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Presence of detergent-resistant microdomains in lysosomal membranes☆

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Abstract

We examined the association of acetyl-CoA:α-glucosaminide *N*-acetyltransferase, a lysosomal enzyme participating in the degradation of heparan sulfate with other components of the lysosomal membrane. We prepared lysosomal membranes from human placenta and treated them with zwitterionic and non-ionic detergents. Membrane proteins were solubilized either in the presence of CHAPS at room temperature or of Triton X-100 at 4 °C. The CHAPS-containing extract was subjected to gel filtration in a column with the nominal size exclusion of 0.6 MDa. Under these conditions the enzyme fractionated near the void volume. To examine the association of the enzyme with detergent-resistant lipid microdomains, the extract that had been prepared with Triton X-100 was subjected to flotation in a density gradient medium. After centrifugation, a major portion of the activity of the acetyltransferase was found at the top of the gradient along with the bulk of alkaline phosphatase. Alkaline phosphatase is a glycosylphosphatidylinositol-anchored protein; possibly a contaminant in the lysosomal fraction originating from the plasma membrane and adventitiously an internal control for the flotation in the gradient. In contrast, acetyltransferase is a genuine lysosomal protein that obligatorily spans the membrane since it transfers acetyl residues from acetyl-CoA in cytosol to glucosaminyl residues in heparan sulfate fragments in the lysosomal matrix. To our knowledge this is the first report on association of a lysosomal membrane protein with detergent-resistant membrane microdomains or rafts.

Keywords: Acetyl-CoA:α-glucosaminide *N*-acetyltransferase; Detergent-resistant microdomains; Lysosomal membrane; Rafts

In biological membranes lipids and proteins are subjected to sorting into various microdomains that act as platforms for the attachment of proteins [1–3]. According to current models of biological membranes such microdomains are enriched in sphingolipids and cholesterol that are organized in a liquid-ordered phase, whereas the surrounding lipid bilayer is in a liquid-disordered state [2,3]. The liquid-ordered areas are resistant to extraction with neutral detergents such as Triton X-100 on ice [4] and are usually referred to as detergent-resistant microdomains (DRMs) or rafts. The size and composition of DRMs appear to vary; larger well-ordered aggregates are known as rafts and transient confinement zones [2]. The DRMs include proteins anchored through glycosylphosphatidylinositol [4,5] and particular transmembrane proteins. They appear to follow distinct exocytic [4,6] and endocytic [7–9] pathways, and to comprise areas serving regulatory and signal transduction functions in the plasma membrane [3]. In the endosomal–lysosomal compartments components of DRMs are subjected to sorting, recycling, and degradation that is considered to occur predominantly in the internal vesicles of the multivesicular bodies [7–9]. This results in a gradient with the minimum concentration of DRMs in the "dense body" lysosomes. In fact it is unclear if "dense bodies" that are found at one end of the sorting biosynthetic and endocytic pathways contain DRMs at all. Therefore, any residual DRMs in preparations containing the bulk of "dense

☆ *Abbreviations:* Acetyltransferase, acetyl-CoA:α-glucosaminide *N*-acetyltransferase, CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; CoA, coenzyme A; DRMs, detergent-resistant microdomains.

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bodies'' could represent a steady-state residue of materials subjected to degradation. Here we show that an enzyme known to participate in the degradation of heparan sulfate in lysosomes that transfers acetyl groups from acetyl-CoA in cytosol to non-reducing termini of heparan sulfate within the organelles is associated with DRM fractions of lysosomal membranes.

Materials and methods

Materials. Fresh placentas were obtained from normal deliveries in local hospitals. Acetyl-CoA and *p*-nitrophenyl substrates were purchased from Sigma–Aldrich Group, Deisenhofen, Germany. Axis-Shield OptiPrep medium was from Progen Biotechnik, Heidelberg, Germany, Coomassie blue (Roti-Blue) from Roth, Karlsruhe, Germany, [^{3}H]glucosamine (specific activity 20–33 Ci/mmol) from ICN Biomedicals, Eschwege, Germany, CHAPS from Calbiochem-Novachem, Bad Soden, Germany, and Percoll from Amersham Pharmacia Biotech, Freiburg i. Br., Germany. Mouse anti-human Lamp-2 monoclonal antibody 2D5 has been described previously [10].

Lysosomal membrane preparations. Fresh human placenta tissue was processed for the Percoll gradient centrifugation [11] as described by Diettrich et al. [12]. ''Dense body'' lysosomes were collected in fractions near the bottom of the tubes and after a hypotonic lysis in 10-fold volume of 10 mM Tris–HCl buffer, pH 7.4, and brief ultrasonication (throughout at 4 °C) the membrane fraction was collected by ultracentrifugation in a Beckman 60Ti rotor for 2 h at 265,000g_{max}. Affinity purification of lysosomal membranes was performed by the method of Diettrich et al. [12].

Gel filtration. An aliquot of the membrane preparation (0.1–0.2 mg protein) was incubated in the presence of 1% (w/v) CHAPS, 0.15 M NaCl, and 10 mM Na-phosphate buffer, pH 6.8, for 20 min at 20 °C. The sample was centrifuged for 5 min at 12,000g_{max} and the sediment was subjected to a second extraction under the same conditions. The combined supernatant fractions were applied to a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with the buffered CHAPS solution. The chromatography was performed using an Äkta explorer apparatus form Amersham Pharmacia Biotech. Fractions of 0.25 mL were collected and examined for enzyme activity and protein.

Flotation of DRMs. Lysosomal membranes (0.1–0.2 mg protein) were incubated in the presence of 1% (v/v) Triton X-100 on ice and the samples were centrifuged for 10 min at 12,000g_{max} at 4 °C. The supernatant was mixed with the Optiprep medium and overlayered as described by Bagnat et al. [6]. The volumes were adopted to 1.1 × 6 cm tubes. The centrifugation (6 h at 265,000g_{max} at 4 °C) was performed in a SW60Ti ultracentrifuge rotor (Beckman Instruments, München, Germany) in a Sorvall Combi Plus ultracentrifuge from Kendro, Osterode, Germany. Six fractions, 0.6 mL each, were collected from the top of the gradient.

Determination of enzyme activity and protein concentration. The activities of β-hexosaminidase (EC 3.2.1.52) and acid β-glucosidase (EC 3.2.1.45) were determined spectrophotometrically with the corresponding *p*-nitrophenyl substrates at 37 °C as previously described [12]. The activity of alkaline phosphatase (EC 3.1.3.1) was determined analogously using *p*-nitrophenyl phosphate, 5 mM. However, the incubation was performed in 50 mM Tris–HCl buffer containing 0.25 M sucrose, 5 mM $MgCl_2$, and 0.1% (v/v) Triton X-100 and absorbance at 405 nm was determined without admixture of any stop buffer. The activity of acetyl-CoA:α-glucosaminide *N*-acetyltransferase (EC 2.3.1.78) was determined using the procedure of Rome et al. [11] with [^{3}H]glucosamine as the acetyl acceptor. Radioactivity was determined using a liquid scintillation analyzer 1600TR Tri-Carb from Canberra Packard (Dreieich, Germany). Protein concentration was determined by the method of Bradford [13].

Polyacrylamide gel electrophoresis and band analysis. Sample preparation and separation in SDS–PAGE was performed as described by Laemmli [14]. The protein was visualized with silver nitrate [15]. Staining with colloidal Coomassie blue was performed as recommended by the manufacturer. For identification a Coomassie blue-stained band was excized and its analysis was performed using standard procedures (trypsin digestion and MALDI-mass spectrometry using a Reflex III mass spectrometer equipped with a Scout 384 ion source from Bruker Daltonik, Bremen, Germany). The obtained peptide mass fingerprint data were interpreted with ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound).

Results and discussion

Distribution of soluble and membrane-associated lysosomal enzymes in Percoll gradient fractions

Lysosomes were prepared from human placenta by fractionation in a self-forming Percoll gradient. The activity of the lysosomal marker enzyme β-hexosaminidase was distributed in two peaks. Similar to results with fibroblasts that had been reported by Rome et al. [11], the peak of the activity near the bottom of the tube contained little protein and was highly enriched in lysosomal marker enzyme activities. The organelles in these fractions have been shown to be represented by mainly ''dense body'' lysosomes [11]. Similar results were obtained in the present fractionation that was adopted to extracts of human placenta (Fig. 1A). At a lower density, another peak of β-hexosaminidase was found that had been considered to represent lower buoyant density tubular and tubulovesicular lysosomes and endosomes [11]. In the fractionation of organelles from human placenta this second peak of β-hexosaminidase activity was partially separated from marker enzymes of other organelles such as mitochondria, ER, and Golgi apparatus (not shown) and also from the bulk of the plasma membrane marker alkaline phosphatase (Fig. 1A).

The distribution of acid β-glucosidase in Percoll gradient fractions (Fig. 1B) resembled that of β-hexosaminidase. The activity ratio of the two enzymes indicated a relative enrichment of the latter in ''dense body'' lysosomes. This was observed in dozens of lysosomal preparations and the difference in the distribution could rely on the known morphological difference between the two populations of lysosomal organelles. As indicated briefly above, in fibroblasts the ''dense body'' acid phosphatase positive lysosomes are represented predominantly by round vesicular organelles, whereas the low buoyant density acid phosphatase positive lysosomes have a tubular appearance [11]. Assuming this difference remains valid for lysosomes from placenta, the relatively higher contents of the membrane-associated acid β-glucosidase in the more buoyant lysosomes could reflect a higher surface to volume ratio in these organelles as compared to ''dense body'' lysosomes.

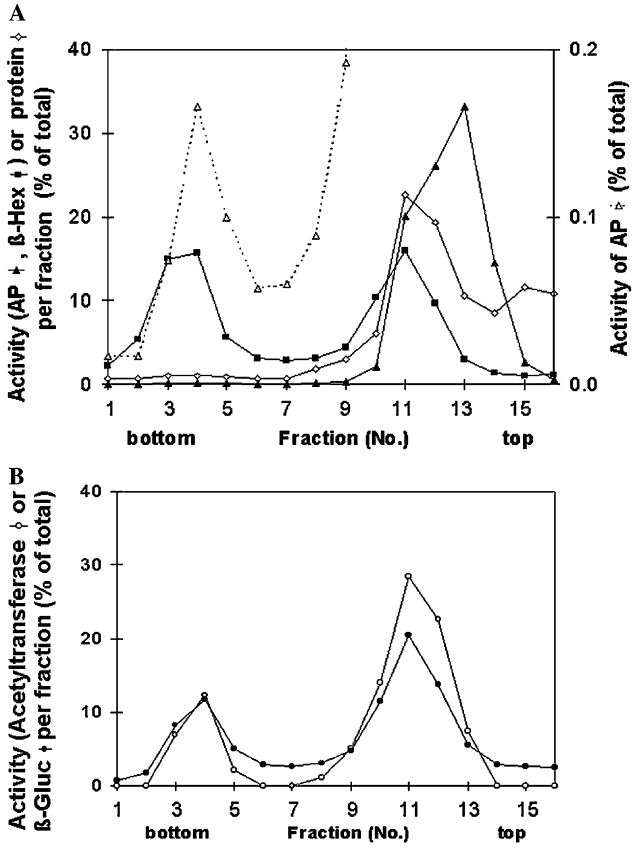

Fig. 1. Distribution of lysosomal and other marker enzyme activities in a Percoll gradient. (A) The activity of β-hexosaminidase (β-Hex) was compared with that of alkaline phosphatase (AP) and (B) the activity of acetyltransferase with that of acid β-glucosidase (β-Gluc). In (A) the dashed line represents an enlarged portion of the alkaline phosphatase curve.

Similar to acid β-glucosidase, the activity of acetyl-CoA:α-glucosaminide *N*-acetyltransferase (referred to as acetyltransferase further on) was found in both populations of the lysosomal organelles (Fig. 1B).

The further above-mentioned finding of the activity of the alkaline phosphatase in the "dense body" lysosomes peak was unexpected, since after the synthesis this glycosylphosphatidylinositol-anchored protein is incorporated into DRMs in the Golgi apparatus and then transferred into the plasma membrane [4,16]. Was the enzymes' presence in the "dense body" lysosomal fraction due to a contamination with fragments of the plasma membrane? We attempted to resolve this issue by affinity purification of the lysosomal membrane fraction using an immobilized *anti*-Lamp-2 monoclonal antibody 2D5. This procedure has been shown to increase the specific activity of the lysosomal membrane marker enzyme acid β-glucosidase [12]. In a typical experiment we observed a 3.2-fold enrichment of acid β-glucosidase and 1.2-fold of alkaline phosphatase. This means that using affinity purification of lysosomal membranes alkaline phosphatase is neither enriched nor removed to a significant amount. It is possible though not proven that a small portion of alkaline phosphatase is present in the lysosomal membrane and that this represents protein delivered to these organelles for degradation. This possibility is reminiscent of the observation by Tanaka et al. [17] on 5′-nucleotidase in rat liver tritosomes. Upon treatment with Triton WR1339 this glycosylphosphatidylinositol-anchored plasmalemmal enzyme has been found in lysosomes that have accumulated Triton WR1339 in both membrane-associated and soluble forms. Their report has indicated that 5′-nucleotidase enters a lysosomal compartment to be released from the membrane and probably degraded completely. Currently, endosomal multivesicular bodies are considered to be the compartment responsible for the degradation of membranes [7,9]. As far as tritosomes are concerned, however, it is not known if they are generated from multivesicular or "dense body" lysosomes or both.

Large molecular complexes in lysosomal membranes

We examined whether there is any molecular association between the membrane bound lysosomal enzymes and alkaline phosphatase. For this purpose the membranes were incubated in the presence of CHAPS and the soluble fraction was subjected to gel filtration. In the presence of 0.1% (w/v) CHAPS the solubilized fraction contained a complex mixture of proteins including acetyltransferase and Lamp-2 (not shown). In the presence of 1% (w/v) CHAPS most of the enzymatic activity of acetyltransferase and a significant portion of alkaline phosphatase were found in the excluded volume (Fig. 2A). However, the majority of the proteins including Lamp-2 and acid β-glucosidase eluted later (Fig. 2B).

The differential behavior of the 1% CHAPS-extractable lysosomal membrane proteins during gel filtration is of considerable interest. The examined proteins represent distinct examples of protein function and insertion in the membrane. The acetyltransferase is probably a polytopic transmembrane protein since it interacts with one of its substrates, acetyl-CoA, at the cytosolic and the other, heparan sulfate, at the luminal side of the membrane [18,19]. Lamp-2 is a type I membrane protein with a single transmembrane domain [20]. Acid β-glucosidase is attached to the luminal side of the lysosomal membrane without having any transmembrane domains; little is known about the nature of the attachment apart from its independence from glycosylation [21]. Before targeting the plasma membrane, alkaline phosphatase is integrated in DRMs in the Golgi apparatus by its C-terminal glycosylphosphatidylinositol anchor [4,16]. The presence of acetyltransferase and of a portion of alkaline phosphatase among proteins excluded from the gel matrix indicated their participation

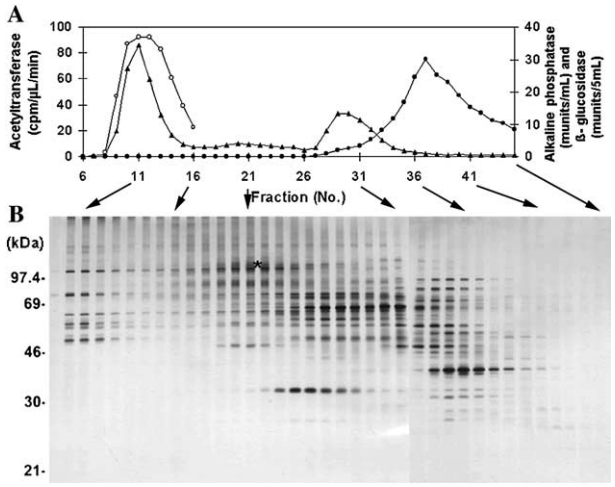

Fig. 2. Gel filtration of a solubilized fraction of lysosomal membrane proteins. Lysosomal membranes were extracted in the presence of 1% (w/v) CHAPS at 20 °C and the soluble fraction was applied to a Superdex 200 column. (A) In aliquots of the fractions enzymatic activities of alkaline phosphatase, acid β-glucosidase, and acetyltransferase were determined. In several experiments, the latter was found only near the exclusion volume. Therefore, here it was not measured beyond fraction No. 16. (B) The separation of proteins was examined by SDS–PAGE and staining using silver nitrate. The protein bands containing Lamp-2 had a characteristic diffuse appearance with a distinct "golden" tint. They were identified in a Western blot (not shown). The asterisk indicates the maximum reaction intensity on the blot.

in homo- or heteromeric complexes with proteins or lipids.

We examined the latter possibility using an established procedure for the isolation of DRMs. We found that, in the presence of Triton X-100, after ultracentrifugation in an OptiPrep gradient both alkaline phosphatase and acetyltransferase floated to the top of the centrifuge tube (Fig. 3A). By the decrease in the activity in fractions of increasing density the two enzymes resembled each other. When the fractionation was repeated with the top fraction, both enzymes were recovered with the highest concentration in the top fraction (not shown).

The activity of alkaline phosphatase that was found in the "dense body" lysosomal fractions represented a minor portion of the total enzyme activity in placenta extracts. Therefore, it was of interest to identify the protein responsible for alkaline phosphatase activity in DRMs in this particular fraction. We prepared a concentrate of proteins from a pool of the top fractions from several OptiPrep gradients, separated them in SDS–PAGE, and stained with Coomassie blue (Fig. 3B, lane C). The band that was stained at approximately 70 kDa was treated with trypsin and the digested material was subjected to MALDI-mass spectrometric analysis. In the mass fingerprint placental alkaline phosphatase (EC 3.1.3.1, SwissProt number P05187) was found with a Z score of 2.22 (a probability of nearly 0.99).

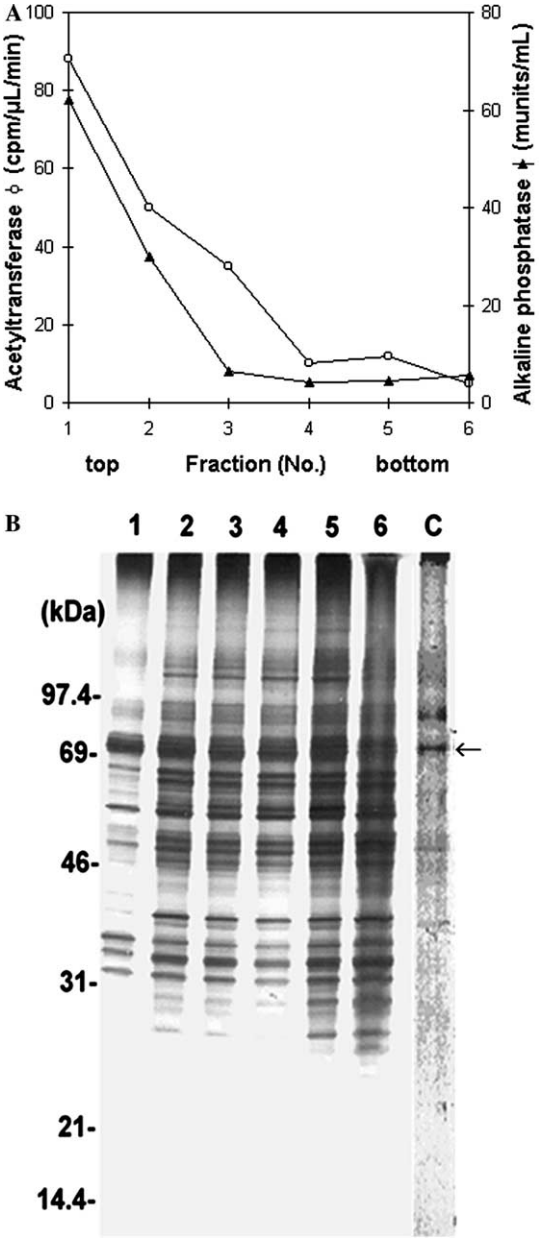

Fig. 3. Association of acetyltransferase and alkaline phosphatase with DRMs. A lysosomal membrane preparation was incubated with 1% (w/v) Triton-X100 at 4 °C and subjected to centrifugation in an OptiPrep gradient. (A) The distribution of acetyltransferase and alkaline phosphatase in the gradient fractions is different from that of the bulk of proteins (B) as determined in SDS–PAGE and silver nitrate staining. Protein in fractions 1–6 (indicated above the lanes) was concentrated by precipitation with cold TCA, 15% (w/v). In lane C protein was concentrated from the top fractions of three gradients and after the separation stained with Coomassie blue.

Conclusions

We present evidence that the characteristic lysosomal enzyme acetyl-CoA:α-glucosaminide *N*-acetyltransferase is associated with DRMs. The association of this enzyme with DRMs indicates that lipid structures of this

kind are physiological components of lysosomal membranes. The observation was not expected, since DRMs are considered to become degraded during the passage through endosomal compartments [8,9], with cholesterol and sphingolipids being subjected to recycling or degradation except in lipid storage diseases [7,22]. The transferase itself is likely to bind a lipid shell that has been proposed to direct incorporation of proteins into DRMs [23]. Defects in the studied transferase cause Sanfilippo type C disease and result in a lysosomal storage of heparan sulfate [24]. This recessively inherited progressively disabling disease, also known as mucopolysacharidosis IIIC, is one of the storage diseases that have eluded the molecular elucidation so far. The present finding may promote the identification of the protein and its gene.

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