FORMATION OF COLLOIDAL CALCIPROTEIN PARTICLES*

Structural Basis of Calcification Inhibition by 2-HS Glycoprotein/Fetuin-A

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The combination of mineral with an organic matrix called “biomineral” is commonplace in biology. Biominerals studied in detail include magnetic crystals in bacteria (1), silica skeletons in diatomaceous algae (2, 3), shells of marine molluscs (4, 5), and skeletons of vertebrate animals (6). Generally, biominerals form in close proximity with biomacromolecules. Ultrastructural analyses suggest that a protein scaffold provides the ordered and spatially restrained framework for crystal deposition. In mammals, collagen is an excellent scaffold for calcification. Noncollagenous proteins control nucleation, growth, shape, and orientation of crystals in the mineral phase (7, 8).

Major mineral ions are equally distributed in the extracellular space of most living organisms. Extracellular fluids are especially supersaturated with regard to calcium and phosphate ions. Therefore, it is surprising that mineralization is restricted to collagenous matrix of the vertebrate skeleton and that once started mineralization does not proceed throughout the organism (9). This suggests that the inhibition of unwanted mineralization is at least as important as the initiation of mineralization. Genetic experimentation with mutant mice indeed suggests that mineralization is the default pathway, which must be actively prevented, not started (10). Unwanted mineralization resulted from the genetic ablation of mineralization inhibitors, pyrophosphate (11, 12) and matrix γ-carboxyglutamic acid (GLA)1-containing protein (MGP) (13). We showed that the lack of α2-HS glycoprotein/fetuin-A (Ahsg) results in severe systemic calcification in mice and humans (15).2 Of note, Ahsg is the only protein inhibitor of calcification known so far that is systemic and present throughout the extracellular space in mammals. Due to its high affinity for the mineral phase of bone, Ahsg accumulates about 100-fold over other serum proteins in bones and teeth (16). This seems paradoxical, considering that Ahsg is an efficient inhibitor of calcification both in vitro and in vivo. Here we studied how Ahsg inhibits the formation of basic calcium phosphate (BCP).

Using electron microscopy and dynamic light scattering, we determined that the inhibition is effected by a transient formation of colloidal diatoms. We suggest that a protein scaffold provides the ordered and spatially restrained framework for crystal deposition. In mammals, collagen is an excellent scaffold for calcification. Noncollagenous proteins control nucleation, growth, shape, and orientation of crystals in the mineral phase (7, 8).

1 The abbreviations used are: GLA, γ-carboxyl glutamic acid; MGP, matrix GLA-containing protein; Ahsg, α2-HS glycoprotein/fetuin-A; BCP, basic calcium phosphate; mAhsg and mAhsg, bovine and mouse Ahsg, respectively; BSA, bovine serum albumin; TEM, transmission electron microscopy; FETUB, fetuin-B; mFETUB and mFETUB, human and mouse FETUB, respectively; HRG, histidine-rich glycoprotein; hHRG, human HRG; KNG, kininogen; KNG, human KNG; MBP, maltose-binding protein; GST, glutathione S-transferase; HS, α-Heremans-Schmid.

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crystal nuclei and thus delays their precipitation. This proposed mechanism of the transient inhibition of BCP precipitation by Ahsg is fundamentally different from previous concepts, namely sequestration of calcium ions by negatively charged proteins like serum albumin or calcium binding through an EF-hand motif.

**EXPERIMENTAL PROCEDURES**

**Precipitation Inhibition Assay**—The precipitation inhibition assay was performed as described (17). Briefly, a buffered salt solution (50 mM Tris/HCl, pH 7.4, 4.8 mM CaCl₂, 2 × 10⁵ cm²/ppm of (CaCl₂·1.6 mM Na₂HPO₄), containing test proteins as indicated in the figure legends was used for 1 min at 37 °C for 90 min. Precipitates were collected by centrifugation (15,000 × g, 5 min at room temperature), dissolved in 1% acetic acid, and quantified by liquid scintillation counting. All incubations were done in triplicates. Bovine serum albumin (BSA; Roth, Karlsruhe, Germany) and bovine fetuin/B/hAhs (Sigma) were used as negative and positive control proteins, respectively.

**Electron Microscopy and Electron Spectroscopy**—For scanning electron microscopy, a supersaturated solution of calcium (2.5 mM CaCl₂) and phosphate (1.8 mM KH₂PO₄) was prepared (18) with and without 200 mM B/hAhs added. After a 90-min incubation at 22 °C, precipitate was spun down, air-dried, and viewed in a Leo series 1400 scanning electron microscope (Leo Electron Microscopy Ltd., Cambridge, UK). For transmission electron microscopy, all proteins were purified by gel filtration (Superdex 200; Amersham Biosciences, Freiburg, Germany) in 50 mM Tris/HCl, pH 7.4. Monomer-containing fractions were collected, and the protein concentration was determined using a dye assay (Roti-Nanounot, Roth, Karlsruhe, Germany). All solutions were microfiltered (0.22 μm) before mixing. Following the precipitation reaction in buffer (5 mM CaCl₂, 3 mM Na₂HPO₄, 50 mM Tris/HCl) at pH 7.4 and 22 and 37 °C, respectively, samples were dialyzed against water (MilliQ; Millipore Corp.) using micro dialysis cartridges. This step was essential for electron microscopy of the precipitation mixture, which would otherwise have been obscured by dried salt. The dialyzed samples were cleared by centrifugation at 10,000 × g. The supernatant and, if formed, the precipitate were transferred to carbon-coated grids. Excess liquid was blotted from the side. The samples were viewed directly in TEM without staining. Supporting films of 7-nm thickness were coated onto freshly cleaved mica using a Balzers BAE 250 vacuum evaporator (Bingen, Germany), floated onto water, and transferred to 300-mesh copper grids. For elemental mapping of carbon, supporting films were made of boron. Energy-filtering transmission electron microscopy was performed on a Leo 912 Omega instrument (tungsten-flame) operated at 100 kV. The bacterial pellet was resuspended in buffer containing 20 mM Tris, pH 7.4, 1 mM EDTA, and 200 mM NaCl (MBP fusion protein, amylose column buffer) or in PBS (GST fusion protein, glutathione-Sepharose column buffer). In the case of small MBP-fused fragments of Ahsg D1, the salt concentration was adjusted to 300 mM to improve amylose binding. The suspension was frozen overnight at −20 °C. The thawed ice-cold suspension was pulse-sonicated three times for 20 s. Then 1% Triton-X100, a protease inhibitor cocktail (Complete, Roche), and protease inhibitors were added, and the suspension was mixed for 10 min at 4 °C. Nuclease digestion of crude protein preparations was critically important for the reproducibility of precipitation assays, because DNA and RNA are potent inhibitors of BCP precipitation (26). Protein preparations, which proved active in the BCP precipitation inhibition assay, were routinely treated with protease K to ensure that the inhibitory activity indeed resided with the protein fraction of each preparation and not with residual contaminating nucleic acids or low molecular weight inhibitors.

After centrifugation for 15 min at 4 °C and 30,000 × g, the supernatant was loaded onto an amylose column. After washing the column with amylose column buffer containing 10 mM maltose, and the GST-fused protein was eluted with buffer containing 20 mM Tris, pH 8, and 10 mM reduced glutathione. Recombinant protein isolated from the bacteria without a denaturating detergent was generally inactive in the BCP precipitation inhibition assay. Therefore, every recombinant protein had to be denatured and refolded (17). The stability and activity of the fusion proteins depended on the refolding procedure. Following is an optimized refolding procedure yielding active protein for both GST and MBP fusion proteins.

All recombinant proteins were solubilized for 2 h at room temperature in a buffer containing 50 mM Tris/HCl adjusted to pH 8, 6 mM urea, and 50 mM dithiothreitol. The denatured protein solution was concentrated to 5 mg/ml using the same buffer with dithiothreitol content reduced to 5 mM. Refolding was initiated by slow dilution of protein...
### Table I

**Oligonucleotide primers used for cloning of cystatin domain-containing fusion proteins of GST or MBP and mouse Ahsg, mouse, rat, and human FETUB, and human KNG**

| Construct | 5’-primer | 3’-primer |
|-----------|-----------|-----------|
| Myc tag blunt/EcoRI | GGGGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | |
| Myc tag EcoRI/Blunt | AATTCATGTTACGTCCTCTTTTCAGGAGATCAGCTCTTGTCCCC | |
| pRSET mAhsg-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| pMAL mAhsg-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| pGEX mAhsg-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| pGEX-mAhsg/4S->E-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| pRSET mAhsg 15–70-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| pGEX-mAhsg 15–70-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| pRSET mAhsg 1–81-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| pGEX mAhsg 1–81-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| pRSET mAhsg 42–81-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| pGEX mAhsg 42–81-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| pRSET mAhsg 42–70-Myc | GGGTGTGACAGAAGCTGATCTCGGAGGAGGACCTGAACATTAG | GATTTTGAAGTGTCTCTTGTCC |
| MBP-mAhsg 42–70-Myc | GGAGATATAGGATGGATCCGCTCCACAAGGTACAGG GCCAACTCGCTCTCTTTTCAGGAGATCAGCTCTTGTCC |
| GST-mFETUB | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| MBP-mFETUB | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| GST-rFETUB | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| GST-hFETUB | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| MBP-hKNG-domain D1 | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| MBP-hKNG-domain D2 | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |
| MBP-hKNG-domain D3 | CCCTTCACACTTCCTCGATCCGATG | GGAATCTACAGATGGGACAGCAG |

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Inhibition of Basic Calcium Phosphate Precipitation by Ahsg—Our previous work has shown that Ahsg is highly effective in vitro to inhibit de novo formation of BCP (17). This inhibition was transient and lasted about 5–6 h at the conditions of our assay. The precipitation delay seems, however, sufficient to prevent generalized calcification in vivo. Our recent finding of severe systemic calcification of most soft tissues in Ahsg-deficient mice validates the biological relevance of this concept. Here we asked how Ahsg interacts with the calcium and phosphate ions or the mineral nuclei to achieve inhibition.

Using scanning electron microscopy, we studied the influence of Ahsg on the morphology of the mineral precipitate. First, we analyzed the precipitate formed from a metastable calcium phosphate solution with or without added native Ahsg (Fig. 1). The exact nature of this precipitate is uncertain because of phase transitions. Therefore, we collectively address the precipitate formed as BCP, regardless of its exact chemical and crystallographic composition of variable proportions of amorphous calcium phosphate, octacalcium phosphate, and apatite. In the absence of Ahsg, copious amounts of BCP formed and appeared as a compact pellet (Fig. 1A). The addition of 200 nM Ahsg to the precipitation mixture barely reduced the amount of precipitate formed. However, the morphology of the BCP precipitate formed at this low concentration of Ahsg was changed into a loose, fluffy precipitate comprising small, 2–15-μm-sized aggregates (Fig. 1B).

A precipitation mix containing 10 μM Ahsg, the nominal serum concentration, was stable for many hours at 37 °C without any precipitate formation. Control incubations containing 10 μM BSA or no protein formed a clearly visible precipitate within 2 h under otherwise identical conditions. The precipitates of both controls were indistinguishable and were microcrystalline as judged by TEM (not shown). Next, the dialyzed supernatants were subjected to TEM analysis. Supernatants of precipitation mixtures, which had been incubated for 2 h at 22 or at 37 °C, respectively in the presence of 10 μM Ahsg contained spherical aggregates with a diameter of 30–150 nm (Fig. 2, A and F). The size and shape of the aggregates were independent of the order of CaCl2 and Na2HPO4 addition. The precipitates were amorphous as judged by the lack of discrete diffraction patterns in TEM (Fig. 2F, inset). We termed these soluble, colloidal spheres “calciprotein particles” in analogy to the well established lipoprotein particles. The supernatant of both controls (with BSA and protein-free, respectively) did not contain any similar particles (not shown).

After 4 h of incubation at 37 °C, small crystalline needles started to grow on the surface of the calciprotein particles (Fig. 2G), but not at 22 °C (Fig. 2B). The fact that a diffraction pattern was obtained from samples incubated for 6 h at 37 °C indicated that crystallization had started (Fig. 2H, inset). A reduced temperature of 22 °C resulted in a delayed transformation of morphology (Fig. 2, A–E). After 23 h at 22 °C, crystallization of needles on the surface of spheres started to appear (Fig. 2C), similar in appearance to the samples harvested at 4 h and 37 °C (Fig. 2G). After 30 h at 37 °C, a solid BCP precipitate had formed. Small crystalline needles were present in the supernatant (Fig. 2F), whereas the precipitate consisted of large clusters of radially oriented needles with a diameter of about 450 nm (Fig. 2F). In summary, the transient inhibition of BCP precipitation by Ahsg relies on the formation of soluble colloidal spheres, “calciprotein particles,” which progressively turn into an insoluble calciprotein precipitate.

Next, we obtained information on the composition of the calciprotein particles by elemental mapping (Fig. 3). This procedure visualizes the electron energy loss at absorption edges characteristic of each element. Imaging of electrons with an energy loss corresponding to, e.g. calcium absorption edge will image the calcium-enriched regions of the specimen (19). Fig. 3 represents the elemental mapping of densely packed calciprotein particles harvested 2 h after the start of a precipitation reaction performed with 10 μM Ahsg at 37 °C. We show the elastically filtered image (mass density, Fig. 3A) and the phosphorus (Fig. 3B), the carbon (Fig. 3C), and the calcium (Fig. 3D) net distribution image of the same region. The elemental mapping indicates that all three elements were evenly distributed within the calciprotein particles.

We analyzed by dynamic light scattering (DLS) the speed of calciprotein particle formation in solution. First, we studied a solution of Ahsg without calcium and phosphate. We detected a major fraction (99.5%) with a hydrodynamic radius (rh) of 4.2 nm corresponding to the Ahsg monomer and a small fraction (0.5%) with rh = 55.4 nm, which we tentatively assigned to Ahsg aggregates (not shown). The addition of calcium resulted in an increase of the gyration radius by about 10%. Compared with the calcium phosphate- and Ahsg-containing samples (below), the scattering intensities were very low (Fig. 4). We observed a sharp rise in light scatter immediately after the addition of calcium and phosphate (Fig. 4). A slow, strongly scattering colloidal matching the size of the calciprotein particles (rh = 40–50 nm) was detected in the solution, which we assigned to the emerging calciprotein particles. Within the first 1 h of incubation, a steep increase in the partial scattering intensity was measured (Fig. 4). During the following 19 h, the increase in intensity was moderate, yet continued. The hydrodynamic size still continued to grow after 1 day, but the intensity decreased (not shown). This is best explained by the complete sedimentation of the newly formed insoluble BCP precipitate.

Inhibition of BCP Precipitation by Proteins of the Cystatin Superfamily—To further analyze the structural requirements for efficient inhibition of BCP precipitation by Ahsg, we conducted a structure-function analysis of Ahsg-related proteins. We produced a series of mutated Ahsg fusion proteins as well as recombinant proteins related to Ahsg and measured their ability to inhibit BCP precipitation. To this end, we employed an established assay measuring co-precipitation of 45Ca in a...
buffered solution containing calcium, phosphate, and test protein (17). In this initial work, we had mapped the basal structural motif mediating the inhibition of spontaneous BCP precipitation to the amino-terminal cystatin-like Ahsg domain D1. Hence, we tested several related proteins of the cystatin superfamily containing structurally related cystatin-like domains. KNG contains three cystatin-like domains, HRG contains two, and Ahsg as well as its relative, the recently discovered FETUB (27), both contain two cystatin-like domains. We previously determined that HRG could inhibit precipitation, albeit with a 2-fold lower molar efficiency than Ahsg (28). We scanned for additional structural features that might contribute to the inhibition of calcification by Ahsg. It is known that post-translational modifications of mineral binding proteins, notably phosphorylation, influence their binding properties (7, 29). We and others reported that human Ahsg and rat Ahsg are transiently serine-phosphorylated (24, 25, 30). To assess the contribution of the resulting additional negative charge of mAhsg in the precipitation inhibition assay, we constructed the phosphorylation-mimicking mAhsg mutant mAhsg/4S/H11022 by a site-directed mutagenesis of putative Ser (positions 120, 291, 294, and 296) phosphorylation sites with Glu. We expressed mAhsg, mAhsg/4S/H11022, human, mouse, and rat FETUB, and the three cystatin-like domains of hKNG in Escherichia coli as untagged or as FLAG-tagged proteins (data not shown) and as Myc-tagged fusion proteins with GST or with MBP. Generally, untagged or FLAG-tagged cystatin-like domains in our hands did not yield sufficient starting material for subsequent purification and functional testing. GST-fused proteins were more highly expressed (15 mg of GST fusion protein/liter of LB medium) but tended to form insoluble inclusion bodies. Therefore, all recombinant proteins had to undergo an unfolding/refolding cycle in redox buffer before functional testing in the precipitation assay.

The top section of Fig. 5 shows the inhibition of BCP precipitation by negative control proteins, BSA and GST, and the positive control protein, bAhsg, at a concentration of 3 μM. Native serum bAhsg was most active in this assay, whereas BSA, GST, and MBP (17) did not significantly inhibit BCP precipitation under identical conditions. Recombinant GST/MBP full-length mAhsg fused to GST or MBP proved active inhibitors of BCP precipitation like native bAhsg (Fig. 5). A comparison of the GST/MBP fusion proteins of mAhsg with the GST/MBP fusion proteins of mFETUB re-
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Fig. 5. Inhibition of BCP formation by natural and mutated forms of cystatin-like domains of cystatin protein family members. Recombinant forms of mAhsg, mFETUB, rFETUB, and hFETUB; and hKNG domains D1, D2, and D3 were expressed in E. coli as fusion proteins with MBP or GST as detailed under “Experimental Procedures.” All proteins were tested at 3 μM final concentration. Results represent triplicate measurements ± S.E. GST-mAhsg/4S-E-myc denotes a mutant form of mouse Ahsg with serine residues 120, 291, 294, and 296 mutated to glutamic acid to mimic serine phosphorylation at these positions.

Next, we asked the question which minimal sequence within the cystatin-like Ahsg domain D1 confers the inhibition of BCP precipitation. To this end, we generated deletion mutants of the Ahsg domain D1 fused to GST. The resulting fusion proteins GST-mAhsg 1–81-Myc and GST-mAhsg 15–70-Myc were fully active in the BCP precipitation assay when compared with the full-length fusion proteins GST-mAhsg-Myc and MBP-mAhsg-Myc, respectively (Fig. 5). Shortening from the amino terminus (GST-mAhsg 42–81-Myc) and additionally from the C terminus (GST-mAhsg 42–70-Myc) caused a progressive loss of inhibitory activity (Fig. 5), which is completely lost in the mutant MBP-mAhsg 1–52 (17).

Structural Requirement for the Inhibition of BCP Precipitation by Cystatin-like Domains—To interpret the results obtained by the precipitation assay in a three-dimensional protein structure context, we aligned the protein sequences of 16 cystatin-like domains, namely of chicken egg white cystatin, human, mouse, and bovine Ahsg, human and mouse fetuin-B, human histidine-rich glycoprotein, and human kininogen using ClustalW software (Fig. 6). The alignments were manually adjusted to match the secondary structure elements identified in the crystal structure of chicken egg white cystatin (21) (Fig. 6A).

Based on the multiple sequence alignment (Fig. 6D), we generated models of each domain by comparative structure modeling using the published crystal structure of chicken egg white cystatin (21) as a template. Table II summarizes the sequence identity and similarity with the template sequence, the G-factors calculated by Procheck software (22), and the Z scores obtained by Prosa II software (23) as indicators of the accuracy of the modeled structures. According to these parameters, all models were free of sterical clashes and well within the confines of theoretical structure prediction.

This segment of the mAhsg domain D1 model (Fig. 6, B and C), which adopts a β-sheet conformation when folded according to the known chicken egg white cystatin template structure (Fig. 6A) or when predicted by the sequence analysis software packages PSIPRED and PHD (31, 32) (data not shown), contains a remarkably high number of acidic amino acids, causing an extended negative surface charge (red residues in Figs. 6C and 7). In mAhsg D1, 6 out of 7 exposed residues in β-strands 2 and 3 of the four-pleated β-sheet are Asp or Glu, forming a contiguous acidic surface. These residues alternate with hydrophobic amino acids in a regular fashion, resulting in an asymmetric distribution of charge on opposing faces of the β-strands. Charged amino acids cluster on the exposed surface of the β-sheet facing the external milieu, whereas hydrophobic or uncharged amino acids cluster on the core side pointing toward the amino-terminal α-helix. No other cystatin-like domain analyzed showed a similarly regular pattern of charge distribution like mAhsg domain D1.

Next we scanned the modeled three-dimensional protein structure of each domain for features that would explain their differential activity in the BCP precipitation assay. Fig. 7 shows a compilation of identical views facing the extending β-sheets of all cystatin-like domains. The modeled structures are oriented in order to allow a top view of the four-pleated β-sheets. They illustrate striking differences in charge density on the extended β-sheets, which can also be detected in the protein sequence alignment (Fig. 6D). The alternating pattern of charges, which leads to a uniformly negative charge flanked by positive charges on the extended β-sheet of Ahsg D1 is absent or grossly distorted in the cystatin-like domains of chicken egg white cystatin, Ahsg domain D2, FETUB, and KNG (Fig. 7). The two cystatin-like domains of HRG show a coherent charged surface like Ahsg D1, but to a lesser extent. This may explain why HRG inhibited the precipitation of BCP, albeit with a 2-fold lower molar efficiency than Ahsg (28), whereas none of the remaining cystatin-like domains inhibited BCP precipitation in this study (Fig. 5).

Discussion

Fetuin/Ahsg is a potent inhibitor of BCP precipitation in vitro and in vivo (17, 33). According to published literature, bovine fetuin/bAhsg binds one calcium ion tightly and five calcium ions more weakly (34). Based on a sequence alignment, it was proposed that Ahsg domain D1 harbors an EF-hand-like calcium binding motif, 92EGDCDFQLK101 (35). The amino acid positions 92–101 would, however, be located within β-strand 3 in our model of Ahsg domain D1 (Fig. 6), a configuration incompatible with a functional EF-hand. Therefore, EF-hand-like calcium binding is unlikely to mediate the efficient inhibition of precipitation of BCP caused by Ahsg. Also, on theoretical grounds, the simple binding of ionic calcium by Ahsg cannot be responsible for the inhibition of BCP precipitation. Considering the ion concentrations of the precipitation assay and assuming that one Ahsg molecule would bind six calcium ions, one can calculate that 3 μM Ahsg (the nominal concentration present in the precipitation mixture) would reduce the free calcium concentration to 6 × 3 = 18 μmol at best. This is insignificant compared with the millimolar concentration of calcium contained in the precipitation mixture and indeed most extracellular fluids of living animals. The solution
would still be supersaturated, and consequently, BCP precipitation would not be delayed. Therefore, the inhibition of BCP precipitation by Ahsg cannot be explained by a reduction of the ion product through calcium ion binding as in the case of albumin (36). Albumin does not have any extended β-sheet structure but is exclusively α-helical in nature (37). In the case of Ahsg, the arrangement of acidic amino acids in domain D1 and their folding into a defined array of charges on an extended β-sheet is, however, a crucial feature of effective inhibition of BCP precipitation. Based on the amount of calcium neutralized by Ahsg binding, we suggest that Ahsg binds BCP, not ionic calcium like serum albumin. Thus, both Ahsg and serum albumin contribute to extracellular calcium homeostasis, albeit on a different level of complexity. In summary, extracellular proteins can bind calcium in three different ways illustrated in Fig. 8. Ahsg binds BCP, whereas albumin and the EF-hand protein SPARC/osteonectin bind free calcium with low and high affinity, respectively.

Our structure-function study of cystatin-like domains of Ahsg, FETUB, HRG, and KNG suggests that the β-sheet in Ahsg domain D1 is critically important for the inhibition of BCP precipitation. It forms a contiguous negatively charged, almost planar structure that is missing in Ahsg domain D2 and in all other modeled cystatin-like domains (Fig. 7). It is tempting to speculate that the acidic residues might occupy the phosphate lattice positions in apatite-like mineral through epitactic lattice matching. We hypothesize that the regularly spaced pattern of charges, characteristic of the extended β-sheet in Ahsg domain D1, is well suited to bind hydroxyapatite, especially at lattice planes with a high calcium content like the (001) plane. This binding mode is reminiscent of globular antifreeze proteins (38), which bind ice through several hydrogen bonding Asn and Gln residues arranged in a flat hydrogen bonding β-strand.

In summary, extracellular proteins can bind calcium in three different ways illustrated in Fig. 8. Ahsg binds BCP, whereas albumin and the EF-hand protein SPARC/osteonectin bind free calcium with low and high affinity, respectively.
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FIG. 7. Surface charge distribution of cystatin-like protein domains. Using the x-ray structure of chicken egg white cystatin (Protein Data Bank accession code 1CEW) (21) as a template, we modeled the cystatin-like domains of mAhsg, mFETUB, hHRG, and hKNG using Modeled software (20). The structures are orientated in order to allow a top view on the four-pleated array depicted in red and basic residues in blue. Note that the accumulation of negative charge on the extended β-sheet of mAhsg D1 is absent in comparable cystatin-like domains.

FIG. 8. Calcium (phosphate) binding mechanisms of extracellular proteins Ahsg, serum albumin, and SPARC/osteonectin. A, in Ahsg domain D1, surface binding of calcium is mediated by negative charges on the extended β-sheet of domain D1 (top) that might occupy PO₄ positions on the (001) face of apatite crystals (bottom), resulting in high affinity binding despite the relatively low serum concentration of Ahsg (10 μM). B, serum albumin binds calcium as counterions to numerous negatively charged amino acids (red) facing the external milieu. The high serum concentration of albumin (1 mM) causes high capacity calcium binding. C, high affinity binding is achieved by a functional EF-hand conformation. The calmodulin-like mode of calcium binding depicted here is established for few extracellular proteins (e.g. SPARC/osteonectin) (14).

Although deletion of the MGP gene in mice results in lethal calcification of the aorta (13). Blocking of MGP glutamic acid carboxylation by warfarin causes vascular calcification in young rats (39). Therefore, glutamate γ-carboxylation is regarded as critical for the function of MGP. The low solubility of MGP (<10 μg/ml) hampers structural research on this important inhibitor molecule, but the related bone-GLA protein/BGP/osteocalcin (40) has been analyzed by CD spectroscopy (41). In the α-helical conformation determined in this study, the spacing of the GLA residues in human BGP ((GLA)PRR/GLA/VC-GLA) results in the clustering of GLA residues on one side of the α-helix. This configuration may also exist in human MGP, which harbors two comparable sequence motifs ((GLA)RIR-GLA) and (GLAILNRLGLA). Intriguingly, the interval of an α-helix of 540 pm corresponds to the distance of the Ca(I) ions in the (001) plane of the apatite structure permitting a tight binding of the GLA residue pairs to the Ca(I) ions on the mineral surface. Besides direct calcium binding, MGP may interfere with osteogenesis by regulating BMP-2 (42). Interestingly, Ahsg can likewise regulate osteogenesis by sequestering transforming growth factor-β and BMP-2 (43).

Here, we present a time-resolved TEM morphological study of direct physical calcification inhibition by Ahsg in vitro (Fig. 2) in the absence of osteogenic cells. Our study details the growth and transformation of the soluble precursors instead of the precipitate, which have been described (6, 26, 44, 45). The most important discovery of this study is that Ahsg forms transiently soluble, colloidal complexes with calcium and phosphate, which we termed calciprotein particles. At 37 °C and 10 μM Ahsg, which correspond to the normal serum temperature and Ahsg concentration, a BCP precipitate formed with a delay of at least 6 h. In biological terms, this suggests that Ahsg coating of BCP nuclei will delay the growth of insoluble crystals long enough to assure the mobilization and removal of otherwise insoluble calcium salts in the form of Ahsg containing calciprotein particles. We hypothesize that phagocytic cells of the reticuloendothelial system (namely macrophages in spleen and liver and osteoclasts in bone marrow) will clear the calciprotein particles, thus mediating the recycling of extracellular calcium and phosphate from BCP. Corroborating this view, the total absence of Ahsg causes severe ectopic calcification of almost every soft tissue in Ahsg−/− knockout mice, and Ahsg deficiency is an independent predictor of vascular calcification in long term dialysis patients (15).

It remains to be determined whether the formation of calciprotein particles also plays a role in the dissolution of bone mineral. The removal of osteoclast bone resorption products involves transcytosis of vesicles containing bone mineral and bone matrix proteins (46, 47). Ahsg is a major noncollagenous protein in bone and teeth (48) and could therefore prevent the
precipitation of calcium salts during the transcytosis and thereafter. If this is so, calciprotein particles should be detectable in the bone remodeling compartment (49) and perhaps in the circulation. The attempts to isolate Ahsg-, calcium-, and phosphate-containing calciprotein particles from blood of normo- and hypercalcemic mice were up until now unsuccessful. However, a high molecular weight complex containing calcium, phosphate, Ahsg/Fetuin-A, and matrix-GLA protein was recently isolated in large amounts from the serum of etidronate-treated rats (51). It is very possible that this complex is identical with the calciprotein particles described in this study.

Our data suggest a novel mechanistic concept on the inhibition of generalization of calcification by serum protein, namely by stabilization of soluble, colloidal particles containing calcium, phosphate, and the mineral-binding protein, Ahsg. We present a model calciprotein particle in Fig. 9. We arrived at this model by comparing the hydrodynamic radius of the Ahsg molecule (4.2 nm) estimated by dynamic light scattering (see Fig. 4) with the radius of 25 nm for an early calciprotein particle (Fig. 2A). A rough estimate of the number of globular Ahsg molecules required to fill the volume of a calciprotein particle arrives at ~100 Ahsg molecules. We adapted the stoichiometry of Ahsg-containing calciprotein from published literature (51), reporting an Ahsg/phosphate ratio of 7.6 mg/mg for soluble high molecular weight complex (in our model equal to early, soluble calciprotein from published literature (51), reporting an Ahsg/phosphate ratio of 7.6 mg/mg for soluble high molecular weight complex 10. Schinke, T., McKee, M. D., and Karsenty, G. (1999) \textit{Nat. Genet.} 23, 277–285.

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