The Serum Protein α₂-HS Glycoprotein/Fetuins Inhibits Apatite Formation in Vitro and in Mineralizing Calvaria Cells

A POSSIBLE ROLE IN MINERALIZATION AND CALCIUM HOMEOSTASIS*

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We present data suggesting a function of α₂-HS glycoproteins/fetuins in serum and in mineralization, namely interference with calcium salt precipitation. Fetuins occur in high serum concentration during fetal life. They accumulate in bones and teeth as a major fraction of noncollagenous bone proteins. The expression pattern in fetal mice confirms that fetuin is predominantly made in the liver and is accumulated in the mineralized matrix of bones. We arrived at a hypothesis on the molecular basis of fetuin function in bones using primary rat calvaria osteoblast cultures and salt precipitation assays. Our results indicate that fetuins inhibit apatite formation both in cell culture and in the test tube. This inhibitory effect is mediated by acidic amino acids clustering in cystatin-like domain D1. Fetuins account for roughly half of the capacity of serum to inhibit salt precipitation. We propose that fetuins inhibit phase separation in serum and modulate apatite formation during mineralization.

Considerable progress has been made toward a molecular understanding of skeletal development through the identification of local factors that control skeletal patterning and bone shape (1). Stages beyond the condensation of mesenchymal cells and the developing chondrogenic phenotype are envisaged as a chain of timed events that lead to a progressive development of the osteoblast phenotype (2). Following that is the assembly of collagen, noncollagenous proteins, and mineral into functional bone tissue with acidic proteins controlling crystal growth at the interphase of the mineral and organic components (3). Most protein components of this assembly are expressed bone tissue-specific. However, serum proteins made in the liver are also known to contribute to the organic phase of bone and dentine (4). The function of serum proteins in biomineralization is poorly understood despite the fact that cell culture systems mimicking bone formation generally include serum.

One particular family of serum proteins, synonymously called α₂-HS glycoproteins or fetuins, are concentrated in the mineral phase of bone (5) and teeth (6). They are structurally well studied. To date complete primary structures are published for human (7), bovine (8), sheep, pig (9), rat (10), and mouse (11) fetuins and the intron-exon structure has been published for the rat gene (12). From N terminus to C terminus two conserved cystatin domains functionally silent in human fetuin (13) are followed by a third, unrelated and more divergent domain rich in proline. Due to the cystatin domains fetuins are grouped within the cystatin superfamily of proteins (14). Fetuins are both N- and O-glycosylated (15, 16) and Ser-phosphorylated (17). Human plasma α₂-HS is proteolytically processed (17).

Throughout all species studied high amounts of fetuins consistently occur in mineralized bone (5, 18–21). Based on this observation a role for fetuins in bone formation and resorption has been suggested (22). Fetuins are abundant in fetal blood and tissues indicating that they may play a more general role during organ development. More specific functions of fetuin like lipid transport or inhibition of insulin receptor tyrosine kinase have been suggested; they are critically reviewed in a recent monograph (23).

Fetuins bind strongly to apatite and can thus be selectively enriched from serum (18). Bovine fetuin has several low affinity calcium binding sites (24) that might mediate apatite binding. However, the molecular basis of apatite binding and the significance of fetuin accumulation in bone have not been elucidated. In this paper we demonstrate that fetuins inhibit apatite precipitation. We propose a biological function for fetuins in the maintenance of high blood calcium levels and in the inhibition of unwanted mineralization.

EXPERIMENTAL PROCEDURES

Materials—α₂-HS glycoprotein (human fetuin) from serum, the corresponding rabbit antisera, and α₂-HS glycoprotein isolated from HepG2 hepatoma medium have been described (17). Human serum was depleted of α₂-HS-glycoprotein by antibody affinity chromatography (17). Mouse fetuin cDNA was kindly provided by Dr. Yang, San Antonio, TX (11). Copy DNAs encoding human and rat fetuins have been described (10). Bovine serum albumin and ovalbumin were obtained from Roth, Karlsruhe, calmodulin, lysozyme and type II-collagenase from Clostridium histolyticum from Sigma, [45Ca]Cl2 and Na[125I] from ICN-Flow, Iodogen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) from Pierce, antibody conjugates from Dako (FITC-conjugated) and Bio-Rad (peroxidase-conjugated).

Recombinant Expression and Purification of Fetuins—The complete cDNAs encoding human and mouse fetuins, respectively, were subcloned into the baculovirus expression vector pVL1393 (Pharmingen) as EcoRI fragments. Rat fetuin was subcloned from pBluescript vector as polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FCS, fetal calf serum; HPLC, high performance liquid chromatography; GEE, glycine ethyl ester; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide.
an EcoRV/NotI fragment into pVL1393 digested with SmaI and NotI. Sf9 cells were co-transfected with pVL1393 vector and Baculogold™ DNA (PharMingen). Virus expressing fetuins were cloned by limited dilution of virus mixtures. Recombinant fetuin proteins were purified from protein-free medium (SF900II, Life Technologies, Inc.) of virus-infected Sf9 cells by a four-step procedure. Protein was concentrated by precipitation with 2 M (NH₄)₂SO₄. The precipitate was dissolved in buffer (50 mM NaH₂PO₄, pH 7, 0.75 M (NH₄)₂SO₄), and applied to a butyl-Sepharose (Pharmacia Biotech Inc) column. Bound protein was eluted at a flow of 2 ml/min with a 30-min linear gradient of 0.75 M ammonium sulfate followed by 50 mM NaH₂PO₄, pH 7. Fractions containing recombinant protein (as determined by ELISA) were pooled and concentrated by precipitation with ammonium sulfate. The pellets were redissolved in 50 mM Tris-HCl, pH 7.4, and fractionated on a Superose 200 (Pharmacia) column. The purified fetuins migrated as single bands upon SDS-PAGE and Coomassie staining. Purification of the fusion proteins was monitored by SDS-PAGE and Western blot analysis using antibody against rat serum fetuin (Dr. Nawratil, Munich).

Bacterial Expression of Mouse Fetuin Deletion Mutants—C-termi-
nally truncated variants of mouse fetuin were expressed in Escheri-
chia coli (XL1-Stratagene) as fusion proteins of the maltose-binding protein (MBP) using the expression vector pMal™-c2 (New England Biolabs). N-terminally truncated variants of fetuin were expressed in E. coli (M15) by digestion of test proteins with proteinase K.

The mouse fetuin cDNA was shortened from the 3'-end by digestion with exonuclease III. The expression vector pMal™-c2 was modified by inserting into XbaI/PstI-digested vector an oligonucleotide hybrid carrying a KpnI restriction site and three TAA stop codons. The hybrid forming oligonucleotides used were 5'-CTAGATGCTACCTAACTAC-
TAA-3' and 5'-CTAGATGCTACCTAACTAC-3'. The modified pMal™-c2 vector was digested with BamHI and KpnI, ligated and transformed into E. coli Bacteria were lysed in buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 0.1% benzamidine, 1 mM PMSF, 1 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 0.1 mM sodium orthovanadate, 0.1 µM sodium molybdate, 1 mM dithiothreitol, 100 units/ml aprotinin, 100 units/ml leupeptin, 100 units/ml pepstatin, 0.2 µg/ml benzamidine, 0.1 mM PMSF, 0.1 mM sodium orthovanadate, 0.1 µM sodium molybdate, 1 mM dithiothreitol), dissolved in 0.5% acetic acid, and quantified by liquid scintillation counting. All incubations were done in triplicate and independently repeated at least two times. To estimate the inhibition of salt precipitation by and fetuin, 100 µg of fetuin was incubated with 1 ml (10 µl) of carrier-free Na[125I] and Iodogen (100 µg/ml) for 10 min.

Precipitates were collected by centrifugation (15,000 g, 30 min), dehydrated, and exposed to BioMAX x-ray film (Kodak) for 3 h. Co-purification of Fetuins with Salts—40 µg of human α2-HS glycoprotein dissolved in 90 µl of phosphate-buffered saline was incubated with 1 ml (10 µl) of carrier-free Na[125I] and iodogen (100 µg/ml) for 10 min.

Precipitates were collected by centrifugation (15,000 g, 30 min), dehydrated, and exposed to BioMAX x-ray film (Kodak) for 3 h.

Inhibition of calcium salt precipitation was determined by incubating at 37 °C for 90 min a buffered salt solution (50 mM Tris-HCl or 20 mM Hepes-NaOH, pH 7.4, 4.8 mM CaCl₂, 2 x 10⁵ cpm [45Ca]Cl₂, 1.6 mM NaH₂PO₄) containing test proteins as indicated in the figure legends. Precipitates were collected by centrifugation (15,000 g, 5 min), and α2-HS coprecipitation was quantified using a gamma-counter.

Inhibition of Apoptosis—To determine the effect of fetuins on the formation of apoptosis, we adapted the assays described (27, 28).

Inhibition of calcium salt precipitation was determined by incubating at 37 °C for 90 min a buffered salt solution (50 mM Tris-HCl or 20 mM Hepes-NaOH, pH 7.4, 4.8 mM CaCl₂, 2 x 10⁵ cpm [45Ca]Cl₂, 1.6 mM NaH₂PO₄) containing test proteins as indicated in the figure legends. Precipitates were collected by centrifugation (15,000 x g, 5 min), and α2-HS coprecipitation was quantified using a gamma-counter.

Proteolytic Digests—Human α2-HS glycoprotein was digested either with chymotrypsin, elastase, proteinase K, trypsin, or endoproteinase Glu-C at a molar ratio of 100:1. All digests proceeded 2 h at 37 °C in buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT). The endoproteinase Glu-C digest was fractionated by HPLC gel filtration. 0.5 ml fractions were collected and assayed for inhibitory potency toward apatite formation. Blot sequencing of peptides separated by nonreducing SDS-PAGE was performed by Dr. R. Kellner at the Central Protein Chemistry Facility, University of Mainz.

Chemical Modification of Fetuin—5 mg of bovine fetuin was chemically modified by incubation for 3 h at 45 °C in 1 ml of buffer (0.2 M Tris-HCl, pH 8.3, 1.5 mM DTT, 6 mM guanidinium hydrochloride). The reduced cysteine residues were carbamylmethyalted by iodoacetamide (final concentration 6 mM) for 30 min at 25 °C in the dark. β-Mercapto-
atoethanol was added to a final concentration of 0.5% to quench residual iodoacetamide, and the reduced/carbamoylated fetuin was desalted by gel filtration. Reduction was assayed by SDS-PAGE, as reduced single chain fetuins display higher apparent molecular weights than nonreduced forms (17). Modification of carboxyamide groups was performed as published (29). 5 mg of bovine fetuin was reacted for 4 h at 37 °C in 1 ml of buffer (12.5 mM glycine ethyl ether (GEE), 50 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 5 mM N-hydroxysuccinimide, pH 4.75). The modified fetuin was dialyzed against 0.5 M NaCl, 0.03% NaN₃, and stored at 4 °C.

Primary Osteoblast Cell Cultures—Calvaria from newborn rats were dissected and cultured with collagenase (type II from C. histolyticum, 2
signals for fetuin mRNA were detected in liver. No other organ expressed fetuin mRNA at levels comparable with liver. Similar hybridization patterns were obtained with 35S-UTP-labeled riboprobes transcribed from the full-length cDNA (1250 base pairs) and from PCR fragments comprising the 3'-terminal half (650 base pairs) of the cDNA (not shown). The 5'-terminal part of fetuins contains two cystatin domains, and riboprobes representing this part of the molecule could possibly cross-hybridize with mRNA for other cystatin-like molecules. The 3'-terminal part of fetuins is, however, specific for this molecule and should only detect fetuin mRNA. The fact that both riboprobe types yielded similar hybridization patterns therefore indicates that our staining was strictly fetuin-specific.

To study the tissue distribution of fetuin protein, we stained sections consecutive to the ones used for in situ hybridization with antibody against mouse fetuin protein. Antibody staining of sections at low concentration of antibody (antiserum diluted 1 in 2000) outlined the skeleton, most notably the skull, the calcified part of the ribs, long bones, and teeth (Fig. 1B), indicating that these tissues contained the highest amounts of fetuin protein. Earlier stages of development showed no detectable signal at this low concentration of antisera. At higher concentration of antibodies (antiserum diluted 1 in 500), many more tissues stained positive for fetuin, among them liver, kidney, hind gut, and skeletal muscle (not shown). Sections stained with preimmune serum diluted 1 in 200 remained unstained (Fig. 1D).

The combined results of in situ hybridization and antibody staining indicate that fetuins are primarily made in the liver and are concentrated in bone and teeth. We sought to determine what function in bone fetuins might serve.

Fetuins Inhibit Apatite Precipitation in Primary Osteoblast Culture—To test the influence of fetuins on actively mineralizing bone cells, we established primary rat calvaria osteoblast cultures. These cultures grow confluent until day 4 in DMEM containing 10% FCS. After this initial culture period the cells were further grown in DMEM supplemented with ascorbate and β-glycerophosphate to induce mineralization. During this second culture period, the cells deposited calcium mineral as revealed by von Kossa staining at day 12. Fig. 2A represents a typical view of von Kossa stained primary osteoblasts at day 12 in culture. When the DMEM culture medium contained 10% FCS and hence large amounts of bovine fetuin, calcium salt deposits were inconspicuous and only microscopically visible (Fig. 2A, top left well). Bone nodule formation commenced and the cells stayed alive in long term culture for up to 3 weeks (longer periods were not tested). When FCS was omitted from the culture medium and instead 0.5% BSA was included (serum-free medium), upon addition of β-glycerophosphate and ascorbate abundant precipitation of calcium salts occurred starting at day 10, and the cells died until day 12 (Fig. 2A, bottom row). When, however, bovine fetuin (Fig. 2A, left panel) or human α2-HS (Fig. 2A, center panel) were included in the serum-free medium, the initial pattern of FCS-containing cell culture was restored in that precipitation of calcium salts was reduced, mineralized primary osteoblasts were formed, and cells were visually indistinguishable from FCS-containing cultures. This inhibition of salt precipitation was also observed with asialofetuin and recombinant human, rat, and mouse fetuin expressed by baculovirus-infected insect cells (not shown). The inhibition was dose-dependent in that higher concentrations of fetuin more consistently inhibited precipitation than lower concentrations. For example, human α2-HS at 2 µM (100 µg/ml) completely inhibited salt precipitation in 6 out of 6 replicates within 3 independent experiments. At 0.02 µM (1 µg/ml), complete inhibition was observed in 4 out of 6 replicates. Bovine
Fetuins Bind to and Inhibit the Formation of Apatite—

Fetuins modulate the formation of calcium mineral and sought to determine the molecular basis for this activity. We deliber-
ately switched between forms of fetuins from different species to confirm that includes ascorbate and β-glycerophosphate but lacked FCS or α2-HS glycoproteins/fetuins. B–D, immunolocalization of α2-HS glycoprotein in primary osteoblast cultures. The cells were incubated in DMEM containing 0.5% BSA and 50 μg/ml α2-HS glycoprotein for 12 days in presence (B, C) or absence (D) of ascorbate and β-glycerophosphate. α2-HS glycoprotein was detected by immunofluorescence with polyclonal antiserum against serum α2-HS as described under "Experimental Procedures." B, phase contrast of the same cells shown in C. Note that calcium precipitates visible as dark spots in phase contrast (B) stained positive for α2-HS.

Fetuins and human serum containing 10% FCS co-precipitated in mixtures of calcium and phosphate in presence of magnesium and carbonate but not in mixtures of magnesium and phosphate (Fig. 3). These data indicate that the binding of fetuins to apatite is due to interactions with calcium ions rather than with phosphate ions. The mineral formed in mixtures of calcium and phosphate under the assay conditions was poorly crystalline apatite as judged by X-ray diffraction of spun and dried precipitate powder (data not shown). Henceforth, we refer to the mineral formed as apatite.

We noticed that considerably less apatite but not calcium carbonate was formed in the presence of fetuins than in the protein-free control incubations. Quantification of the salt precipitates under modified assay conditions (Tris-buffered precipitation mixture with added [45Ca]Cl2) revealed that, for example, human α2-HS inhibited the precipitation of apatite in a dose-dependent manner. Fig. 4A shows that the transition between no inhibition and maximum inhibition of apatite formation was sudden, resulting in a steep slope of the sigmoid dose-response curve and concomitant large error. The dose response for precipitation inhibition was similar for all forms of α2-HS glycoproteins/fetuins tested, in that half-maximum inhibition occurred around 0.5 μM (Table I). Fig. 4B illustrates the time course of a precipitation reaction. In protein-free control incubations the precipitation reached its maximum after 2 h (Fig. 4B, □). When α2-HS was included in the precipitation mixture a dose-dependent delay of precipitation occurred, and the maximum of precipitation was achieved after 4 h (1 μM α2-HS, Fig. 4B, ◊) at 10 h, respectively (5 μM α2-HS, Fig. 4B, ◊). Adding α2-HS to the precipitation mixture delayed but did not completely stop the formation of apatite. Accordingly, α2-HS added to the apatite mixture and not dissolve the mineral (data not shown).

Fetuins Inhibit Apatite Formation in Serum—It is known that serum contains inhibitors of salt precipitation (31). We therefore sought to determine the contribution of fetuins to the overall inhibitory effect of human serum. The results obtained with human serum containing 10 μM α2-HS (500 μg/ml, measured by ELISA) depleted to less than 0.15 μM and reconsti-
tuted serum are shown in Fig. 5. These experiments were performed in buffer containing the major inorganic components at their respective serum concentrations. Due to restraints of the experimental setup, the maximum serum content of the precipitation mixture was 30%. This amount of serum corresponded to 3 μM α₂-HS and inhibited the formation of apatite by 95% when compared with the protein-free control incubation (Fig. 5). Isolated α₂-HS at 3 μM alone inhibited by 75%, indicating that non-dialyzable serum components other than α₂-HS also inhibit the formation of apatite. Serum depletion of α₂-HS by affinity absorption caused a marked reduction in serum's inhibitory activity that could be restored by adding back the original amounts of α₂-HS. This was also observed when serum was added to the precipitation mixture at 15 and 5% final concentration. In summary, the results illustrated in Fig. 5 demonstrate that (i) the capacity of serum to inhibit apatite precipitation can be partially accounted for by the amount of α₂-HS present in this serum; (ii) when α₂-HS is removed from the serum, the capacity of the remainder to inhibit apatite precipitation drops by 50% demonstrating that α₂-HS significantly contributes to the inhibition of calcium salt precipitation in serum.

Inhibition of Apatite Formation by Fetuins Is Largely Independent of Post-translational Modification—Next we determined if forms of fetuins from different species also differing with respect to post-translational modification inhibited the precipitation of apatite better than others. To this end we included commercial preparations of bovine fetuin (complex glycosylation) and asialofetuin as well as human α₂-HS isolated from serum (dephosphorylated (13)) and from HepG2 cell culture medium (phosphorylated (17)). We expressed human, rat, and mouse fetuins in baculovirus-infected insect cells, and we expressed mouse fetuin in E. coli cells that do not glycosylate eukaryotic proteins. We determined the N-linked and O-linked carbohydrate structure of several forms of fetuins by lectin blotting and tested the forms of fetuin thus characterized for their ability to inhibit the formation of apatite.

Table I shows that bovine fetuin, asialofetuin, and forms of human α₂-HS purified from serum, HepG2 hepatoma cell cul-

![Graph](Image)
ture medium, or from culture supernatant of baculovirus-infected insect cells (Sf9) similarly inhibited the precipitation of apatite with IC50 values of about 0.5 μM. Likewise, recombinant forms of rat and mouse fetuins expressed in insect cells inhibited the precipitation of apatite in a dose-dependent manner with IC50 values of about 0.5 μM. As the forms of fetuins tested differed with respect to glycosylation and phosphorylation, we conclude that these post-translational modifications do not greatly affect the inhibitory activity of fetuins. A fusion protein between maltose-binding protein and mouse fetuin (MBP mouse fetuin, crude) expressed in the cytoplasm of E. coli did not inhibit the formation of apatite unless the protein was refolded in the presence of glutathione (Table I, MBP mouse fetuin, refolded).

Several control proteins were also tested. Bovine serum albumin, a protein similarly acidic like fetuins, did not inhibit the formation of apatite below 10 μM. Lysozyme, a protein with high affinity for hydroxyapatite (32), and calmodulin, a well known high affinity for hydroxyapatite (32), and calmodulin, a well known

Amino Acids in Cystatin Domain 1 Mediate the Inhibition of Apatite Formation by Fetuin—Fig. 6A demonstrates that blocking of Glu and Asp residues by glycine ethyl ester (GEE) and carboxidiimide (EDC) abolished the inhibitory activity indicating that acidic amino acid residues are required for the inhibition of apatite precipitation. Likewise, the reduction and alkylation of disulfide bonds by dithiothreitol (DTT) and iodoacetamide in the presence of guanidinium hydrochloride (GdnHCl) completely abolished the inhibitor activity (Fig. 6B). Denaturation of bovine fetuin with GdnHCl alone and subsequent removal of GdnHCl by dialysis did not destroy the protein’s ability to inhibit apatite precipitation (Fig. 6B).

To identify minimum primary sequences of the fetuin molecules responsible for the inhibition of apatite precipitation, we generated proteolytic fragments. Limited proteolysis of human α2-HS and bovine fetuin with trypsin, chymotrypsin, elastase, and proteinase K all destroyed the ability to inhibit the precipitation of apatite. The results for human α2-HS are shown in Fig. 7A. Limited proteolysis of human α2-HS with Staphylococcus V8 protease (Endo Glu-C), however, generated a mixture of fragments of roughly 10, 20, and 30 kDa molecular mass that could still inhibit salt precipitation (Fig. 7B).

Fig. 6. Chemical modification abolishes inhibition of apatite formation by bovine fetuin. A, the carboxyterminal groups of bovine fetuin were modified by glycine ethyl ester (GEE) alone or by GEE in combination with the activating agent, ethylenediamine (EDC). B, bovine fetuin was denatured using guanidinium hydrochloride (GdnHCl), reduced by dithiothreitol (DTT) and carboxymethylated by iodoacetic acid (IAA) as described under “Experimental Procedures.” The influence of these modifications was assayed in the apatite precipitation assay as described in Fig. 4. Assays were done in triplicate; bars indicate the standard error of the mean.

Fig. 7. Proteolysis abolishes inhibition of apatite formation by human α2-HS. A, 50 μg of human α2-HS was digested with the proteases indicated at a molar ratio of 100:1 in buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT) for 2 h at 37°C. The buffer was adjusted to final concentrations of 4.8 mM CaCl2, 1.6 mM Na2HPO4 containing a spike of [45Ca]Cl2. The final concentration of α2-HS was 2 μM. Incubation was for 90 min at 37°C, and apatite formed was quantified by liquid scintillation counting of the insoluble pellet. Proteases alone did not inhibit the formation of apatite (not shown). B, 2 mg of human α2-HS was digested with endoproteinase Glu-C as described above. The sample was fractionated on HPLC gel filtration, and 0.5 ml fractions were collected. 50 μl of each fraction was assayed for inhibition of apatite precipitation in buffer (50 mM Tris-HCl, pH 7.4, 4.8 mM CaCl2, 1.8 mM Na2HPO4 and a spike of [45Ca]Cl2), 5 μl of each fraction was analyzed by SDS-PAGE and visualized by silver staining. A similar result was obtained with bovine fetuin.
or phosphate to the culture medium, whereas the formation of nodules and the expression of bone-specific proteins are independent of an external phosphate source (36). Serum-free culture conditions demonstrate that conditions of ion supersaturation causing spontaneous salt precipitation can exist in this system (Fig. 2A). It is known that phase separation in supersaturated solutions of calcium and phosphate is slowed down in the presence of serum proteins (31). Serum albumin has been described as an inhibitor of crystal formation in serum, but serum albumin alone could not account for whole serum’s ability to inhibit salt precipitation (37). Our data indicate that α2-HS glycoproteins/fetuins are efficient inhibitors on a molar basis and could account for the remainder of serum’s inhibitory activity. Fetuins can prevent phase separation in serum efficiently as the concentration causing half-maximum inhibition of apatite formation in vitro (0.5 μM) is 20-fold below the observed serum concentration (10 μM). Specifically, fetuins might be the major inhibitors of salt precipitation during fetal life, when serum albumin concentration is low. This conclusion gains support from the observation that serum Ca\(^{2+}\) concentration and fetuin concentration closely correlate during fetal development in any single one species and also when several species are compared (24).

Common structural features of protein inhibitors of salt precipitation can be stretches of poly(Asp) and extensive serine/threonine phosphorylation (27), γ-carboxyglutamatic acid (28), and sequences that contain no specific motifs but are generally acidic (3) or highly charged like the N-terminal fragment of salivary statherin (38). Fetuins contain clusters of Asp residues in both cystatin domains 1 and 2 (9) and are also Ser-phosphorylated (17). The inhibition of apatite formation by fetuins was abolished after chemical blocking of carbohydrate groups with glycin ethyl ester, indicating that aspartic and glutamic acid residues might confer calcium binding. The combined results from chemical modification of fetuin (Fig. 6), limited proteolysis (Fig. 7), and C-terminal deletion mutagenesis (Fig. 8) restrict the amino acid clusters to the N-terminal half of domain D1. Acidic amino acids conserved in all known fetuin domains D1 are Asp\(^{15}\)-Asp-X-(Asp/Glu)-X-Glu\(^{20}\), Asp\(^{46}\) and (Asp/Glu\(^{63}\))-X-Glu-X-Asp-X-Glu\(^{66}\). Neither Asp\(^{15}\)-Glu\(^{20}\) alone (see MBP-mf52) nor a synthetic peptide comprising Ile\(^{62}\)-Arg\(^{81}\) of human MBP was able to inhibit apatite formation equally without refolding in the presence of glutathione.

**DISCUSSION**

We developed antibodies against mouse fetuin and studied its tissue distribution on longitudinal whole body sections by immunocytchemistry and in situ hybridization. Our results demonstrate that fetuin mRNA and protein are made in the liver and that fetuin protein is transported to the bone to be sequestered in the mineralized matrix of the developing bone. These findings corroborate earlier physiological experiments in rabbits (5) and immunodetection of fetuin in human (33) and rat tissues (34). The accumulation of fetuin in bone suggests a relatively high affinity of these serum proteins for calcium which should represent an aspect of a more basic involvement in bone formation or remodelling.

Rat calvaria cells are an established model system of osteogenesis and mineralization in vitro (35). In this system mineralization commences after the addition of β-glycerophosphate

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α₂-HS Glycoprotein/ Fetuin Inhibit Apatite Formation

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2-HS Glycoprotein/Fetuin Inhibit Apatite Formation

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