Effect of a cholesterol-rich lipid environment on the enzymatic activity of reconstituted hyaluronan synthase

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Abstract

Hyaluronan synthase (HAS) is a unique membrane-associated glycosyltransferase and its activity is lipid dependent. The dependence however is not well understood, especially in vertebrate systems. Here we investigated the functional association of hyaluronan synthesis in a cholesterol-rich membrane-environment. The culture of human dermal fibroblasts in lipoprotein-depleted medium attenuated the synthesis of hyaluronan. The sequestration of cellular cholesterol by methyl- β -cyclodextrin also decreased the hyaluronan production of fibroblasts, as well as the HAS activity. To directly evaluate the effects of cholesterol on HAS activity, recombinant human HAS2 proteins with a histidine-tag were expressed as a membrane protein by using a baculovirus system, then successfully solubilized, and isolated by affinity chromatography. When the recombinant HAS2 proteins were reconstituted into liposomes composed of both saturated phosphatidylcholine and cholesterol, this provided a higher enzyme activity as compared with the liposomes formed by phosphatidylcholine alone. Cholesterol regulates HAS2 activity in a biphasic manner, depending on the molar ratio of phosphatidylcholine to cholesterol. Furthermore, the activation profiles of different lipid compositions were determined in the presence or absence of cholesterol. Cholesterol had the opposite effect on the HAS2 activity in liposomes composed of phosphatidylethanolamine or phosphatidylserine. Taken together, the present data suggests a clear functional association between HAS activity and cholesterol-dependent alterations in the physical and chemical properties of cell membranes.

Keywords: Hyaluronan; Biosynthesis; Synthase; Cholesterol; Lipid

1. Introduction

Hyaluronan (HA) is a simple polysaccharide composed of repeating disaccharide units in which *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcUA) are linked together by alternating β -1,3 and β -1,4 linkages [1]. HA biosynthesis is primarily regulated by HA synthase (HAS), which catalyzes the transfer of both UDP-GlcNAc and UDP-GlcUA substrates into newly synthesized HA [2]. The HAS gene was first characterized in the bacterium streptococci [3], and three different HAS enzymes (HAS1, HAS2, and HAS3) were later discovered in vertebrates [4]. Structurally, all HAS enzymes are membrane-associated proteins composed of multiple membrane-spanning regions with hydrophobic amino acid clusters and large cytoplasmic loops. Unlike typical glycosyltransferases, it has been proposed that these enzymes localize in the plasma membrane and face its catalytic sites toward the cytoplasm. Purification and characterization of HAS have been accomplished in bacterial recombinant proteins [5], and recent *in vitro* reconstitution studies using a bacterial recombinant HAS protein have shown that a phospholipid environment is required for the full activity of this HAS enzyme [5, 6]. In these cell-free reactions, administration of caldiolipin increased the activity of solubilized recombinant HAS in a dose-dependent manner. Therefore, the three-dimensional structure required for full enzymatic activity may be ensured by an interaction with lipids, and due to similarities in primary structure and configuration, the activities of mammalian HAS enzymes may also have been affected by the microenvironment of the lipids.

Cholesterol is a major lipid component in the plasma membrane of eukaryotic cells and plays an essential role in maintaining membrane fluidity and architecture [7]. In principal, cholesterol

consists of pure hydrocarbons in the form of a steroid ring structure and effectively fills the existing space between phospholipids. The proper distribution of cholesterol and other lipid constituents determines the physical membrane properties and is critical for numerous cellular functions [7]. Cholesterol-rich microdomains also play a role in the lipid environment for optimization of enzyme activity and formation of ordered platforms for assembly of cell signaling molecules [8]. This opens the possibility that the activity of HAS enzymes may also be modulated by cholesterol-enriched ordered microenvironments, in particular by restricted lipid fluidity and increased stiffness of membrane lipid bilayers. However, there has been no direct demonstration of cholesterol-dependent regulation of HAS activities to date.

In the present study, we aimed at obtaining further insight into the lipid-dependence of HA biosynthesis by investigating the effect of cholesterol on this process.

2. Materials and Methods

2.1. Materials

The phospholipids used were listed in the Supplementary Table S1. Reagents were supplied by Sigma-Aldrich unless stated otherwise.

2.2. Determination of HA concentrations

The HA content in the conditioned medium of exponentially growing cultures was measured by a modified competitive ELISA-like assay as described previously [9] (see Supplementary materials).

2.3. Cholesterol measurement

Human dermal fibroblasts (2×10^6 cells in 100-mm-diameter dishes) were cultured for 2 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS or lipoprotein depleted serum from fetal bovine (LPDS), and then further incubated in the presence or absence of 10 mM methyl- β -cyclodextrin (Me β CD) for 24 h. Total lipids were extracted with chloroform/methanol/water (2:2:1) by phase separation, as described by the standard Bligh-Dyer method [10]. Total cholesterol content was measured using the Cholesterol E-Test Wako (Wako Pure Chemical Ind., Osaka, Japan), according to the manufacturer's instructions. Cell proliferation was determined using the premix WST-1 reagent (Takara Biochemicals, Shiga, Japan) according to the manufacturer's instructions.

2.4. Expression of human HAS2 in insect cells

The baculoviral construct for expression of human HAS2 proteins with a C-terminal tetra-histidine (His)-tag was produced using the BaculoDirect™ baculovirus expression system (Invitrogen). Briefly, recombinant baculovirus DNA encoding human HAS2 was directly transfected into *Sf21* cells. Baculovirus was harvested 5–7 days after incubation and then further amplified in infected *Sf21* cells to obtain a high-titer virus. Recombinant baculovirus was infected into *Sf21* cells (9×10^6 cells in 150-mm-diameter dishes) and cultured for 3 days at 27 °C in Grace's insect cell culture medium (Invitrogen) supplemented with 10% FBS, 0.3% yeastolate and 0.3% lactoalbumin hydrolysate. The cells infected with the recombinant virus were harvested, washed twice with PBS, and then disrupted by sonication in lysis buffer (10 mM Hepes-NaOH,

pH 7.1, 0.5 mM DTT, 0.25 M sucrose). Cell lysates were ultracentrifuged in a Beckman TLA 100.3 rotor at 100,000 g for 1 h to give high speed pellets, and stored at -80 °C.

2.5. Extraction and purification of recombinant HAS2

Membrane pellets were solubilized using Preserve X (0.1% w/v) (QBI Life Sciences, Madison, WI) in extraction buffer (200 mM Hepes-NaOH, pH 7.8, 150 mM NaCl, a cocktail of protease inhibitors; Roche Diagnostics Corp., Mannheim, Germany) at 4 °C for 2 h with gentle mixing. Insoluble membrane components were sedimented by centrifugation at 13,000 g for 10 min, and imidazole was then added to the supernatant to a final concentration of 20 mM to minimize nonspecific binding of *Sf21* cell proteins to the His-tagged protein. The final extract was applied directly to a His-Spin-Trap column (GE Healthcare UK Ltd., Buckinghamshire, UK), which had been equilibrated with the equilibrium buffer (50 mM NaH₂PO₄, pH 7.8, 300 mM NaCl, 10 mM MgCl₂, 0.1 mM DTT, a cocktail of protease inhibitors, 50 mM imidazole, 0.1% Preserve X). The His-tagged proteins were then washed with equilibrium buffer containing 80 mM imidazole, and eluted with equilibrium buffer containing 250 mM imidazole.

2.6. Liposome preparation and reconstitution of HAS2-liposome complexes

Lipid solutions composed of various proportions of PC, PE, PS and cholesterol were prepared at 2 mg/ml in chloroform, followed by evaporation with dry nitrogen stream. The lipid film was thoroughly dried to remove residual chloroform by placing the test tube on a vacuum pump overnight, and then hydrated in 1 mM DTT at above the gel-liquid crystal transition temperature (T_m) of the lipid. The liposome suspension was sonicated and extruded through 0.3 µm filters

(IsoporeTM membrane filter; Millipore Corp., Billerica, MA) to produce a uniform sized liposome, which was then stored at -80 °C.

Purified HAS2 proteins were reconstituted into liposomes by a detergent dilution method [11]. Recombinant HAS2 proteins solubilized in a detergent-containing solution were mixed with a liposome solution (25 mM Hepes–NaOH, pH 7.2, 15 mM MgCl₂, 5 mM DTT, 0.4 mg/ml lipid), and then the HAS2-liposome mixtures were incubated for 2 h at 4 °C with gentle mixing on a rotating wheel. The mixture was diluted in the reconstitution buffer (25 mM Hepes buffer, pH 7.2, 15 mM MgCl₂, 5 mM DTT) and centrifuged at 100,000 g for 1 h at 4 °C. The pellet was resuspended by pipetting in the reconstitution buffer. Reconstitution of HAS2 recombinant proteins into liposome vesicles was assessed as follows. The HAS2-liposome mixtures were loaded on top of a 5-step sucrose gradient consisting of 50, 40, 30, 20 and 10% sucrose. Each step in the gradient was prepared with sucrose and 25 mM Hepes buffer (pH 7.2) containing 150 mM NaCl, 15 mM MgCl₂, 5 mM DTT, and protease inhibitors. Centrifugation was done in a Beckman TLA 100 rotor at 100,000 g for 3.5 h at 4 °C. Liposome vesicles were separated into different fractions from protein fractions according to their densities. Six fractions were collected, starting from the top of each centrifuge tube. Each fraction was analyzed by western blotting with an anti-His-tag antibody.

2.7. SDS-PAGE and western blotting

Proteins from each sucrose gradient fraction were separated by 10% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes. Blots were probed with a primary antibody against tetra-His-tag (QIAGEN, Valencia, CA), and detected with a secondary antibody

conjugated to horseradish peroxidase (Dako Japan Inc., Kyoto, Japan). Imaging was obtained on ECL film (GE Healthcare UK Ltd.) developed by ECL reagent.

2.8. HA synthase assay and kinetic study

HAS activity was monitored in a cell-free HA synthesis system using UDP-[¹⁴C] GlcUA (300 mCi/mmol; American Radiolabeled Chemical, Inc., St. Louis, MO) and UDP-[¹⁴C] GlcNAc (288 mCi/mmol; PerkinElmer Life and Analytical Sciences, Wellesley, MA) as donors, as described previously [9]. For kinetic studies, recombinant HAS2 proteins were reconstituted into liposomes composed of DPPC/cholesterol (9:1, mol/mol) mixtures or DPPC alone. Synthase activity was determined by calculating the amounts of incorporated UDP-[¹⁴C]GlcUA or UDP-[¹⁴C]GlcNAc using their known specific radioactivities. The second substrate concentration was held constant at 1.0 mM. K_m values for the UDP-GlcNAc and UDP-GlcUA substrates were obtained by measuring the synthase activity as a function of UDP-sugar concentration. Michaelis-Menten plots were generated by titration of UDP-sugar. Lineweaver-Burk plots of $1/v$ versus $1/[S]$ gave apparent K_m values of recombinant HAS2 proteins for UDP-GlcNAc and UDP-GlcUA. Protein content was determined using a Micro-BCA protein assay reagent kit (Pierce, Rockford, IL).

2.9. Statistical analysis

Statistical analysis was performed by using the two-tailed Student's t-test. The results are reported as mean $\pm$ SD.

3. Results

3.1. HA biosynthesis depends on cellular cholesterol content

In this study, we aimed to elucidate the requirement of cholesterol for HA biosynthesis. Cellular cholesterol content is controlled by the uptake from plasma lipoproteins as well as its synthesis in the endoplasmic reticulum. Thus, we first evaluated the HA production of human dermal fibroblasts cultured in a medium containing LPDS (Fig. 1). The HA production was significantly reduced at 7 days after cultivation in the absence of lipoprotein (Fig. 1A). Inversely, the suppressed HA production was somewhat restored later on by the addition of exogenous cholesterol (data not shown). This is consistent with a previous report showing that HA accumulation in aortic smooth muscle cell cultures was dependent on cellular or membrane cholesterol content [12]. Since HA biosynthesis is closely coupled with cell proliferation, we then examined the influence of lipoprotein-depletion on cell growth. During the experimental period, the proliferation rate of cells under lipoprotein depletion was compatible with normal culture conditions (Fig. 1B), suggesting that the absence of lipoprotein from the culture medium affected cellular cholesterol content and HA production of dermal fibroblasts without any growth suppression.

To acutely deplete free cholesterol from membranes with high specificity, cells were incubated with 10 mM Me β CD which is a cholesterol-sequestering drug. The Me β CD treatment significantly reduced both HA production and cellular cholesterol content of cells cultured in normal medium (Fig. 1C and E). Lastly, we measured HAS activity in membrane preparations of cells treated with or without Me β CD (Fig. 1D). In agreement with the suppression of HA synthesis,

there was an approximate 70% reduction in HAS activity observed in Me β CD-treated cells, but this suppressive effect was not obvious in the LPDS-substituted medium. Tamni and her co-worker have recently shown that cholesterol depletion down-regulated HAS2 mRNA expression by reduced phosphorylation of Akt [13]. To confirm whether the reduction in HAS2 mRNA levels is mainly responsible for the suppression of HA synthesis by the cholesterol depletion, we analyzed exogenous mouse and endogenous rat Has2 gene expression in Has2 transfectants of rat 3Y1 cells after treatment with various doses of Me β CD (Fig. S1). Unexpectedly, exogenous mouse Has2 expression was markedly increased by treatment with high dose of Me β CD, while HA production was greatly inhibited. These results prompted us to investigate whether the cholesterol-dependent regulation of HA synthesis is attributed to the direct modification of the enzymatic activity of HAS.

3.2. Influence of a cholesterol-rich lipid environment on HAS activity

To investigate the influence of a cholesterol-rich lipid environment on the activity of HAS enzyme, the active HAS enzyme system was reconstituted *in vitro* from recombinant enzymes. Recombinant human HAS2 protein with a C-terminal His-tag was expressed as a membrane protein by a baculovirus system. The recombinant protein was successfully solubilized from isolated membranes using a detergent and subsequently affinity-purified using nickel chelate chromatography². Detergent-solubilized and purified HAS2 proteins were reconstituted into liposomes composed of two different PCs, DMPC and DSPC, in mixtures with different molar ratios of cholesterol. In both cases, the reconstitution of liposomes caused a significant increase in

relative HAS activity as compared with the detergent-solubilized proteins (Fig. 2A and B). DMPC/cholesterol (9:1, mol/mol) significantly increased relative HAS activity as compared with the phospholipid alone (Fig. 2A). When the HAS2 proteins were reconstituted into liposomes composed of DSPC and cholesterol, the highest activities were recorded for DSPC/cholesterol (9:1, mol/mol), followed by DSPC/cholesterol (7:3, mol/mol) mixtures (Fig. 2B). In contrast, no significant increase in HAS activity was detected for a DSPC/cholesterol (1:1, mol/mol) mixture, suggesting biphasic effects of cholesterol on HAS activity.

3.3. Effects of phospholipid structure on HAS activity

To assess whether the enhancement of HAS activity by the cholesterol depletion would be affected by the relative ability of phospholipids to form ordered lipid domains, a panel of different PCs with differing abilities to form ordered domains by themselves and with cholesterol were examined. The cholesterol-dependency of HAS activity increased with PC type in the order of DMPC=DPPC>DSPC>DOPC, as demonstrated by the incorporation of sugar nucleotide substrates (Fig. 2C).

Next, the influence of the phospholipid head group structure on HAS activity was tested using acyl chain-matched species. Four species of PE or PS were used for the preparation of liposomes with or without 10% (mol/mol) cholesterol. When the HAS2 proteins were reconstituted into phospholipids alone, the highest activity was observed for DMPE with two saturated myristoyl acyl chains. The acyl chain-matched DMPS gave intermediate HAS activity, and the DMPC gave low activity (Fig. 2). Notably, the addition of cholesterol to DMPS or DMPE resulted in a significant

decrease in HAS activity in both cases. Similar negative effects of cholesterol were observed in the mixture of DOPS or DOPE, each with two unsaturated chains. These findings therefore suggest that the phospholipid head group and acyl chain have significant effects on the cholesterol-dependence of HAS activity.

3.4. Kinetic properties of recombinant HAS2 in cholesterol-containing liposomes

The HAS2 proteins were reconstituted into DPPC liposomes and the kinetic profiles of the enzyme were compared in the presence and absence of cholesterol. Kinetics showed that the $V_{\max}$ for UDP-GlcNAc in DPPC/cholesterol (9:1, mol/mol) mixtures was significantly higher than the level observed in DPPC alone (Fig. 3A and Table 1). In contrast, the recombinant HAS2 protein showed similar affinity and $V_{\max}$ value for UDP-GlcUA regardless of cholesterol addition (Table 1).

We then excluded the possibility that the enhanced activity obtained in cholesterol-containing PC liposomes might be attributed to the enhanced association of HAS2 protein with liposomes. The association yield of the HAS2 recombinant protein to DPPC/cholesterol or DPPC liposomes was determined by the western blot analysis after ultracentrifugation on a discontinuous sucrose gradient. Most HAS2 proteins were recovered in liposome-containing fractions independently of cholesterol content (Fig. 3B). Taken together, current observations suggest that the cholesterol-dependency of HA synthesis is partly attributed to the modification of enzymatic activity.

4. Discussion

In order to gain further insight into the role of the lipid environment controlling HA biosynthesis, the present study was undertaken to evaluate the influence of cholesterol on HA biosynthesis and HAS activity. Exposure to a cholesterol-depleting agent, which induces a perturbation of membrane microdomains, significantly decreased HAS activity as well as HA production. Our current data demonstrated that the suppressive effect of HA production by the cholesterol depletion is not due to the decreased Has2 gene transcription in the Has2 transfectants (Fig. S1). Thus, membrane cholesterol may have other effects on translation, posttranslational modification, and the enzyme activity of HAS2. In this study, we revealed the cholesterol-dependent activation of recombinant human HAS2 reconstituted in PC liposomes, implying that the cholesterol-dependent regulation of HA synthesis is partially attributed to the direct modification of the HAS enzymatic activity.

A single HAS molecule has the ability to catalyze the transfer of GlcNAc and GlcUA into newly synthesized HA [2]. Here, kinetic profiles of HAS2 enzymes that were reconstituted into DPPC liposomes showed an increased $V_{\max}$ value for UDP-GlcNAc in the presence of cholesterol, supporting cholesterol-dependent enhancement of HAS2 activity. In contrast, the HAS2 protein showed a similar $V_{\max}$ for UDP-GlcUA regardless of the addition of cholesterol. This may imply that the critical elements for GlcNAc transferase are structurally flexible and stabilized in a cholesterol-dependent manner. In contrast, the catalytic elements for GlcUA transferase may be stable in conformation under cholesterol-free conditions. Therefore, the optimal conformation for HAS protein activity may be one stabilized in a cholesterol-rich lipid environment. Recent studies

have indicated that HAS2 activity is regulated by dimerization [14]. Thus, it is also possible that cholesterol may affect the dimerization status resulting in alteration of HAS2 enzymatic activity. Together with these kinetic studies, understanding the three-dimensional structures and dimerization status of HAS proteins will give insight on the molecular dynamics in cholesterol-dependent changes in HAS activity.

Together with other lipids, cholesterol shifts the T_m upwards, thus decreasing membrane fluidity [15]. One might therefore suppose that the transition of HAS to its highly active form may be enhanced by a decrease in the fluidity of its membrane microenvironment. For instance, a decrease in membrane fluidity increases the V_{max} of membrane-associated Na^+ and K^+ -ATPase [16]. In the present study, the highest HAS activity was recorded in a saturated PC/cholesterol (9:1, mol/mol) mixture, while no significant effects were observed at an equimolar ratio, suggesting that the order of HAS activity was biphasic with the order of lipid fluidity. A lipid environment that gives proper fluidity may ensure maximal HAS enzyme activity. Another membrane parameter that may explain the cholesterol-dependent activation of HAS is the phase separation between lipids in different physical states [17]. Cholesterol differs in biological features from most phospholipids and has an important effect on phase behavior. These characteristics allow phospholipids to easily pack tightly together [18, 19]. Consequently, a phase in which acyl chains are highly ordered is separate from a disordered phase by the addition of cholesterol to a pure phospholipid bilayer. Although the HAS-localized membrane compartment has not yet been resolved, our preliminary finding that the HAS activity associates with the raft-rich and detergent-resistant membrane fraction supports a selective localization of the highly active form in the ordered phase of phospholipids. Further investigation is necessary in order to clarify the exact

subcellular compartment of HA biosynthesis.

Phospholipids in the plasma membranes are asymmetrically distributed across the lipid bilayer [20]. Saturated PCs are major phospholipids in the outer leaflet of plasma membranes, whereas PEs and PSs are enriched in the inner leaflet. Cholesterol is also predicted to associate more strongly with the outer leaflet than the inner leaflet of plasma membrane bilayers [21]. It is therefore important to know whether suitable lipid configurations in both the inner and outer leaflets are necessary for understanding the cholesterol-dependent enhancement of HAS activity.

In conclusion, HAS activity is associated with a cholesterol-dependent alteration of physical and chemical membrane properties. The lipid environment surrounding HAS proteins should support them as a scaffold and simultaneously enable the necessary conformational changes and dimerization to occur. Here, we propose that a lipid environment providing the proper fluidity and suitable composition optimizes the activity of HAS enzymes. Further studies are being planned to elucidate the relationship between the functionally relevant changes of HAS and its local membrane environment.

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Footnotes

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; DMPC, dimyristoyl PC; DPPC, dipalmitoyl PC; DSPC, distearoyl PC; DOPC, dioleoyl PC; DMPS, dimyristoyl PS; DPPS, dipalmitoyl PS; DSPS, distearoyl PS; DOPS, dioleoyl PS; DMPE, dimyristoyl PE; DPPE, dipalmitoyl PE; DSPE, distearoyl PE; DOPE, dioleoyl PE.

² A. Murakawa, *et al.*, manuscript in preparation.

References

- [1] T.C. Laurent, J.R.F. Fraser, Hyaluronan, FASEB. J. 6 (1992) 2397-2404.
- [2] P.H. Weigel, V.C. Hascall, M. Tammi, Hyaluronan synthases, J. Biol. Chem. 272 (1997) 13997-14000.
- [3] P.L. DeAngelis, J. Papaconstantinou, P.H. Weigel, Isolation of a *Streptococcus pyogenes* gene locus that directs hyaluronan biosynthesis in acapsular mutants and in heterologous bacteria, J. Biol. Chem. 268 (1993) 19181-19184.
- [4] N. Itano, K. Kimata, Mammalian hyaluronan synthases, IUBMB Life 54 (2002) 195-199.
- [5] V.L. Tlapak-Simmons, B.A. Baggenstoss, T. Clyne, P.H. Weigel, Purification and lipid dependence of the recombinant hyaluronan synthases from *Streptococcus pyogenes* and *Streptococcus equisimilis*, J. Biol. Chem. 274 (1999) 4239-4245.
- [6] P.H. Weigel, Z. Kyosseff, L.C. Torres, Phospholipid dependence and liposome reconstitution of purified hyaluronan synthase, J. Biol. Chem. 281 (2006) 36542-36551.
- [7] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221-17224.
- [8] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569-572.
- [9] N. Itano, T. Sawai, M. Yoshida, P. Lenas, Y. Yamada, M. Imagawa, T. Shinomura, M. Hamaguchi, Y. Yoshida, Y. Ohnuki, S. Miyauchi, A.P. Spicer, J.A. McDonald, K. Kimata, Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties, J. Biol. Chem. 274 (1999) 25085-25092.

- [10] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911-917.
- [11] W. Jiskoot, T. Teerlink, E.C. Beuvery, D.J. Crommelin, Preparation of liposomes via detergent removal from mixed micelles by dilution. The effect of bilayer composition and process parameters on liposome characteristics, *Pharm. Weekbl. Sci.* 8 (1986) 259-265.
- [12] S.W. Sakr, S. Potter-Perigo, M.G. Kinsella, P.Y. Johnson, K.R. Braun, Y. Goueffic, M.E. Rosenfeld, T.N. Wight, Hyaluronan accumulation is elevated in cultures of low density lipoprotein receptor-deficient cells and is altered by manipulation of cell cholesterol content, *J. Biol. Chem.* 283 (2008) 36195-36204.
- [13] A. Kultti, R. Karna, K. Rilla, P. Nurminen, E. Koli, K.M. Makkonen, J. Si, M.I. Tammi, R.H. Tammi, Methyl-beta-cyclodextrin suppresses hyaluronan synthesis by down-regulation of hyaluronan synthase 2 through inhibition of Akt, *J. Biol. Chem.* 285 (2010) 22901-22910.
- [14] E. Karousou, M. Kamiryo, S.S. Skandalis, A. Ruusala, T. Asteriou, A. Passi, H. Yamashita, U. Hellman, C.H. Heldin, P. Heldin, The activity of hyaluronan synthase 2 is regulated by dimerization and ubiquitination, *J. Biol. Chem.* 285 (2010) 23647-23654.
- [15] J.R. Silvius, D. del Giudice, M. Lafleur, Cholesterol at different bilayer concentrations can promote or antagonize lateral segregation of phospholipids of differing acyl chain length, *Biochemistry* 35 (1996) 15198-15208.
- [16] C. Bookstein, M.W. Musch, P.K. Dudeja, R.L. McSwine, Y. Xie, T.A. Brasitus, M.C. Rao, E.B. Chang, Inverse relationship between membrane lipid fluidity and

- activity of Na⁺/H⁺ exchangers, NHE1 and NHE3, in transfected fibroblasts, *J. Membr. Biol.* 160 (1997) 183-192.
- [17] S.L. Veatch, From small fluctuations to large-scale phase separation: lateral organization in model membranes containing cholesterol, *Semin. Cell Dev. Biol.* 18 (2007) 573-582.
- [18] F.T. Presti, R.J. Pace, S.I. Chan, Cholesterol-phospholipid interaction in membranes. 2. Stoichiometry and molecular packing of cholesterol-rich domains, *Biochemistry* 21 (1982) 3831-3835.
- [19] A.H. Etemadi, Membrane asymmetry. A survey and critical appraisal of the methodology. II. Methods for assessing the unequal distribution of lipids, *Biochim. Biophys. Acta* 604 (1980) 347-422.
- [20] V. Kiessling, J.M. Crane, L.K. Tamm, Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking, *Biophys. J.* 91 (2006) 3313-3326.
- [21] J.L. Delaunay, M. Breton, G. Trugnan, M. Maurice, Differential solubilization of inner plasma membrane leaflet components by Lubrol WX and Triton X-100, *Biochim. Biophys. Acta* 1778 (2008) 105-112.

Figure Legends

Fig. 1. Effects of cholesterol depletion on HA production and HAS activity. Human dermal fibroblasts were cultured in DMEM containing 10% FBS (*open bars*) or LPDS (*closed bars*) for the indicated days, and HA production (A) and cell growth curve (B) were determined. Fibroblasts were cultured for 2 days in DMEM containing 10% FBS (*open bars*) or LPDS (*closed bars*) and then treated with 10 mM Me β CD for 24 h. HA production (C), relative HAS activity (D), and cholesterol content (E) were then assayed. Data are presented as mean $\pm$ SD from three independent experiments (* p < 0.05, ** p < 0.01).

Fig. 2. Effects of cholesterol on HAS2 activity in various lipids. Purified recombinant HAS2 proteins were reconstituted in PC liposomes. The liposomes were composed of different molar ratios of DMPC/cholesterol (A) or DSPC/cholesterol (B). Relative HAS activities were determined without liposomes (*white bars*) and with liposomes in the absence (*gray bars*) or presence of cholesterol (*black bars*). Similarly, the recombinant HAS2 proteins were reconstituted into cholesterol-containing liposomes composed of the different molecular species of PC (C), PS (D), or PE (E). Relative HAS activities were determined without liposomes (*white bars*), and with liposomes in the absence (*gray bars*) or presence (*black bars*) of 10% cholesterol. The data is presented as a mean $\pm$ SD from the three independent experiments (* p < 0.05, ** p < 0.01).

Fig. 3. Kinetic profiles of HAS2 reconstituted into liposomes. (A) Purified recombinant HAS2 proteins were reconstituted into liposomes composed of DPPC (*open circles*) or

DPPC/cholesterol (9:1, mol/mol; *closed circles*) and assayed for its kinetic properties. HAS2 activity was determined at various concentrations of UDP-GlcNAc, while holding UDP-GlcUA concentration at 1.0 mM. Values represent the averages of three independent experiments. (B) Association of HAS2 proteins with liposome vesicles was assessed by discontinuous sucrose density gradient centrifugation. Purified HAS2 proteins were reconstituted into liposomes composed of DPPC or DPPC/cholesterol. Six fractions were collected starting from the top of each centrifuge tube. Protein samples in each fraction were analyzed for HAS2 by SDS-PAGE followed by western blotting.

Table 1
Effects of cholesterol on the kinetic properties of reconstituted HAS2.

Lipid composition	UDP-GlcNAc		UDP-GlcUA	
	K_m	V_{max}	K_m	V_{max}
	μM	$nmol/\mu g/h$	μM	$nmol/\mu g/h$
w/o lipid	59.0 ± 2.6	28.6 ± 10.3	58.6 ± 15.9	28.1 ± 9.4
DPPC	31.4 ± 12.3	173.8 ± 10.7	13.2 ± 3.0	102.7 ± 42.2
DPPC/cholesterol	37.1 ± 9.8	$256.1 \pm 37.5^*$	14.1 ± 2.3	118.9 ± 32.9

Values represent the averages of three independent experiments.

* $p < 0.05$ versus DPPC alone.

Figure 1

Figure 2

Figure 3