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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-ß-cyclodextrin also decreased the hyaluronan production of fibroblasts, as well as the HAS activity. To directly evaluate the effects of cholesterol on HAS activity, a recombinant human HAS2 protein with a histidine-tag was 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.

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.

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.

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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-ß-cyclodextrin (MeßCD) 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 NaH_2PO_4, pH 7.8, 300 mM NaCl, 10 mM $MgCl_2$, 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 (Isopore™ 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 $MgCl_2$, 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 $MgCl_2$, 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 $MgCl_2$, 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