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A Polymorphism in Thrombospondin-1 Associated with Familial Premature Coronary Heart Disease Causes a Local Change in Conformation of the Ca^{2+}-binding Repeats*

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A single nucleotide polymorphism that substitutes a serine for an asparagine at residue 700 in the Ca^{2+}-binding repeats of thrombospondin-1 is associated with familial premature coronary heart disease. We expressed the Ca^{2+}-binding repeats alone (Ca) or with the third epidermal growth factor-like module (E3Ca), without (Asn-700) or with (Ser-700) the disease-associated polymorphism. The intrinsic fluorescence of a single tryptophan (Trp-698) adjacent to the polymorphic residue was quenched cooperatively by adding Ca^{2+}. The third epidermal growth factor-like repeat dramatically altered the Ca^{2+}-dependent fluorescence transition for the Asn-700 constructs; the half-effective concentration (EC_{50}) of Ca Asn-700 was 390 μM, and the EC_{50} of E3Ca Asn-700 was 70 μM. The Ser-700 polymorphism shifted the EC_{50} to higher Ca^{2+} concentrations (Ca Ser-700 EC_{50} of 950 μM and E3Ca Ser-700 EC_{50} of 110 μM). This destabilizing effect is due to local conformational changes, as the Ser-700 polymorphism did not influence the secondary structure of E3Ca or Ca as assessed by far UV circular dichroism. At 200 μM Ca^{2+}, in which both E3Ca Asn-700 and Ser-700 are in the Ca^{2+}-replete conformation at 37 °C, the fluorescence of E3Ca Ser-700 reverted to the Ca^{2+}-depleted spectrum at 50 °C compared with 65 °C for E3Ca Asn-700. These findings indicate that the Ser-700 polymorphism subtly but significantly sensitizes the calcium-binding repeats to removal of Ca^{2+} and thermal denaturation.

Cardiovascular disease is a leading cause of death in Western societies with over 50% of the cases due to coronary heart disease (CHD)[1] (1). Some patients who develop CHD prematurely (before age 45 in men and before age 50 in women) have a family history of the disease, suggesting genetic bases for premature CHD. A recent case control study (2) identified a single nucleotide polymorphism in thrombospondin-1 (TSP-1) that was strongly associated with familial premature CHD in patients homozygous for the single nucleotide polymorphism. The single nucleotide polymorphism results in the substitution of a serine for an asparagine at residue 700 of TSP-1. TSP-1 is a 450-kDa trimeric extracellular matrix glycoprotein that previously has been observed in atherosclerotic plaques and intimal hyperplasia (reviewed in Ref. 3). During arterial injury or upon stimulation with growth factors *in vitro*, TSP-1 expression in smooth muscle cells is increased (4–7), and TSP-1 and platelet-derived growth factors synergistically enhance smooth muscle cell migration (8). Patients having two Ser-700 alleles also had 2-fold lower levels of plasma TSP-1 than control patients (2).

A TSP-1 monomer contains an N-terminal module, an oligomerization sequence, a procollagen module, three properdin (type 1) modules, three EGF-like (type 2) modules, a number of Ca^{2+}-binding (type 3) repeats, and a long C-terminal sequence (Fig. 1*A*). The Ca^{2+}-binding and C-terminal sequences are unique to TSPs and are highly conserved. For instance, the alignment of human TSP-1 and *Drosophila* TSP demonstrates exact spacing of 16 cysteines in the Ca^{2+}-binding repeats. TSP-1 has an additional cysteine in the C-terminal globe that favors the isomerization of disulfides (9) and the formation of disulfide-linked complexes with thrombin (10) and von Willebrand factor (11, 12). Ca^{2+} (~5 mM) prevents formation of thrombin-TSP-1 complexes (10). In the absence of Ca^{2+}, rotary shadowing microscopy has shown that the C terminus of TSP-1 adopts an extended conformation (13–16) that is sensitive to proteolytic degradation. Thus, the structure, stability, and cysteine reactivity of TSP-1 C-terminal sequences are Ca^{2+}-sensitive.

The Ser-700 polymorphism localizes to the beginning of the Ca^{2+}-binding repeats (Fig. 1*B*). The homologous region in TSP-5 (cartilage oligomeric matrix protein (COMP)) is linked to two related autosomal dominant syndromes of skeletal dysplasia, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (EDM1) (17, 18). Mutations in COMP localize to the Ca^{2+}-binding repeats and C-terminal sequence and are missense mutations or small insertions/deletions that often affect aspartate and asparagine residues important for binding Ca^{2+} (18–26). Two PSACH-causing missense mutations in COMP have been identified within 10 residues of the aspartate that occupies the position of the N700S polymorphism of TSP-1 (20) (Fig. 1*B*). Several studies on PSACH and EDM1 mutations have shown via electron microscopy that mutant proteins have altered structures (27) and bind a decreased number of Ca^{2+} ions with altered affinity (27–30). Mutant COMP accumulates in the endoplasmic reticulum (ER) of chondrocytes with type IX collagen (31) and chaperone proteins (32, 33).

We hypothesized that, similar to COMP mutations, the Ser-

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[1] The abbreviations used are: CHD, coronary heart disease; TSP, thrombospondin; COMP, cartilage oligomeric matrix protein; PSACH, pseudoachondroplasia; EDM1, multiple epiphyseal dysplasia; ER, endoplasmic reticulum; MOPS, 4-morpholinepropanesulfonic acid; EGF, epidermal growth factor.

700 polymorphism alters the conformation of the Ca^{2+}-binding repeats of TSP-1. We characterized segments of TSP-1 comprised of the Ca^{2+}-binding repeats (Ca) and the Ca^{2+}-binding repeats with the third EGF-like module (E3Ca) as without (Asn-700) and with (Ser-700) the polymorphism associated with familial premature CHD. We found that the Ser-700 polymorphism causes a subtle local change in conformation that destabilizes the protein in response to lowering Ca^{2+} concentration or increasing temperature.

EXPERIMENTAL PROCEDURES

Cloning of Ca Asn-700, Ca Ser-700, E3Ca Asn-700, and E3Ca Ser-700 into the pAcGP67.coco Transfer Vector—To facilitate baculovirus-mediated protein expression, we used the pAcGP67.coco transfer vector, in which cloning sites are flanked by 5′ DNA encoding a signal sequence and 3′ DNA encoding a polyhistidine tag (34). The Ser-700 polymorphism was introduced by PCR mutagenesis into a construct called E3CaG Asn-700 that consisted of the last EGF-like module (E3), the Ca^{2+}-binding repeats (Ca), and the C-terminal globe and contained residues 648–1170 (12). Using DNA encoding E3CaG Asn-700 as a template, Primer 1 and Primer 2, containing a 3′ *Avr*II site, were used to amplify by PCR a product that encoded residues 648–700. Primer 3, containing a 5′ *Spe*I site, and Primer 4 were used to generate a PCR product encoding residues 700–1170. The PCR products were digested with *Avr*II or *Spe*I and were ligated together using the compatible cohesive ends of *Avr*II and *Spe*I to generate DNA that encodes E3CaG containing serine at residue 700 (E3CaG Ser-700). A second amplification of DNA encoding E3CaG Ser-700 employed Primers 1 and 4 with *Bam*HI and *Nsi*I sites, respectively. This PCR product was digested with *Bam*HI and *Nsi*I and inserted into the pAcGP67.coco baculovirus vector (34) linearized with *Bam*HI and *Pst*I using compatible cohesive ends of *Nsi*I/*Pst*I.

DNA encoding Ca Asn-700 or Ser-700 (residues 689–945) then was generated by PCR amplification using a template that encoded E3CaG Asn-700 or E3CaG Ser-700. The forward primer contained an *Xma*I site, and the reverse primer contained an *Nsi*I site. E3Ca (residues 648–945) constructs also were generated by PCR amplification using the previously mentioned Primer 1 containing restriction site *Bam*HI and the reverse primer described for the Ca constructs. The PCR products were inserted into *Xma*I (for Ca constructs) or *Bam*HI (for E3Ca constructs) and *Pst*I (using compatible cohesive ends of *Nsi*I) sites of pAcGP67.coco. Correct sequencing of PCR-amplified DNAs was verified by automated DNA sequencing.

Expression and Purification of Recombinant Proteins—Recombinant infectious viruses were generated as described (34). Passage 3 of the virus ($>10^8$ plaque-forming units/ml) was used to infect High-Five insect cells (Invitrogen) at a multiplicity of infection of 5 in SF-900 II serum-free medium at 22 °C. Conditioned medium, after 60–65 h, was harvested and dialyzed into 10 mM MOPS, 0.3 M NaCl, and 2 mM Ca^{2+} (pH 7.5). Dialyzed medium was incubated with Ni^{2+}-nitrilotriacetic acid resin overnight at 4 °C, a column was poured with protein-bound resin, and the protein was eluted in a buffer containing 300 mM imidazole. Purified protein was dialyzed into 10 mM MOPS, 0.15 M NaCl, and 2 mM Ca^{2+}, pH 7.5. The protein was stored in aliquots at −80 °C and thawed at 25 °C prior to use.

Intrinsic UV Fluorescence and Titration with Ca^{2+}—Prior to fluorescence assays, all recombinant proteins were treated with 4 mM EDTA to remove the Ca^{2+}. The protein then was dialyzed at 4 °C into a buffer containing 5 mM MOPS and 0.1 M NaCl (pH 7.5). Dialyzed wild-type or polymorphic proteins were titrated with Ca^{2+} at 37 °C and excited at 295 nm in an Aminco SLM 8100 fluorometer in 1-cm path length cells. Intrinsic fluorescence was measured from 310 to 400 nm, and the spectra were recorded at each Ca^{2+} concentration from 0 mM to saturating Ca^{2+} concentration. Reversal of the change also was determined by the addition of EDTA in excess of the saturating Ca^{2+} concentration. The change in fluorescence intensity (ΔF) relative to the Ca^{2+}-depleted protein from 0 mM to saturation was calculated as $\Delta F = (F_o - F)/(F_o)$, where F_o is the total fluorescence at 0 mM Ca^{2+} and F is the total fluorescence at a given Ca^{2+} concentration. The EC_{50} of calcium-sensitive structural transition was determined from the graph of ΔF *versus* Ca^{2+} concentration. The Hill coefficient was determined by calculating the slope of the line generated by $\log[\Delta F/(1 - \Delta F)]$ *versus* $\log[Ca^{2+}]$, and the values used for ΔF were taken from 30–70% of saturation. The E3Ca proteins in 0.2 mM Ca^{2+} also were excited at 295 nm at varying temperatures (25, 37, 42, 50, and 65 °C), and emission from 310–400 nm was recorded at each temperature.

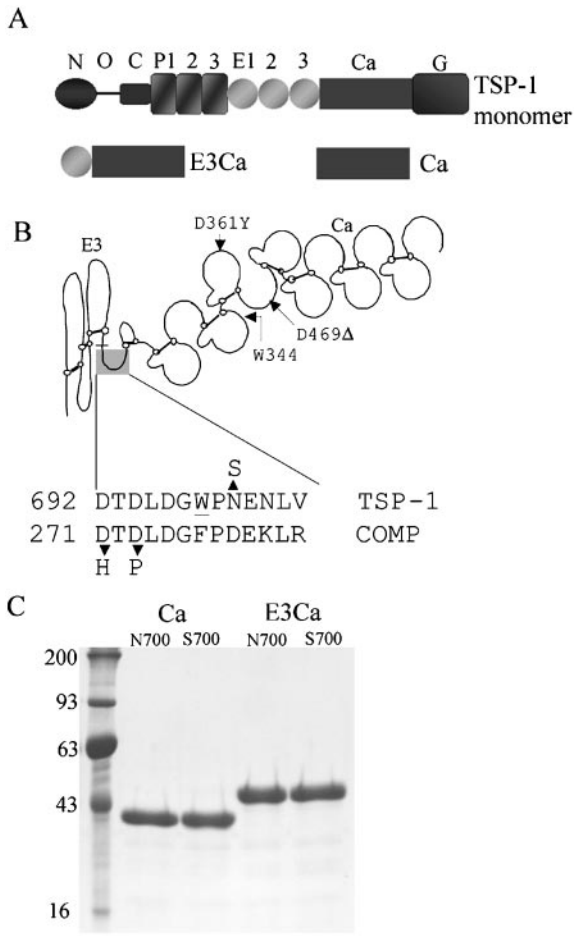

FIG. 1. **Schematic of TSP-1 modules and expressed proteins (*A*), localization of key residues in E3Ca (*B*), and SDS-PAGE of expressed proteins (*C*).** *A*, modules E3Ca and Ca containing either asparagine or serine at residue Ser-700 were used in this study. *N*, N-terminal module; *O*, oligomerization sequence; *P123*, properdin or TSP type 1 modules; *E123*, EGF-like modules; *G*, C-terminal globe. *B*, schematic of E3Ca. A *line* denotes the junction between the last EGF repeat, E3, and the Ca^{2+}-binding repeats. The sequence of the first *loop* containing residue Trp-698 (*underlined* "*W*") and the Ser-700 polymorphism is shown in alignment with the homologous sequence in COMP along with the PSACH and EDM1 mutations that have been identified in this sequence. *Arrows* denote Trp-344 (*W344*) of COMP as well as the PSACH mutation D469Δ and the EDM1 mutation D361Y studied by Thur *et al.* (28). Disulfides (*thick lines*) are depicted as deduced for TSP-2 (44). *C*, Ca Asn-700 (*N700*), Ca Ser-700 (*S700*), E3Ca Asn-700 (*N700*), and E3Ca Ser-700 (*S700*) were run on a 12% polyacrylamide gel and stained with Gel-Code Blue. The Ca and E3Ca proteins had predicted molecular masses of 30 and 34 kDa, respectively, but migrated very close to the ovalbumin 43-kDa molecular mass marker.

Far UV CD—Prior to CD spectral analysis, all recombinant proteins were treated with EDTA and dialyzed as described above. At 37 °C, dialyzed Asn-700 and Ser-700 proteins were titrated with Ca^{2+}, and CD spectra were collected in the far UV region (260–200 nm) in an AVIV 62 DS CD spectrophotometer at each Ca^{2+} concentration. The reversibility was determined by the addition of excess EDTA. The mean residue weight ellipticity was calculated using the mean residue weight for each protein. The fractional change in mean residue weight ellipticity at 220 nm (ΔE) was calculated using the equation $\Delta E = (E_0 - E)/(E_0 - E_2)$, where E_0 is the ellipticity at 0 mM Ca^{2+}, E is the ellipticity at a given Ca^{2+} concentration, and E_2 is the ellipticity at 2 mM Ca^{2+}. The EC_{50} of the transition was determined from the graph of ΔE *versus* Ca^{2+}. The Hill coefficient was determined by calculating the slope of the line generated by $\log[\Delta E/(1 - \Delta E)]$ *versus* $\log[Ca^{2+}]$.

RESULTS

Expression of Recombinant C-terminal Constructs—Baculovirus expression of TSP-1 fragments E3Ca and Ca (Fig. 1*A*)

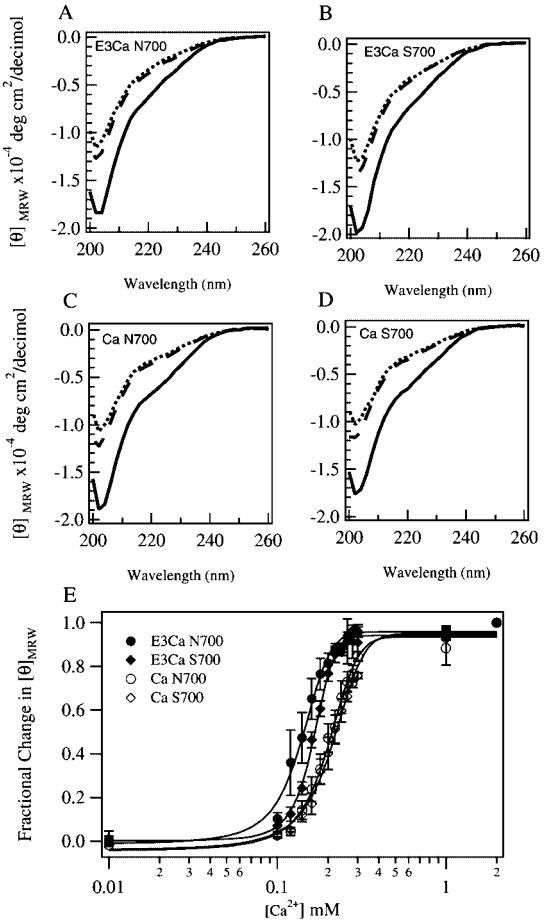

FIG. 2. **CD spectra and Ca^{2+} titrations of expressed proteins.** CD spectra for E3Ca Asn-700 (*N700*, *panel A*), E3Ca Ser-700 (*S700*, *panel B*), Ca Asn-700 (*N700*, *panel C*), and Ca Ser-700 (*S700*, *panel D*) were recorded in the presence (*solid line*) and absence (*dashed line*) of Ca^{2+} as well as in excess EDTA (*dotted line*). Increasing concentrations of Ca^{2+} were added, and the ellipticity at 220 nm was recorded. The fractional change in ellipticity was calculated as described under "Experimental Procedures" and plotted *versus* Ca^{2+} (*panel E*). E3Ca Asn-700 (*N700*) is represented by *closed circles* (●), E3Ca Ser-700 (*S700*) by *closed diamonds* (◆), Ca Asn-700 (*N700*) by *open circles* (○), and Ca Ser-700 (*S700*) by *open diamonds* (◇).

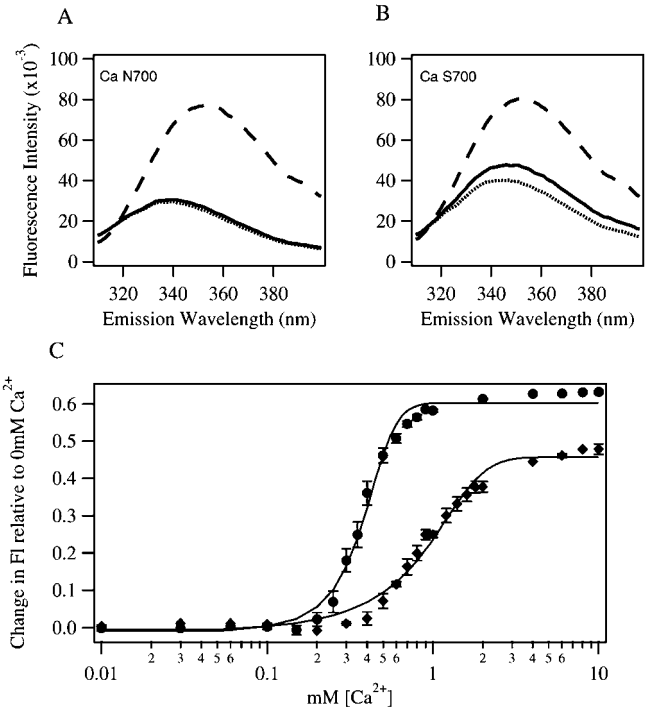

FIG. 3. **Fluorescence spectra and Ca^{2+} titrations of Ca Asn-700 and Ca Ser-700.** Tryptophan 698 in Ca Asn-700 (*N700*, *panel A*) and Ca Ser-700 (*S700*, *panel B*) was excited at 295 nm in 5 mM MOPS, 0.1 M NaCl in the absence (*dashed line*) and presence (*solid line*) of 2 and 10 mM (*dotted line*) Ca^{2+}. Intrinsic fluorescence was measured from 310 to 400 nm, and spectra were recorded at each Ca^{2+} concentration from 0 to 10 mM Ca^{2+}. Using the area under the curve and the equation described under "Experimental Procedures," Ca^{2+} titration curves were generated (*panel C*) with Ca Asn-700 represented by *closed circles* (●) and Ca Ser-700 represented by *closed diamonds* (◆).

with either asparagine or serine at residue 700 resulted in a high yield of protein (30–50 mg/liter conditioned medium). The N-terminal secretion signal targeted protein into the medium, and the C-terminal polyhistidine tag allowed for purification over a Ni^{2+}-nitrilotriacetic acid column. The polyhistidine tag was not removed for subsequent studies. All proteins migrated more slowly than predicted by molecular weight standards (Fig. 1*C*). This is likely because of the high aspartate content (17%). The presence of the Ser-700 polymorphism did not alter the migration of the protein by SDS-PAGE. There were no observable differences in the expression levels of Asn-700 and Ser-700 proteins at 22 °C, the temperature used to infect High-Five insect cells with baculovirus.

Ca^{2+} Titration of Protein Secondary Structure as Assessed by Far UV CD—We performed far UV CD on Ca and E3Ca to determine whether the Ser-700 polymorphism conferred a change in secondary structure. Similar to what was reported for full-length TSP-1 (35), we observed that two conformations exist for both Ca Asn-700 and E3Ca Asn-700 (one conformation in the presence of Ca^{2+} and one in its absence (Fig. 2, *A* and *C*)). The shape of the CD spectra for both Ca and E3Ca with a trough of negative ellipticity at 202 nm also was similar to Ca^{2+}-binding repeats of COMP (Fig. 2, *A* and *C*) (28–30). The addition of 2 mM Ca^{2+} resulted in greater negative ellipticity for Ca and E3Ca (Fig. 2). This change in ellipticity was reversible upon addition of excess EDTA. The presence of the Ser-700 polymorphism did not alter greatly the shape of the CD spectra for either Ca Ser-700 or E3Ca Ser-700 (Fig. 2, *B* and *D*) in the presence or absence of Ca^{2+}.

The proteins were titrated with Ca^{2+} using ellipticity at 220 nm as a measure. The fractional change in ellipticity was calculated for each protein and plotted *versus* Ca^{2+} concentration (Fig. 2*E*). The EC_{50} of Ca Asn-700 was determined to be 213 ± 14 μM (x ± S.E., n = 4). The calculated Hill coefficient of 4.6 ± 0.1 indicates positive cooperativity. Ca Ser-700 had an EC_{50} of 218 ± 13 μM and a Hill coefficient of 4.8 ± 0.1. E3Ca Asn-700 had an EC_{50} of 150 ± 15 μM with a Hill coefficient of 5.1 ± 0.2. E3Ca Ser-700 had an EC_{50} of 168 ± 7 μM with a Hill coefficient of 6.1 ± 0.5. Thus, the Ca proteins titrated at a significantly higher Ca^{2+} concentration than the E3Ca proteins, but the polymorphism did not alter the Ca^{2+} concentration at which the secondary structure transition of the Ca^{2+}-binding repeats occurs. The presence of the Ser-700 polymorphism, however, caused a significant increase in the cooperativity of the transition in E3Ca.

The Ser-700 Polymorphism Alters Conformation and Calcium Sensitivity of a Region in the Ca^{2+}-binding Repeats as Assayed by UV Fluorescence—A sole tryptophan (Trp-698), located 2 residues from the Ser-700 polymorphism in the Ca^{2+}-binding repeats (Fig. 1*B*), was used as a reporter for local changes in conformation. Ca Asn-700 and Ca Ser-700 were excited at 295 nm specifically to excite the tryptophan, and emission spectra from 310 to 400 nm in varying concentrations of Ca^{2+} were collected for both proteins (Fig. 3, *A* and *B*). The addition of Ca^{2+} caused alterations in two spectral parameters,

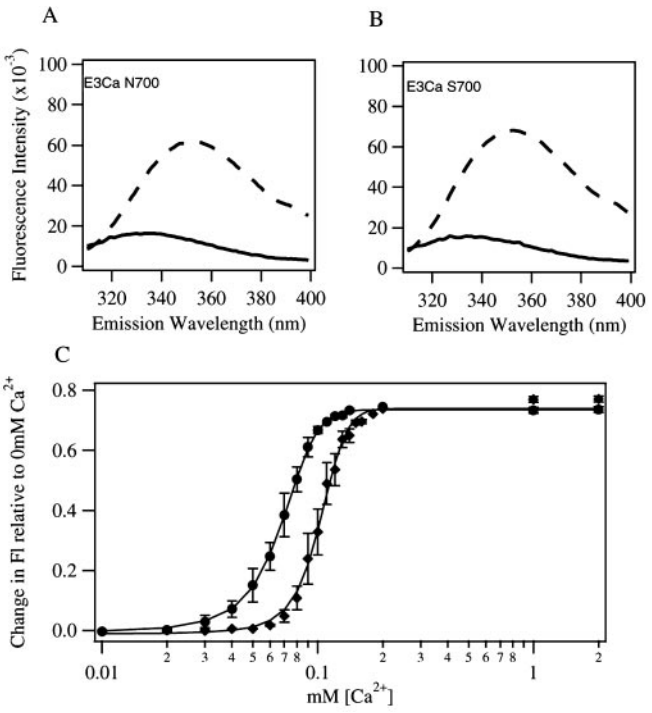

FIG. 4. **Fluorescence spectra and Ca^{2+} titrations of E3Ca Asn-700 and E3Ca Ser-700.** Tryptophan 698 in Ca Asn-700 (*N700*, *panel A*) and Ca Ser-700 (*S700*, *panel B*) was excited at 295 nm in 5 mM MOPS, 0.1 M NaCl in the absence (*dashed line*) and presence (*solid line*) of 2 mM Ca^{2+}. Intrinsic fluorescence was measured from 310 to 400 nm, and spectra were recorded at each Ca^{2+} concentration from 0 to 2 mM Ca^{2+}. Using the area under the curve and the equation described under "Experimental Procedures," Ca^{2+} titration curves were generated (*panel C*) with E3Ca Asn-700 represented by *closed circles* (●) and E3Ca Ser-700 represented by *closed diamonds* (◆).

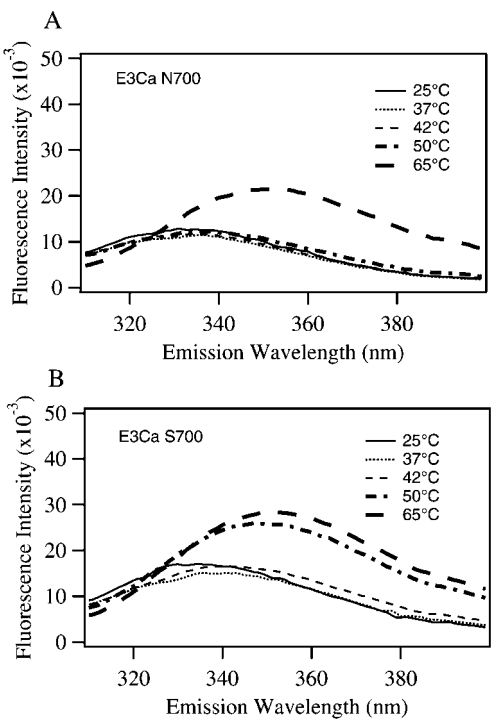

FIG. 5. **The effect of temperature on E3Ca Asn-700 and E3Ca Ser-700 in 0.2 mM Ca^{2+}.** The effects of temperature were measured using intrinsic fluorescence. E3Ca Asn-700 (*N700*, *panel A*) and E3Ca Ser-700 (*S700*, *panel B*) were excited at 295 nm, and emission spectra were recorded from 310 to 400 nm at the following temperatures: 25, 37, 42, 50, and 65 °C.

fluorescence intensity and the wavelength of peak intensity (λ_{max}). In the absence of Ca^{2+}, Ca Asn-700 and Ser-700 both had a λ_{max} of 354 nm. Upon addition of 2 mM Ca^{2+} to Ca Asn-700, the fluorescence intensity was quenched 2.6-fold, and the λ_{max} underwent a blue shift to 340 nm (Fig. 3*A*). The addition of 2 mM Ca^{2+} to Ca Ser-700 caused 1.7-fold quenching of fluorescence and a shift in the λ_{max} to 348 nm (Fig. 3*B*). The addition of 10 mM Ca^{2+} to Ca Ser-700 caused further quenching (from 1.7- to 2.0-fold) and a further shift in the λ_{max} to 344 nm.

In the absence of Ca^{2+}, both E3Ca Asn-700 and E3Ca Ser-700 (Fig. 4, *A* and *B*) had a λ_{max} of 353 nm, very similar to the λ_{max} of the Ca proteins in 0 mM Ca^{2+}. In the presence of 2 mM Ca^{2+}, both proteins had a λ_{max} of 334 nm. This value, lower than the λ_{max} of the Ca proteins in the presence of Ca^{2+}, was not influenced by the polymorphism. Also in contrast to Ca proteins, the fold changes (~4-fold) in fluorescence of E3Ca Asn-700 and E3Ca Ser-700 were similar. Changes in the fluorescence of Ca and E3Ca upon addition of Ca^{2+} were reversible (data not shown) when excess EDTA was added.

The fractional change in total fluorescence for each protein was calculated at each Ca^{2+} concentration from 0 to saturating Ca^{2+} and then plotted *versus* Ca^{2+} concentration (Figs. 3*C* and 4*C*, *mM [Ca^{2+}]*). The titration curves were sigmoidal and exhibited positive cooperativity. The titrations were influenced by the presence or absence of E3 and the Ser-700 polymorphism. The EC_{50} for Ca Asn-700 was 390 ± 20 μM (x ± S.E., n = 4). The presence of the Ser-700 polymorphism caused the EC_{50} to more than double to 950 ± 10 μM. The Hill coefficient for Ca Asn-700 was calculated to be 2.5 ± 0.2. The calculated Hill coefficient for Ca Ser-700 was lower, 1.4 ± 0.1. The altered EC_{50} and cooperativity suggest that the Ser-700 polymorphism causes a local structural change in the Ca^{2+}-binding repeats. Inspection of the titration curve also suggests that the fluorescence characteristics of Ca Asn-700 and Ser-700 proteins at saturating Ca^{2+} would be different even at very high Ca^{2+} concentration.

Although the presence of the Ser-700 polymorphism did not cause a detectable conformation change in E3Ca in the absence or presence of 2 mM Ca^{2+} as assessed by intrinsic UV fluorescence (Fig. 4, *A* and *B*), the two proteins titrated at different Ca^{2+} concentrations (Fig. 4*C*). The EC_{50} of the calcium-sensitive structural transition was 70 μM ± 2 for E3Ca Asn-700 (x ± S.E., n = 4) and 110 μM ± 6 for E3Ca Ser-700. The Hill coefficient for E3Ca Ser-700 was 5.3 ± 0.4, greater than the Hill coefficient for E3Ca Asn-700, 3.8 ± 0.3.

The Ser-700 Polymorphism Causes Thermal Instability—The intrinsic UV fluorescence of E3Ca Asn-700 and E3Ca Ser-700 in a Ca^{2+} concentration of 0.2 mM was measured at varying temperatures (25, 37, 42, 50, and 65 °C). The concentration of 0.2 mM was selected because this is the lowest Ca^{2+} concentration at which both proteins are in a Ca^{2+}-replete conformation at 37 °C as assessed by intrinsic UV fluorescence (Fig. 3*C*). E3Ca Asn-700 in 0.2 mM Ca^{2+} had a λ_{max} of 333–334 nm at 25 and 37 °C. E3Ca Ser-700 had a λ_{max} of 334 and 336 nm at 25 and 37 °C, respectively, slightly higher than E3Ca Asn-700. Increasing the temperature resulted in an increase in fluorescence and a red shift in the λ_{max} to ~352 nm (Fig. 5, *A* and *B*). These changes occurred at lower temperatures for E3Ca Ser-700, for which the red shift was apparent at 42 °C. An increase in fluorescence and a further shift in the λ_{max} were noted for E3Ca Ser-700 at 50 and 65 °C, respectively. The λ_{max} and fluorescence intensity of E3Ca Asn-700 remained constant through 50 °C. At 65 °C, the λ_{max} of E3Ca Asn-700 underwent a red shift to 352 nm, and the fluorescence intensity increased.

DISCUSSION

The Ser-700 polymorphism that localizes to the Ca^{2+}-binding repeats of TSP-1 has been associated with familial prema-

ture CHD (2), raising the question of if or how this polymorphism alters the structure and function of TSP-1. The results described above indicate that the Ser-700 polymorphism is associated with a perturbation in the local conformation of the Ca^{2+}-binding repeats of TSP-1 at low concentrations of Ca^{2+} or high temperatures.

The Ca^{2+}-binding repeats are highly conserved across TSP family members and species. The importance of this conservation is demonstrated by the finding that missense mutations or minor expansions/deletions in the Ca^{2+}-binding repeats of COMP (TSP-5) cause two forms of autosomal dominant skeletal dysplasias, pseudoachondroplasia and multiple epiphyseal dysplasia (17, 18). These mutations often change aspartate or asparagine residues that are likely important for binding Ca^{2+}. The Ser-700 polymorphism is similar to COMP mutations in that it localizes to the Ca^{2+}-binding repeats of TSP-1 and changes an asparagine to serine at a site where there is either an aspartate or asparagine in all TSPs (36, 37). The structural consequences of the Ser-700 polymorphism on the Ca^{2+}-binding repeats were studied by intrinsic fluorescence and far UV CD to compare Ca^{2+}-sensitive structural changes with and without the polymorphic residue.

Intrinsic fluorescence was due to a single tryptophan, Trp-698, within the first presumptive Ca^{2+}-binding loop of the Ca region and fortuitously only 2 residues away from the Ser-700 polymorphism (Fig. 1*B*). A construct similar to our Ca construct but derived from COMP and containing PSACH and EDM1 mutations (Fig. 1*B*) has been expressed in bacterial or mammalian human embryonic kidney cells and assessed for altered protein structure using similar techniques (28–30). The single tryptophan, Trp-344, of the Ca construct from COMP is in the fourth presumptive Ca^{2+}-binding loop, not in the same Ca^{2+}-binding loops that harbor the D361Y EDM1 mutation and the D469Δ PSACH mutation tested (Fig. 1*B*) (28). Comparing the Ca constructs of both TSPs, Trp-698 of TSP-1 titrated at a higher Ca^{2+} concentration than Trp-344 of COMP (0.4 *versus* 0.2 mM) (28). Ca Ser-700 had its fluorescence transition at 0.95 mM. This increase is similar to the Ca^{2+}-binding repeats of COMP with the D361Y EDM1 mutation that had a transition at 1.1 mM (28). Interestingly, the Ca^{2+}-binding repeats harboring the more severe PSACH mutation D469Δ had a fluorescence transition similar to Ca of wild-type COMP, indicating that the location of the tryptophan may be critical for detection of conformational changes by intrinsic fluorescence.

The far UV CD spectra of Ca and E3Ca had sharp minima at 202 nm and were similar to the Ca^{2+}-binding repeats of COMP (28–30). The Ca^{2+}-binding repeats of TSP-1 and COMP exhibited transitions at 0.2 mM Ca^{2+} in this study and 0.3 mM Ca^{2+} in a study by Thur *et al.* (28), respectively. The Ser-700 polymorphism did not alter the Ca^{2+} concentration at which the far UV CD transition for Ca occurs. Similarly, the COMP Ca^{2+}-binding repeats with and without COMP mutations D361Y, D469Δ, and D446N have been analyzed by far UV CD (28, 29). In both such studies, the COMP mutations did not alter greatly how the spectral changes were titrated by Ca^{2+}. Therefore, in the absence of a major change in the CD, the Ser-700 polymorphism of TSP-1 and the D361Y disease-causing COMP mutation both cause a local structural change as assessed by fluorescence.

The adjacent EGF-like module influenced the conformation of the Ca^{2+}-binding repeats. The module affected the secondary structure at intermediate Ca^{2+} concentrations (Fig. 2*E*) such that the far UV CD transition for E3Ca occurred at 1.5-fold lower Ca^{2+} than the transition for Ca. E3 also caused a blue shift in the λ_{max} of Trp-698 in the presence of Ca^{2+}. The fluorescence transition for E3Ca occurred at 6-fold lower Ca^{2+} concentrations and was associated with increased positive cooperativity compared with Ca. The fluorescence transition of E3Ca also occurred at a lower Ca^{2+} concentration than the far UV CD transition, whereas the opposite is true for Ca, suggesting that E3 influences the structure of the adjacent first Ca^{2+}-binding repeat (Repeat 1) that contains both Trp-698 and the Ser-700 polymorphism (Fig. 1*B*). Despite the stabilizing effect of the third EGF-like module on the Ca^{2+}-binding repeats, the presence of the Ser-700 polymorphism still altered the titration of Trp-698. This finding suggests that the Ser-700 polymorphism alters the affinity and cooperativity of Ca^{2+} binding to Repeat 1 in intact TSP-1.

Alteration of a potential Ca^{2+}-binding residue not only alters local Ca^{2+} binding but also sensitizes the Ca^{2+}-binding repeats to heating. Temperature has a quenching effect on tryptophan fluorescence regardless of protein structure (38). Although decreasing the temperature below 20 °C causes a blue shift in the λ_{max} of *N*-acetyl-L-tryptophanamide in viscous solvents (39), a red shift is not seen when the temperature is increased above 20 °C (39). Therefore, in MOPS-buffered saline at the temperature range studied, a change in temperature should not alter the λ_{max} unless there is a change in protein structure. Our studies were carried out in 0.2 mM Ca^{2+}, the lowest calcium concentration at which both E3Ca Asn-700 and E3Ca Ser-700 are found in calcium-replete conformations at 37 °C as assessed by intrinsic fluorescence. Increasing the temperature caused the Trp-698 in the E3Ca proteins to have increased fluorescence intensity that underwent a red shift, thereby mimicking the fluorescence pattern seen in the absence of Ca^{2+}. The temperature-induced change occurred at lower temperatures for polymorphic E3Ca Ser-700 than wild-type E3Ca Asn-700.

The pathophysiology of PSACH is likely related to the instability of COMP and its accumulation with type IX collagen and chaperone proteins in ER vesicles of chondrocytes (31–33). Familial premature CHD associated with the Ser-700 polymorphism requires that both alleles encode the polymorphism, whereas PSACH and EDM1 are autosomal dominant syndromes. Because COMP is a pentamer, a mutation in one allele statistically is predicted to result in only 3.1% of the homopentamers containing all wild-type subunits. TSP-1 is a trimeric protein. If only one allele of TSP-1 encoded the Ser-700 polymorphism, then statistically 12.5% of the homotrimers would contain asparagine as residue 700 in all three subunits. TSP-1 also can heterotrimerize with TSP-2 (40), thereby potentially further diluting the number of TSP trimers composed of subunits containing the Ser-700 polymorphism. One may speculate that a threshold of "normal" TSP molecules protects against disease by proteins carrying destabilizing subunits. Alternatively, the Ser-700 polymorphism in TSP-1 may be less destabilizing than any of the PSACH and EDM1 mutations. It is unknown if trimeric TSP-1 containing residue Ser-700 in all subunits is destabilized in the ER. However, the observation that patients with both Ser-700 alleles have significantly lower levels of plasma TSP-1 than control patients (2) suggests that a secretion defect is present in cells that contribute to the pool of plasma TSP-1. The Ca^{2+} concentration of the ER has been estimated to be ~100–800 μM depending upon the cell type and the method of measurement (41–43). Our fluorescence results demonstrate that the Ser-700 polymorphism causes a local change in conformation at the lower end of this Ca^{2+} range. Therefore, it is possible that, similar to mutant COMP, TSP-1 Ser-700 is retained in the ER because of protein aggregation with other extracellular matrix molecules and chaperone proteins. Future studies are needed both to determine whether structural alterations caused by the Ser-700 polymorphism

alter the secretion of intact TSP-1 and to relate such results to coronary artery lesions of patients with familial premature CHD associated with the Ser-700 polymorphism.

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