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ENDOCYTOSIS OF HYALURONIDASE- 1 BY THE LIVER.

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SYNOPSIS

It has been suggested that intracellular hyaluronidase-1 (Hyal-1), considered as a lysosomal enzyme, originates from the endocytosis of the serum enzyme. To check this proposal we have investigated the uptake of recombinant human hyaluronidase-1 (rhHyal-1) by mouse liver and its intracellular distribution, making use of centrifugation methods. Experiments were performed on wild type mice injected with ¹²⁵I-rhHyal-1 and on null mice (Hyal-1^{-/-}) injected with the unlabelled enzyme. Mice were euthanized at increasing times after injection. Activity of the unlabelled enzyme was determined by zymography. Intracellular distribution of the Hyal-1 was investigated by differential and isopycnic centrifugation. Results indicated that rhHyal-1 is endocytosed by the liver, mainly by sinusoidal cells and follows the intracellular pathway described for many endocytosed proteins that find themselves eventually in lysosomes. However, Hyal-1 endocytosis has some particular features. Endocytosed rhHyal-1 is quickly degraded. Its distribution after differential centrifugation differs from the distribution of β -galactosidase, taken as reference enzyme of lysosomes. After isopycnic centrifugation in a sucrose gradient, endocytosed rhHyal-1 behaves like β -galactosidase soon after injection but Hyal-1 distribution is markedly less affected than the distribution of β -galactosidase by a prior injection of Triton WR-1339 into the mice. This agent is a specific density perturbant of lysosomes. Behaviour in centrifugation of endogenous liver Hyal-1, identified by HA zymography exhibits some kinship with the behaviour of the endocytosed enzyme, suggesting that it could originate from an endocytosis of the serum enzyme. Overall, these results could be explained by supposing that active endocytosed

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Hyal-1 is mainly present in early lysosomes. Although its degradation half-time is short, Hyal-1 could exert its activity owing to a constant supply of active molecules from the blood.

INTRODUCTION

Hyaluronan (HA) catabolism depends mainly on two somatic hyaluronidases: hyaluronidase-1 (Hyal-1) and hyaluronidase-2 (Hyal-2) [1,2,3]. Hyal-1 exhibits a maximum activity at acidic pH and is considered a lysosomal enzyme [2,3] whereas the location and activity of Hyal-2 are 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], has by far the largest 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 the plasma enzyme recovered by endocytosis after its secretion. Recent *in vitro* findings of Harada and Takahashi [9] favour such a hypothesis: these authors have shown that Hyal-1 added to the medium of cultured cells is internalised and becomes functional intracellularly. It is to be noted that, contrary to most lysosomal enzymes, the plasma levels of Hyal-1 are not elevated in patients with mucopolidoses II and III [10]. This suggests that liver Hyal-1 has to follow another pathway to reach lysosomes than the classical intracellular phosphomannosyl recognition pathway. Endocytosis of the serum enzyme is a possibility.

To resolve this issue, we have injected recombinant human Hyal-1 (rhHyal-1) into mice. Centrifugation results presented here 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, the liver endogenous Hyal-1 exhibits a distribution profile, after differential and isopycnic centrifugation, distinct from that of reference lysosomal enzymes but presenting some kinship with the distribution of injected rhHyal-1. It is suggested that the liver enzyme originates from a continuous endocytosis of Hyal-1 secreted in the serum.

EXPERIMENTAL

Tissue fractionation

Experiments were performed with male NMRI mice and C57BL/6 wild type (Hyal-1^{+/+}) or null (Hyal-1^{-/-}) mice. The latter were a kind gift of Prof. Barbara Triggs-Raine, University of Manitoba, Winnipeg, Canada. Animals were cared for using protocols approved by the FUNDP Commission d'Ethique en Expérimentation Animale. Mice were injected i.v. via the tail vein with rhHyal-1, a gift of Dr Greg Frost (Halozyme, San Diego, CA, USA). In some experiments, rhHyal-1 was labelled with ¹²⁵I prior to injection using the chloramine T method [11]. In some experiments, mice were also injected with Triton WR-1339 (17 mg in 0.2 ml saline) 4 days before rhHyal-1 injections. A Hyal-1 null mouse was injected with reconstituted lyophilised wild-type murine serum. All mice were euthanized at selected 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 homogeniser consisting of a smooth-walled glass tube fitted with a Teflon pestle (Arthur Thomas, Philadelphia, NJ, U.S.A). Fractionation of the homogenate by differential centrifugation was achieved by the method of de Duve *et al* [12]. A nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a soluble fraction S were successively isolated as described by these authors. 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 39 000 rev./min for 150 min for L fraction and overnight for P fraction, in the Beckman rotor SW 55Ti. After centrifugation in a SW 55 Ti Beckman rotor, fractions were collected by slicing the tube with a device similar to that described by Beaufay *et al.* [13].

Enzyme measurements

Acid β -galactosidase was assayed by incubating the granules at 37° C in a volume of 0.2 ml containing 0.5 mM 4-methylumbelliferyl- β -D-galactopyranoside, 50 mM NaCl, 50 mM acetate buffer (pH 5) and 0.05% Triton X-100. N-Acetylglucosaminidase was measured by incubating the granules at 37°C in a volume of 0.2 ml containing 0.5 mM 4-methylumbelliferyl-N acetyl- β -D-glucosaminide, 50 mM acetate buffer (pH 5) 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 released fluorogenic group (4-methyl umbelliferone) was determined with a Versa Fluor fluorimeter (Bio-Rad, Nazareth-Eke, Belgium), excitation filter 170-2420 and emission filter 170-2421). For assay of alkaline phosphodiesterase, the granules were incubated at

25°C in a volume of 0.25 ml containing 1.5 mM p-nitrophenylthymidine-5'-phosphate, 100 mM glycine buffer (pH 9.6) and 2 mM zinc acetate. The reaction was stopped by adding 1 ml of 0.1 M NaOH. 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]. That is, granule samples were added to a loading buffer containing 4% SDS, 20% glycerol, 250 mM Tris-HCl buffer (pH 6.8), and 0.01% Bromophenol Blue. Separation gels consisted of 10% polyacrylamide to which 0.17 mg/ml of human umbilical cord hyaluronan (Sigma-Aldrich, Bornem, Belgium) was added. Proteins were separated by SDS-PAGE on ice at 200 mA under a constant voltage of 80 volts. After electrophoresis, SDS gel was rinsed with distilled water and incubated for 2 h in a 3% Triton X100 solution on a rocking table. The gels were subsequently equilibrated with the assay buffer (100 mM formate, 150 mM NaCl, pH 3.7) 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 (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% 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. Proteins were determined using the method of Lowry *et al.* [15] and radioactivity was counted with a gamma counter. (Master Gamma, LKB, Pharmacia)

Isolation of liver cells

Separation of hepatocytes and sinusoidal cells was performed after collagenase perfusion according to Seglen [16] with slight modifications as described by Lecocq *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 rhHyal-1 labelled with ^{125}I , injected into NMRI mice or with unlabelled rhHyal-1 injected into C57BL/6 Hyal-1^{-/-} (null) mice. The intracellular journey of endocytosed Hyal-1 was followed by analytical centrifugation techniques. Liver homogenates were prepared at increasing times after injection and fractionated by differential and isopycnic centrifugation. Distribution of endocytosed Hyal-1 in the fractions was established by determining the radioactivity or hyaluronidase activity and compared with that of marker enzymes: β -galactosidase and β -N-acetylglucosaminidase for lysosomes, alkaline phosphodiesterase for plasma membrane and endocytic vesicles [20]. Centrifugation experiments were also carried out to determine the intracellular localisation of endogenous liver Hyal-1.

Uptake of ^{125}I -rhHyal-1 by the liver

The total radioactivity recovered in liver and in blood plasma at increasing times after ^{125}I -rhHyal-1 injection, is shown in Fig 1. ^{125}I -rhHyal-1 was quickly endocytosed, and reaches a maximum within a few minutes after injection, which is maintained for at least 30 min. After that, liver radioactivity markedly decreases and corresponds to only a small percentage of the injected dose after 60 min. The proportion of acid-soluble radioactivity, resulting from a proteolysis of ^{125}I -rhHyal-1 increases almost linearly with time, suggesting that the labelled enzyme begins to be degraded soon after it is endocytosed.

Differential centrifugation

Livers isolated 2, 10 and 30 min after injection were fractionated by differential centrifugation according to de Duve et al. [12]. A nuclear fraction N, a heavy mitochondrial fraction M, a light mitochondrial fraction L, a microsomal fraction P and a soluble fraction S were isolated. The mean proportion of radioactivity and of β -galactosidase, reference enzyme of lysosomes, recovered in the total sedimentable fractions for the three fractionations, was $80.5 \pm 6.4\%$ and $86.4 \pm 6.0\%$ respectively. Their distributions in the different sedimentable fractions are illustrated in Fig 2A. A relatively high amount of radioactivity is constantly associated with N fraction. This fraction is very heterogeneous and contains nuclei as the main components along with large sheets of plasma membrane fragments, collagen fibers, undisrupted cells. It is possible that radioactivity found in the fraction results from a binding of ^{125}I -rhHyal-1 to the large plasma membrane fragments present in this fraction. A significant proportion of

radioactivity is recovered in P fraction but it decreases with time. Like β -galactosidase (lysosomes), radioactivity present in the post nuclear sedimentable structures is mainly found in the mitochondrial fractions M and L. but the partitioning of β -galactosidase and radioactivity between these fractions differ, the lysosomal enzyme sediments mainly in M fraction, while the radioactivity is mainly recovered in L fraction. These results illustrate that, like several proteins that are endocytosed by the liver [21,22], ^{125}I -rhHyal-1 is first associated with endocytic vesicles (P fraction) but is very quickly transferred to endosomes (P and L fractions) and lysosomes (L and M fractions). The rapidity of the transfer suggests that the uptake of ^{125}I -rhHyal-1 is carried out by sinusoidal cells [22].

Isopycnic centrifugation

To identify the localisation of ^{125}I -rhHyal-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 Fig 2A, 2 min after injection, most of the radioactivity is almost equally distributed between P and L fractions. Fig 2B illustrates the distribution after isopycnic centrifugation in a sucrose gradient of the radioactivity recovered in these fractions 2 min after injection of ^{125}I -rhHyal-1. The distribution curves of radioactivity are the same in the two fractions and coincide with the distribution of alkaline phosphodiesterase (plasma membrane, endocytic vesicles). In L, a comparison has also been established with the distribution of β -galactosidase (lysosomes) present in high amounts in the fraction. An overlapping of the two distribution curves is apparent but not exact. Median equilibrium density computed from the density distribution histogram of the fractions is about 1.16 g/cm^3 for the radioactivity and for alkaline phosphodiesterase, while it is 1.18 g/cm^3 for β -galactosidase.

At longer time intervals after injection, radioactivity is mostly associated with the mitochondrial fractions M and L with it predominating in L fraction (Fig 2A). Fig 2C illustrates the distributions of radioactivity present in these fractions 30 min after injection of ^{125}I -rhHyal-1. A comparison can be made with the distribution of β -galactosidase, reference enzyme of lysosomes. The similarity between the distribution curves of the hydrolase and radioactivity is clearly apparent. The median equilibrium density of both components is around 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 having received an injection of Triton

WR-1339, a non-ionic detergent. 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 the presence in these organelles of a compound, for example an endocytosed molecule [24,25]. As seen in Fig 2C, after Triton WR-1339 injection, a large proportion of β -galactosidase recovered in M and L fractions has shifted towards the low density end of the gradient, the median equilibrium density being close to 1.10 g/cm^3 . The distribution of radioactivity recovered in L and M fractions is markedly less affected by the detergent injection (median equilibrium densities 1.15 g/cm^3 and 1.17 g/cm^3 respectively).

Endocytosis of unlabelled rhHyal-1

Studying liver incorporation of ^{125}I -rhHyal-1 by 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 internalisation owing 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, devoid of Hyal-1 and to which unlabelled rhHyal-1 or serum of wild type mice was injected. The fate of the internalised enzyme was followed by determining its activity using 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 baseline, 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 null mice (data not shown)

Liver fractions were isolated by differential centrifugation. Forty-five min after injection, Hyal-1, activity was barely detectable (data not shown), indicating that by that time, rhHyal-1 taken up by the liver has been probably degraded to a large extent as is seen for ^{125}I -rhHyal-1 (see Fig 1). Fig 3A illustrates the activity of rhHyal-1 recovered in the fractions, 4 min (a) and 30 min (b) after injection. In these experiments, enzymatic activity was measured with an equal amount of protein for each fraction. To allow a comparison with the data obtained with the radioactive enzyme results illustrated in Fig 2 A are shown again in Fig 3, but now expressed in 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 P fraction, a second weak band is observed in the gel characterizing a compound of lower molecular weight. It could result from a deglycosylation of the enzyme after its internalisation, the glycosyl part of rhHyal-1 corresponding to about 10 % of the molecular mass of the molecule [26]. To summarise, the distributions after differential centrifugation observed after injection of unlabelled rhHyal-1, identified by the activity of the enzyme, are comparable with the distributions obtained with the labelled enzyme. An experiment has been done by injecting reconstituted lyophilized mouse serum that contains endogenous Hyal-1, to a null mouse. The animal was sacrificed 30 min after injection. The reaction product is recovered in the four sedimentable fractions particularly in L fraction. These results indicate that the mouse serum Hyal-1 can be endocytosed by the liver as the human recombinant enzyme. Early (4 min) after rhHyal-1 injection analysis of P and L fractions, by isopycnic centrifugation, has been carried out. Results (Fig 3B) illustrate that the enzyme present in P distributes like alkaline phosphodiesterase while the enzyme recovered in L distributes like β -galactosidase indicating a rapid transfer of the endocytosed protein from endocytic vesicles to the lysosomal compartment. After 30 min (Fig 3C), when rhHyal-1 activity is mainly present in L fraction, its distribution after isopycnic centrifugation is similar to that of the two lysosomal enzymes β -galactosidase and N-acetylglucosaminidase. However, the distribution shift caused by a Triton WR-1339 injection was markedly less pronounced for internalised rhHyal-1 than for the *bona fide* lysosomal enzymes as it is seen for the radioactive enzyme. In conclusion, centrifugation experiments concerning the subcellular localisation of endocytosed rhHyal-1 give similar results irrespective of whether ^{125}I -rhHyal-1 or unlabelled active rhHyal-1 is injected into the mice.

Cell specificity of rh Hyal-1 endocytosis

Numerous purified lysosomal enzymes, when injected to animals, are preferentially endocytosed by hepatic sinusoidal cells [27,28]. To determine if Hyal-1 had a similar fate, its activity was measured in preparations of purified hepatocytes and purified sinusoidal cells obtained 15 min after injection of rhHyal-1 into null mice. As shown in Fig. 4, the concentration of rhHyal-1 in sinusoidal cells largely exceeds its concentration in hepatocytes.

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

Fractions P and L were then examined in more details. First, the distribution of Hyal-1 recovered in the P fraction was analysed using isopycnic centrifugation in a sucrose gradient. As shown in Fig. 5B, the distributions of Hyal-1, alkaline phosphodiesterase and β -galactosidase (the latter is in low amount in P) were broadly similar. The L fraction, which contains a high amount of lysosomes, was examined likewise. Hyal-1 was recovered in the same regions of the gradient as the reference lysosomal enzymes β -galactosidase and N-acetylglucosaminidase or the plasma membrane marker alkaline phosphodiesterase (Fig.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 low density zones of the gradient.

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

Our results 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. A possibility is that it is particularly sensitive to the action of hydrolases present in the lysosomes. In favor 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 Fig.6, the activity of the enzyme decreases 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 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 those lysosomes that contain the bulk of the typical markers β -galactosidase and N-acetylglucosaminidase.

Firstly, after differential centrifugation, Hyal-1 is recovered mainly in the light mitochondrial fraction L while β -galactosidase predominates in the heavy mitochondrial fraction M. 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 manoeuvre 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 ^{125}I -rhHyal-1 injection into wild type mice and by the fast decrease of liver activity after rhHyal-1 injection into null 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]. The 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, the lysosomes are recovered in the total mitochondrial fraction ML, where early lysosomes being mainly found in the L fraction, late lysosomes that 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 is likely 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 acid hydrolases present in early and late lysosomes and is unable to cross the lysosomal membrane. As a consequence, it accumulates mainly 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 the blood makes a simple fluid phase endocytosis (pinocytosis) improbable. On the other hand like many lysosomal enzymes, Hyal-1 is a glycosylated protein containing mannose in the glycosyl part of the molecule [26]. After injection it may thus behave as several purified lysosomal enzymes [27] which are taken up by a receptor-mediated endocytosis involving the mannose receptor on liver sinusoidal cells. The degradation half-life time of lysosomal enzymes after endocytosis depends on the type of hydrolase being injected: e.g., 2.2 days for β -glucuronidase and 0.4 days for N-acetylglucosaminidase [27]. Our results suggest that degradation is much faster for endocytosed Hyal-1.

Liver Hyal-1

After differential centrifugation, endogenous liver Hyal-1 recovered from the mitochondrial fractions M+L sediments almost totally in the L fraction. After isopycnic centrifugation of L, the enzyme is present in the density zone where lysosomes equilibrate but its distribution profile is not affected by a prior Triton WR-1339 injection. This behaviour suggests that part of liver Hyal-1 is located in early lysosomes similar to endocytosed Hyal-1. A large proportion of endogenous Hyal-1 was also found in the P fraction: this could result from a localisation of the enzyme in the endocytic vesicles/endosomes it has to travel through before reaching the lysosomal system. The almost equal distribution of Hyal-1 between L and P may translate 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 to mention that previous observations [32] suggest that liver Hyal-1 has to follow another pathway to reach lysosomes than the classical intracellular

phosphomannosyl recognition pathway. 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. Previous observations [32] did suggest that liver Hyal-1 had to follow a different pathway to reach lysosomes compared with the classical intracellular phosphomannosyl recognition pathway. Interestingly, Nielsen *et al.* [33] have recently 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 multiligand receptor. This system allows cathepsin B to be efficiently endocytosed and intracellularly active. If Hyal-1 behaves similarly, the potential receptor (mannose receptor?) for its endocytosis remains to be identified.

It has long been recognised that the liver is the main site of 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/stabilin-2 [34]. Liver Hyal-1 is thought to be an essential actor too [3]. According to our results, 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. This is ascertained by the effect of Triton WR-1339 on the distribution of these osidases after isopycnic centrifugation in our experiments and those of 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 null 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 upregulation of other HA-degrading mechanisms such as the cell surface-anchored enzyme Hyal-2. It has been recently shown by Jadin *et al.* that a lack of Hyal-2 in mice results in HA accumulation in liver sinusoidal cells [14]. Further

studies will be required to determine how HARE, Hyal-1, Hyal-2, and possibly additional actors interact in liver HA catabolism.

HEADING TITLE

ENDOCYTOSIS OF HYALURONIDASE- 1 BY THE LIVER.

KEYWORDS: Hyaluronidase-1, endocytosis, liver, lysosomes, hyaluronan

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LEGENDS

Fig 1. Radioactivity present in liver and blood

(A) : Radioactivity was measured in homogenate of mouse liver and blood at increasing times after ^{125}I -rhHyal-1 injection. The values are given as percentages of the injected dose. The value in the blood was estimated by supposing that blood represents 10% of animal mass. (B) Evolution of acid-soluble radioactivity with time, given as a percentage of the total radioactivity recovered in the homogenate, after ^{125}I -rhHyal-1 injection.

Fig 2. Distribution of radioactivity after differential and isopycnic centrifugation

The distributions were obtained with livers originating from mice killed at increasing times after ^{125}I -rhHyal-1 injection. (A): Distribution of radioactivity after differential centrifugation of liver homogenates. The values are given as percentages of the amount present in the total sedimentable components of the homogenate (N+M+L+P). N, nuclear fraction, M, heavy mitochondrial fraction, L, light mitochondrial fraction, P, microsomal fraction. For the sake of comparison, the mean distribution of β -galactosidase (lysosomes) in the three fractionation experiments is presented (mean \pm S.D.). (B):

Distribution after isopycnic centrifugation of radioactivity recovered in P and L fractions, 2 min after ^{125}I -rhHyal-1 injection. For the sake of comparison, distributions of alkaline phosphodiesterase (marker for plasma membrane and endocytic vesicles) sedimenting in P and β -galactosidase (marker for lysosomes) sedimenting in L are presented. (C): Distribution after isopycnic centrifugation of radioactivity and β -galactosidase recovered in L and M fractions, 30 min after ^{125}I -rhHyal-1 injection to a normal mouse or to a mouse injected with Triton WR-1339 (17 mg in 0.2 ml saline), four days before receiving ^{125}I -rhHyal-1 injection. Ordinate : 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). In abscissa, fractions are numbered from the top of the gradient.

Fig 3. HA zymography of liver fractions isolated by differential and isopycnic centrifugation after injection of rhHyal- 1 into Hyal-1^{-/-} null mice

(A) 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 lyophilised mouse serum (c). N, nuclear fraction, M, heavy mitochondrial fraction, L, light mitochondrial fraction, P, microsomal fraction. In these experiments, HA zymography has been done with an equal amount of protein for each fraction. To allow a comparison with the results obtained with the radioactive enzyme presented in Fig. 2, the percentages of radioactivity recovered in each fraction, indicated in Fig 2, have been reported to the percentage of protein found in the fraction (relative specific activity) and presented here. (B): HA zymography of fractions obtained by isopycnic centrifugation of P and L isolated 4 min after injection of rhHyal-1. For the sake of comparison, distributions of alkaline phosphodiesterase sedimenting in P and of β -galactosidase recovered in L are presented. (C): HA zymography of fractions obtained by isopycnic centrifugation of L fraction isolated 30 min after injection to a Hyal-1^{-/-} mouse or to a Hyal-1^{-/-} mouse injected with Triton WR-1339 (17 mg in 0.2 ml of saline), four days before receiving the rhHyal-1 injection. Centrifugation conditions as described in legend of Fig 2. For the sake of comparison, distributions of the two lysosomal enzymes : β -galactosidase and N-acetylglucosaminidase recovered in L are presented.

Fig.4 HA zymography of homogenates enriched in parenchymal (PC) or in non parenchymal cells (NPC) after injection of rhHyal-1 to a null rhHyal-1 (/)

Preparations were obtained from the liver of a null mouse sacrificed 15 min after an injection of rhHyal-1. HA zymography was performed with a same amount of protein found in homogenates of each preparation. If we suppose that the concentration of proteins is roughly the same in the two kinds of cells, HA zymography illustrates the concentration of rhHyal-1 in each preparation.

Fig 5. HA zymography of liver fractions isolated by differential and isopycnic centrifugation.

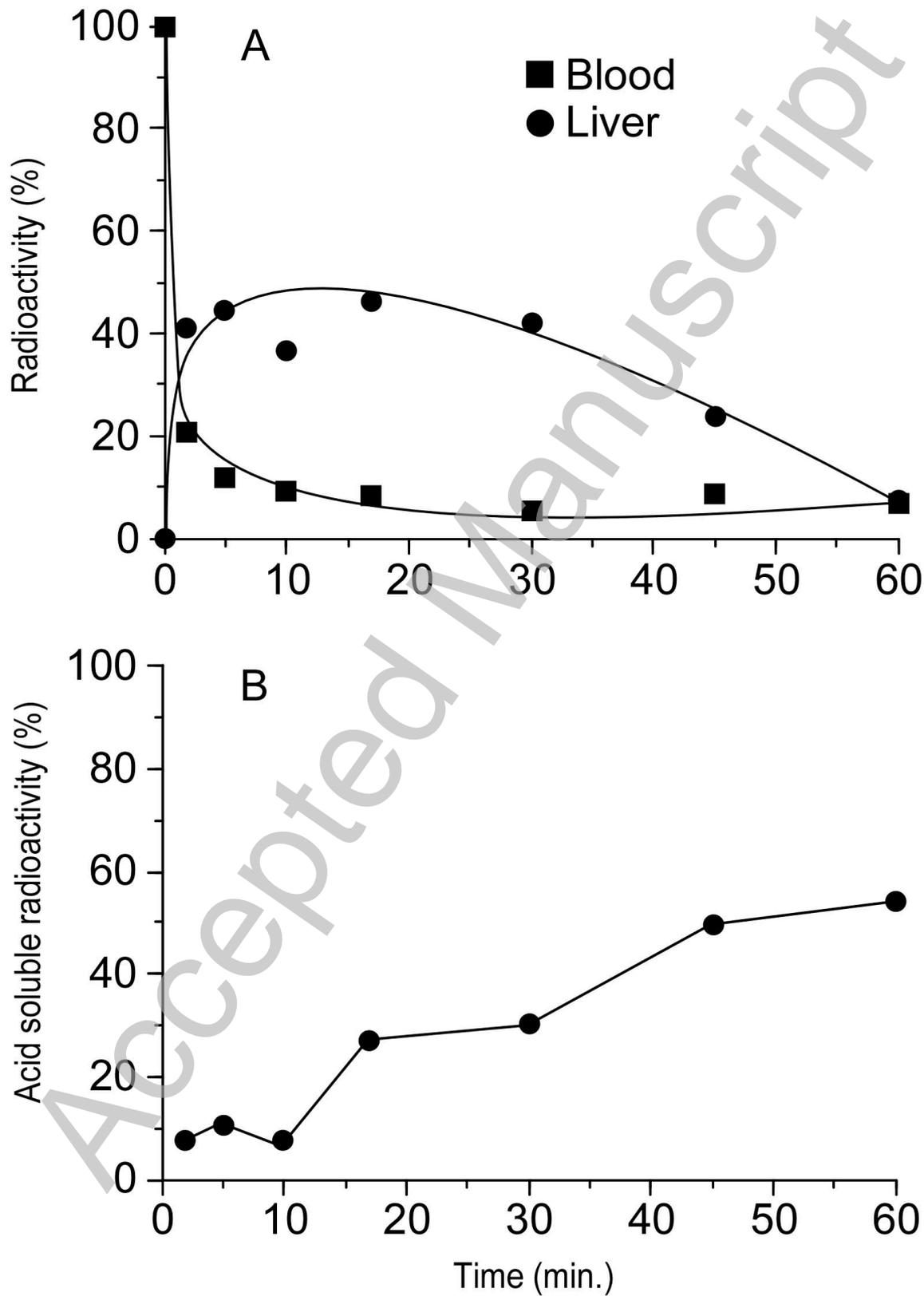
(A) Differential centrifugation. (B) Isopycnic centrifugation of P fraction. (C) : Isopycnic centrifugation of L fraction isolated from the liver of a normal mouse or from the liver of a mouse injected with Triton WR-1339 (17 mg in 0.2 ml of saline). Distributions of reference enzymes alkaline phosphodiesterase (plasma membrane, endocytic vesicles), β -galactosidase and N-acetylglucosaminidase (lysosomes) are indicated. Centrifugation conditions as described in legend of Fig.2

Fig 6. Effect of lysosomal hydrolases on Hyal-1 activity.

(A) rhHyal-1 incubated at 37°C° in 0.05 M sodium acetate buffer (pH 5) for increasing times with purified rat-liver lysosomes obtained after Triton WR-1339 injection (18,19). P fraction used as source of endogenous Hyal 1 was incubated at 37°C° at pH 5 (acetate buffer, 0.05 M) for increasing times without (B) or with (C) purified lysosomes obtained after a Triton WR-1339 injection [19]. After incubation, HA zymography was performed on the preparations. Moreover, activity of two lysosomal enzymes β -galactosidase and cathepsin C was measured on the preparations. (D)

ACCEPTED MANUSCRIPT

FIG 1



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FIG 2

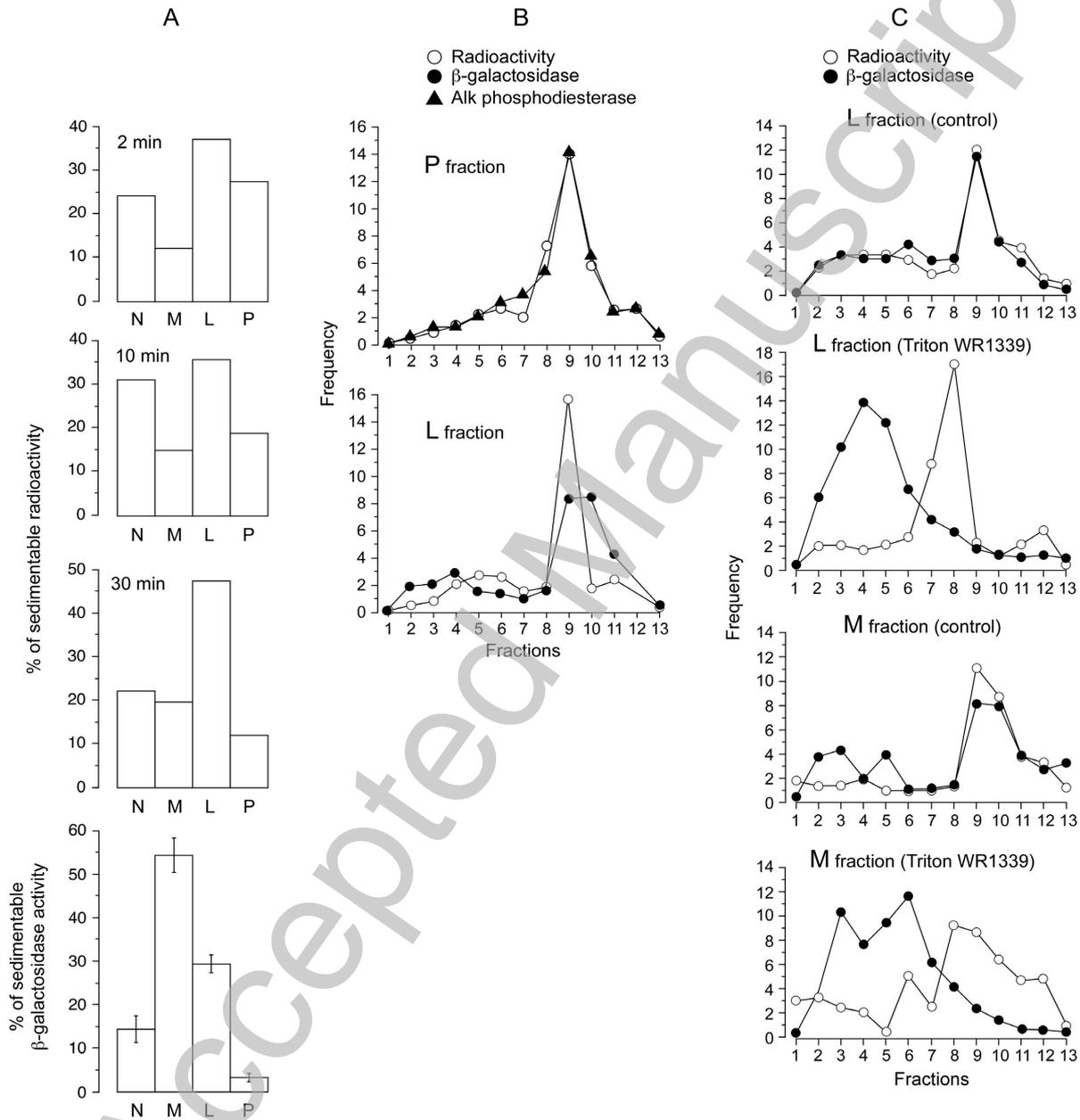


FIG 3

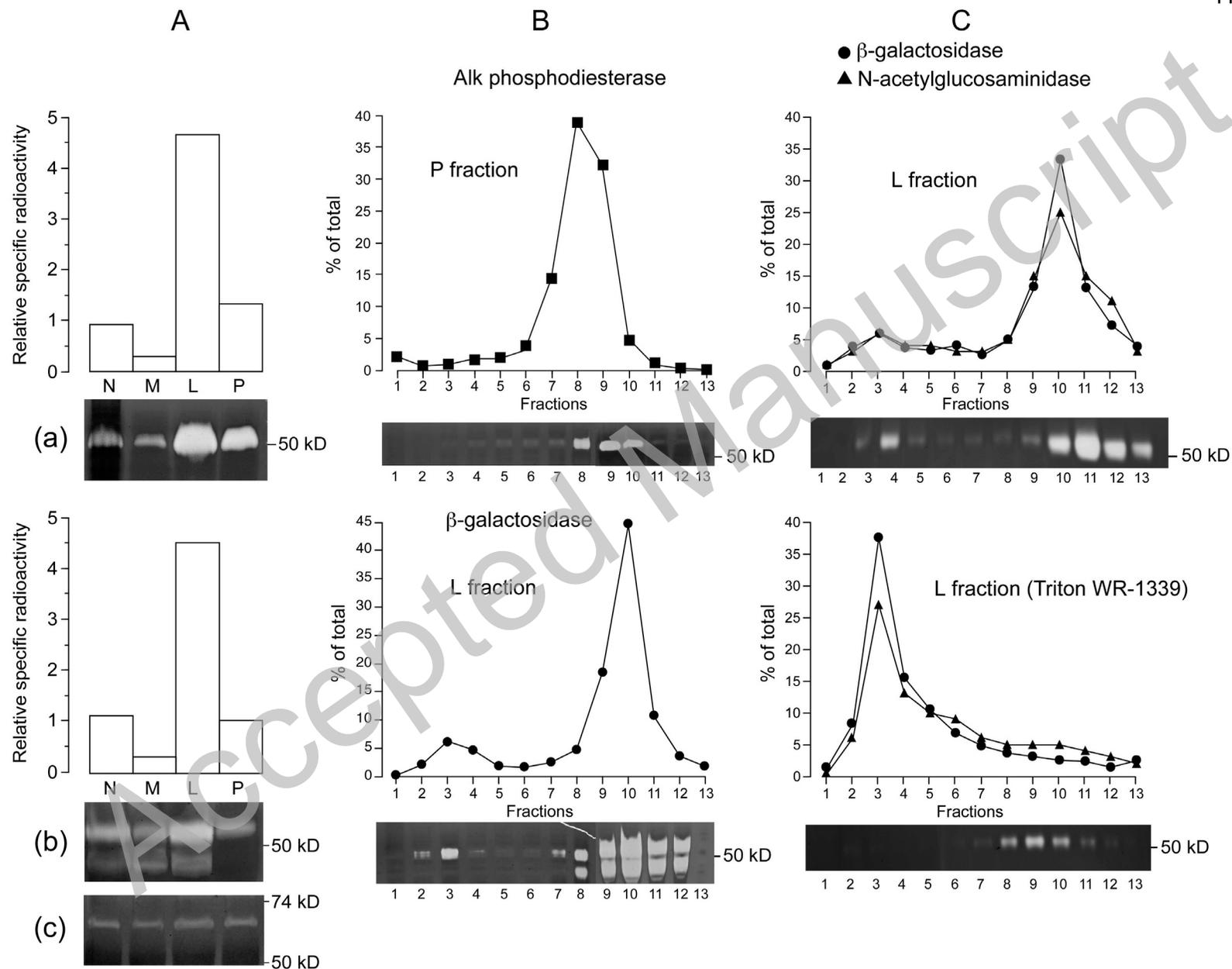


FIG 4

PC

NPC



— 50 kD

FIG 5

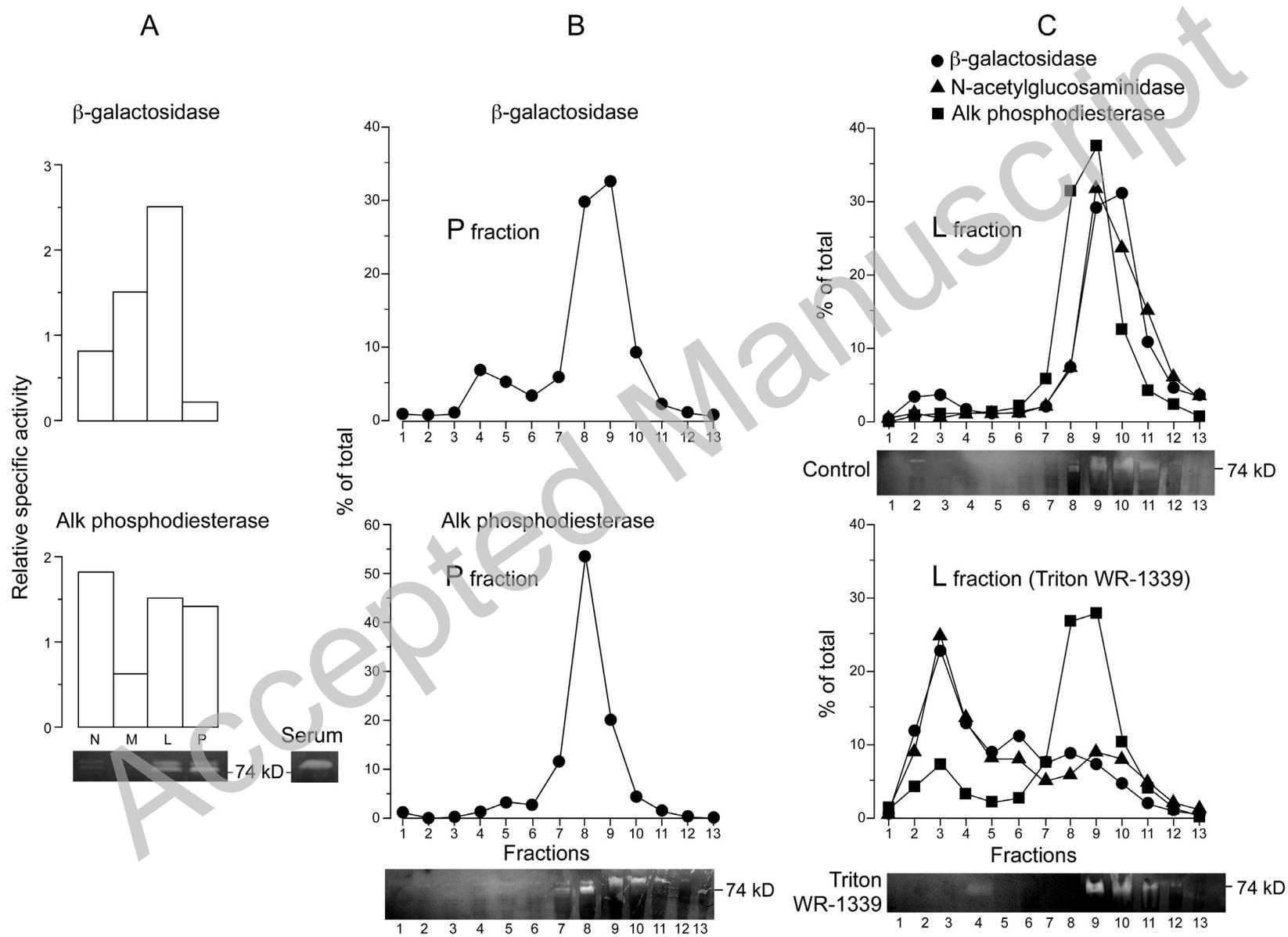
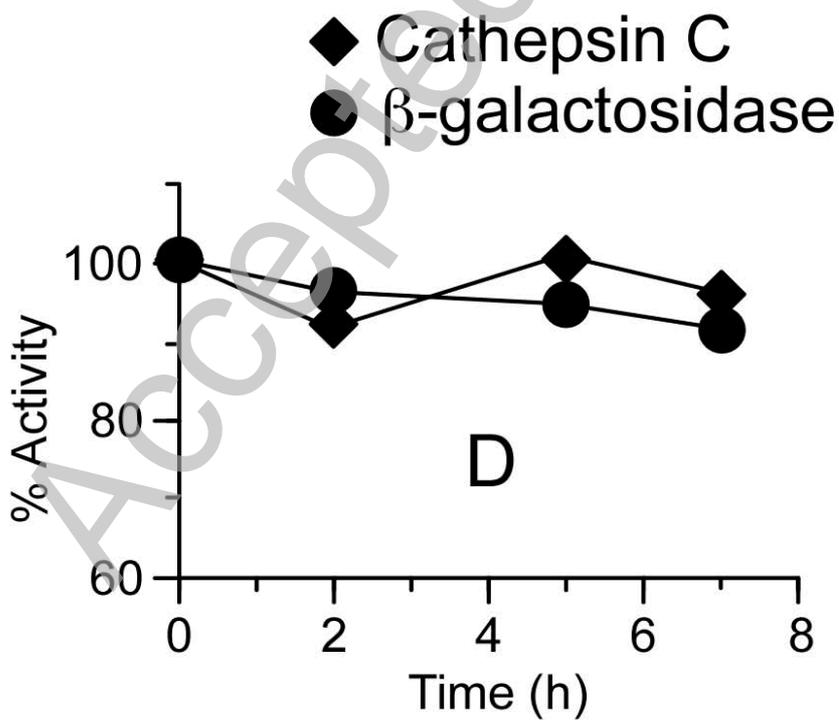
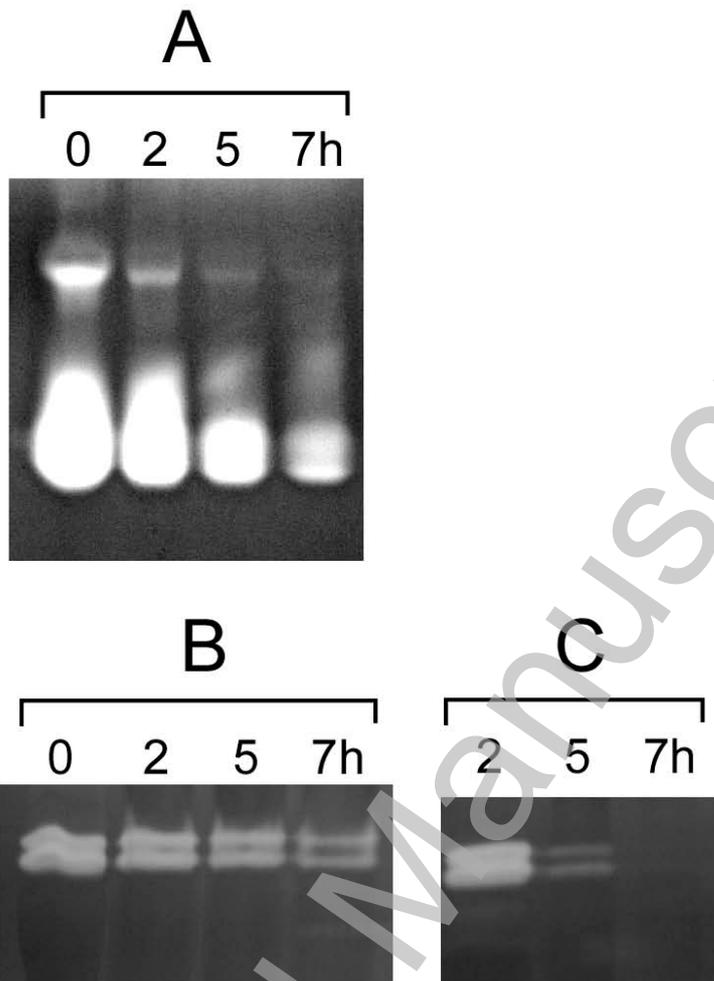


FIG 6



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