Asporin competes with decorin for collagen binding, binds calcium and promotes osteoblast collagen mineralization

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INTRODUCTION

Decorin and biglycan are the best characterized members of the SLRP (small leucine-rich repeat proteoglycan/protein) homology class I family [1,2]. One of the features of SLRPs are the conserved cysteine residues that form two intra-chain bridges near the N-terminal and one bridge near the C-terminal end of the protein. Asporin, the third class I SLRP, differs from decorin and biglycan in not being a proteoglycan. The N-terminal domain of asporin (the glycosylated domain in decorin and biglycan) has a contiguous polyaspartate sequence that is polymorphic in the human population (8–19 residues encoded by alleles D8–D19) [3,4]. The D14 allele (14 aspartate residues) was linked to predisposition for knee joint osteoarthritis, whereas the D13 allele (13 aspartate residues) was more prevalent in healthy individuals. This finding appears to be true in Asian populations, possibly due to ethnic background or environment [5,6]. The difference in the number of aspartate residues has been reported to differentially alter TGF-β (transforming growth factor-β)-driven chondrogenic differentiation [5], but the molecular background of this is not known.

Polyaspartate sequences in proteins (e.g. osteopontin) or peptides can bind calcium and regulate hydroxyapatite deposition in vitro [7,8]. The studies also suggest that different conformations of polyaspartates, conveyed by the number of such residues and the flanking moieties, favour different interactions during specific steps of a growing hydroxyapatite crystal [7–10]. Since bone mineralization occurs in the collagen fibres [11], it is of interest to discern the roles of proteins with polyaspartic domains and potential collagen-binding sites.

Many SLRPs (e.g. decorin) bind collagen and it is likely that asporin also shares this property [12,13]. We have demonstrated that decorin binds collagen type I via a short SYRIADTNIT sequence in LRR (leucine-rich repeat) 7 [12]. Fibromodulin binds collagen via a RLDGNEIKR sequence [14] and shares the binding site with lumican [15]. Decorin does not compete with fibromodulin and lumican for binding to collagen [13], but the binding competition between class I SLRPs has not been reported.

Asporin is expressed in the peristem and in dental follicles during development [16,17]. This is where osteoblast progenitor cells arise and their expression of asporin coincides with the temporal onset of osteoblast-driven bone mineralization. Progenitor cells express the characteristic transcription factor Runx2 (Cbfa1), without which bone mineralization cannot take place [18]. Under the influence of Runx2 and BMPs (bone morphogenetic proteins), osteoprogenitors differentiate to osteoblasts and produce collagen type I, osteopontin, osteocalcin, alkaline phosphatase and other bone matrix-related proteins [19,20]. After the build-up of non-mineralized matrix, the actual calcification of collagen commences by progressive nucleation of hydroxyapatite crystals within collagen fibrils. Since the collagen fibril structure appears to affect the biomineralization process [21], we hypothesized that the initial deposition of hydroxyapatite in the fibrils may be regulated by asporin, provided that it contains a collagen-binding domain and that the asporin polyaspartate domain can bind calcium. We now demonstrate two functional domains in asporin, binding to collagen and calcium respectively; and how asporin compares with the homologous proteins decorin and biglycan, in terms of collagen binding competition and influence on osteoblast collagen mineralization activity.

EXPERIMENTAL

Expression of recombinant His-tagged asporin and GST (glutathione transferase)-tagged asporin fragments in bacteria

Human asporin cDNA from the D13 allele (GenBank® accession AF316824) was used as a template in PCR, to amplify cDNAs for full-length asporin or asporin fragments. Full-length cDNAs were cloned into the pET-27b(+) expression vector. Recombinant His-tagged asporin was produced in E. coli Rosetta-gami® as described previously [22]. The purified recombinant asporin was characterised by western blot [23].

Key words: asporin, biomineralization, calcium, collagen binding, decorin, small leucine-rich repeat proteoglycan/protein (SLRP).
vector (Novagen) and the fragments were cloned into the pGEX-4T3 expression vector (Amersham Biosciences). Asporin fragments were: AspN-3, amino acids 33–162; AspN-6, amino acids 160–232; AspN-7, amino acids 230–302; and AspN-10, amino acids 299–379. Constructs were sequenced, transfected into Rosetta-gami™ Escherichia coli (Novagen), and proteins were expressed and purified according to the manufacturer’s instructions. Full-length proteins were purified under denaturing conditions, and fragments were purified in native conditions (as described in [12]), and dialysed against PBS. Full-length asporin was purified further by the presence and location of cysteine bridges were confirmed by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS) (as described in [3]).

Expression of recombinant decorin, biglycan and lumican in mammalian cells, and fibromodulin in bacteria

The proteins were expressed and purified as described previously [12–15].

ITC (isothermal titration calorimetry)

Calorimetry was performed using the VP-ITC (Microcal). Asporin and bovine acid-solubilized collagen (PureCol from Inamed, Leimuiden, The Netherlands) were dialysed against 20 mM phosphate buffer (pH 7.4). Asporin (1 μM) was titrated into the 0.2 μM collagen solution in the sample cell, by injections of 10 μl aliquots, at 30 °C. Calorimetry data were corrected for the titrant heat of dilution, \( K_w \) was calculated using a one-binding-site algorithm, with the Origin software v.7 (OriginLab, Northampton, MA, U.S.A.) provided by Microcal.

Solid-phase collagen-binding assay

Coating of 96-well Maxisorp plates (Nunc, Roskilde, Denmark) with 10 μg/ml acid-solubilized collagen diluted in 0.012 M HCl was performed. Wells were washed [PBS with 0.5 mg/ml BSA and 0.05 % (v/v) Tween 20] and blocked for 1 day with non-fat skimmed milk. After washing, GST-tagged fibromodulin fragments and/or His-tagged asporin, decorin or biglycan were added. For competition experiments, asporin or decorin (20 nM each) was incubated with the inhibitors. After incubating for 4 h at 37 °C, the wells were washed and protein binding was detected using rabbit anti-GST antibody (ab9085; Abcam, Cambridge, U.K.) or rabbit anti-His antibody (ab9108; Abcam), which had been diluted 1:500, incubated for 1 h. Secondary antibody was an anti-rabbit alkaline phosphate-conjugated antibody (ab6722; Abcam), diluted 1:1000, incubated for 1 h. Enzyme activity was measured using 1 mM PNPP (p-nitrophenyl phosphate) as the substrate (Sigma). Colour development was read at 405 nm. The data were analysed with GraphPad Prism v.5 (GraphPad Software, La Jolla, CA, U.S.A.).

Collagen in vitro fibrillogenesis assay

The method used has been described previously [12]. Briefly, pepsin-extracted acid-solubilized collagen (Vitrogen) was neutralized and diluted in 150 mM NaCl buffered with 20 mM Heps (pH 7.4). Asporin or decorin was added, and the solution was incubated at 37 °C (inducing collagen fibrillogenesis) in a spectrophotometer, with readings of increasing absorbance (correlating with increasing fibril formation) being recorded continuously at λ = 400 nm over 12 h.

Calcium overlay assay

The method used has described previously [22]. Briefly, proteins were separated by SDS/PAGE (10 % linear gels) and transferred on to a nitrocellulose membrane, which then was soaked (1 h) in a buffer containing 60 mM KCl, 5 mM MgCl2 and 10 mM Heps (pH 7.4) with two buffer changes. The membrane was incubated in a buffer with 1 μCl/ml of \( ^{45} \text{Ca}^2+ \) for 10 min (with or without non-radioactive 10 mM CaCl2). Then, the membrane was washed with 50 % ethanol for 5 min, dried and the isotope was quantified with an FLA-3000 bioimaging analyser (Fuji Photo Film, Tokyo, Japan).

Dot-blot calcium-binding assay

Varying amounts of asporin were spotted on to a nitrocellulose membrane and dried, and the calcium overlay assay was performed as described above.

Calculated nodule formation in cultures of osteoblastic cells

The method was described previously [23,24]. The MG63 cell line (CRL-1427 from the A.T.C.C., Manassas, VA, U.S.A.) is derived from an osteosarcoma. Cells were cultured in EMEM (Eagle’s minimum essential medium; Gibco, Invitrogen) with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 1.5 g/l sodium bicarbonate, supplemented with 10 % (v/v) heat-inactivated fetal bovine serum. To induce the osteoblast-like phenotype and formation of calcified bone-like nodules, the cells were grown to confluence and supplemented with 100 nM dexamethasone and 6 mM CaCl2, replenished each day. For the assays, the cells were also supplemented daily with increasing concentrations of asporin, decorin, biglycan, lumican or fibromodulin. Nodules were quantified on day 2 of culture: after PBS washes, Alizarin Red was applied for 5 min and then rinsed with water (using a method described previously [17]). Staining for collagen and asporin was done with rabbit anti-collagen antibody (a gift from Professor Kristofer Rubin, Department of Medical Biochemistry and Microbiology, University of Uppsala, Uppsala, Sweden) or in-house anti-asporin antibody diluted 1:500 in PBS with 0.5 mg/ml BSA and 0.05 % (v/v) Tween 20, followed by secondary Texas Red-conjugated antibody diluted 1:1000. Staining was recorded using an Axioshot fluorescence microscope (Zeiss) with a Plan-APCHROMAT ×10/0.45 objective lens, an Axiocol (Zeiss) camera and Axiovision 3.0 software (Zeiss).

Quantitative real-time RT–PCR (reverse transcription–PCR)

RNA was extracted with TRIZol® (Invitrogen) and RNaseasy (Qiagen). CDNA was made with an iScript™ cDNA synthesis kit (Bio-Rad). Real-time PCR was performed using the following primers: \( \text{Osterix: } \) CCTCTGGCGGGACTCAACACG and TAAAGGAGGCTGTTAAGCAT; \( \text{Runx2: } \) ACCAGATGG-GACTGTGTTTAC and CCTGTAACCTTGCCTACTTGTTT; \( \text{Gusb (internal control): } \) TGGCCTACTTGAAGATTGTG and GCTCACAAGGTCACAGG. Primers and cDNA were mixed with Power SYBR® Green Master Mix (Applied Biosystems) and the reactions were carried out with an ABI-PRISM 7900HT cycler (Applied Biosystems).

RESULTS AND DISCUSSION

Asporin with intra-chain cysteine bridges can be expressed in bacteria

To express asporin, we used commercially available Rosetta-gami™ E. coli that are deficient in thioredoxin reductase and...
glutathione reductase, thus allowing the formation of cysteine bridges. Asporin was expressed as a His-tagged protein, and purification by Ni-NTA (Ni²⁺-nitrilotriacetate) affinity Sepharose yielded the expected 50 kDa protein (Figures 1A and 4A). This protein was digested with trypsin and identified as full-length asporin (68% sequence coverage) by MALDI–TOF-MS (see Supplementary Figure S1). In addition, smaller 38 and 27 kDa proteins were apparent on the gel and were analysed by MALDI–TOF-MS. These proteins were identified as fragments of asporin lacking the N-terminal portions of the protein. To purify full-length asporin from the fragments, we used MonoQ anion-exchange chromatography (Figure 1A) and recovered approx. 150 μg of full-length asporin from a 10 litre bacterial culture. This is a very low level of expression compared with other recombinant SLRPs that are expressed in milligram amounts using the same procedure.

Asporin expressed by eukaryotic cells contains three intra-chain cysteine bridges: two near the N-terminal end and one near the C-terminal end of the protein. To confirm the presence and proper location of the cysteine bridges in asporin, we first analysed the protein on reducing and non-reducing gels. The protein migrated faster on the non-reducing gel, indicating a more compact tertiary structure imposed by cysteine bridges; also, a faint band on the reducing gel that migrated as fast as the protein on the non-reducing gel probably indicates a small fraction of asporin with re-oxidized cysteine bridges that reform in the slightly alkaline pH of the Laemmli SDS/PAGE buffer system (Supplementary Figure S1). The presence of cysteine bridges could be traced further by MALDI–TOF–MS; an asporin sample reduced with DTT (dithiothreitol), alkylated with iodoacetamide and trypsin-digested contains peptides with m/z 2700.08 and 2328.11. These correspond to the N-terminal sequences SHFFPFDLFPMC (cysteine residues are underlined), or amino acids 62–82 and 83–103 in the recombinant protein. In contrast, asporin untreated with DTT, after trypsin digestion, contains a peptide with m/z 4795.08, which covers the sequence SHFFPFDLPMCPEG-CQCYSR...VVHCSGLTSLVPTNIPFPDTR (observing the trypsin cleavage site SR...VV). This correlates well with the theoretical m/z 4794.20, showing that the two trypsin-digested peptides are interconnected with a cysteine bridge (GCQ to HCS). (The theoretical m/z was calculated with the assumptions that two cysteine bridges are present within the peptide and that the peptide is cleaved by trypsin in two parts (SR...VV) but these are covalently interconnected by one cysteine bridge (GCQ to HCS), and the second cysteine bridge exists between MCP and QCY.)

Similarly, the third cysteine bridge in asporin present near the C-terminal end would interconnect two trypsin-digested peptides VGVNDFCPTVPK and CVLRSR (amino acids 325–336 and 364–368). Indeed, in the asporin sample reduced with DTT, these two peptides are separated (m/z 1332.68 and 634.44), whereas the non-reduced asporin sample contains a peptide with m/z 1850.01 (deviation from theoretical m/z is 0.07; assuming intra-chain cysteine bridge). This shows that also the third intra-chain cysteine bridge is present in our bacterially expressed asporin (for all MS data, see Supplementary Figure S1).

### Asporin has a high affinity for collagen

To determine the binding of asporin to collagen, we used ITC. Asporin bound to collagen at K₆₅ ≈ 30 nM (Figures 1B and 1C). The collagen binding was also analysed in a solid-phase assay, using collagen-coated plates, where asporin bound with K₆₅ ≈ 4 nM (Figure 1D). These experiments were performed with three different preparations of asporin, all giving similar results. The larger K₆₅ in calorimetry may be related to the fibril formation of collagen in the conditions used in the experiment.

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**Figure 1 Expression and collagen binding of asporin**

(A) D13 asporin was expressed as a His-tagged protein in bacteria and affinity-purified on Ni-NTA Sepharose (his-tag), followed by MonoQ anion-exchange chromatography (MonoQ). Bands indicated by arrows were identified to represent asporin or fragments of it by MALDI–TOF–MS of trypsin digests. The 50 kDa band is full-length asporin. (B) ITC of asporin–collagen binding. D13 asporin (1 μM) was titrated into collagen solution (0.2 μM) in stepwise injections. Phosphate buffer (20 mM; pH 7.4) was used. The peaks show heat development for each consecutive injection of asporin. (C) Experimental details are the same as in (B) but asporin (1 μM) was titrated into a blank buffer (without collagen). (D) Solid-phase assay of asporin–collagen binding. Coating of 96-well microtitre plates with pepsin extracted collagen type I in 0.012 M HCl was performed and then the plates were blocked with non-fat dried skimmed milk powder. Asporin was added to the wells at increasing concentrations (plotted on a semi-logarithmic scale). After washing, bound protein was detected first with an anti-His antibody, followed by an alkaline phosphatase-conjugated secondary antibody, and the phosphatase substrate. Colour development was determined by measuring the absorbance at 405 nm. Results are the means±S.D. for triplicate samples.
(neutral buffer and 30°C), or to the different buffers used in the experiments. Nevertheless, the interaction between the two matrix proteins is strong in both cases.

Asporin–collagen interaction is inhibited by decorin, but not by biglycan, and the interaction leads to inhibited collagen fibrillogenesis

Asporin belongs to the class I SLRP family, which also includes decorin and biglycan. We therefore investigated whether these SLRPs could compete for collagen binding. In a solid-phase assay, decorin inhibited asporin–collagen binding with $K_i \approx 30$ nM (asporin concentration $= 20$ nM) (Figure 2A). However, asporin was a more potent inhibitor of 20 nM decorin, with $K_i \approx 10$ nM (Figure 2B). Biglycan had no effect on asporin or decorin binding to collagen, even at a 10-fold molar excess (results not shown). Finally, asporin inhibited in vitro collagen fibrillogenesis, in a dose-dependent mode, comparable with that of decorin reported earlier [12] (Figure 2C).

Binding of asporin and decorin to the same sites on collagen is likely to have a role in regulating the development of ECMs (extracellular matrices) and in modulating their physical properties and functions. We have previously described a similar competition between the class II SLRPs lumican and fibromodulin. The differential binding of fibromodulin or lumican seems to be related to the formation of progressively thicker collagen fibrils during the development of tendons [15,25].

The collagen-binding domain of asporin is located in LRRs 10–12 near the C-terminal end of the protein

To identify the collagen-binding domain(s) in asporin, we expressed four consecutive fragments of asporin in bacteria. The recombinant asporin fragments were tagged with GST to allow purification and binding analysis with GST antibodies. The proteins were AspnN-3 (spanning the N-terminus to LRR 3), Aspn4-6 (LRR 4–6), Aspn7-9 (LRR 7–9) and Aspn10-12 (LRR 10–12) (Figures 3A and 3B). Collagen binding of these fragments was determined in a solid-phase assay, using collagen-coated plates. Aspn10-12 had the highest collagen affinity with $K_d \approx 30$ nM and a sigmoidal binding curve, whereas Aspn4-6 and Aspn7-9 bound weakly (Figure 3C). Aspn-N-3, with the polyaspartic domain, had no collagen affinity (Figure 3C), showing that the polyaspartic domain can be exposed for interaction with other molecules/proteins while asporin is bound to collagen. GST alone had no collagen affinity (results not shown). Binding of full-length asporin to collagen could only be inhibited by the Aspn10-12 fragment with $K_i \approx 70$ nM, whereas the other fragments were ineffective (Figure 3D). This confirms the collagen-binding specificity of asporin LRR 10–12.

Asporin binds calcium in the polyaspartic domain

The N-terminal asporin polyaspartate sequence may bind calcium, similar to the polyaspartate in osteopontin. We analysed the binding of radioactive $^{45}\text{Ca}^{2+}$ to asporin, as well as to other SLRPs, including mammalian-expressed and glycosylated biglycan, decorin and lumican, as well as bacterial-expressed fibromodulin (without tyrosine sulfations). After SDS/PAGE (Figure 4A), the proteins were transferred on to a nitrocellulose membrane and incubated with $^{45}\text{Ca}^{2+}$. Calcium bound only to asporin and no binding to the other SLRPs was detected (Figures 4B and 4C). Notably, calcium did not bind to the N-terminally truncated 38 kDa asporin fragment that we included in the assay (arrow in Figure 4C). $^{45}\text{Ca}^{2+}$ binding to asporin was abolished by excess unlabelled calcium chloride (Figure 4D), verifying the binding specificity.

To rule out the influence of SDS on the binding, we also performed a dot-blot asporin calcium overlay assay. Increasing amounts of the protein were spotted on to a nitrocellulose membrane, and after incubation with $^{45}\text{Ca}^{2+}$, asporin showed dose–response-type calcium binding (Figure 4E). These observations indicate that the polyaspartate domain interacts with calcium, and represents one major functional difference between the homologous decorin and biglycan. It
Asporin binds collagen and regulates biomineralization

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Figure 3 Expression and collagen binding of asporin fragments

(A) Recombinant asporin fragments of consecutive parts of asporin (as depicted on the cartoon) were expressed in bacteria as GST-tagged proteins, electrophoresed on linear SDS/10% polyacrylamide gels and stained with Coomassie Blue. The gel image shows total lysate of bacteria. (B) Asporin fragments purified on glutathione affinity Sepharose and electrophoresed on linear SDS/10% polyacrylamide gels and stained with Coomassie Blue. For both (A) and (B), molecular masses in kDa are indicated on the left-hand side. (C) Solid-phase assay of collagen binding by asporin fragments. Coating of 96-well microtitre plates with pepsin extracted collagen type I in 0.012 M HCl was performed, the plates were then blocked with non-fat dried skimmed milk powder, and proteins were added to the wells at increasing concentrations (plotted on a semi-logarithmic scale). AspnN-3 (△), Aspn4–6 (■), Aspn7–9 (▲) and Aspn10–12 (○) were tested. After washing, the binding was detected with an anti-GST antibody, followed by an alkaline phosphatase-conjugated secondary antibody, and phosphatase substrate. Colour development was determined by measuring the absorbance at 405 nm. Results are the means±S.D. for triplicate samples. (D) Solid-phase assay of the inhibition of asporin–collagen binding by asporin fragments. The assay was performed as in (C) but asporin (20 nM) was incubated with increasing concentrations of asporin fragments: AspnN-3 (△), Aspn4–6 (■), Aspn7–9 (▲) and Aspn10–12 (○). Binding of asporin was detected with an anti-His antibody, an alkaline phosphatase-conjugated secondary antibody and a phosphatase substrate.

Asporin, but not other SLRPs, induces collagen mineralization driven by an osteoblast cell line MG63 and triggers increased levels of osteoblastic transcription factors

We investigated how asporin influences the calcification of collagen in vitro, since asporin has both collagen-binding and calcium-binding properties. Also, asporin is expressed in tissues where osteoblast progenitor cells reside and migrate into the mineralizing tissue while differentiating to an osteoblastic phenotype.

We used MG63 cells that differentiate into an osteoblastic phenotype after treatment with dexamethasone and calcium chloride [23,24]. Under these conditions, the cells form distinct bone-like nodules that stain with Alizarin Red, indicating the presence of calcium. To analyse the possible effects on nodule formation of the presence of asporin, we supplemented the dexamethasone-induced cells with increasing concentrations of asporin. This supplementation accelerated the nodule formation and nodule size already at a low concentration (10 nM) with a potentiating effect at higher concentrations; 100 nM induced an approx. 10-fold higher amount of nodules compared with the dexamethasone-induced control (Figure 5A). Compared with asporin, other SLRPs such as decorin, biglycan, fibromodulin or lumican had no effect on nodule formation during 1 week in culture (results not shown).

We also analysed selected markers of an osteoblastic phenotype in the cell cultures. The mRNA levels of the osteoblast transcription factors Osterix and Runx2 were higher in the presence of asporin (Figure 5B).

Lastly, we tested if asporin-induced calcification could be inhibited by decorin (Figure 5C) or the collagen-binding asporin fragment Aspn10–12 (Figure 5D). Both proteins efficiently reduced the number of asporin-induced calcified deposits: decorin at an approx. 5-fold higher concentration and Aspn10–12 at an approx. 10-fold higher concentration than asporin. Other asporin fragments (AspnN-3, Aspn4-6 and Aspn7-9) did not inhibit the calcification process, and none of the tested inhibitors had any calcification-promoting effects (results not shown).

Asporin is a potent inducer of collagen mineralization, and may be implicated in the progression of certain pathological conditions

We show that asporin has two, previously unknown, functional domains: an N-terminal calcium-binding polyaspartate domain and a C-terminal collagen-binding domain. These two domains appear to work in concert to trigger biomineralization of collagen. This is a unique function of an SLRP, and from what is reported so far, asporin is the only SLRP able to regulate collagen
calcification. This function is similar to other polyacidic domain proteins, including osteopontin and bone sialoprotein (or polyaspartic peptides) that can bind calcium and influence hydroxyapatite crystal formation in collagen [7,8,10,26]. However, we should point out that some SLRP glycosylation patterns that vary in different tissues and age may, due to altered acidic properties, gain calcium-binding properties under certain conditions.

Asporin–collagen binding and asporin-induced collagen mineralization can be inhibited by decorin (Figures 2A and 5C) that binds near to the gap region of collagen [27]. Decorin inhibits collagen mineralization [28], but gains an inverse, calcification-promoting effect when the collagen-binding peptide of decorin is coupled with a polyacidic sequence of bone sialoprotein [29]. Therefore we suggest that asporin (binding, as decorin, close to the gap region of collagen) directly regulates the initial deposition of hydroxyapatite in the collagen gap regions, rather than acting secondarily by inhibiting BMP-2 or TGF-β activity, as reported previously [5,17]. In this context, it has to be considered that TGF-β binds to many other matrix proteins, including decorin and fibromodulin [30], and the much more abundant collagen [31] and fibronectin [32], which questions the physiological relevance of asporin as a TGF-β inhibitor.

The role of asporin in biomineralization is consistent with its expression pattern found in osteoblast progenitor cells [16]. These cells regulate intramembranous bone formation, and are also involved in healing of bone fractures. Similarly, asporin may be one of the key proteins implied in calcification of blood vessels, some tumours like breast adenocarcinoma, or in osteoarthritis.

**Figure 4 Calcium binding by asporin and other SLRPs**

(A) SDS/PAGE (10% linear gels), stained with Coomassie Blue, of recombinant proteins used in the assay. (B) The proteins from (A) were blotted on a nitrocellulose membrane, the membrane was overlaid with 45Ca2⁺ (1 μCi/ml) for 10 min and washed with 50% (v/v) ethanol, and the isotope was detected by radiography. (C) Overlay of (A) and (B). Arrow points to the 38 kDa fragment of asporin that does not bind calcium. (D) Experimental details are the same as in (B) but with addition of non-radioactive calcium chloride (10 mM) as a competitor. (E) Dot-blot of asporin and calcium binding. Protein in increasing amounts was spotted on a nitrocellulose membrane that was overlaid with 45Ca2⁺ (1 μCi/ml) and treated as in (B).

**Figure 5 Collagen calcification assay**

(A) MG63 cells were cultured in EMEM with 10% heat-inactivated fetal calf serum, supplemented with dexamethasone (100 nM) and calcium chloride (6 mM). After 2 days, nodules were observed (arrows) that stained with Alizarin Red. Scale bar, 50 μm. Cells were cultured with different concentrations of D13 asporin (50 or 100 nM). Nodule formation was quantified in duplicate wells in a six-well plate and is shown in the graph. (B) Quantitative PCR for Osterix and Runx2 transcripts in cells cultured with asporin. The control was cell culture without asporin. The scatter plot represents values from duplicate biological samples with further duplicated technical samples. (C) Cells were cultured with 20 nM asporin (control, left panel) or with 200 nM Aspn10-12 (right image). After 2 days, cells were stained with Alizarin Red for visualization of calcified nodules; quantities are inserted in the pictures. Scale bar, 50 μm. (D) Cells were cultured with 20 nM asporin (control, left image) or with 20 nM asporin and 200 nM Aspn10-12 (right image). After 2 days, cells were stained with Alizarin Red for visualization of calcified nodules; quantities are inserted in the pictures. Scale bar, 50 μm.
AUTHOR CONTRIBUTION
Sebastian Kalamajski designed and performed the research, analysed the data and wrote the paper. Åke Oldberg designed and performed the research, analysed the data and wrote the paper. Karin Lindblom performed the research and analysed the data. Dick Heinegard designed the research, analysed the data and wrote the paper.

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SUPPLEMENTARY ONLINE DATA

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PROTEIN SEQUENCE

MALDI-TOF SPECTRUM ON REDUCED AND ALKYLATED TRYPSIN-DIGESTED ASPORIN

MALDI-TOF SPECTRUM ON NON-REDUCED TRYPSIN-DIGESTED ASPORIN

PURIFIED RECOMBINANT ASPORIN run on 10% linear SDS-PAGE reducing and non-reducing gels stained with Coomasie R-250.

Figure S1 Protein sequence of asporin with intra-chain cysteine bridges, and analysis by SDS/PAGE and MALDI–TOF–MS

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