Influences of the N700S Thrombospondin-1 Polymorphism on Protein Structure and Stability*\textsuperscript{1,2}

C. Britt Carlson\textsuperscript{1}, Yuanyuan Liu, James L. Keck, and Deane F. Mosher\textsuperscript{2}

From the Departments of Medicine and Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706

Thrombospondins (THBSs)\textsuperscript{3} are multimodular, secreted proteins characterized by a signature domain comprising a unique set of 13 calcium-binding repeats flanked by epidermal growth factor (EGF)-like and lectin-like modules. A polymorphism that changes a conserved Asn to Ser at residue 700 in the most N-terminal calcium-binding repeat of THBS-1 (repeat 1C) is found in 8–10% of European populations and has been linked to increased risk of premature coronary artery disease. The Ser substitution leads to altered stability in the EGF-like and wire modules of the THBS-1 signature domain as assessed by differential scanning calorimetry carried out in 2 mM or 200 \textmu M calcium. Studies of the melting profiles of the THBS-2 signature domain proteins with Asn or Ser at position 702 (homologous to 700 in THBS-1) revealed that the impact of the Ser allele is similar in both THBS-1 and THBS-2. Structure determination of the Ser\textsuperscript{702} THBS-2 variant in 2 mM calcium showed that repeat 1C contains two bound calcium ions as in the crystal of the Asn\textsuperscript{702} protein, including the ion that is coordinated by Asn\textsuperscript{702}, and is associated with changes in conformation of repeat 1C and the adjacent EGF-like modules. The Ser substitution leads to the decreased ability of soluble THBS-2 signature domain protein to bind 4B6.13, a conformation-sensitive monoclonal antibody that recognizes an epitope in repeat 1C. These results indicate that although THBS harboring the Ser allele binds a full complement of calcium ions, repeat 1C is altered, leading to destabilization of surrounding structures.

Thrombospondins (THBSs)\textsuperscript{3} are secreted metalloglycoproteins found in organisms as diverse as \textit{Drosophila}, \textit{Ciona}, and\textsuperscript{*}\textsuperscript{1}

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\textsuperscript{3}The atomic coordinates and structure factors (code 2RPH) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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\textsuperscript{2}To whom correspondence should be addressed: 4285 Medical Sciences Center, 1300 University Ave., University of Wisconsin, Madison, WI 53706. Tel.: 608-262-1576; Fax: 608-263-4969; E-mail: dfmosher@wisc.edu.

\textsuperscript{3}The abbreviations used are: THBS, thrombospondin; EGF, epidermal growth factor; r.m.s. deviation, root mean square deviation; COMP, cartilage oligomeric matrix protein; ELISA, enzyme-linked immunosorbent assay; DSC, differential scanning calorimetry; TBS, Tris-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.
ture of the THBS-1 and THBS-2 signature domains, these genetic changes map to the wire and lectin-like modules (4, 5).

The polymorphic residue in THBS-1 (N700S) associated with premature coronary artery disease is at position 10 of wire repeat 1C (Fig. 1C) near the interaction sites among EGF3, the wire, and the lectin-like module (5). Remarkably, position 10 of all 13 wire repeats of all five vertebrate THBSs is an Asn or Asp (2, 5). Previous experiments of THBS-1 constructs revealed that the Ser-containing proteins were more sensitive to heat (12) and proteolysis (6). A study of the effects of calcium ions on THBS-1 constructs containing EGF3 and the wire revealed that a change in calcium concentration is associated with a change in the fluorescence of Trp698. This transition occurs at higher calcium concentrations and with greater cooperativity in the Ser700 protein than the Asn700 protein (13). The homologous residue in THBS-2, Asn702, coordinates a calcium ion through a water molecule (Fig. 1, B and 1 C), a finding consistent with the altered kinetics of a calcium-induced conformational change associated with the Ser700 allele (5, 13). It is not known whether the Asn to Ser change in THBS-1 causes a loss of a calcium-binding site or changes after filling of the site. It is also not known how the Ser allele impacts stability and structure of specific parts of the protein, which could lead to the changes in protein function implied in the link to disease.

In this study, we sought to answer these questions about the structural consequences of the THBS-1 N700S single nucleotide polymorphism by investigating the changes in thermal stability of the THBS-1 signature domain and investigating the homologous N702S change in the signature domain of THBS-2, for which, unlike THBS-1, there is a crystal structure (5) and an informative conformation-sensitive monoclonal antibody (14). We found that the presence of the Ser allele alters the structure and stability of wire repeat 1C and the EGF-like modules, compared with the Asn-containing proteins.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning**—The pAcGP67.coco baculovirus transfer vector encoding a secretion signal peptide 5’ to the cloning site followed by DNA encoding a short linker and six-histidine tag was used to enable baculovirus-driven protein expression, secretion, and subsequent purification (15). Signature domain THBS-2 comprises residues 551–1172 with ADP and ARGHH-HHHHH N- and C-terminal tails. The Ser700 allele was introduced by PCR mutagenesis into DNA encoding the signature domain THBS-2. Correct orientation and sequence of PCR-amplified DNA were verified by sequencing. Asn700 and Ser700 THBS-1 signature domain proteins (residues 549–1170 with ADP and NAAGHHHHHHH N- and C-terminal tails), 4C-1, the lectin-like module with 10 adjacent wire repeats protein (residues 775–1170 ADP and NAGHHHHHHH N- and C-terminal tails), EGF3-Wire-1, the third EGF-like module with the calcium-binding wire protein, (described previously (12)), and EGF123-1, the three tandem EGF-like modules protein (residues 550–690 with ADP and ALEVPRGSAAGHHHHHHH N- and C-terminal tails) were constructed in a similar fashion.

**Expression and Purification of Recombinant Proteins**—The Asn702 and Ser702 THBS-2 proteins were expressed by infecting High Five insect cells in SF900II serum-free medium (Invitrogen) at 27 °C with high titer virus (>1 E10⁶ plaque-forming units/ml) at a multiplicity of infection of ~5. Conditioned medium was collected ~65 h post-infection. Histidine-tagged proteins were purified from the medium in the presence of 2 mM CaCl₂ using Ni²⁺-nitrilotriacetic acid resin (Qiagen) and, if necessary, an anion-exchange HiTrap Q HP column (GE Healthcare), as described previously (5). Protein concentration was determined using absorbance at 280 nm less the absorbance at 320 nm and divided by the calculated extinction coefficient of 1.24 ml mg⁻¹ cm⁻¹ (THBS-2 proteins), 1.2 ml mg⁻¹ cm⁻¹ (THBS-1 signature domain proteins), 1.33 ml mg⁻¹ cm⁻¹ (4C-1), 2.42 ml mg⁻¹ cm⁻¹ (E123-1), or 0.81 ml mg⁻¹ cm⁻¹ (EGF-Wire-1) (16).

**Differential Scanning Calorimetry (DSC)**—DSC experiments were performed at the University of Wisconsin Biophysics Instrumentation Facility using a Microcal VP-differential scanning calorimeter equipped with Origin 7 software. Proteins (8.3–25 μM) were dialyzed into 10 mM MOPS, 150 mM NaCl, 2 mM or 200 μM CaCl₂, pH 7.5. Scans were conducted from 15 to 95 °C at a rate of 60 °C h⁻¹, except EGF123-1, which was scanned from 15 to 85 °C. Appropriate buffer scans were used for reference subtraction and data were normalized using protein concentrations, including an estimated 2% dilution upon introduction into the DSC sample cell. Area-fitting analyses were performed with the DSC Origin 7 software package. Manual-fit baselines were subtracted from sample scans that were corrected for reference sample and concentration. This curve was then fitted to a non-2-state model with various numbers of peaks. Fits were refined using several iterations until the χ² value stabilized.

**Enzyme-linked Immunosorbent Assay (ELISA)**—Asn702 and Ser702 THBS-2 signature domain protein were coated onto 96-well microtiter plates at 10 μg ml⁻¹ in Tris-buffered saline (TBS) containing 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, plus 2 mM CaCl₂. The plates were blocked with 5% bovine serum albumin in TBS plus 0.05% Tween-20 (TBST). The monoclonal antibody 4B6.13 (14) was diluted in TBST containing various concentrations of CaCl₂ and then incubated with the plate for 2 h. Alkaline phosphatase-conjugated secondary antibody was added for a 1-h incubation. Sigma 104 AP substrate at 1 mg ml⁻¹ in TBS, pH 9.0, was added to each well and color development was monitored at 405 nm. As a control, we used polyclonal rabbit anti-human THBS-2 antibodies (18) to confirm that antigen protein was adsorbed to the plate and present at similar concentrations. The proteins were tested in competition ELISA as described (14) to assess if soluble antigen was able to compete with surface-adsorbed antigen for binding of the monoclonal antibody. All ELISA experiments were repeated, each with triplicate data points, on multiple occasions. Data are expressed as the mean of the different experiments ± S.E.

**Protein Crystallization**—The Ser702 THBS-2 signature domain was crystallized using the hanging drop vapor diffusion method at room temperature. Protein was first exchanged into 5 mM MOPS, pH 7.5, 2 mM CaCl₂, 0.1 mM sodium acetate. Protein was then concentrated to 7–10 mg ml⁻¹ using Ultrafree-0.5 centrifugal filter units (Millipore). Drops were made from 0.9 μl of protein mixed with 0.9 μl of mother liquor (100 mM Tris-Cl, pH 8.5–9.0, 200 mM sodium acetate, 30% polyethylene glycol
4000) and 0.1 μl of 1 M glycine. Crystal formation was facilitated by streak seeding from Asn702 THBS-2 signature domain crystals (5). Prior to data collection, crystals were stabilized by transfer to a cryoprotectant solution of the above mother liquor conditions supplemented with 25% ethylene glycol and then frozen directly in liquid nitrogen.

**Protein Crystal Data Collection and Analysis**—Data were collected using a Proteum CCD detector with x-rays generated by a Microstar rotating anode (Bruker AXS, Madison, WI). Data were indexed and scaled using HKL2000 (19). The structure was solved to 2.9-Å resolution using AMORE (20) for molecular replacement with the Asn702 THBS-2 signature domain protein as a search model (5) (Table 1). Refinement with REFMAC5 (21) produced interpretable 2Fo − Fc and Fo − Fc electron density maps. The model was improved by rounds of refinement with REFMAC5 and manual rebuilding using O (22) and Coot (23) to a final R_free of 22.7% (R_free = 27.7%, refer to Table 1). There was one molecule per asymmetric unit. 73.7% of all residues fall in the most favored Ramachandran category, with 23.8% in the allowed category, 2.1% in the generously allowed category, and 0.4% in the disallowed category. Superpose and root mean squared analyses of Ca carbons were calculated using the CCP4 suite of programs (21).

**Coordinates**—Model coordinates and structure factors have been deposited in the Protein Data Bank (code 2RHP).

**RESULTS**

The Polymorphism Leads to Altered Thermal Stability of the EGF-like Modules and the Wire—To examine the impact of the Ser allele on stability of the signature domain and its specific parts, we examined the thermal melting profiles of THBS-1 and THBS-2 proteins having Asn or Ser at the position of the THBS-1 polymorphism (700 of THBS-1 and 702 of THBS-2).

The DSC profile of the Asn700 THBS-1 signature domain in 2 mM calcium had four apparent melting events at 46.1, 53.7, 72.1, and 88.6 °C (Fig. 2A). The first two peaks were not reversible upon cooling and reheating, whereas the second two peaks were reversible (not shown). To identify the origins of these peaks, we studied various truncations of the THBS-1 signature domain (Fig. 2A). A construct comprising the three tandem EGF-like modules of THBS-1 (EGF123-1) melted reversibly with one peak at 68.0 °C. The EGF3-wire construct (EGF3-wire-1) melted reversibly with one peak at 87 °C. Because secretion of the lectin-like module requires a portion of the wire that mediates the wire repeat 9C-lectin interaction (4), we examined a construct containing the lectin-like module and portion of the calcium-binding wire that includes repeats 4C to 13C (4C-1), including the wire repeat 9C-lectin interface (Fig. 1A). This protein melted with a non-reversible peak at 47.5 °C, followed by the beginning of a second peak at >90 °C, which was reversible. Alignment of the DSC curves (Fig. 2A) suggests that the first or second peak of the signature domain likely represents the melting of the wire repeat 9C-lectin interface, and the third and fourth peaks likely represent the melting of the EGF-like modules and the wire. Based on previous studies of the THBS-2 signature domain constructs (24) (see below), we suspect that the other of the first two peaks represents melting of the wire repeat 1C-lectin interface.

Compared with the Asn700 THBS-1 protein, the Ser700 THBS-1 protein also had two non-reversible melting events at 47.3 and 53.8 °C, but instead of the 72 and 88.6 °C peaks, there was a broad two-part reversible peak from 75 to 95 °C (Fig. 2A). Thus, the reversible high temperature melting events attributed to the EGF-like and wire modules are impacted by the amino acid substitution, whereas the non-reversible low temperature events are not.

Previous studies with a THBS-1 construct comprising EGF3 and the wire demonstrated that the Ser allele is more susceptible to heat denaturation in 200 μM calcium, compared with the Asn-containing protein (12). To examine if this is also seen in the signature domain protein, the thermal stability of Asn- and Ser-containing proteins were examined at 200 μM calcium, using DSC. At 200 μM calcium, the Asn700 protein melted with two apparent peaks at 43.5 and 69.4 °C. The Ser700 protein melted with an apparent three-part peak from 40 to 70 °C (Fig. 2B). Lowering the calcium concentration to 200 μM, therefore, lowers the temperatures of the reversible transitions and, as in 2 mM calcium, the Ser700 protein was more sensitive to heating than the Asn700 protein.

The four peaks seen in the THBS-1 proteins contrasts with DSC studies on the signature domain of THBS-2, which melts with two peaks (24, 25) (Fig. 2C). The first peak (non-reversible) of the THBS-2 protein has been assigned to the melting of the lectin-wire interfaces and the second peak (reversible) has been assigned to the melting of the wire itself (24, 25). To test the possibility that the two THBS-2 peaks are each compound transitions comprising two melting events, we used area-fitting analyses assuming a non-two-state model based on the non-reversible status of the low temperature events. We fit the Asn702 THBS-2 melting profiles to a four-event model and a two-event model. The Asn702 THBS-2 curve modeled as two events (51.0 and 78.8 °C) (Fig. 2D), compatible with the hypothesis that the transitions seen for both THBS-1 and THBS-2 proteins are reporting four (or more) events.

The DSC profile of the THBS-2 signature domain with the homologous Asn to Ser change, N702S, also fit best modeled as four events. When modeled as two events (50.0 and 77.6 °C), the Ser702 THBS-2 curve had a χ² value of 2.3 × 10⁴ (not shown) that decreased to 7.5 × 10⁴ when modeled as four events (50.0, 51.0, 66.5, and 78.8 °C) (Fig. 2E). However, the melting events are altered in the Ser702 protein, compared with the Asn702 protein (Fig. 2C). The largest changes are seen in the reversible high temperature events where the Asn702 82.7 °C event is shifted to 78.8 °C in the Ser702 protein, and the Asn702 78.8 °C event is shifted to 66.5 °C in the Ser702 protein. Smaller changes are seen in the nonreversible low temperature events, where the Asn702 53.1 °C event is shifted to 51.0 °C in the Ser702 protein and the Asn702 51.0 °C event is shifted to 50.0 °C in the Ser702 protein. Thus, the major changes in the DSC pattern due to Ser702 are of the reversible high temperature events, and as with the THBS-1 protein, indicate changes in stability of the EGF-like and wire modules.

**Mutation of Asn to Ser Does Not Lead to a Loss of Calcium**—A possible structural explanation for observed changes in protein
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stability in the Ser-containing proteins is that the Ser<sup>700</sup> allele and Ser<sup>702</sup> mutant are associated with a loss of a bound calcium ion. Residue 702/700 is located at position 10 of the consensus sequence found in both C-type and N-type wire repeats (Fig. 1C). Asp or Asn are invariably present at this position, and the residue coordinates calcium directly or through a water molecule, (Fig. 1, B and C) (5). The 76% identity between the sequences of THBS-1 and THBS-2 signature domain proteins and the changes in kinetics of the conformational transition induced by calcium and reported by adjacent Trp<sup>698</sup> in Ser<sup>700</sup> THBS-1 proteins (13) argue for a similar calcium-binding role for Asn<sup>700</sup> in THBS-1 (Fig. 1C).

To examine the impact of the N700S polymorphism on calcium coordination, we first attempted to study the Asn<sup>700</sup> and Ser<sup>700</sup> THBS-1 signature domain proteins by crystallography. Our attempts to crystallize the Asn<sup>700</sup> signature domain of THBS-1, including the C992S derivative used to stabilize and crystallize a portion of the THBS-1 signature domain (4), were unsuccessful. We therefore addressed this question by solving the crystal structure of the Ser<sup>702</sup> THBS-2 signature domain in 2 mM calcium. The Ser<sup>702</sup> protein crystallized under the same conditions used for the Asn<sup>702</sup> protein (5) used streak seeding of the Asn<sup>702</sup> crystals to nucleate growth. Ser<sup>702</sup> crystals grew to a smaller size than Asn<sup>702</sup> protein, and were solved at a maximum resolution of 2.9 Å (Table 1), as compared with 2.6 Å for the Asn<sup>702</sup> protein (5).

Electron density for all 30 of the calcium ions, including the ion coordinated by Asn<sup>702</sup> (Figs. 3, A and B) are present in the Ser<sup>702</sup> variant, indicating that the Asn to Ser amino acid change does not result in a loss of calcium coordination. The Ser<sup>702</sup> side chain is present in the same space occupied by the Asn<sup>702</sup> side chain, although strong density for the terminal oxygen of Ser<sup>702</sup> is lacking, which indicates a dynamic structure for the residue. The electron density for the coordinating water molecule seen in the Asn<sup>702</sup> structure.

structure is not visualized in the Ser<sup>702</sup> structure. Bond lengths and geometries between the calcium and remaining coordinating residues (Asp<sup>694</sup>, Asp<sup>696</sup>, Asp<sup>698</sup>, Trp<sup>700</sup>, and Asp<sup>718</sup>) are very similar to those of the Asn<sup>702</sup> protein.

The Ser<sup>702</sup> Mutation Leads to Altered Structure—Superposition of the Asn<sup>702</sup> and Ser<sup>702</sup> THBS-2 signature domain structures showed an overall root mean square deviation (r.m.s. deviation) value of 0.31 Å for all 621 Ca atoms (Fig. 3, C and D).
This analysis revealed that repeat 1C (36 Ca atoms) has a modestly higher r.m.s. deviation value than the entire wire module (264 Ca atoms) (rmsd_{wire1C} = 0.34 Å, rmsd_{wire} = 0.29 Å). The lectin-like module (215 Ca atoms) had an overall r.m.s. deviation of 0.18 Å, in contrast to the EGF-like modules (142 Ca atoms), which had an overall r.m.s. deviation of 0.40 Å.

Hot-spots of differences lay at the EGF1-EGF2 and EGF2-wire interfaces (supplemental Fig. S1). Specifically, at the EGF2-wire interface, the Ala603–Pro608 loop, which extends down to contact the wire repeat 10N-13C hairpin turn, shows substantial movement, as evidenced by relatively high r.m.s. deviation values (average 0.77 Å) and weak electron density in the Ser702 crystal (supplemental Fig. S2). In addition, there were other distant sites where r.m.s. deviation values were relatively high (Fig. 3D). All of these residues are surface-accessible (supplemental Fig. S1), and some contact with symmetry-related molecules in the crystal lattice. No increases in r.m.s. deviation values were observed at sites of intermodular interactions between the wire and lectin-like modules.

Because residues in wire repeat 1C make close contacts with a neighboring protein molecule in crystals of Asn702 and Ser702 proteins, which could influence conformation in the crystal, we probed the structures of adsorbed and soluble THBS-2 signature domains with 4B6.13, a conformation-sensitive monoclonal antibody that requires calcium and the presence of EGF3, the wire, and the lectin-like module of human THBS-2 (14) for binding. This antibody recognizes residues Leu703 and His722, which are proximal to the site of the N702S amino acid change (5). The side chains of Leu703 and His722 are slightly different in the two crystals (Fig. 3C). Both the Ca atom and the side chain of Leu703 are shifted in the Ser702 THBS-2 structure relative to the Asn702 THBS-2 structure. The Ca atom of His722 of Ser702 THBS-2 overlays well with the Asn702 THBS-2 His722, but the side chain is rotated slightly. The Leu703–His722 Cβ distance is 13.9 Å, instead of the 13.5 Å distance seen in the Asn702 protein.

As previously described (14), 4B6.13 binding to Asn702 protein transitioned from 0 to 100% binding within a range of 140–200 μM calcium with a midpoint at 150 μM. In contrast, the Ser702 protein did not bind 4B6.13 at any calcium concentration tested (Fig. 4A). Direct ELISAs performed with polyclonal antibodies confirmed similar levels of Asn702 and Ser702 adsorption to the ELISA plates (not shown). To avoid possible effects in the direct ELISA due to altered protein conformation upon adsorption to the ELISA plates, competition ELISAs were performed in 2 mM calcium. The Asn702 signature domain competed with recombinant full-length THBS-2 for binding to 4B6.13, with half-inhibition at 0.1 μM (Fig. 4B). The Ser702 signature domain competed with
recombinant full-length THBS-2 for 4B6.13 binding minimally and only at high concentrations. For 20% inhibition, greater than 100-fold higher concentrations of the Ser702 protein than the concentration of the Asn702 signature domain protein was required (Fig. 4B). The fact that the Ser702 THBS-2 protein does compete for 4B6.13 binding, however, suggests that there may be a subpopulation (~1%) of the protein that is in the conformation recognized by 4B6.13.

DISCUSSION

The postulated roles of THBSs are many, yet the functions of these unusual calcium-binding proteins remain enigmatic. Knock-out mice have been developed for Thbs-1 (26), Thbs-2 (27), Thbs-3 (28), and COMP (THBS-5) (29). In addition, Thbs-4 knock-out mice have been produced commercially. Each of the knock-out strains has only slight abnormalities, and all strains survive to adulthood and reproduce. The mild phenotypes seen with these animals lacking individual THBSs and the observations that mutations or polymorphisms of THBSs are associated with disease in heterozygotes suggest that the role of genetically altered THBS-1 and THBS-5/COMP in diseases is a result of having disrupted THBSs rather than an absence of THBS activities.

We used the THBS signature domain to study the effects of an Asn to Ser amino acid change resulting from the premature coronary artery disease-associated polymorphism of THBS-1. The Asn700 THBS-1 signature domain melts with four peaks in DSC. Using smaller constructs of the signature domain, we attributed each of these peaks to the melting of specific parts of the signature domain. EGF123-1 reversibly melts at 68.0 °C and likely corresponds to the reversible peak seen at 72.1 °C in the complete signature domain. Past studies have shown that the addition of EGF-like modules can stabilize the wire, thus increasing the melting temperature in the longer protein constructs (24, 25). Deconstruction of the THBS-2 DSC profile indicates that the THBS-2 signature domain melts with two compound peaks, and suggests that the first THBS-2 peak corresponds to the first two peaks of THBS-1, whereas the second THBS-2 peak corresponds to the second two peaks of THBS-1. The four peaks seen in the THBS-1 signature domain and deconvoluted

TABLE 1
Crystallography statistics
Highest resolution shell (2.97-2.90 Å) is indicated in parentheses.

| Data collection       |       |
|-----------------------|-------|
| Space group           | I\textsubscript{2}22 |
| Cell dimensions       | 93.89 122.65 155.38 |
| a, b, c (Å)           | 90.00 90.00 90.00 |
| α, β, γ (°)           | 1.54   |
| Wavelength (Å)        | 80-2.9 (2.97-2.90) |
| Resolution (Å)        | 0.137 (0.372) |
| R<sub>sym</sub> (I/σ(I)) | 9.7 (3.0) |
| Completeness (%)      | 98.1 (99.6) |
| Redundancy            | 6.8 (6.8) |
| Refinement            |       |
| Resolution (Å)        | 20-2.9 |
| Cut-off (σ(F))        | None used |
| No. reflections       | 18,868 |
| R<sub>_meas</sub>/R<sub>_free</sub> | 22.7/27.7 |
| B-factors             |       |
| Protein               | 37.9   |
| Ligand/ion            | 50.0   |
| Water                 | 34.0   |
| Wilson                | 63.2   |
| R.m.s. deviations     |       |
| Bond lengths (Å)      | 0.006  |
| Bond angles (°)       | 1.052  |

![Figure 3. Electron density and superposition analysis of Asn702 and Ser702 THBS-2 signature domain proteins.](image)
addition of 2 mM calcium to the Asn700 and Ser700 proteins, although the Ser700 protein exhibited differences in the titration in the range of 50–150 μM calcium (12, 13). The quenching, which likely is tied to the Trp698 main chain coordination of the same calcium ion that is coordinated by the Asn700 side chain (5), is highly cooperative with a Hill coefficient of 3.5–5.0 (12). Effects of calcium on a set of truncations indicate that adjacent repeats 2N, 3C, and 4C interact allosterically with the repeat 1C (13). The Trp698 quenching experiments and the crystallographic results with the THBS-2 constructs, therefore, are consistent in indicating that the expected two calcium ions bind to wire repeat 1C at calcium concentrations approaching 2 mM.

Disruption of 4B6.13 binding to the Ser702 protein and differences between the Asn700 and Ser702 THBS-2 signature domain crystal structures indicated a change in local structure upon introduction of the polymorphic allele. Residue 702 is located next to Leu703, which along with His722, is required for formation of the epitope for 4B6.13 (14). Because of the proximity of residue 702 to the 4B6.13 epitope comprising residues 703 and 722, the decreased ability of the Ser702 THBS-2 protein to bind 4B6.13 could conceivably be due to a contribution of the Asn702 side chain to the 4B6.13 epitope. However, although part of the Asn main chain is surface accessible, the side chain is buried in the structure of the calcium-replete THBS-2 signature domain (5). Thus, we think that Asn702 does not contribute directly to the epitope, but changes the conformation of Leu703 and His722 as shown in Fig. 2C. Supporting this hypothesis, preliminary studies of a THBS-2 signature domain variant in which Leu697 of repeat 1C, which is also not on the surface, is changed to Pro, thus mimicking the L272P mutation in THBS-5/COMP associated with pseudoachondroplasia (31), show that this amino acid change also leads to a dramatic decrease in 4B6.13 binding (data not shown).

Although crystal lattice contacts may suppress or enhance effects due to the Asn to Ser amino acid change, we can make tentative conclusions based on comparing the modest resolution Asn702 and Ser702 THBS-2 signature domain structures. Relatively high r.m.s. deviation values point to alterations in individual surface residues throughout the protein, particularly in wire repeat 1C and the EGF-like modules. Of note, the N700S polymorphism is immediately adjacent to the 13-residue insert that is found in repeat 1C of THBS-1 and THBS-2 (Fig. 1, B and C). The insert interacts extensively with EGF3.

The changes seen in surface residues and in the stability of the EGF-like modules shown by DSC suggest that long-range effects are propagated N terminally. This may extend outside the signature domain, and result in changes in the heparin-binding properties of Asn700 and Ser700 THBS-1, which are believed to be occurring at the N terminus of the molecule (6). A possible mechanism for increased risk for coronary artery disease, therefore, is that structural changes caused by the Ser702 allele impact the entire protein and its function. The fact that the Ser700 allele has a prevalence of 8–10% of Europeans (7, 32) suggests, however, that earlier in the history of this population, such structural and functional alterations conferred an evolutionary advantage.

The current results provide insight into the pathology of the THBS-5/COMP mutations. Many of the THBS-5/COMP
mutations change residues that directly coordinate calcium (11). Structural studies using THBS-5/COMP constructs containing disease-associated mutations have illustrated the diversity of effects on protein structure and calcium-binding induced by the mutations (33–37). As previously stated, Asn<sup>700</sup> of the THBS-1 protein and Asn<sup>702</sup> of THBS-2 occurs at position 10 in repeat 1C of the wire. Interestingly, there are three mutations at position 10 in THBS-5/COMP (D310V in repeat 2N and D479H/D479Y in repeat 11C) associated with disease (38). Similar to Asn<sup>702</sup>, the THBS-2 residues at these positions, Asp<sup>738</sup> and Asp<sup>907</sup>, coordinate a calcium ion through a water molecule. However, Asp<sup>738</sup> and Asp<sup>907</sup> also coordinate an additional calcium ion; Asp<sup>738</sup> provides main chain coordination for the calcium ion found between repeats 2N and 3C, and Asp<sup>907</sup> provides side chain coordination for the calcium ion found between repeats 10N and 11C. The THBS-5/COMP mutations result in severe phenotypes leading to multiple epiphysyal dysplasia or pseudoachondroplasia skeletal dysplasias. The discrepancies between the presumed advantageous effect of the Ser<sup>700</sup> THBS-1 polymorphism (in wire repeat 1C) that led to its prevalence in European populations and the disease caused by the mutation at homologous residues in wire repeats 2N and 11C in THBS-5/COMP may be because the polymorphic residue is involved in the coordination of only one calcium ion, whereas the mutated THBS-5/COMP residues coordinate two calcium ions.

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