently that a lack of Hyal-2 in mice results in HA accumulation in liver sinusoidal cells. Further studies will be required to determine how HARE, Hyal-1, Hyal-2, and possibly additional factors, interact in liver HA catabolism.

AUTHOR CONTRIBUTION
Marie-Christine Gasingirwa, Bruno Flamion, Robert Watiaux and Michel Jadot designed the research. Marie-Christine Gasingirwa, Jacqueline Thirion, Joannine Mertens-Strijthagen and Simone Watiaux-De Coninck performed the research. All authors analysed the results. Robert Watiaux, Michel Jadot and Bruno Flamion wrote the paper.

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