Human histidine-rich glycoprotein expressed in SF9 insect cells inhibits apatite formation

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Abstract Histidine-rich glycoprotein (HRG) is structurally related to the α_2-HS glycoprotein/fetuin family of mammalian plasma proteins; both belong to the cystatin superfamily of proteins. We expressed recombinant human HRG and α_2-HS in Sf9 insect cells for functional analysis. Recombinant HRG bound heparin and fibrinogen while α_2-HS did not. Both proteins inhibited the formation of apatite, recombinant HRG (IC_{50} ~1 μM) with 2-fold lower molar activity than α_2-HS (IC_{50} ~0.5 μM). The inhibition in vitro of apatite formation suggests a new function for plasma HRG protein, inhibition of phase separation in blood vessels.

Key words: Serum protein; Calcium homeostasis; Histidine-rich glycoprotein; α_2-HS-glycoprotein

1. Introduction

Histidine-rich glycoprotein (HRG), also called histidine–proline-rich glycoprotein, is a mammalian plasma protein belonging to the cystatin superfamily [1]. It is known to interact with several ligands including metal ions [2], heme [3], heparin [4], plasminogen [5], fibrinogen [6] and thrombospondin [7]. The physiological role of histidine-rich glycoprotein is still unclear [8], although an involvement in T-cell regulation [9] and fibrinolysis [5] have been suggested. Primary structures were determined for the human [10] and the rabbit [11] HRG while partial amino acid sequences are known for the bovine protein [12]. The sequence data suggest a common multidomain structure for both proteins. The aminoterminal part of the molecule contains two tandemly arranged cystatin-like domains, while the carboxyterminal part contains a region unusually rich in histidine arranged between two proline-rich regions [11]. Amino- and carboxyterminal parts of the molecule are linked by a disulfide bond [12].

The overall structural features of HRG are similar to α_2-HS glycoprotein/fetuin, another member of the cystatin superfamily. Collectively called fetuins, these proteins consist of two aminoterminally located cystatin-like domains followed by a proline-rich carboxyterminal domain connected to the aminoterminal domain by a disulfide bond [13]. The most striking difference between HRG and fetuin is that HRG contains the histidine-rich region with 12 tandem repeats of the sequence Gly–His–His–Pro–His. This region is proposed to mediate binding of HRG to heparin and metal ions and it might target the protein to (sub)endothelial surfaces. Unlike HRG fetuin has never been shown to interact with heparin, components of the fibrinolytic system or extracellular matrix. Fetuin does, however, accumulate in bones and teeth by interacting with the anorganic phase [14]. Based on this observation we have recently shown that fetuins inhibit apatite formation in vitro and in mineralising calvaria cells suggesting a possible role of these proteins in calcium homeostasis and mineralisation [15]. We generated by homologous recombination mice lacking the fetuin gene, *Ahsg* [Jahnen-Dechent et al., in preparation]. The mice were phenotypically normal showing no defect causally related to phase separation or spontaneous calcification in blood. We reasoned that proteins structurally similar to fetuins might exert similar biological roles to act as backup systems. Intrigued by the similar structures of HRG and fetuin we examined the influence of HRG on apatite formation. We show that recombinant human HRG expressed in Sf9 insect cells is indeed able to inhibit apatite formation in vitro in a similar fashion as human α_2-HS glycoprotein/fetuin. Therefore, HRG might contribute to the inhibition of phase separation and unwanted calcification in blood vessels, by virtue of its affinity to heparin-like molecules and fibrinogen possibly localized at sites of vascular repair.

2. Materials and methods

2.1. Protein expression in Sf9 insect cells

The cDNA encoding human histidine-rich glycoprotein has been described [10]. It was subcloned into the baculovirus transfer vector pVL1393 as an *Eco*RI fragment. Sf9 cells were contransfected with this plasmid and Baculogold™-DNA (Pharmingen). Recombinant virus expressing HRG was cloned by limited dilution. Baculovirus-mediated expression of human α_2-HS–glycoprotein/fetuin in insect cells has been described [15]. Proteins were expressed in serum-free medium SF900II (Life Technologies, Inc.). HRG as well as fetuin were purified by immunoaffinity chromatography from the medium 6 days after infection. Bound proteins were eluted with 0.2 M glycine, pH 2.3, and subsequently desalted utilizing Centricon spin dialysis units (Amicon).

2.2. Electrophoresis and blotting procedures

SDS-PAGE and chemiluminescence immunoblots were done as described [16]. Polyclonal antiserum raised against human HRG has been described [10]. Lectin blots with *Sambucus nigra* agglutinin and *Galanthus nivalis* agglutinin were performed using the DIG-glycan differentiation kit (Boehringer Mannheim) according to the manufacturer's protocol.

2.3. Binding experiments

Heparin–Sepharose and fibrinogen were obtained from Sigma. Binding to heparin was analyzed by passing supernatant of infected Sf9 cells over a heparin–Sepharose containing column. The supernatants were diluted 5-fold in 50 mM HEPES, pH 6.8 or pH 7.4. Bound

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Abbreviations: HRG, histidine-rich glycoprotein; α_2-HS, α_2-HS–glycoprotein; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; DIG, digoxigenin; Tris, *tris*-(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)

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proteins were eluted by 2 M NaCl in buffer. The fractions were analyzed by SDS-PAGE and subsequent immunoblotting. Binding to fibrinogen was analyzed by a modified ELISA technique. Fibrinogen was coated overnight onto the surface of microtiter plates at a concentration of 10 μg/ml. Free binding sites were blocked with incubation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100, 0.25% gelatin). The plates were then incubated with supernatant of infected cells in a 2^n dilution series starting with a 20-fold dilution. Incubations were performed in the presence of 0.5 mM $ZnCl_2$ or 1 mM EDTA. Fibrinogen-bound proteins were detected by incubation with antisera against HRG and fetuin, respectively, at a 5000-fold dilution in buffer. Bound antibodies were visualized by peroxidase-conjugated anti-rabbit IgG antibodies utilizing the substrate 2,2′-azino-di[3-ethylbenzthiazoline sulfonate].

2.4. Inhibition of apatite formation

A buffered salt solution (50 mM Tris-HCl, pH 7.4, 4.8 mM $CaCl_2$, 2×10^6 cpm $^{45}CaCl_2$, 1.6 mM Na_2HPO_4) with increasing amounts of purified HRG, fetuin or bovine serum albumin was incubated at 37°C for 90 min. Precipitates were collected by centrifugation, dissolved in 0.5% acetic acid and analyzed by liquid scintillation counting. All incubations were done in triplicate and repeated at least two times.

2.5. Extraction of proteins from bone

Human epiphysial bone remnants trimmed during routine surgery (Klinik für Unfallchirurgie, Universitätsklinik Mainz) were ground to a powder in liquid nitrogen. Bone proteins were extracted according to the procedure of Termine et al. [17]. The second extract done with guanidine and EDTA was dialyzed against water and analyzed by SDS-PAGE and subsequent immunoblotting.

3. Results

Human histidine-rich glycoprotein and human α_2-HS–glycoprotein were expressed in Sf9 insect cells by infection with recombinant baculovirus. Fig. 1 (left panel) shows a stained polyacrylamide gel analysis of HRG isolated from plasma (lane 1) and from the supernatant of virus infected Sf9 cells (lane 2). The apparent molecular mass of recombinant HRG was smaller in comparison to the HRG purified from plasma. Under reducing conditions, recombinant HRG migrated at 66 kDa whereas the molecular mass of the reduced plasma form was 78 kDa as published [18]. We have previously determined that a similarly reduced apparent molecular mass of recombinant α_2-HS (45 kDa) compared to plasma α_2-HS (60 kDa) was due to incomplete N-glycosylation in the Sf9 expression system [15]. Therefore we analyzed the carbohydrate moieties of purified HRG by lectin blotting using *Sambucus nigra* agglutinin (SNA) and *Galanthus nivalis* agglutinin (GNA). Fig. 1 shows that plasma HRG, like the positive control proteins, α_2-HS and bovine fetuin (not shown), were all detected by SNA indicating complex N-glycosylation with terminal sialic acid residues. In contrast, the recombinant form of HRG was not detected by SNA, but reacted positive with GNA, which is specific for terminal mannose residues. Thus, the recombinant proteins expressed in Sf9 insect cells are partially glycosylated lacking terminal sialic acid, which results in the reduced apparent molecular masses observed upon reducing SDS-PAGE.

Next we assayed the binding of recombinant HRG and α_2-HS to heparin using affinity chromatography and immunoblotting. As shown in Fig. 2A, recombinant HRG bound strongly to heparin–Sepharose at pH 6.8 (Fig. 2A, lane 3) and, to a lesser extent at pH 7.4 (Fig. 2A, lane 6) as described earlier for plasma HRG [19]. In the same assay α_2-HS failed to bind heparin–Sepharose at pH 6.8 or at pH 7.4, but was quantitatively recovered in the unbound column effluent in each case (Fig. 2A, lanes 2 and 5). Binding of proteins to fibrinogen was analyzed by a modified ELISA technique. Fig. 2B illustrates that recombinant HRG bound fibrinogen in a concentration-dependent and saturable manner (Fig. 2B, □,■). Furthermore, the binding was enhanced in the presence of divalent Zn^{2+} ions (Fig. 2B, ■ vs. □). In contrast, α_2-HS did not bind fibrinogen in the absence of Zn^{2+} and only weakly in the presence of Zn^{2+} ions (Fig. 2B ● vs. ○). In summary, our results indicate that recombinant HRG has the same functional properties as the native protein with respect to heparin and fibrinogen binding.

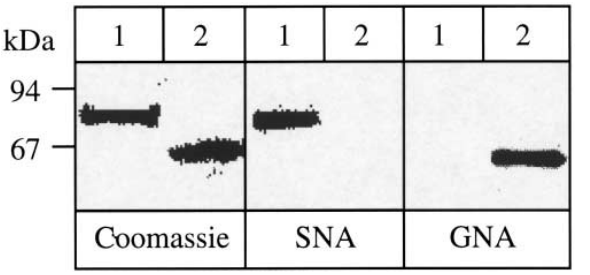

Fig. 1. Carbohydrate analysis of plasma and recombinant HRG. 1 μg of purified plasma HRG (lane 1) and recombinant HRG (lane 2) were subjected to SDS-PAGE and stained with Coomassie Blue (left panel). Two identical replicas were blotted onto nitrocellulose and incubated with the DIG-conjugated lectins *Sambucus nigra* agglutinin, SNA (center panel) and *Galanthus nivalis* agglutinin, GNA (right panel) utilizing the DIG glycan differentiation kit (Boehringer Mannheim).

Extending previous investigations [15] we asked, if purified recombinant HRG could inhibit the formation of apatite. Fig. 3 illustrates a typical precipitation assay where HRG dose-dependently inhibited the formation of apatite with an IC_{50} of ~1 μM. As a positive control, α_2-HS likewise inhibited with an IC_{50} value of ~0.5 μM. In this assay BSA, a negative control protein at concentrations up to 10 μM did not inhibit apatite precipitation. Pretreatment of the test proteins with proteinase K completely abolished the inhibitory activity indicating that indeed the protein moieties accounted for the inhibition of apatite formation (data not shown).

We asked, if the ability to bind nascent apatite demonstrated in Fig. 3 would be sufficient to target HRG to bone tissue. α_2-HS glycoprotein/fetuin is known to accumulate in bone mineralized matrix while no predominant localisation of HRG outside the plasma pool has been reported to date. To this end we analyzed human cortical bone extracts by immunoblotting. Fig. 4 shows that the antiserum raised against human plasma HRG readily detected HRG in serum, but not in bone extract while α_2-HS was readily detected in both samples. Therefore, HRG does not seem to concentrate in bones, although it can interact with apatite.

4. Discussion

Like most body fluids blood contains calcium and phosphate ions at relatively high concentrations raising the possibility that a spontaneous formation of apatite could occur very easily under (patho)physiological conditions. Proteins have been identified to inhibit the formation of calcium salts in body fluids, saliva [20], pancreatic juice [21] and urine [22]. Plasma proteins have also been described to inhibit apatite formation in vitro [23] and albumin has been shown to con-

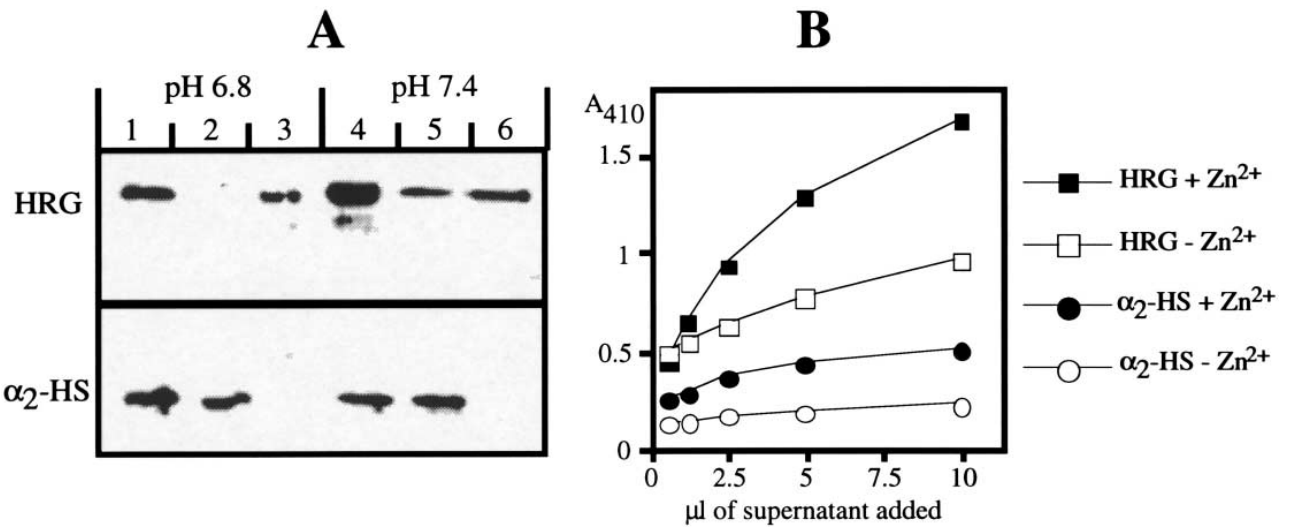

Fig. 2. Binding of recombinant HRG and α_2-HS to heparin and fibrinogen. A: Supernatant of Sf9 cells infected with virus expressing HRG (upper panel) or α_2-HS (lower panel) was diluted 5-fold in 50 mM HEPES, adjusted with NaOH to pH 6.8 (lanes 1–3) or 7.4 (lanes 4–6) and applied onto a column containing heparin–Sepharose. Bound proteins were eluted with 2 M NaCl in buffer. 10 μl of the loaded solution (lanes 1 and 4), the flow-through (lanes 2 and 5) and the eluate (lanes 3 and 6) were subjected to SDS-PAGE and subsequently blotted onto nitrocellulose. HRG and α_2-HS were detected by antisera as described in Fig. 1. B: Fibrinogen was coated onto microtiter plates at a concentration of 10 μg/ml. Supernatant of Sf9 cells infected with virus expressing HRG (□,■) or α_2-HS (○,●) was applied at a 2^n serial dilution in incubation buffer in the presence (●,■) or absence (○,□) of 0.5 mM $ZnCl_2$. Bound proteins were detected with antisera against HRG or α_2-HS (both at a 5000-fold dilution) and subsequent incubation with peroxidase-labelled anti-rabbit IgG antibodies. Absorbance at 410 nm was measured after incubation with the substrate 2,2′-azino-di[3-ethylbenzthiazoline sulfonate].

tribute to this effect because of its high concentrations in blood [24]. The need for and the existence of inhibitory mechanisms of ectopic calcification has been unequivocally demonstrated by recent genetic experimentation. The targeted disruption of the gene for matrix GLA (gamma-glutamic acid) protein in mice resulted in spontaneous, lethal calcification of arteries and cartilage [25]. We have recently demonstrated that fetuin is an effective inhibitor of apatite precipitation, as the inhibitory activity of total serum protein drops about 30% after depletion of fetuin by affinity absorption [15] or in transgenic mice lacking the fetuin gene [Jahnen-Dechent et al., in preparation]. Obviously, the remainder of plasma proteins can compensate the loss of fetuin. Therefore, we started to identify further proteins contributing to the overall inhibitory activity of serum. Here we show that HRG, like α_2-HS a protein of the cystatin superfamily, can also inhibit apatite formation in vitro. The data presented in this paper raise the possibility that proteins of the cystatin superfamily in general serve a function as inhibitors of phase separation. Notably, a third member of this family, salivary cystatin, is an inhibitor of salt precipitation in saliva [26] and a fourth cystatin-like protein with unknown function, SPP24 [27], is expressed in liver and in bone.

Both HRG and α_2-HS/fetuin presumably contribute to a larger pool of plasma inhibitors yet they might exert their functions in separate locations due to their respective binding characteristics. Like plasma HRG, recombinant HRG avidly

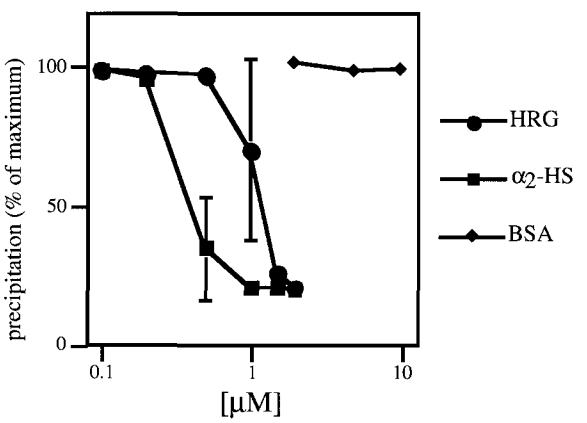

Fig. 3. Inhibition of apatite formation by recombinant HRG and α_2-HS. A buffered salt solution (50 mM Tris-HCl, pH 7.4, 4.8 mM $CaCl_2$, 2×10^6 cpm $^{45}CaCl_2$, 1.6 mM Na_2HPO_4) also containing at the indicated concentration purified HRG (●), α_2-HS (■) or bovine serum albumin (◆) was incubated at 37°C for 90 min. Precipitates were collected by centrifugation, dissolved in 0.5% acetic acid and analyzed by liquid scintillation counting. Incubations were done in triplicate. Bars indicate the SEM within triplicates. Note that triplicate readout was essentially identical at low and high protein inhibitor concentrations.

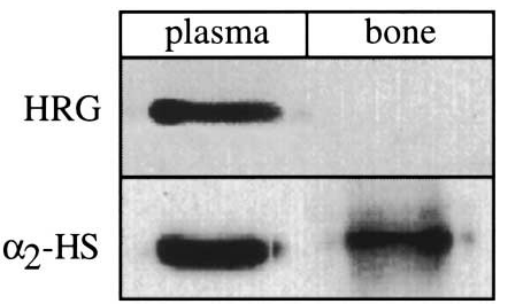

Fig. 4. Detection of HRG and α_2-HS in plasma and bone. Proteins were extracted from adult human bone. 0.02 μl human plasma (lane 1) and 1 μg of the extracted bone proteins (lane 2) were subjected to SDS-PAGE and subsequently blotted onto nitrocellulose. HRG and α_2-HS were detected by antibodies and enhanced chemoluminescence.

bound heparin and fibrinogen. HRG therefore likely accumulates at sites of tissue damage with concomitant exposure of subendothelial surfaces. At these sites HRG could prevent ectopic calcification by inhibiting apatite precipitation. In contrast, α_2-HS/fetuin does not bind to heparin and fibrinogen and therefore remains in circulation until it is eventually sequestered to the mineral phase upon passage through bone tissue.

In summary this paper describes a hitherto unknown in vitro activity of HRG, namely inhibition of spontaneous apatite formation. The (patho)physiological relevance of this finding needs further examination.

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