Calcium Stabilizes Fibrillin-1 against Proteolytic Degradation*

(Received for publication, July 23, 1996, and in revised form, October 14, 1996)

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The calcium-binding epidermal growth factor (cbEGF)-like domain is a structural motif that is present in many matrix proteins throughout the animal kingdom from invertebrates to mammals. This module has been demonstrated to bind calcium in the micromolar range. However, little is known about the functional consequences of calcium binding to proteins that contain this structural element.

We used fibrillin-1, an extracellular matrix protein consisting of ~60% cbEGF-like motifs, as a model system to study stabilizing effects of calcium in protease degradation assays. Authentic human fibrillin-1 and recombinant human fibrillin-1 subdomains, spanning the whole molecule, showed significantly slower proteolytic degradation in the presence of CaCl₂ than in the presence of EDTA, demonstrating that calcium stabilizes the structure of fibrillin-1 and protects the molecule against proteolytic degradation.

Information about cleavage sites protected by calcium was obtained with a new recombinant subdomain, rF17 (Asp952–Val1527), comprising the longest stretch of cbEGF-like motifs in the center of the fibrillin-1 molecule. The most sensitive sites for trypsin and endoprotease Glu-C were observed in cbEGF-like motifs in the center of the fibrillin-1 molecule, showing significantly slower proteolytic degradation in the presence of CaCl₂ than in the presence of EDTA, demonstrating that calcium stabilizes the structure of fibrillin-1 and protects the molecule against proteolytic degradation.

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A structural motif found in the epidermal growth factor (EGF) is widely distributed throughout the animal kingdom as a subdomain in a variety of extracellular proteins. These proteins include functionally diverse members such as the fibrillin and fibulin families (1–9), nidogen/entactin (10, 11), blood coagulation factors (reviewed in Ref. 12) and anticoagulants (13), and proteins for specification of cell fate (14–17). A characteristic pattern of amino acid residues (D/N)(D/N)(Q/E)X₉₋₁₀(D*/N*)₂₋₃(X/V)ₙ residues with an asterisk may be β-hydroxylated) has been identified in a subset of EGF-like motifs that is responsible for calcium binding. Calcium binding to this type of motif has been demonstrated with blood clotting factors IX and X (18, 19), with anticoagulants Protein C (20) and Protein S (21), and the microfibrillar protein fibrillin-1 (22–24).

Little is known about the functional significance of these motifs and the consequences of calcium binding on the properties of proteins containing these motifs. It has been suggested that calcium binding to cbEGF-like motifs mediates protein-protein interactions. For example, mutations of amino acid residues crucial for calcium binding affect the clotting activity of factor IX (25). The interaction of Protein S with complement C4b-binding protein is mediated by this type of module (26), and the interaction of the Drosophila transmembrane protein Notch with Delta and Serrate depends on two tandemly repeated cbEGF-like motifs (27). Possibly, cbEGF-like motifs are also involved in mediating the calcium-dependent interaction of fibrillin-1 with fibulin-2 (28).

The fibrillin family consists of two members, fibrillin-1 and fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29). Both proteins consist of repetitive motifs: 47 of them are EGF-like repeats in fibrillin-1 and 50 of them are cbEGF-like repeats in fibrillin-2, both of which are integral constituents of the 10–12-nm diameter extracellular matrix microfibrils (5, 29).

Mutations in fibrillin-1 result in Marfan’s syndrome, a connective tissue disorder affecting the cardiovascular, skeletal, and ocular systems (reviewed in Ref. 30). On the other hand, mutations in fibrillin-2 give rise to congenital contractual arachnodactyly, a disorder characterized primarily by joint contractures and other skeletal features (31). Most of the more than 50 known mutations in fibrillin-1 and the two mutations in fibrillin-2 occur in cbEGF-like repeats and are predicted to disturb calcium binding, suggesting an important role of the cbEGF-like repeats in these proteins.

In this study, we investigated the effect of calcium binding on the stability of fibrillin-1 in protease degradation assays. These results demonstrate that calcium stabilizes fibrillin-1 against proteolytic degradation. This may be a general functional aspect for cbEGF-like motifs in other proteins. We hypothesize that mutations in fibrillin-1, and possibly in other proteins, that disturb calcium binding to cbEGF-like motifs render the molecules more susceptible to proteolytic degradation. Progression of the disease.

EXPERIMENTAL PROCEDURES

Radiolabeling and Purification of Fibrillin-1—Confluent normal skin fibroblasts from neonatal foreskins in 225-cm² flasks (Costar Corp.) were washed twice with phosphate-buffered saline (9.6 mM phosphate, pH 7.3, 2.7 mM KCl, 137 mM NaCl) and then incubated for 48 h with 20 ml of Dulbecco’s modified Eagle’s medium (without L-methionine and L-cysteine) containing Tran³⁵S-label (2.2 mCi/ml, 50 mCi/ml or 100 μCi/ml L-²²⁵Smethionine and L-³⁵S-cysteine, ICN). 40 ml of the labeled medium was treated with 2 μl disopropyl fluorophosphate (Sigma) and then passed over 10 ml of gelatin-Sepharose 4B (Pharmacia Biotech Inc.), equilibrated with Dulbecco’s modified Eagle’s medium, to remove fibronectin. After the flow-through was concentrated to ~20 ml by ultrafiltration, 5 ml of monoclonal antibody (mAb) 26-Sepharose (24).
was added and rocked gently overnight at 4 °C. The resin was washed with Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.4, 150 mM NaCl) including 0.05% (v/v) Tween 20. Bound protein was eluted with 0.1 mM glycine HCl, pH 2.5, and immediately adjusted to pH 8.0 with saturated Tris solution. Aliquots of each 1-ml fraction were counted in a scintillation counter.

**Design and Production of Recombinant Fibrillin-1 Subdomain rF17**—To express a subdomain of fibrillin-1 coding for Asp952–Val1527 (rF17), a 7189-base pair Nhel-Bsu36I fragment from plasmid pCis-rF11H (24) was religated with complementary oligonucleotides DR41 (5′-CTAAGCTTACGTCGAAACCATGCTCC-3′) and DR42 (5′-CTCAGGTCGACGTCGAAACCATGCTCC-3′), resulting in pCis-rF17H. The 1759-base pair Nhel-NotI fragment was then subcloned into Nhel-NotI-restricted pCEP4/H2II14 (32) and designated pCEPSPrF17H. Correct ligation of the construct was confirmed by DNA sequencing (~300 base pairs of both ends of the insert).

Transfection of pCEPSPrF17H into 293EBNA cells (Invitrogen) was performed following established procedures (33). Selection with hygromycin B (Calbiochem) was started 2 days after transfection at 0.5 mg/ml for 4 days and then reduced to 0.25 mg/ml thereafter. For production of serum-free medium, the cells were grown in 500-cm² plates (Nunc) to confluence, washed twice with phosphate-buffered saline, and incubated for 48 h with Dulbecco’s modified Eagle’s medium without fetal calf serum.

**Purification of Recombinant Subdomains**—Recombinant subdomains rF6 and rF11 were as described and characterized in detail previously (24). The affinity purification method on mAb 26 (rF11) and mAb 69 (rF6) were used. Briefly, serum-free medium containing rF6 or rF11 (1-liter volume) was concentrated to ~50 ml, treated with diisopropylfluorophosphate (2 mM), dialyzed against TBS, and passed over mAb 26-Sepharose (rF11) or mAb 69-Sepharose (rF6) equilibrated with TBS. After extensive washing with TBS, the recombinant subdomains were eluted with 0.1 mM glycine HCl, pH 2.5, and neutralized immediately with saturated Tris solution. Fractions containing rF6 or rF11 were pooled, concentrated by ultrafiltration, and dialyzed against TBS.

For purification of rF17, which has a C-terminal tag of 6 histidine residues, serum-free medium (2-liter volume) was supplemented with 0.5 mM phenylmethylsulfonyl fluoride, concentrated to 50 ml, and dialyzed against equilibration buffer containing 5 mM imidazole, rF17 was displaced from the column by an imidazole gradient (5–250 mM) in equilibration buffer containing 5 mM imidazole. After a 20-min incubation at 20°C, trypsin (EC 3.4.21.4; Sigma, treated with tosyllysyl chloromethyl ketone; Sigma), endoproteinase Glu-C, or elastase at an enzyme/substrate ratio of 1:20 (w/w) at 37 °C.

Analysis of Proteolytic Degradation—After proteolytic degradation, aliquots of radiolabeled fibrillin-1 (50 μl), rF6 and rF11 (20 μl), and rF17 and serum albumin (7.5 μl) were separated by SDS gel electrophoresis. Homogeneous gels with acrylamide concentrations of 5% (w/v) for radiolabeled fibrillin-1, 7.5% (w/v) for rF6 and rF11, and 11% (w/v) for rF17 and serum albumin were used. After electrophoresis, the gels were fixed for 20 min in 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) H₂O and then exposed to x-ray film (Amersham Corp.) for radiolabeled fibrillin-1 or stained in 0.2% (w/w) Cooamassie Brilliant Blue R-250 (Bio-Rad) in fixing solution. The radioactive gel was dried under vacuum and then exposed to x-ray film (X-Omat AR, Eastman Kodak Co.).

For Edman degradation of proteolytic products, the protein bands were transferred after SDS gel electrophoresis to Immobilon-P (Millipore Corp.) in 10 mM sodium borate, pH 9.2, at 0.4 A for 30 min. The protein bands of interest were excised and then analyzed on the protein sequencer.

**RESULTS**

The experiments described were designed to study the functional contribution of calcium binding to cbEGF-like motifs. Since the fibrillins, a family of large extracellular matrix proteins, contain multiple (43) cbEGF-like repeats in tandem arrays, we used fibrillin-1 as a prototype protein for our investigations.

To isolate fibrillin-1 from cell culture sources, confluent normal skin fibroblasts were labeled with [35S]methionine/ [35S]cysteine for 48 h. To reduce the background, fibronectin was depleted from the radiolabeled cell culture medium by gelatin-Sepharose. Finally, fibrillin-1 was purified from the medium by affinity chromatography on mAb 26, which is specific for fibrillin-1.

Incubation of radiolabeled fibrillin-1 with trypsin in the presence of 5 mM CaCl₂ or 5 mM EDTA resulted in significantly slower degradation with calcium, demonstrating that calcium stabilizes fibrillin-1 and protects the molecule against proteolysis (Fig. 1a). Already, after an incubation time of 10 min, fibrillin-1 was strongly degraded in the presence of EDTA. On the other hand, bovine serum albumin, a disulfide-bonded non-calcium-binding plasma protein, showed faster degradation in the presence of calcium as compared with EDTA (Fig. 1b), which is probably due to the stabilizing effect of calcium on trypsin (36). Thus, the differences observed in fibrillin-1 degradation in the presence of calcium and EDTA may even be underestimated. Also, the considerably slower degradation of serum albumin in the presence of calcium or EDTA indicates that the extended thread-like shaped fibrillin-1 molecule (37) is much more susceptible to degradation than the globular shaped serum albumin.

Recently, we demonstrated correct structural and functional properties of recombinantly expressed subdomains of fibrillin-1 (24, 28). To extend the studies described above, we used recombinant subdomains rF6 and rF11, which span the whole fibrillin-1 molecule, for protease degradation assays with different proteases (Fig. 2). Consistently, with trypsin, chymotrypsin, endoprotease Glu-C, and elastase, rF11 (the N-terminal half) and rF6 (the C-terminal half) were stabilized and protected against proteolysis in the presence of calcium versus EDTA. Often, the subdomains were completely digested after 23 h of incubation in the presence of EDTA, whereas in the presence of calcium, the subdomains remained relatively intact. These data indicate that essentially all types of amino acid residues (basic, acidic, and hydrophobic) within the fibrillin-1 molecule are more susceptible to proteolytic attack in the absence of calcium.

D. R. Keene, C. D. Jordan, D. P. Reinhardt, C. C. Ridgway, R. N. Ono, G. M. Corson, M. Fairhurst, M. D. Sussman, V. A. Memoli, and L. Y. Sakai, submitted for publication.
Role of Calcium in Fibrillin-1

Calcium binding to fibrillin is now well established and has been demonstrated with fibrillin from cell culture sources (3), with pepsin fragments of microfibrils (22), with recombinant subdomains of fibrillin-1 (24, 40), and with synthetic peptides (23, 41). Although little is known about the contribution of calcium to the biology of the fibrillin, it has been demonstrated that calcium mediates protein-protein interaction (28) and that calcium plays a role in maturation of a precursor fibrillin (42) and in stabilization of fibrillin molecules and microfibrils (40, 43, 44).

In this study, we tested possible stabilizing effects of calcium on fibrillin-1 in protease degradation assays using neutral proteases. We found consistently that radiolabeled authentic fibrillin-1 as well as recombinant subdomains that span the full length of fibrillin-1 are significantly more susceptible to proteolytic degradation in the absence of calcium. These results clearly demonstrate that calcium stabilizes fibrillin-1 against proteolysis. Degradation by serine proteases of fibrillin and its assembly products, the microfibrils, was reported previously (45). The contribution of calcium to protease susceptibility of fibrillin and microfibrils, however, was not tested.

To analyze regions that are protected by calcium, we produced a new recombinant subdomain of fibrillin-1 that contains the longest stretch of cbEGF-like motifs in the molecule and the preceding 8-cysteine motif at the N-terminal end. Structural characterization of this subdomain by rotary shadowing revealed an extended shape similar to regions in fibrillin purified from cell culture (37). The analysis of degradation products demonstrated that labile sites that are protected by calcium are located in the regions between cysteines 1 and 2 and between cysteines 3 and 4 of cbEGF-like motif 17. It was shown that the structural change upon calcium binding is localized to the N-terminal pocket of a single cbEGF-like repeat of factor X (38) or factor IX (39). Labile sites Met1034 and Asn1046 are located near this region in cbEGF-like motif 17 and thus, enhanced proteolytic degradation upon removal of calcium can be explained by a structural change and by reduced steric hindrance. Other degradation products were observed in the regions between cysteines 5 and 6 of cbEGF-like motifs 12 and 17 (Ser1103 and Thr1318) and in the interdomain region (Ile1019), connecting the third 8-cysteine motif with cbEGF-like motif 11. However, no structural changes in these regions upon calcium

when bound to fibrillin-1 (Fig. 4). SDS gel electrophoresis of protease-treated rF17 under nonreducing conditions often resulted in one band corresponding to full-length rF17 (data not shown). These data demonstrate that most of the cleavage sites are located within disulfide-bonded loops and not between neighboring cbEGF-like repeats, which are not connected by disulfide bridges.

The major protein bands obtained after a short (5–30 min) digest with trypsin or endoprotease Glu-C in the presence of EDTA were analyzed by Edman degradation (Fig. 4). Protease-sensitive sites, which are protected by calcium, were determined in cbEGF-like repeat 11 (Met1034 and Asn1046), repeat 12 (Ser1103), and repeat 17 (Thr1318) and in the C-terminal part of the third 8-cysteine motif (Ile1019). Met1034 and Asn1046 originate from the regions between cysteines 1 and 2 and between cysteines 3 and 4 of cbEGF-like motif 11, respectively. These regions are close to the calcium-binding pocket (Fig. 5) (38, 39).

DISCUSSION

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binding have been observed in single cbEGF-like motifs (38, 39), and no stabilizing ligand for the calcium ion originates in this region in a pair of repeats (cbEGF-like repeats 32 and 33) (43). As suggested by Wu et al. (41), additional coordination sites for calcium binding could be provided by an aspartate or asparagine residue at position 240 or 26 from the last cysteine of the preceding N-terminal cbEGF-like repeat, thus stabilizing the loop between cysteines 5 and 6 of this repeat. However, no appropriate aspartate or asparagine residue is located in the last loop of cbEGF-like repeats 12 and 17.

The lack of explanation for these labile sites may suggest that tandemly repeated cbEGF-like repeats of n > 2 are stabilized by calcium in a yet unknown manner. Therefore, the stabilizing effect of calcium binding on the last loop of the previous repeat remains to be established.

We suggest that the protective nature of calcium against proteolytic degradation of cbEGF-like repeats may be a general function of calcium binding to this type of repeat. In fibrillins, as well as in other proteins containing these repeats, this could preserve important spacer functions of tandem stretches between functional domains or protect protein-binding domains against proteolytic attack. For example, the spacer between protein-binding domains in nidogen, consisting of five consecutive EGF-like repeats, two of which are of the calcium-binding type, could be stabilized by calcium. Another example is the binding of the Drosophila transmembrane protein Notch (cbEGF-like motifs 11 and 12) to Delta and Serrate (27), where calcium binding, in addition to mediating the protein-protein interaction, could also preserve the binding domain against proteolytic degradation.

The results presented in this study are of particular interest for the pathology of genetic diseases in which certain missense
mutations in cbEGF-like motifs are known and predicted to disrupt calcium binding. These disorders include Marfan’s syndrome (30), hemophilia B (46), and Protein S deficiency (47). In Marfan’s syndrome, most of the over 50 mutations known today (reviewed in Ref. 30) occur in cbEGF-like motifs and are predicted to disrupt calcium binding by changes in amino acid residues important for calcium binding or by cysteine substitutions resulting in an uneven number of cysteine residues. In fact, mutation analysis with synthetic peptides demonstrated that a mutation affecting the asparagine residue predicted to be β-hydroxylated (N2144S) (48) in cbEGF-like motif 32 of fibrillin-1 decreased calcium binding by >5-fold (23). We suggest that mutations that affect calcium binding to individual cbEGF-like motifs render the molecule more susceptible to proteolysis. This could affect the biology of fibrillin on different levels, as single molecules or as aggregated microfibrils. Mutant fibrillin molecules could be degraded intracellularly or extracellularly, before they are incorporated into microfibrils, which would lead in turn to a diminished number of microfibrils in affected individuals. If mutant fibrillin molecules are incorporated into microfibrils, a dual mechanism may be possible. Although different models for the assembly of fibrillin into microfibrils have been published, they all place the majority of the cbEGF-like motifs into the interdomain regions (23, 24, 43). Calcium binding to these cbEGF-like repeats has been suggested to rigidify the region between two cbEGF-like domains (40, 43) and to play a role in lateral packing of the fibrillin molecules within microfibrils (39, 44). Microfibrils isolated from a cell line that was established from an individual carrying the N2144S mutation appeared disorganized in their interdomain regions (23), similar to the appearance of the interdomain regions of microfibrils from normal individuals after treatment with EDTA (44). These data, combined with our observations described here, suggest that mutations that disturb calcium binding may result in (i) a local loosening of the compact interdomain regions of the microfibrils and (ii) proteolysis of loose fibrillin molecules in the interdomain region.

In our experiments, often, the degraded subdomains are still held together by disulfide bonds, demonstrating that cleavages occur within disulfide-bonded loops and only to a lesser extent between individual motifs. Thus, if indeed Marfan’s microfibrils are more susceptible to proteolysis, it is expected that the microfibrils with endogenous degradation sites would not be fully cleaved immediately. Rather, Marfan’s microfibrils (composed of 50% normal fibrillin-1 and 50% mutant fibrillin-1) would display small “Achilles heels,” endogenous sites for potential degradation. Endogenous degradation at these sites might impair important functions like protein binding or physically weaken the microfibrils. In affected individuals, this might lead to a slow but steady decline of the microfibrils, which could explain the progressive nature of Marfan’s syndrome. Currently, we are investigating this hypothesis with recombinant subdomains harboring typical mutations in cbEGF-like motifs.

Mutations in a central region of fibrillin-1 (8-cysteine motif 3 and cbEGF-like repeat 11) have been suggested to result in neonatal Marfan’s syndrome (49), a severe form of the disease where children often die within the first year after birth. Immunofluorescence labeling of fibrillin in cell lines established from individuals with neonatal Marfan’s syndrome often shows stippled, frayed, and very short fibrils (50–52). This is in contrast to labeling patterns observed in cell lines obtained from classic Marfan’s individuals, where the integrity of the fibrillin network often appears relatively normal, but reduced in amount. These observations led to the suggestion that the neonatal region is essential for microfibril formation (53). Interestingly, three out of five labile sites observed in our study are located in the neonatal region of the molecule. This region may be particularly sensitive to proteolytic degradation when mutations causing neonatal Marfan’s syndrome impair (or influence) calcium binding to this region. It is possible that fibrillin molecules aggregate to form the nuclei for fibril formation (clumps in immunofluorescence images), but the elongation of the microfibrils might be hindered due to the lack of bound calcium and/or endogenous degradation within the neonatal region. Alternatively, since one of the observed sensitive sites (Ile1012) is located in the interdomain region connecting the third 8-cysteine motif with the downstream cbEGF-like motif and therefore is not located within a disulfide-bonded loop, proteolytic degradation at or close to this site would cause the microfibrils to fall apart rapidly. This would explain the frayed and short microfibrils observed in immunofluorescence experiments with neonatal Marfan’s cell lines and the rapid progression of neonatal Marfan’s syndrome.

Acknowledgments—We are grateful to Jay E. Gambee for protein sequencing and Douglas R. Keene and Catherine Ridgway for rotary shadowing.

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