Biochemical and Biophysical Research Communications 411 (2011) 175–179

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hyal2 is a glycosylphosphatidylinositol-anchored, lipid raft-associated hyaluronidase

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ARTICLE INFO

Article history:
Received 7 June 2011
Available online 25 June 2011

Keywords:
Hyaluronidase
Glycosylphosphatidylinositol
Lipid raft

ABSTRACT

The rapid turnover rate of hyaluronan (HA), the major unbranched glycosaminoglycan of the extracellular matrix, is dependent on hyaluronidases. One of them, hyaluronidase-2 (Hyal2), degrades HA into smaller fragments endowed with specific biological activities such as inflammation and angiogenesis. Yet the cellular environment of Hyal2, a purported glycosylphosphatidylinositol (GPI)-anchored protein, remains uncertain. We have examined the membrane association of Hyal2 in MDA-MB231 cancer cells where it is highly expressed and in COS-7 cells transfected with native or fluorescent Hyal2 constructs. In both cell types, Hyal2 was strongly associated with cell membrane fractions from which it could be extracted using a Triton X-114 treatment (hydrophobic phase) but not an osmotic shock or an alkaline carbonate solution. Treatment of membrane preparations with phosphatidylinositol-specific phospholipase C released immunoreactive Hyal2 into the aqueous phase, confirming the protein is attached to the membrane through a functional GPI anchor. Hyal2 transfected in COS-7 cells was associated with detergent-resistant, cholesterol-rich membranes known as lipid rafts. The cellular immunofluorescent pattern of Hyal2 was conditioned by the presence of a GPI anchor. In summary, the strong membrane association of Hyal2 through its GPI anchor demonstrated in this study using biochemical methods suggests that the main activity of this enzyme is located at the level of the plasma membrane in close contact with the pericellular HA-rich glycocalyx, the extracellular matrix, or possibly endocytic vesicles.

1. Introduction

The rapid turnover rate of hyaluronan (HA), the major unbranched glycosaminoglycan of the extracellular matrix, is dependent on hyaluronidases. Two of them, Hyal1 and Hyal2, constitute the main catabolic machinery in somatic tissues [1]. We have recently shown that liver sinusoidal cells quickly engulf plasma Hyal1 and use it to degrade HA within intracellular acidic vesicles which do not correspond to *bona fide* lysosomes [2]. On the other hand, the cellular environment of Hyal2 remains uncertain. Initially proposed as a lysosomal hyaluronidase [3], Hyal2 was later shown to be linked to a glycosylphosphatidylinosotol (GPI) anchor in bronchial epithelial cells [4,5] but not in chondrocytes [6]. This gave rise to a controversy which has not been completely resolved [7]. Hyal2 was suggested as a major producer of proinflammatory HA fragments in various tissues [8,9]; some of its additional, likely non-enzymatic, functions may comprise regulating glycocalyx thickness [10] and stabilizing red blood cell membranes [11]. Therefore, we decided to examine the membrane association of Hyal2 more closely, using MDA-MB231 cancer cells with high expression of Hyal2 and COS-7 cells transfected with native or fluorescent Hyal2 constructs. We found that Hyal2 was strongly associated with lipid rafts.

Abbreviations: AA, amino acid; DRMs, detergent-resistant membranes; EGFP, enhanced green fluorescent protein; GPI, glycosylphosphatidylinositol; HA, hyaluronan; Hyal2, hyaluronidase-2; MLP, combined heavy mitochondrial (M), light mitochondrial (L), and microsomal (P) subcellular fractions; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C.

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doi:10.1016/j.bbrc.2011.06.125

2. Material and methods

2.1. Cell culture

MDA-MB231 human breast cancer cells and monkey COS-7 cells (both from ATCC/LGC Standards) were cultured in DMEM (Lonza) supplemented with 10% FBS and 15 mM Hepes.

2.2. Antibodies

The following antibodies were used: rabbit polyclonal anti-rat Hyal2 (P16) produced in our laboratory [10]; rabbit polyclonal anti-human Hyal2 (Abcam); anti-calreticulin (Affinity Bioreagents); anti-AKT (Cell Signaling Technology); anti-flotillin-1 (BD Biosciences); anti-enhanced green fluorescent protein (EGFP) from Sigma–Aldrich; horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse immunoglobulin G (Dako); and Alexa 594-tagged anti-rabbit immunoglobulin G (Molecular Probes).

2.3. Plasmid construction and transfection

The full-length human, mouse and rat Hyal2 cDNAs (GenBank IDs: NM003773, NM010489, and AF034218 [10], respectively) were cloned between *Bam*HI and *Xho*I sites of a pcDNA3.1 vector using standard protocols [10]. A GPI anchor-deleted rat Hyal2 protein (Hyal2ΔGPI) was produced by deletion of the sequence corresponding to the last 25 amino acid (AA) C-terminus of the precursor chain. In another construction, the cDNA sequence encoding EGFP was inserted within the rat Hyal2 or Hyal2ΔGPI sequences immediately after the sequence corresponding to the signal peptide. Two fluorescent forms of Hyal2 (Hyal2-EGFP and Hyal2ΔGPI-EGFP) were thus created leaving the signal peptide intact. Overexpression of the proteins in COS-7 cells was achieved using the pcDNA1/Amp (Invitrogen) vector and transient transfection with Fugene-6 reagent (Roche). Cells were incubated during 24 h and processed for experiments.

2.4. Subcellular fractionations

Cells were scraped in ice-cold PBS and centrifuged; the pellet was swollen in a hypotonic buffer (20 mM Tris–HCl pH 7.6, 10 mM EDTA, 10 mM EGTA, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, and proteases inhibitors) and lyzed by Dounce homogenization on ice. The soluble and membrane fractions were obtained by ultracentrifugation as described [12]. In other experiments, cells were homogenized in a 0.25 M sucrose/3 mM imidazole, pH 7.4, buffer with a Dounce system. After centrifugation at 500*g* for 2 min at 4 °C, the pellet was subjected to differential ultracentrifugation according to the de Duve protocol [13] in order to obtain the nuclear (N) and post-nuclear (E) fractions, the latter subdivided into heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and soluble (S) fractions. In some cases, a combined MLP fraction of 50 μg proteins was subjected to a 1 h osmotic shock in H_2O on ice. After centrifugation at 100,000*g* for 20 min at 4 °C, either the pellet and supernatant were analyzed by SDS–PAGE (osmotic shock experiments), or the pellet was resuspended in sodium carbonate buffer (100 mM, pH 11.5), incubated on ice for 30 min, and centrifuged as described above, generating a second-generation pellet and supernatant in carbonate buffer.

2.5. Western blots

Various subcellular fractions adjusted to 10 μg of proteins were separated by SDS–PAGE (10% acrylamide/bisacrylamide) according to Laemmli using a Mini Gel Protean set (BioRad) and were blotted on polyvinylidone difluoride membranes (Amersham Biosciences). The proteins were immunodetected with primary antibodies for 2 h at room temperature, followed by incubation with horse radish peroxidase-conjugated anti-immunoglobulin G antibodies. Detection was done using an Enhanced Chemi-Luminescence Western blotting detection system (Perkin Elmer).

2.6. Endoglycosidase treatment and immunoprecipitation

Cells were labeled for 18 h with [^{35}S] methionine and processed for subcellular fractionation in a sucrose/imidazole buffer as described above. MLP fractions were incubated either in 50 mM sodium citrate (pH 5.5) with 20 mU/ml endoglycosidase H or in 50 mM sodium acetate (pH 6.0) with 10 mU/ml endoglycosidase F (both enzymes from New England Biolabs) overnight at 37 °C. After preincubation with protein A-agarose (Roche Applied Science), supernatants were incubated with P16 antibodies followed by protein A-agarose. Immunoprecipitates thus obtained were washed 5× with PBS containing 1% Triton, 0.1% SDS, and 0.5% deoxycholate and boiled with an equal volume of Laemmli sample buffer, followed by SDS–PAGE and Cyclone phosphorimager (Canberra Packard) analysis.

2.7. Detergent phase separation with Triton X-114

The extraction of membrane proteins was realized as described previously [14]. Western blot was performed on the aqueous and detergent phases.

2.8. Triton X-100 and n-octylglucoside treatments

MLP fractions of 50 μg proteins were incubated in 50 μl sucrose (0.25 M)/imidazole (3 mM) buffer, pH 7.4, with or without 60 mM octylglucoside (30 min on ice) or Triton X-100 (1 h at either 4 °C or 37 °C). After centrifugation at 100,000*g* for 20 min at 4 °C, the pellets were resuspended in the same buffers and samples were analyzed by SDS–PAGE.

2.9. Phosphatidylinositol specific-phospholipase C (PI-PLC) treatment

To cleave the GPI anchor, an MLP fraction of 50 μg proteins was incubated with 20 U/ml *Bacillus cereus* PI-PLC (Sigma–Aldrich) at 37 °C for 2h 30 min in 20 mM Tris HCl, pH 7.5. After centrifugation at 100,000*g* for 20 min, the pellets were resuspended in the buffer and samples were analyzed by Western blot.

3. Results

3.1. Expression and membrane association of Hyal2 in MDA-MB321 cells

The anti-human Hyal2 antibody detected a major protein band with an apparent molecular size of 52 kD in the membrane but not in the soluble fraction of MDA-MB231 cells (Fig. 1A). Further subcellular ultracentrifugation analysis confirmed that the signal was concentrated in a membrane-rich fraction, MLP, with no detection in the soluble fraction, S (Fig. 1B). This pattern of Hyal2 detection mirrored that of Flotillin-1, a typical integral membrane protein associated with caveolae and lipid rafts, and was clearly distinct from that of AKT, a soluble serine/threonine protein kinase found in the cytosol (Fig. 1B). When the MLP fraction was treated with Triton X114, Hyal2 was greatly enriched in the detergent phase, highlighting a strong hydrophobic behavior (Fig. 1C). Flotillin-1 behaved in a similar fashion although the enrichment in the detergent phase was less than for Hyal2 (Fig. 1C). We decided to examine the membrane association of Hyal2 in a system where the protein could be expressed at a high level with or without the GPI anchor. To that aim we used COS-7 cells transfected with different rat Hyal2 cDNA constructs. Of note, our laboratory has developed an effective rabbit polyclonal antiserum (P16) directed against a 16-AA peptide sequence located close to the C-terminus of rat Hyal2 [10].

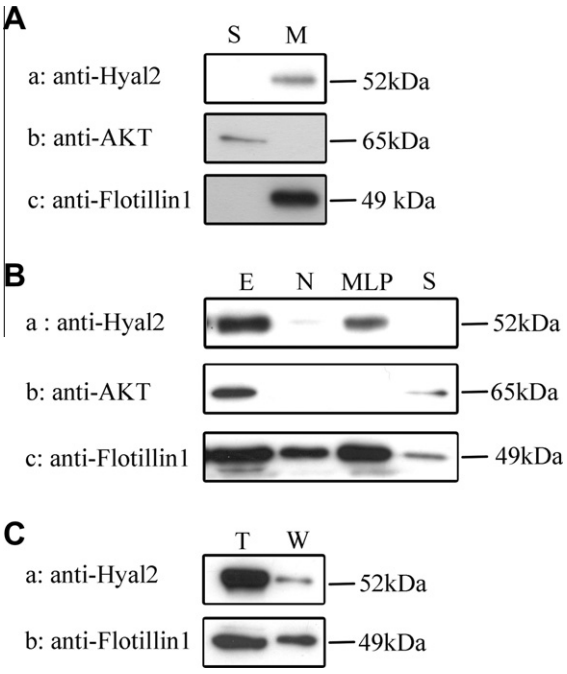

Fig. 1. MDA-MB231 cells. Western blots of Hyal2, AKT (a soluble protein), and Flotillin-1 (a lipid raft-associated protein) in: (A) a separation between soluble (S) and membrane (M) fractions; (B) a subcellular fractionation obtained by differential ultracentrifugation, comprising the nuclear (N) and post-nuclear (E) fractions, the latter subdivided into MLP (heavy mitochondrial [M], light mitochondrial [L], and microsomal [P]) and S (soluble) fractions; (C) an MLP fraction treated with Triton X-114 and separated into the detergent phase (T) and the aqueous phase (W).

3.2. Expression and membrane association of Hyal2 following transfection in COS-7 cells

The rat Hyal2 cDNA was cloned from a rat kidney RNA library. The predicted full-length open reading frame encodes a 473-AA protein. Sequence alignments with mouse Hyal2 showed 85% identity and with human Hyal2, 67%. The analysis of the primary structure of Hyal2 (NCBI Reference Sequence: NP742037.2) yielded a high probability for a signal peptide, a single EGF-like domain, and a C-terminal segment of 27 AA bound to be replaced by a GPI anchor in the endoplasmic reticulum. There are also three potential *N*-glycosylation sites. Homogenates of COS-7 cells transfected with the Hyal2 cDNA were probed with the P16 antibody. The patterns of membrane association (Fig. 2A) and subcellular detection (Fig. 2B) were very similar to those observed in MDA-MB231 cells (Fig. 1A and B), apart from the observation of a second minor band of 62 kD in most, but not all, Hyal2 transfection experiments in COS-7 cells or in HEK293 cells (data not shown). The nature of this less abundant immunoreactive 62-kD band is so far unresolved since in vitro translation of the rat Hyal2 cDNA yielded a single band of the expected size (data not shown), and both the minor and the main species were *N*-glycosylated to the same extent in transfected COS-7 cells (Fig. 2C) and HEK293 cells (data not shown). The present study is restricted to the analysis of the main 52-kD protein resulting from Hyal2 transfection in COS-7 cells, which mirrors the single band of expression of Hyal2 in MDA-MB231 cells.

To determine the strength of the Hyal2 linkage to the membranes, MLP fractions of COS-7 cells were subjected to an osmotic shock, and the relative amount of Hyal2 released into the soluble fraction was evaluated. This amount was low (Fig. 2D), similar to what was observed in MDA-MB231 cells (Fig. 1A). In addition, treatment of the MLP fraction with a strongly alkaline carbonate solution could not solubilize the protein (Fig. 2E), indicating a true membrane docking. Finally, most of Hyal2 partitioned into the detergent phase of a Triton X-114 extract (Fig. 2F), exactly as observed with endogenous Hyal2 in MDA-MB231 cells (Fig. 1C).

3.3. Association of Hyal2 with detergent-resistant membranes (DRMs) and lipid rafts

GPI-linked proteins can exhibit strong membrane association when they are part of DRMs, commonly known as lipid rafts. When MLP fractions of MDA-MB231 and transfected COS-7 cells were treated with the neutral non-ionic detergent Triton X-100 at 4 °C, the majority of Hyal2 was present in the insoluble (pellet) fraction, demonstrating that Hyal2 is a Triton-insoluble protein, but when the procedure was repeated at 37 °C, much more protein was present in the soluble fraction than in the pellet (Fig. 3A). This behavior is typical of DRM-associated proteins, also known as lipid raft proteins [15].

3.4. Association of Hyal2 with DRMs is dependent on a GPI anchor

Based on its primary structure, Hyal2 is probably linked to cellular membranes through a GPI anchor. Two types of experiments were performed to test this hypothesis. First, MLP fractions of transfected COS-7 cells were treated with octylglucoside, a high critical micellar concentration detergent. This type of detergent is able to solubilize GPI-linked proteins that are insoluble in Triton X-100 but can be released by PI-PLC in various cell types [15–17]. Treatment of MLP fractions with 60 mM octylglucoside indeed released the majority of Hyal2 from the pellet (Fig. 3B). Second, MLP fractions of COS-7 cells and MDA-MB231 cells were incubated in parallel with PI-PLC then centrifuged at 100,000*g*. As a result, antigenic Hyal2 was shifted from the pellet into the supernatant (Fig. 3C), confirming that cleavage of a GPI moiety had resulted in release of Hyal2 from the membrane environment. Flotillin-1, a lipid raft-associated protein that bears no GPI anchor, did not follow this pattern (Fig. 3C). The GPI-dependent association of Hyal2 with DRMs was supported by observations made in COS-7 cells with a truncated construct of Hyal2 which is devoid of a GPI anchor (Hyal2ΔGPI). The main species of this construct expressed in COS-7 cells was present in the aqueous phase of Triton X-114 extracts (Fig. 3D).

3.5. The GPI anchor drives Hyal2 to the plasma membrane

In further experiments, chimeric constructs of Hyal2 and Hyal2ΔGPI tagged with EGFP were transfected into COS-7 cells and their expression was observed using confocal microscopy (Supplementary material). Both constructs colocalized with calreticulin, a marker of the endoplasmic reticulum, but only Hyal2-EGFP was additionally localized to the Golgi apparatus and the plasma membrane. When cells were not permeabilized before addition of antibodies, the anti-EGFP antibody yielded a speckled pattern at the surface of Hyal2-EGFP-transfected cells; this was not observed with Hyal2ΔGPI-EGFP, confirming that a GPI anchor is required to drive Hyal2 expression to the plasma membrane (Supplementary material).

4. Discussion

This study explored the association of the somatic hyaluronidase Hyal2 with cell membranes, using human cancer cells (MDA-MB231) with high expression of endogenous Hyal2 and COS-7 cells transfected with various constructs of rat Hyal2, with or without the original GPI anchor. Rat, mouse and human Hyal2

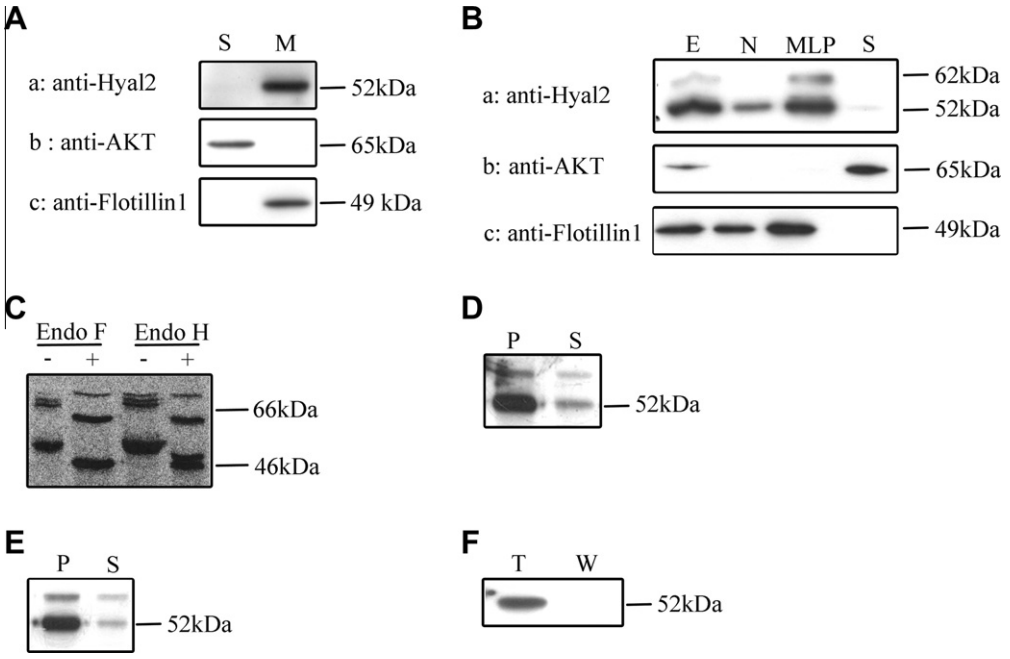

Fig. 2. COS-7 cells transfected with rat Hyal2. Western blots of Hyal2, AKT (a soluble protein), and Flotillin-1 (a lipid raft-associated protein) in: (A and B) the same subcellular fractions as in Fig. 1; (C) MLP fractions treated (+) or untreated (−) with endoglycosidase F or H – in these experiments Hyal2 is detected using immunoprecipitation; (D) an MLP fraction subjected to an osmotic shock then separated into a pellet (P) and a soluble fraction (S) by centrifugation; (E) the pellet of an MLP fraction subjected to an osmotic shock, then treated with a 100-mM sodium carbonate buffer at pH 11.5 and centrifuged into a pellet (P) and a supernatant (S); (F) an MLP fraction treated with Triton X-114 and separated into the detergent phase (T) and the aqueous phase (W).

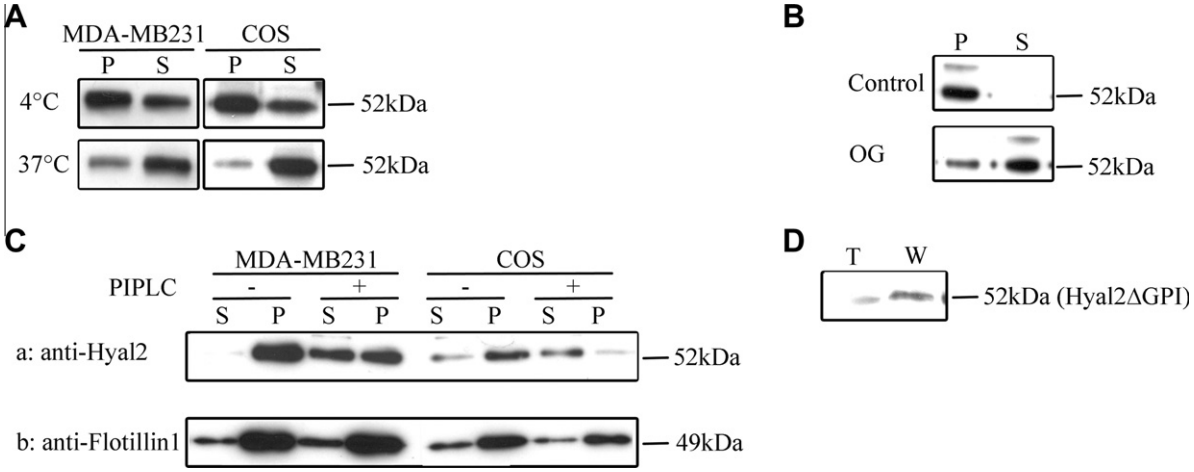

Fig. 3. Western blots of Hyal2 and Flotillin-1 (a non-GPI-anchored, lipid raft-associated protein) in transfected COS-7 cells and MDA-MB231 cells. (A) Detection of Hyal2 in the non-soluble pellet (P) and soluble supernatant (S) of MLP fractions treated with the non-ionic detergent Triton X-100 at 4 °C and 37 °C; (B) Detection of Hyal2 in the non-soluble pellet (P) and soluble supernatant (S) of MLP fractions of transfected COS-7 cells treated with the high critical micellar concentration detergent octylglucoside (OG); (C) Detection of Hyal2 and Flotillin-1 in MLP fractions of transfected COS-7 cells and MDA-MB231 cells. The fractions were incubated (+) or not (−) with PI-PLC and centrifuged into a pellet (P) and a soluble supernatant (S); (D) Detection of Hyal2 in an MLP fraction of COS-7 cells transfected with Hyal2ΔGPI, a form of Hyal2 devoid of the GPI anchor. The fraction was extracted with Triton X-114 before separation into a detergent (T) and an aqueous phase (W).

proteins are highly similar; rat Hyal2 constructs were used because they can be detected reliably with a custom-made anti-rat Hyal2 antibody (P16). A series of biochemical experiments confirmed that Hyal2 is intrinsically associated with cell membranes and especially with DRMs also known as lipid rafts. This association cannot be released through an osmotic shock, highly alkaline treatments, or the non-ionic detergent Triton X-100 but can be broken by the high critical micellar concentration detergent octylglucoside [15–17] or through the action of PI-PLC, confirming that Hyal2 is attached to the membrane through a GPI anchor and faces the external milieu. This was corroborated with the use of fluorescent confocal microscopy and Hyal2-EGFP constructs which do not require specific antibodies (cf. Supplementary material).

The presence of Hyal2 in lipid rafts is a key observation that supports its close interaction with the main HA receptor, CD44, which is also a lipid raft protein in several cell types [18]. A functional Hyal2-CD44 interface has been discovered by several investigators, including ourselves, in many different cell types [10,19,20]. Lipid rafts are critical platforms for the interaction between extracellular clues and intracellular signals; we have shown previously that overexpression of Hyal2 disturbs the interactions of CD44 with HA and with its intracellular effector

ezrin/radixin/moesin [10]. In addition, CD44 shedding from lipid rafts is induced by overexpression of Hyal2 [10] as well as by membrane cholesterol depletion with methyl-β-cyclodextrin [21]. CD44 shedding has been repeatedly linked to tumor cell migration and invasiveness [22]. Therefore, the HA-Hyal2-CD44-lipid raft connection is likely of high significance.

Our observations support previous descriptions of Hyal2 at the cell surface [4,5,8,23–25] but not the suggestion that the majority of the protein resides in lysosomes [3] or the cytoplasm [6]. At the cell surface, Hyal2 would be well poised to exert the various functions that have been demonstrated for this ubiquitous protein, i.e. cleave extracellular or pericellular HA into biologically active (angiogenic, inflammatory) fragments [9,19,20], interact with tumor growth factor-β and mediate some of its intracellular actions [24], act as a receptor for ovine oncogenic retroviruses [25], and, for platelet Hyal2, cleave the extracellular HA cables that bind leukocytes [8]. In tumor cells, Hyal2 may be associated with the process of intravasation [23]. With regard to the role of Hyal2 in HA degradation, our data are compatible with a model of mostly intracellular degradation [1]. Even if the bulk of HA cleavage by Hyal2 occurs in acidic pockets at the cell surface [19], this enzyme is unable to cut HA below a molecular size of ~20 kD [20,26]. Therefore, to be fully degraded, Hyal2-generated fragments need to be endocytosed, perhaps with a portion of Hyal2 [24], into acidic vesicles containing Hyal1 [2]. In addition, recombinant Hyal2 was shown to be ~400-fold less active than the sperm hyaluronidase Spam1/PH20 [26], which is also GPI-anchored. Thus, the role of Hyal2 in the catabolic machinery of HA may be limited to a kind of pretreatment of the largest HA molecules while its position at the cell surface may be critical for other, likely non-enzymatic, functions [10,11].

Acknowledgments

Benedicte Andre was supported by Belgian FRS-FNRS (Fonds National pour la Recherche Scientifique) through a FRIA grant.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.125.

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