A single nucleotide polymorphism that results in substitution at residue 700 of a serine (Ser-700) for an asparagine (Asn-700) in thrombospondin-1 is associated with familial premature coronary artery disease. The polymorphism is located in the first of 13 Ca\(^{2+}\)-binding motifs, within a consensus sequence in which Asn-700 likely coordinates Ca\(^{2+}\). Equilibrium dialysis of constructs comprised of the adjoining epidermal growth factor-like module and the Ca\(^{2+}\)-binding region (E3Ca) demonstrated that E3Ca Ser-700 binds significantly less Ca\(^{2+}\) than E3Ca Asn-700 at low [Ca\(^{2+}\)]. The hypothesis that this difference is due to loss of a binding site in Ser-700 protein was tested with truncations of E3Ca containing four (Tr4), three (Tr3), two (Tr2), or one (Tr1) N-terminal Ca\(^{2+}\)-binding motifs. The Ser-700 truncation constructs bound 1 fewer Ca\(^{2+}\) than matching Asn-700 constructs.

Intrinsic fluorescence of a tryptophan at residue 698 (Trp-698) in the most N-terminal motif was cooperatively quenched by the addition of Ca\(^{2+}\) to Asn-700 Tr2, Tr3, and Tr4 constructs. In Ser-700 constructs, quenching of Trp-698 was incomplete in the Tr2 and Tr3 constructs and complete only in the Tr4 construct. Ca\(^{2+}\)-induced quenching of Ser-700 constructs required higher [Ca\(^{2+}\)] and was slower as shown in stopped-flow experiments than quenching of Asn-700 constructs. Such differences were not found with Tb\(^{3+}\), which quenched the fluorescence of Asn-700 and Ser-700 constructs equivalently. Thus, the Ser-700 polymorphism alters a rapidly filled, high affinity Ca\(^{2+}\)-binding site in the first Ca\(^{2+}\)-binding motif. Slower Ca\(^{2+}\) binding to adjoining motifs partly compensates for the change.

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A single nucleotide polymorphism in the coding region of thrombospondin-1 has been associated with familial premature coronary artery disease when homozygous (1). The encoded protein, TSP-1, is a 450-kDa trimeric extracellular matrix glycoprotein, identified as a protein secreted from platelets upon exposure to thrombin (2, 3). TSP-1 has since been observed in fibrin clots, atherosclerotic plaques, and intimal hyperplasia (reviewed in Ref. 4). TSP-1 belongs to the thrombospondin family of extracellular proteins that includes five vertebrate members (TSP-1–5), at least two chordate TSPs (5), a Drosophila TSP (6, 7), and a shrimp TSP (8). A TSP-1 monomer contains an N-terminal module, an oligomerization sequence, a procollagen module, three properdin modules, three EGF-like modules, several Ca\(^{2+}\)-binding repeats, and a leucine-rich C-terminal globular domain (4, 9). The Ca\(^{2+}\)-binding and C-terminal sequences are highly conserved among all TSP family members. Alignment of human TSP-1 and Drosophila TSP demonstrates exact spacing of 16 cysteines in the Ca\(^{2+}\)-binding repeats. The disulfide bond pattern for the homologous region in TSP-2 has been found to be sequential (10), thereby separating the Ca\(^{2+}\)-binding repeats into putative Ca\(^{2+}\)-binding motifs (Fig. 1).

The crystal structure of the most C-terminal six motifs and the C-globe of TSP-1 indicates that a 12-residue sequence coordinates Ca\(^{2+}\) via two novel Ca\(^{2+}\)-binding motifs, an N-type and a C-type motif (9). The N-type motif binds 1–2 Ca\(^{2+}\) using the 12-residue sequence, whereas the C-type motif binds 2 Ca\(^{2+}\), utilizing both the 12-residue sequence and the residues C-terminal to the 12-residue sequence (Fig. 1). The integrity of each Ca\(^{2+}\)-binding sequence seems important to the structure and function of this region of TSPs since missense mutations in the Ca\(^{2+}\)-binding region of TSP-5 (also known as cartilage oligomeric matrix protein, or COMP) cause two forms of skeletal dysplasias, pseudochondrodysplasia (PSACH) and multiple epiphyseal dysplasia 1 (EDM1) (11, 12). Many of these mutations affect residues potentially important in coordinating Ca\(^{2+}\) to TSP-5. Studies of recombinant TSP-5 with PSACH or EDM1 mutations have shown that mutant proteins bind a decreased number of Ca\(^{2+}\) (13–16).

The polymorphism of TSP-1 associated with coronary artery disease changes residue 700 from an asparagine (Asn-700) to a serine (Ser-700) and falls in the ninth position of a putative Ca\(^{2+}\)-binding motif (Fig. 1). An asparagine or aspartic acid is found at the position of the polymorphic residue in all TSPs. The fluorescence of a nearby tryptophan at residue 698 (Trp-698), which occupies the seventh position in the putative motif, is cooperatively quenched upon the addition of Ca\(^{2+}\) (17). Ca\(^{2+}\)-induced quenching of Trp-698 in recombinant constructs comprised of the third EGF-like module and the Ca\(^{2+}\)-binding repeats (E3Ca) occurs with EC\(_{50}\) values of 70 and 110 \(\mu\)M for Asn-700 and Ser-700 protein, respectively (17). The results suggest that the Ser-700 polymorphism alters the Ca\(^{2+}\) sensitivity of the Ca\(^{2+}\)-binding repeats, but it is not known whether the residues around the N700S polymorphism constitute a Ca\(^{2+}\)-binding site. This region has been considered to be a linker sequence between the third EGF-like repeat and the Ca\(^{2+}\)-binding repeats (9, 18). We noted that the linker can be modeled to be a C-type motif if one considers the 14 residues.
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**EXPERIMENTAL PROCEDURES**

**Cloning of Common and Polymorphic Truncation Constructs—**To facilitate baculovirus-mediated protein expression and subsequent purification, 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 (20). E3Ca Asn-700 and E3Ca Ser-700 were made as described previously (17). Truncated proteins were designed to preserve the disulfide connectivity (10). DNA encoding residues 648–717 (Tr1), 648–740 (Tr2), 648–753 (Tr3), and 648–776 (Tr4) that contained the third EGF-like module and truncations of the Ca²⁺-binding repeats were generated by PCR amplification using E3Ca Asn-700 or E3Ca Ser-700 as template. The limits of these proteins are depicted in Fig. 1. The forward primer contained a BamHI site, and the reverse primer contained a PstI site. PCR products were inserted into BamHI and PstI sites of pAcGP67.coco. Correct sequences of PCR-amplified DNAs were verified by DNA sequencing.

**Expression and Purification of Reombinant Proteins—**Reombinant, infectious virus were generated as described previously (20). Passage three of the virus (>10⁶ plaque-forming units/ml) was used to infect High-Five insect cells (Invitrogen) at a multiplicity of infection of five in SF-900 II serum-free medium at 22°C. After 60–65 h, conditioned medium was harvested and dialyzed into 10 mM MOPS, 0.3 M NaCl, and 2 mM CaCl₂ (pH 7.5). Dialyzed medium was incubated with Ni²⁺-nitrilotriacetic acid resin overnight at 4°C, a column was poured with protein-bound resin, and protein was eluted in buffer containing 5 mM MOPS and 0.1 M NaCl (pH 7.5). Ca²⁺-depleted protein (45 µl) was then dialyzed at 37°C for 4 h into the same buffer (350 µl) containing (Ca²⁺) varying from 10 µM to 2 mM plus trace [⁴⁵Ca] (Amersham Biosciences) (21, 22). Following dialysis, the radioactivity of the protein and buffer samples was determined by scintillation counting, and the protein concentration of the protein samples was determined by BCA protein assay (Pierce). To calculate the [Ca²⁺] in the protein sample, enrichment of Ca²⁺ in the protein sample (counts in the protein sample divided by counts in the buffer sample) was multiplicated by the [Ca²⁺] of the buffer. Subtraction of the buffer [Ca²⁺] yielded the concentration of protein bound Ca²⁺, and division by protein concentration yielded the number of Ca²⁺ bound to one molecule of protein. The Ca²⁺ binding data were fitted to sigmoid curves that was used to relate the number of bound Ca²⁺ at various [Ca²⁺] to spectroscopic changes at the same [Ca²⁺].

**Intrinsic UV Fluorescence and Titration with Ca²⁺ or Tb³⁺—**Prior to fluorescence assays, recombinant proteins were treated with 4 mM EDTA to remove the Ca²⁺ and then dialyzed at 4°C into buffer containing 5 mM MOPS and 0.1 M NaCl (pH 7.5), as described above. Dilutions of proteins were made on 1× SF-900 II serum-free medium at 22°C. After 60–65 h, conditioned medium was harvested and dialyzed into 10 mM MOPS, 0.15 M NaCl, and 2 mM CaCl₂, pH 7.5. Protein was stored in aliquots at −80°C and thawed in a 25°C water bath prior to use. All proteins were produced in yields ranging from 15 to 30 mg/liter conditioned medium and were soluble at concentrations of >1 mg/ml.

**Ca²⁺ Binding—**To determine the number of Ca²⁺ bound to Asn-700 and Ser-700 proteins, we performed equilibrium dialysis. Proteins were initially treated with 4 mM EDTA to remove Ca²⁺ and then dialyzed exhaustively at 4°C into buffer containing 5 mM MOPS and 0.1 M NaCl and 2 mM CaCl₂, pH 7.5. Protein was stored in aliquots at −80°C and thawed in a 25°C water bath prior to use. All proteins were produced in yields ranging from 15 to 30 mg/liter conditioned medium and were soluble at concentrations of >1 mg/ml.

**RESULTS**

The Ser-700 Polymorphism Subtly Alters the Titration of Ca²⁺ Binding to E3Ca—Equilibrium dialysis with ⁴⁵Ca²⁺ was performed on E3Ca Asn-700 and polymorphic E3Ca Ser-700, constructs of TSP-1 that contain the third EGF-like repeat together with the complete Ca²⁺-binding region. Ser-700 protein reproducibly bound 1–2 fewer Ca²⁺ than [Ca²⁺] of 60–200 µM (Fig. 2A). At saturating [Ca²⁺] of >250 µM, the assay was less reproducible, and there was overlap between the two proteins in the estimates of the number of Ca²⁺ bound. E3Ca Asn-700
were calculated to be 160 half-maximal binding for E3Ca Asn-700 and E3Ca Ser-700.

The Ser-700 Polymorphism—Binding of 45Ca2+ to Asn-700 constructs by equilibrium dialysis was sigmoidal for Tr4 Asn-700, Tr3 Asn-700, and Tr2 Asn-700 (Fig. 3, A–C). Removal of motifs from Asn-700 proteins caused a decrease in the maximal number of Ca2+ bound (Table I). Tr4 Asn-700 bound 5.9 ± 0.2 Ca2+, Tr3 Asn-700 bound 4.0 ± 0.1 Ca2+, and Tr2 Asn-700 bound 3.0 ± 0.1 Ca2+. The concentration of half-maximal binding occurred from 150 to 250 μM [Ca2+], depending on the size of the truncation (Table I). The calculated Hill coefficients for Tr4 Asn-700, Tr3 Asn-700, and Tr2 Asn-700 indicated positive cooperativity (Table I). Although the Hill coefficients did not significantly differ depending on the truncation size, all were lower than the Hill coefficient for Ca2+ binding to E3Ca Asn-700 (Table I). The titration curve for Tr1 Asn-700 (Fig. 3D) did not reach a plateau at high [Ca2+] (>800 μM), and at this input concentration, Tr1 Asn-700 bound 1.2 ± 0.1 Ca2+ (X ± S.E., n = 4).

In contrast to the subtle differences of Ca2+ binding to E3Ca Asn-700 and E3Ca Ser-700 (Fig. 2A), greater differences in Ca2+ binding to Ser-700 as compared with Asn-700 truncation constructs were observed (Fig. 3 and Table I). For all truncated proteins, the presence of the Ser-700 polymorphism caused a decrease in the number of bound Ca2+ as compared with wild type at all [Ca2+] tested. The titration curves for Ser-700 truncations were also shifted to higher [Ca2+]. For instance, the titration curve for Tr4 Ser-700 was sigmoidal (Fig. 3A) and reached a binding plateau of 4.8 ± 0.5 Ca2+, lower than that for Tr4 Asn-700. The EC50 of Ca2+ binding for Tr4 Ser-700 was 420 ± 40 μM, higher than that observed for the Tr4 Asn-700. Ca2+ bound to Tr3 Ser-700, but the titration curve did not reach a plateau at high [Ca2+] (Fig. 3B). There was little binding of Ca2+ to Tr2 Ser-700, except at [Ca2+] greater than 600 μM (Fig. 3C), and Tr1 Ser-700 did not bind Ca2+ in the concentration range accessible to study by equilibrium dialysis (Fig. 3D).

Ca2+-induced Quenching of tryptophan fluorescence depends on the size of the truncation and the presence of the Ser-700 polymorphism—Since the presence of the Ser-700 polymorphism in truncation constructs caused a decrease in the number of Ca2+ bound, we next investigated whether this altered the ability of the proteins to achieve the Ca2+-bound conformation as assessed by the fluorescence properties of Trp-698. Asn-700 and Ser-700 truncation constructs were excited at 295 nm to excite Trp-698 specifically, and emission spectra were collected at 310–400 nm in the presence of various [Ca2+]. In the absence of Ca2+, all truncated constructs emitted at a peak wavelength (λmax) of ~350 nm (Fig. 4, A–H). In the
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TABLE I

Ca\(^{2+}\)-binding parameters of Asn-700 and Ser-700 proteins

| Fluorescence | EC\(_{50}\) (\(\mu M\)) | \(n_H\) | Ca\(^{2+}\) binding | EC\(_{50}\) (\(\mu M\)) | \(n_H\) | \(B_{\text{max}}\) |
|--------------|--------------------------|--------|---------------------|--------------------------|--------|-----------------|
| E3Ca Asn-700 | 70\(^a\)                  | 3.8 ± 0.3\(^a\) |                      | 160 ± 10                 | 3.5 ± 0.2 | 25 ± 1          |
| E3Ca Ser-700 | 110\(^a\)                | 5.3 ± 0.4\(^a\) |                      | 170 ± 10                 | 3.9 ± 0.4 | 23 ± 1          |
| Tr4 Asn-700  | 80 ± 1                   | 2.4 ± 0.1 |                      | 220 ± 10                 | 2.2 ± 0.2 | 5.9 ± 0.2       |
| Tr4 Ser-700  | 240 ± 1                  | 3.0 ± 0.2 |                      | 420 ± 40                 | 2.6 ± 0.4 | 4.8 ± 0.5       |
| Tr3 Asn-700  | 100 ± 10                 | 2.5 ± 0.2 |                      | 160 ± 10                 | 2.5 ± 0.3 | 4.0 ± 0.1       |
| Tr3 Ser-700  | 320 ± 10                 | 1.7 ± 0.2 |                      | NC                       | NC      | 3.5 ± 0.5\(^b\) |
| Tr2 Asn-700  | 150 ± 10                 | 2.0 ± 0.1 |                      | NC                       | NC      | 3.0 ± 0.1       |
| Tr2 Ser-700  | 590 ± 20                 | 1.2 ± 0.1 |                      | NC                       | NC      | 1.3 ± 0.3\(^b\) |
| Tr1 Asn-700  | 280 ± 10                 | 1.1 ± 0.1 |                      | NC                       | NC      | 1.2 ± 0.1\(^b\) |
| Tr1 Ser-700  | NC                       | NC      |                      | NC                       | NC      | NC              |

\(^{a}\) Data from Ref. 17.

\(^{b}\) The value at 2 mM, the highest [Ca\(^{2+}\)] tested in the equilibrium dialysis assay.

The fractional change in total fluorescence for each protein relative to 0 \(\mu M\) [Ca\(^{2+}\)] was calculated at each [Ca\(^{2+}\)] and then plotted versus [Ca\(^{2+}\)] (Fig. 5). With the exception of Tr2 Ser-700 and both Tr1 proteins, the titration curves for each protein were sigmoidal and exhibited positive cooperativity. Similar to E3Ca Asn-700, which titrated at 70 \(\mu M\) (17), Tr4 Asn-700 had an EC\(_{50}\) of 80 ± 1 \(\mu M\) (Table I). Further truncation caused the fluorescence of Tr3 Asn-700, Tr2 Asn-700, and Tr1 Asn-700, to titrate at increasing [Ca\(^{2+}\)]. The cooperativity of the fluorescence change for Tr4 Asn-700 (2.4 ± 0.1) was decreased as compared with E3Ca Asn-700 (3.8) (17). Although Tr3 Asn-700 exhibited similar cooperativity as Tr4 Asn-700, further truncation to Tr2 and Tr1 Asn-700 resulted in decreased cooperativity (Table I). The presence of the Ser-700 polymorphism caused the fluorescence of the truncated pro-

FIG. 3. Ca\(^{2+}\) binding to truncated proteins. Ca\(^{2+}\) binding to common and polymorphic Tr4 (A), Tr3 (B), Tr2 (C), and Tr1 (D) was measured by equilibrium dialysis with \(^{45}\)Ca\(^{2+}\). Closed black symbols represent wild-type proteins, and open gray symbols represent polymorphic proteins. Ca\(^{2+}\) binding data were fit to a sigmoid plot represented by a black and a gray line for Asn-700 and Ser-700 proteins, respectively.

FIG. 4. Fluorescence spectra of truncated proteins. Tryptophan fluorescence of Tr4 Asn-700 (A), Tr4 Ser-700 (B), Tr3 Asn-700 (C), Tr3 Ser-700 (D), Tr2 Asn-700 (E), Tr2 Ser-700 (F), Tr1 Asn-700 (G), and Tr1 Ser-700 (H) was excited at 295 nm in 5 mM MOPS, 0.1 M NaCl in the absence (dashed line) and presence of 3 mM (solid line) Ca\(^{2+}\). Intrinsic fluorescence was measured from 310 to 400 nm, and spectra were recorded at each [Ca\(^{2+}\)] from 0 mM to saturating [Ca\(^{2+}\)].
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Teines to titrate at higher [Ca^{2+}] than the Asn-700 proteins, and this difference was magnified in each successive truncation (Table I). Consistent with this trend, Tr4 Ser-700 titrated at higher [Ca^{2+}] (240 ± 1 μM) as compared with E3Ca Ser-700 (110 μM) (17). Similar to E3Ca proteins, the cooperativity of the fluorescence transition for Tr4 Ser-700 (3.0 ± 0.2) was higher than that for Tr4 Asn-700 (2.4 ± 0.1). However, further truncation resulted in decreased cooperativity for Tr3 Ser-700 and Tr2 Ser-700 as compared with matching Asn-700 constructs (Table I). The presence of the Ser-700 polymorphism in Tr1 resulted in minimal titration of the tryptophan fluorescence at only high [Ca^{2+}] (Fig. 5).

The Tr1 Constructs Contain an intact Tb^{3+}-binding Site—The results indicate that Asn-700 constructs with two or more Ca^{2+}-binding motifs bind Ca^{2+} with high affinity, resulting in quenching and a blue shift in the fluorescence of Trp-698. However, the 5-fold change in intrinsic fluorescence upon the addition of Ca^{2+} was not present in the Tr1 constructs. Although the equilibrium dialysis data indicated that Tr1 Asn-700 contains binding sites for Ca^{2+}, the affinity was too weak to characterize binding rigorously. To characterize metal-binding site(s) in the Tr1 protein more definitively, we performed Tb^{3+}-binding studies on Tr1 Asn-700 and Tr1 Ser-700. The lanthamide Tb^{3+} (λ_{ex} = 488, λ_{max} = 543) emits phosphorescence when excited due to resonant energy transfer from nearby tryptophans (23). Thus, Tr1 proteins were excited at 295 nm to excite Trp-698, and emission spectra were collected at 310–560 nm in the presence of various Tb^{3+}. Upon the addition of 30 μM Tb^{3+}, tryptophan fluorescence of Tr1 Asn-700 and Tr1 Ser-700 (310–400 nm) was similarly quenched (Fig. 6, A and B). In contrast to the Ca^{2+}-induced quenching observed with the E3Ca proteins and the longer Asn-700 truncations, there was no blue shift in the λ_{max}. The addition of Tb^{3+} also resulted in an emission peak at 543 nm (Fig. 6, A and B) that increased in amplitude with increasing [Tb^{3+}]. Titration of Trp-698 fluorescence with Tb^{3+} resulted in EC_{50} values of 12.0 ± 3.0 and 12.9 ± 3.5 μM for Tr1 Asn-700 and Tr1 Ser-700, respectively. The transition was not cooperative as the Hill coefficients for Tr1 Asn-700 and Tr1 Ser-700 were 1.3 ± 0.1 and 1.1 ± 0.1, respectively. Analysis of the peak at 543 nm revealed similar EC_{50} values for Tr1 Asn-700 and Tr1 Ser-700 (data not shown).

Thus, these results indicate that Tr1 proteins bind Tb^{3+}, resulting in quenching of Trp-698 fluorescence due to energy transfer to the bound metal, and that the binding is insensitive to the Ser-700 polymorphism.

The Ser-700 Polymorphism Alters the Rate of Ca^{2+} Binding—Since the residues around Trp-698 contain a metal ion-binding site that regulates the number and average affinities of bound Ca^{2+}, we hypothesized that N700S polymorphism may alter the rate of Ca^{2+} binding to E3Ca and truncated constructs. Therefore, we analyzed Ca^{2+}-induced quenching of E3Ca Asn-700, E3Ca Ser-700, Tr4 Asn-700, Tr4 Ser-700, Tr3 Asn-700, and Tr2 Asn-700 by stopped-flow fluorescence. These constructs exhibited at least 4-fold quenching of tryptophan fluorescence upon the addition of Ca^{2+} (Fig. 3) (17), providing a robust signal to monitor as a function of time. Kinetic traces were obtained at high [Ca^{2+}] as compared with protein concentration, conditions for pseudo-first order kinetics. Fig. 7 shows representative traces from Tr4 Asn-700 and Tr4 Ser-700.

All traces were biphasic over concentrations between 125 μM and 4 mM. Upon the addition of Ca^{2+}, there was an initial rapid decrease in fluorescence followed by a slower decrease in fluorescence. For Asn-700 constructs, ~80% of the total change in amplitude was due to the first phase at concentrations between 0.125 and 1 mM [Ca^{2+}]. This increased to ~90% at concentrations greater than 1 mM. In addition, for Asn-700 constructs at
higher [Ca\(^{2+}\)], part of the first phase was not detected as it occurred too quickly to be followed by our instrument. For E3Ca Ser-700 and Tr4 Ser-700, the first phase represented less than 70% of the total change in amplitude at [Ca\(^{2+}\)] less than or equal to 1 mM; this increased to ~80% at concentrations greater than 1 mM (data not shown).

The rate of the first phase (k\(_{\text{obs}}\)) for Asn-700 proteins increased linearly with increasing [Ca\(^{2+}\)] (Fig. 8A) and was dependent on the level of truncation. The k\(_{\text{obs}}\) at each [Ca\(^{2+}\)] for E3Ca Asn-700 was slightly higher than k\(_{\text{obs}}\) for Tr4 Asn-700. The k\(_{\text{obs}}\) of Tr3 Asn-700 was similar to that of Tr4 Asn-700, but both were higher than k\(_{\text{obs}}\) of Tr2 Asn-700 for each [Ca\(^{2+}\)] (Fig. 8A). The presence of the Ser-700 polymorphism in E3Ca Ser-700 and Tr4 Ser-700 caused a decrease in k\(_{\text{obs}}\) such that the k\(_{\text{obs}}\) of both proteins were lower than the k\(_{\text{obs}}\) of Asn-700 Tr4, Tr3, or Tr2 proteins (Fig. 7B). For Ser-700 proteins, k\(_{\text{obs}}\) also increased with increasing [Ca\(^{2+}\)], but the dependence of k\(_{\text{obs}}\) with [Ca\(^{2+}\)] was not linear, and the curves of E3Ca Ser-700 and Tr4 Ser-700 were shifted to higher [Ca\(^{2+}\)] as compared with Asn-700 proteins (Fig. 7B).

There were small differences between the rates of the slow phase (k\(_{\text{slow}}\)) for wild-type constructs (Fig. 8C). The k\(_{\text{slow}}\) of Asn-700 proteins increased mildly between ~1 and 8 s\(^{-1}\) as a function of [Ca\(^{2+}\)], values that are ~20-fold lower than the k\(_{\text{slow}}\) at similar [Ca\(^{2+}\)]. The k\(_{\text{slow}}\) was also dependent on the level of truncation, with Tr2 Asn-700 and Tr3 Asn-700 exhibiting slower rates than E3Ca Ser-700 and E3Ca Asn-700 at Ca\(^{2+}\) between 1 and 4 mM (Fig. 7C). The rate constant of the slow phase (k\(_{\text{slow}}\)) did not significantly differ between Asn-700 and Ser-700 proteins at each [Ca\(^{2+}\)] (Fig. 8D).

**DISCUSSION**

As shown in Fig. 1, two novel but related Ca\(^{2+}\)-binding motifs occur in TSP-1 (9). In both N- and C-type motifs, aspartate residues at positions 1, 3, and 5 coordinate the first Ca\(^{2+}\). The residue at position 7, which is variable among the motifs, provides a main chain carbonyl that contributes to coordinating the first Ca\(^{2+}\). At position 9, an aspartate or asparagine bridges a water molecule to the first Ca\(^{2+}\). The 6-fold coordination of the first Ca\(^{2+}\) is completed by an aspartate at position 12 (9). The second Ca\(^{2+}\) is coordinated by aspartates at positions 3, 5, and 12 that also participate in coordination of the first Ca\(^{2+}\). In C-type motifs, the second Ca\(^{2+}\) is additionally coordinated by residues C-terminal to the 12-residue sequence (9). Equilibrium dialysis of E3Ca indicated that 23–25 Ca\(^{2+}\) ions bind to the Ca\(^{2+}\)-binding repeats. This is higher than what has been observed for TSP-1, TSP-5, and TSP-5 constructs (13–16, 21) but is in agreement with the values predicted by the crystal structure (9) and what has been determined for TSP-2 constructs by atomic absorption spectroscopy (22).
Trp-698, which is quenched upon the addition of Ca\(^{2+}\), is at position 7 of a putative motif (Fig. 1) and would be expected to coordinate Ca\(^{2+}\) via its main chain carbonyl. The N700S polymorphism resides at position 9, where the side chain would be expected to coordinate Ca\(^{2+}\) through water (9). However, these residues have been considered to be part of a linker region (9, 18) between the third EGF-like repeat and the Ca\(^{2+}\)-binding repeats. To test the hypothesis that the linker binds Ca\(^{2+}\), we generated truncations on the disulfide bond pattern that included the linker or the linker and up to three adjacent Ca\(^{2+}\)-binding motifs. The shortest Asn-700 construct, Tr1, which contains the EGF-like module and residues 692–717, was found to bind both Ca\(^{2+}\) and Tb\(^{3+}\) as assayed by equilibrium dialysis and luminescence, respectively. Tb\(^{3+}\) bound to Tr1 Asn-700 and Tr1 Ser-700 with much higher affinity than Ca\(^{2+}\) binding to either construct, similar to what has been observed with other proteins such as calreticulin (24). Thus, Tr1 contains a strong Tb\(^{3+}\)-binding site, and in the case of the Asn-700 protein, a weaker Ca\(^{2+}\)-binding site.

The observations that Trp-698 in Tr1 Asn-700 is not fully quenched even at high [Ca\(^{2+}\)] of >3 mM and Tr1 Asn-700 does not bind Ca\(^{2+}\) with high affinity indicate that either the Ca\(^{2+}\)-binding motif is incomplete or additional motifs of TSP-1 are required for high affinity Ca\(^{2+}\) binding to Tr1. Inspection of the linker sequence of TSP-1 reveals that it can be modeled as a C-type motif if residues 701–714 are considered to be an insert (Fig. 1). Our group is in the process of determining the crystal structure of a Ca\(^{2+}\)-replete portion of TSP-2 that contains the EGF-like modules, the Ca\(^{2+}\)-binding repeats, and the lectin-like module. The linker region in this TSP-2 construct binds 2 Ca\(^{2+}\) with the coordination predicted for a C-type motif, and the insert is accommodated as a loop. TSP-1 and TSP-2 have 90% sequence identity and identical spacing in the Ca\(^{2+}\)-binding region. Therefore, it is likely that the linker of TSP-1 also functions as a C-type motif and binds 2 Ca\(^{2+}\) cooperatively in conjunction with adjacent motifs. Tr1 includes only the N-terminal half of the motif plus the insert (Fig. 1) and therefore is an incomplete C-type motif. The addition of residues 719–728 and the adjacent N-type Ca\(^{2+}\)-binding motif to the linker (Tr2 Asn-700) restored cooperative Ca\(^{2+}\)-induced quenching of Trp-698 fluorescence and high affinity binding of approximately 3 Ca\(^{2+}\) when measured by equilibrium dialysis. These results indicate that the N-terminal 2 Ca\(^{2+}\)-binding motifs participate in a linked set of relatively high affinity sites that, when occupied with Ca\(^{2+}\), quenches Trp-698 in the first motif. The increasing Hill coefficients upon the addition of more Ca\(^{2+}\)-binding motifs, for both quenching of Trp-698 and binding of Ca\(^{2+}\), indicate additional cooperativity among binding sites in N-terminal motifs.

The presence of the Ser-700 polymorphism caused loss of a Ca\(^{2+}\)-binding site as Tr1 Ser-700 did not bind Ca\(^{2+}\) in the range of [Ca\(^{2+}\)] that was tested, and Tr2 Ser-700, Tr3 Ser-700, Tr4 Ser-700, and E3Ca Ser-700 bound on average 1 fewer Ca\(^{2+}\) than Asn-700 constructs. The Ser-700 polymorphism in the most N-terminal Ca\(^{2+}\)-binding motif also influenced high affinity Ca\(^{2+}\)-binding as evidenced by the shift of the Ca\(^{2+}\)-titration curves of Ser-700 proteins to higher [Ca\(^{2+}\)] as compared with Asn-700 counterparts. In addition, complete quenching of Trp-698 fluorescence in E3Ca Ser-700 required 5–6 Ca\(^{2+}\) rather than the 3–4 Ca\(^{2+}\) required for E3Ca Asn-700. The N700S polymorphism localizes to position 9, where an aspartate or asparagine coordinates Ca\(^{2+}\) through a bridging water molecule (9). The presence of a serine rather than an asparagine at position 9 likely disrupts this water bridge, resulting in loss of a high affinity Ca\(^{2+}\)-binding site. However, the results indicate that binding of Ca\(^{2+}\) to adjacent motifs allows the region around Trp-698 to adopt the Ca\(^{2+}\)-replete conformation.

In equilibrium fluorescence studies, Trp-698 quenching in Tr4 Asn-700 titrated similarly to E3Ca Asn-700, and by stopped-flow fluorescence, the \(k_{\text{obs}}\) of Tr4 Asn-700 was also similar to \(k_{\text{obs}}\) for E3Ca Asn-700 at [Ca\(^{2+}\)] less than 4 mM. In the presence of the Ser-700 polymorphism, residues present in the largest truncation, Tr4, and high [Ca\(^{2+}\)] were necessary to induce a Ca\(^{2+}\)-bound conformation that results in quenching of Trp-698 fluorescence. However, Tr4 Ser-700 had a \(k_{\text{obs}}\) that was much lower than even Tr2 Asn-700, and even the presence of all the Ca\(^{2+}\)-binding motifs in E3Ca Ser-700 was not sufficient to restore \(k_{\text{obs}}\) to that observed for Asn-700 protein containing only the first two motifs. Thus, residues in the first Ca\(^{2+}\)-binding motif regulate the rate of formation of the Ca\(^{2+}\)-bound conformation, as monitored by Trp-698. The Ser-700 polymorphism slows the rate of the first conformational change and decreases the affinity of Ca\(^{2+}\)-binding under equilibrium conditions.

Our results demonstrating rapid, high affinity Ca\(^{2+}\)-binding to the N-terminal motifs of TSP-1 can be compared with studies of the Ca\(^{2+}\)-binding region of TSP-5 (cartilage oligomeric matrix protein). Similar to Trp-698 in E3Ca (17), Trp-344 at position 7 in motif 4 of TSP-5 is cooperatively quenched in the presence of Ca\(^{2+}\) (13). The intrinsic fluorescence of Trp-344 of TSP-5 has been analyzed in the presence a mutation, D361Y, at position 1 in the adjacent motif 5. Ca\(^{2+}\)-titration of Trp-344 fluorescence in a TSP-5 fragment reveals that the D361Y mutation caused a transition at 5-fold higher Ca\(^{2+}\) (~1 mM) as compared with the wild-type TSP-5 fragment (~0.2 mM) (13). In addition, the D361Y mutation shifted the EC\(_{50}\) of Ca\(^{2+}\)-binding by equilibrium dialysis to higher Ca\(^{2+}\). In contrast, the D469A mutation at position 12 in motif 10 did not alter the titration curves of the fluorescence or Ca\(^{2+}\)-binding (13), suggesting that mutations in N-terminal motifs more greatly influence high affinity Ca\(^{2+}\)-binding. The importance of the N-terminal motifs in high affinity binding is further demonstrated by a TSP-5 truncation construct that contains only the last 6 Ca\(^{2+}\)-binding motifs. This truncated protein binds less Ca\(^{2+}\) with decreased affinity as compared with a construct containing the entire Ca\(^{2+}\)-binding region (16).

The Ca\(^{2+}\)-titration differences between Asn-700 and Ser-700 protein occur in the range of 100–800 \(\mu\text{M}\) [Ca\(^{2+}\)] that is found in the endoplasmic reticulum (reviewed in Ref. 25). However, differences in the rate of Ca\(^{2+}\)-binding were observed at all [Ca\(^{2+}\)], including high diffusible [Ca\(^{2+}\)] similar to those found in the extracellular fluid (26). Thus, the Ser-700 polymorphism may potentially exert effects both during trafficking of TSP-1 to the cell surface and after secretion of TSP-1 into the extracellular space. The trafficking hypothesis is suggested due to possible analogies between the polymorphism in TSP-1 and mutations in TSP-5 that cause TSP-5 to accumulate in the endoplasmic reticulum of chondrocytes in patients with PSACH or EDM1 (27, 28). Patients with mutant TSP-5 have decreased levels of plasma TSP-5 (29), and similarly, patients homozygous for the allele encoding the Ser-700 polymorphism also have decreased levels of plasma TSP-1 (1), suggesting a related problem with secretion. The Ser-700 polymorphism that causes loss of a Ca\(^{2+}\)-binding site and slows the rate of Ca\(^{2+}\)-induced protein folding could influence TSP-1 maturation and transit through the endoplasmic reticulum. The Ser-700 polymorphism alters the conformation of full-length TSP-1, inasmuch as polymorphic TSP-1 is more sensitive to unfolding by urea and guanidine as assessed by intrinsic fluorescence.

\(^{2}\) J. L. Keck, C. B. Carlson, and D. F. Mosher, unpublished observations.
and circular dichroism (30). It will therefore be key to learn whether TSP-1 is accumulated in the endoplasmic reticulum in lesions of patients with familial premature coronary artery disease.

In the extracellular space, the Ser-700 polymorphism may alter interactions of the C-terminal region of TSP-1 with hemostatic proteins such as thrombin (31) and von Willebrand factor (32, 33), which bind covalently to TSP-1 via disulfide bonding at the C terminus. Recently, the presence of the Ser-700 polymorphism in TSP-1 has been shown to increase binding of TSP-1 to fibrinogen on the platelet surface, resulting in increased platelet aggregation (30). Increased platelet aggregation is one mechanism by which the Ser-700 polymorphism may cause disease by exerting its effects extracellularly. Further studies are required to learn how loss of a high affinity Ca$^{2+}$-binding site associated with slowed kinetics alters the function of TSP-1, leading to familial premature coronary artery disease. Such studies may also uncover beneficial effects of the polymorphism that account for the frequency of $\approx10\%$ for the Ser-700 allele in Caucasian populations (1, 34).

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