THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 2, Issue of January 10, pp. 1231–1236, 1997
Printed in U.S.A.

Calcium Stabilizes Fibrillin-1 against Proteolytic Degradation*

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

Dieter P. Reinhardt‡§, Robert N. Ono‡, and Lynn Y. Sakai‡¶

From the ‡Shriners Hospital for Children and the ¶Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201

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 $CaCl_2$ 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 (Asp^{952}–Val^{1527}), comprising the longest stretch of cbEGF-like motifs in the center of the fibrillin-1 molecule. The most sensitive sites for trypsin and endoproteinase Glu-C were observed in cbEGF-like motifs 11 (Met^{1034} and Asn^{1046}), 12 (Ser^{1103}), and 17 (Thr^{1318}). Since most of the currently known mutations in fibrillin-1 are found within cbEGF-like motifs and are predicted to disrupt calcium binding, we suggest that these mutations render fibrillin-1 more susceptible to proteolytic cleavage, and this might be one of the reasons why these mutations result in Marfan's syndrome.

A structural motif found in the epidermal growth factor (EGF)[1] 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)X(D/N)(Q/E)X_n(D*/N*)X_m(Y/F); 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, and 43 have the consensus sequence for calcium binding. The cbEGF-like repeats are dispersed over both entire molecules in nine stretches of 1–12 tandemly repeated motifs.

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 contractural 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. Progressive degradation of the molecules or their assembly products could thus lead to the pathology of the disease.

EXPERIMENTAL PROCEDURES

Radiolabeling and Purification of Fibrillin-1—Confluent normal skin fibroblasts from neonatal foreskins in 225-cm^2 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 Tran^{35}S-label (2.2 mBq/ml = 60 μCi/ml L-[^{35}S]methionine and L-[^{35}S]cysteine; ICN). 40 ml of the labeled medium was treated with 2 μl/ml diisopropyl 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)

* This work was supported by Deutsche Forschungsgemeinschaft Fellowship Re 1021/1-1 (to D. P. R.) and by the Shriners Hospitals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 503-221-3782; Fax: 503-221-3451; E-mail: dpr@shcc.org.

[1] The abbreviations used are: EGF, epidermal growth factor; cbEGF, calcium-binding epidermal growth factor; mAb, monoclonal antibody; TBS, Tris-buffered saline.

was added and rocked gently overnight at 4 °C. The resin was washed with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) including 0.05% (v/v) Tween 20. Bound protein was eluted with 0.1 M 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 Asp^{952}–Val^{1527} (rF17), a 7189-base pair *Nhe*I-*Bsu*36I fragment from plasmid pCis-rF11H (24) was religated with complementary oligonucleotides DR41 (5′-CTAGCTGATATCCGCCTGGAAACCTGCTTCC-3′) and DR42 (5′-TCAGGAAGCAGGTTTCCAGGCGGATATCAG-3′), resulting in pCis-rF17H. The 1759-base pair *Nhe*I-*Not*I fragment was then subcloned into *Nhe*I-*Not*I-restricted pCEP4/γ2III4 (32) and designated pCEPSP-rF17H. Correct ligation of the construct was confirmed by DNA sequencing (~300 base pairs of both ends of the insert).

Transfection of pCEPSP-rF17H 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-cm^2 plates (Nunc) to confluency, 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) was utilized. Briefly, serum-free medium containing rF6 or rF11 (1-liter volume) was concentrated to ~50 ml, treated with diisopropyl fluorophosphate (2 μl/ml), 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 M 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 (20 mM phosphate, pH 7.2, 1 M NaCl). The dialyzed medium was passed over a 1-ml cobalt-loaded chelating column (HiTrap chelating, Pharmacia Biotech Inc.). After washing with equilibration buffer containing 5 mM imidazole, rF17 was displaced from the column by an imidazole gradient (5–250 mM) in equilibration buffer. Fractions containing rF17 were pooled, concentrated by ultrafiltration, and dialyzed against TBS.

Characterization of Recombinant Subdomain rF17—Purified rF17 was analyzed on a protein sequencer (Applied Biosystems Model 475) according to the manufacturer's instructions. Rotary shadowing of rF17 was performed as described previously (34). Determination of the molecular mass was done by SDS gel electrophoresis (35). For analysis of *N*-glycosylation, rF17 was treated with *N*-glycanase (Genzyme Corp.) as instructed by the supplier. The relative amount of *N*-linked glycosyl chains was then determined by SDS gel electrophoresis.

Proteolytic Degradation of Fibrillin-1 and Recombinant Subdomains of Fibrillin-1—Aliquots (50 μl, ~6000 cpm) of affinity-purified and radiolabeled fibrillin-1 were supplemented with 10 μg of bovine serum albumin (Sigma) and adjusted to a final concentration of 5 mM $CaCl_2$ or 5 mM EDTA. After a 20-min incubation at 20 °C, trypsin (EC 3.4.21.4; treated with tosylphenylalanyl chloromethyl ketone; Sigma) was added at an enzyme/substrate ratio of 1:100 (w/w) (adjusted to the amount of serum albumin present in the sample) and incubated at 37 °C. The incubation was terminated by adding 1 volume of 5-fold concentrated SDS sample buffer including 100 mM dithiothreitol to 4 volumes of the sample and then heating the sample for 3 min at 95 °C.

For proteolytic digestion of recombinant subdomains, 0.7 mg/ml rF6 or rF11 or 1.1–1.3 mg/ml rF17 in TBS was supplemented with either 5 mM $CaCl_2$ or 5 mM EDTA and incubated for 20 min at 20 °C. Then, the recombinant fragments were incubated with trypsin (treated with tosylphenylalanyl chloromethyl ketone), α-chymotrypsin (EC 3.4.21.1; treated with tosyllysyl chloromethyl ketone; Sigma), endoproteinase Glu-C (staphylococcal serine proteinase, EC 3.4.21.19; Boehringer Mannheim), and elastase (EC 3.4.21.36; Sigma) from porcine pancreas at enzyme/substrate ratios of 1:100 (w/w) for rF6 and rF11 and 1:80 (w/w) for rF17. Termination of the reactions was done as described above. As controls, 1.1 mg/ml bovine serum albumin supplemented with 5 mM $CaCl_2$ or 5 mM EDTA in TBS was digested with trypsin, α-chymotrypsin, 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) H_2O and then either incubated in Amplify (Amersham Corp.) for radiolabeled fibrillin-1 or stained in 0.2% (w/v) Coomassie 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 [^{35}S]methionine/[^{35}S]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.[2]

Incubation of radiolabeled fibrillin-1 with trypsin in the presence of 5 mM $CaCl_2$ or 5 mM EDTA resulted in significantly slower degradation with calcium, demonstrating that calcium stabilizes fibrillin-1 and protects the molecule against proteolysis (Fig. 1*a*). 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. 1*b*), 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, endoproteinase 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

[2] 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.

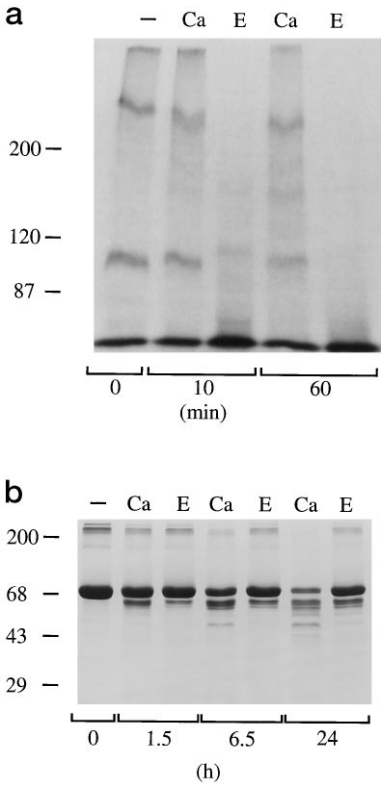

FIG. 1. **Influence of calcium on the protease susceptibility of fibrillin-1.** Fibrillin-1 labeled with [^{35}S]cysteine and [^{35}S]methionine (50-μl aliquots, ~6000 cpm) (*a*) and disulfide-bonded non-calcium-binding bovine serum albumin (7.5-μl aliquots, 1.12 mg/ml) (*b*) were incubated in the presence of 5 mM $CaCl_2$ (*Ca*) or 5 mM EDTA (*E*) with trypsin (0.1 μg/50 μl in *a* and at an enzyme/substrate ratio of 1:20 w/w in *b*) for the time periods as indicated. The reaction was stopped by adding 0.25 volume of 5-fold concentrated SDS sample buffer including 100 mM dithiothreitol and heating the samples for 3 min at 95 °C. The aliquots were separated by SDS gel electrophoresis on a 5% (*a*) or an 11% (*b*) (w/v) acrylamide gel and then analyzed by fluorography (*a*) or Coomassie Blue staining (*b*). The positions of globular marker proteins (in kilodaltons) are indicated. The experiment was repeated with identical results.

calcium. Control experiments (data not shown) with bovine serum albumin, as described above, showed slightly higher activities for α-chymotrypsin and elastase in the presence of calcium. For endoproteinase Glu-C, degradation patterns were identical or only slightly enhanced in the presence of EDTA.

To obtain sequence information about protease cleavage sites, which are usually protected by calcium, we recombinantly produced the longest stretch of 12 tandemly repeated cbEGF-like motifs preceded by the third 8-cysteine motif in fibrillin-1 for calcium protection assays. This recombinant subdomain (rF17; Fig. 3) was secreted by episomally transfected human 293 cells in amounts of 10 μg/ml/day. Edman degradation and amino acid analysis of rF17 were as expected from the cDNA. The electrophoretic mobility on SDS gels was somewhat slower than calculated. Treatment of rF17 with *N*-glycanase prior to SDS gel electrophoresis reduced its apparent molecular mass by ~10%, indicating that most, if not all, of the five potential *N*-glycosylation sites present in rF17 are occupied by *N*-linked glycosyl chains. Similar observations with rF6, rF11, and other recombinant fibrillin-1 subdomains were reported previously (24). Rotary shadowing of rF17 (data not shown) demonstrated extended thread-like particles similar to regions in fibrillin previously isolated from cell cultures (37).

Incubation of subdomain rF17 with trypsin or endoproteinase Glu-C at various incubation times in the presence of $CaCl_2$ or EDTA demonstrated again the protective nature of 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 endoproteinase 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 (Met^{1034} and Asn^{1046}), repeat 12 (Ser^{1103}), and repeat 17 (Thr^{1318}) and in the C-terminal part of the third 8-cysteine motif (Ile^{1019}). Met^{1034} and Asn^{1046} 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). The other labile sites originate from the last loop between cysteines 5 and 6 of cbEGF-like repeat 12 (Ser^{1103}) and repeat 17 (Thr^{1318}) or from the interdomain region between the third 8-cysteine motif and cbEGF-like repeat 11 (Ile^{1019}). These regions are some distance away from the calcium-binding pocket (Fig. 5).

DISCUSSION

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 fibrillins, 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 11. 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 Met^{1034} and Asn^{1046} are located near this region in cbEGF-like motif 11, 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 (Ser^{1103} and Thr^{1318}) and in the interdomain region (Ile^{1019}) connecting the third 8-cysteine motif with cbEGF-like motif 11. However, no structural changes in these regions upon calcium

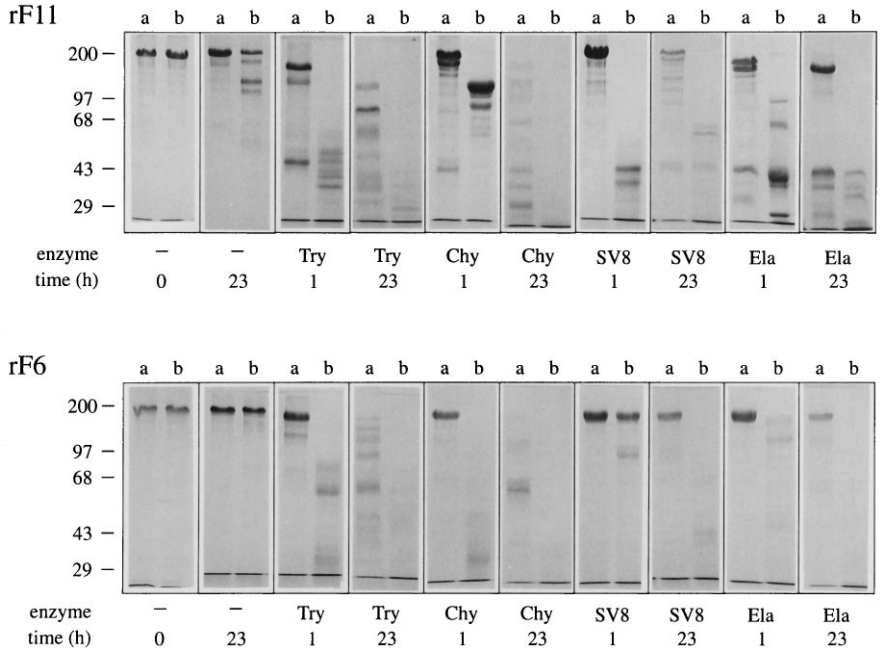

FIG. 2. **Influence of calcium on the protease susceptibility of recombinant subdomains of fibrillin-1.** Aliquots (20 μl, 0.7 mg/ml) of recombinant subdomains rF11 (*upper panel*) and rF6 (*lower panel*) were incubated in the presence of 5 mM $CaCl_2$ (*lanes a*) or 5 mM EDTA (*lanes b*) with trypsin (*Try*), α-chymotrypsin (*Chy*), endoproteinase Glu-C (*SV8*), or elastase from porcine pancreas (*Ela*) at an enzyme/substrate ratio of 1:100 (w/w) at 37 °C for the time periods as indicated. Termination of the reactions was performed as described in the legend of Fig. 1. The samples were analyzed by SDS gel electrophoresis (7.5% (w/v) acrylamide) and Coomassie Blue staining. The positions of globular marker proteins (in kilodaltons) are indicated. This experiment was repeated with identical results, and similar experiments further substantiated these results.

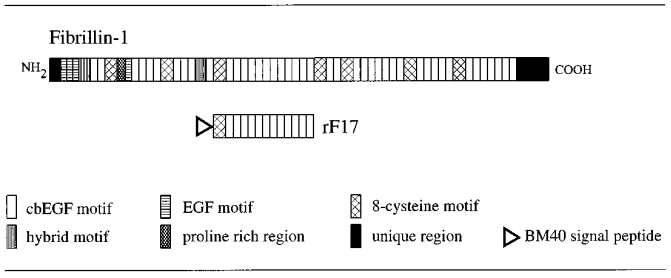

FIG. 3. **Schematic graph of recombinant subdomain rF17 as compared with full-length fibrillin-1.**

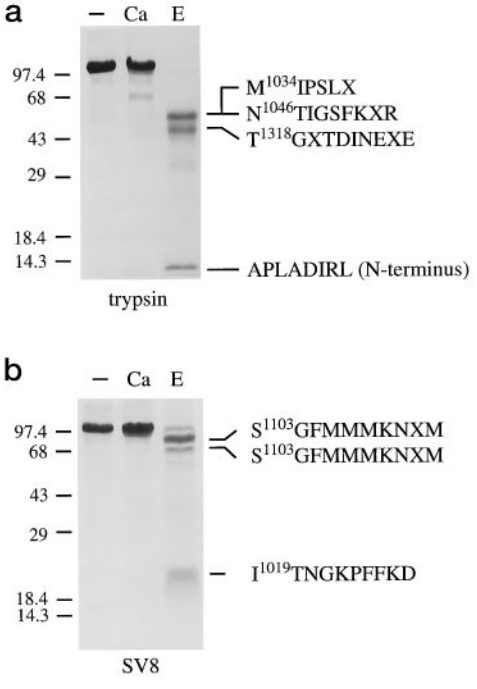

FIG. 4. **Analysis of proteolytic cleavage sites that are protected by calcium.** Recombinant subdomain rF17 (1.1 mg/ml in *a* and 1.3 mg/ml in *b*) was incubated for 5 min with trypsin (enzyme/substrate ration = 1:80 (w/w)) (*a*) or for 30 min with endoproteinase Glu-C (*SV8*; enzyme/substrate ratio = 1:100 (w/w)) (*b*) at 37 °C in the presence of 5 mM $CaCl_2$ (*Ca*) or 5 mM EDTA (*E*). Aliquots (7.5 μl) were separated under reducing conditions by SDS gel electrophoresis (11% (w/v) acrylamide) and then stained with Coomassie Blue. The experiments were repeated with identical results. Additional experiments with extended incubation times further confirmed these results. In a second experiment, the degradation products were transferred after SDS gel electrophoresis to Immobilon-P, and individual bands were analyzed by Edman degradation. N-terminal sequences of the degradation products and the positions of globular marker proteins (in kilodaltons) are indicated. The positions of the labile sites identified are demonstrated in Fig. 5 schematically.

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 −4 or −6 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

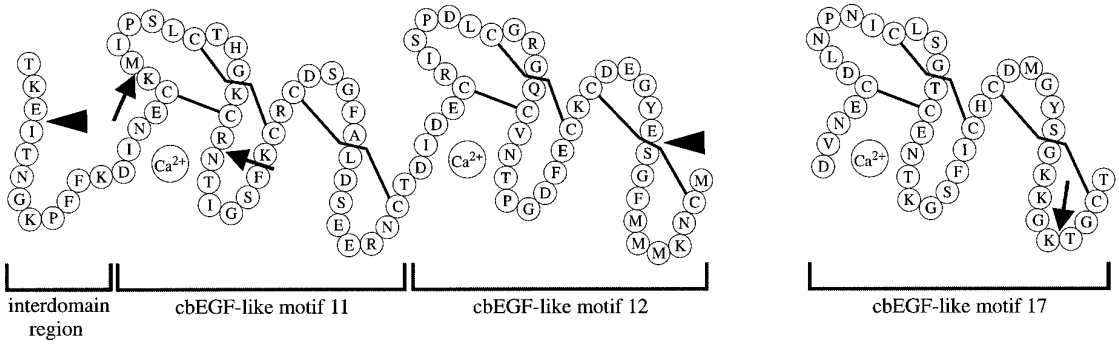

FIG. 5. **Mapping of calcium-protected labile sites onto cbEGF-like repeats of fibrillin-1.** cbEGF-like repeats 11, 12, and 17 are indicated. Each amino acid residue is represented by a *circle* and is identified by the one-letter code. *Lines* represent disulfide bridges. The N-terminal positions of degradation products obtained with trypsin (*arrows*) and with endoproteinase Glu-C (*arrowheads*) are indicated.

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 interbead domains (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 interbead domains (23), similar to the appearance of the interbead 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 interbead regions of the microfibrils and (ii) proteolysis of loose fibrillin molecules in the interbead 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 (Ile^{1019}) 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.

REFERENCES

1. Lee, B., Godfrey, M., Vitale, E., Hori, H., Mattei, M. G., Sarfarazi, M., Tsipouras, P., Ramirez, F., and Hollister, D. W. (1991) *Nature* **352,** 330–334
2. Maslen, C. L., Corson, G. M., Maddox, B. K., Glanville, R. W., and Sakai, L. Y. (1991) *Nature* **352,** 334–337
3. Corson, G. M., Chalberg, S. C., Dietz, H. C., Charbonneau, N. L., and Sakai, L. Y. (1993) *Genomics* **17,** 476–484
4. Pereira, L., D'Alessio, M., Ramirez, F., Lynch, J. R., Sykes, B., Pangilinan, T., and Bonadio, J. (1993) *Hum. Mol. Genet.* **2,** 961–968
5. Zhang, H., Apfelroth, S. D., Hu, W., Davis, E. C., Sanguineti, C., Bonadio, J., Mecham, R. P., and Ramirez, F. (1994) *J. Cell Biol.* **124,** 855–863
6. Argraves, W. S., Tran, H., Burgess, W. H., and Dickerson, K. (1990) *J. Cell Biol.* **111,** 3155–3164
7. Pan, T.-C., Kluge, M., Zhang, R.-Z., Mayer, U., Timpl, R., and Chu, M.-L. (1993) *Eur. J. Biochem.* **215,** 733–740
8. Pan, T.-C., Sasaki, T., Zhang, R.-Z., Fässler, R., Timpl, R., and Chu, M.-L.

(1993) *J. Cell Biol.* **123,** 1269–1277
9. Zhang, R.-Z., Pan, T.-C., Zhang, Z.-Y., Mattei, M.-G., Timpl, R., and Chu, M.-L. (1994) *Genomics* **22,** 425–430
10. Durkin, M. E., Chakravarti, S., Bartos, B. B., Liu, S. H., Friedman, R. L., and Chung, A. E. (1988) *J. Cell Biol.* **107,** 2749–2756
11. Mann, K., Deutzmann, R., Aumailley, M., Timpl, R., Raimondi, L., Yamada, Y., Pan, T.-C., Conway, D., and Chu, M.-L. (1989) *EMBO J.* **8,** 65–72
12. Stenflo, J. (1991) *Blood* **78,** 1637–1651
13. Lundwall, Å., Dackowski, W., Cohen, E., Shaffer, M., Mahr, A., Dahlbäck, B., Stenflo, J., and Wydro, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 6716–6720
14. Wharton, K. A., Johansen, K. M., Xu, T., and Artavanis-Tsakonas, S. (1985) *Cell* **43,** 567–581
15. Yochem, J., Weston, K., and Greenwald, I. (1988) *Nature* **335,** 547–550
16. Coffman, C., Harris, W., and Kintner, C. (1990) *Science* **249,** 1438–1441
17. Ellisen, L. W., Bird, J., West, D. C., Soreng, A. L., Reynolds, T. C., Smith, S. D., and Sklar, J. (1991) *Cell* **66,** 649–661
18. Persson, E., Selander, M., Linse, S., Drakenberg, T., Öhlin, A.-K., and Stenflo, J. (1989) *J. Biol. Chem.* **264,** 16897–16904
19. Handford, P. A., Baron, M., Mayhew, M., Willis, A., Beesley, T., Brownlee, G. G., and Campbell, I. D. (1990) *EMBO J.* **9,** 475–480
20. Öhlin, A.-K., Linse, S., and Stenflo, J. (1988) *J. Biol. Chem.* **263,** 7411–7417
21. Dahlbäck, B., Hildebrand, B., and Linse, S. (1990) *J. Biol. Chem.* **265,** 18481–18489
22. Glanville, R. W., Qian, R.-Q., McClure, D. W., and Maslen, C. L. (1994) *J. Biol. Chem.* **269,** 26630–26634
23. Handford, P., Downing, A. K., Rao, Z., Hewett, D. R., Sykes, B. C., and Kielty, C. M. (1995) *J. Biol. Chem.* **270,** 6751–6756
24. Reinhardt, D. P., Keene, D. R., Corson, G. M., Pöschl, E., Bächinger, H. P., Gambee, J. E., and Sakai, L. Y. (1996) *J. Mol. Biol.* **258,** 104–116
25. Rees, D. J. G., Jones, I. M., Handford, P. A., Walter, S. J., Esnouf, M. P., Smith, K. J., and Brownlee, G. G. (1988) *EMBO J.* **7,** 2053–2061
26. Dahlbäck, B., Frohm, B., and Nelsestuen, G. (1990) *J. Biol. Chem.* **265,** 16082–16087
27. Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. (1991) *Cell* **67,** 687–699
28. Reinhardt, D. P., Sasaki, T., Dzamba, B. J., Keene, D. R., Chu, M.-L., Göhring, W., Timpl, R., and Sakai, L. Y. (1996) *J. Biol. Chem.* **271,** 19489–19496
29. Sakai, L. Y., Keene, D. R., and Engvall, E. (1986) *J. Cell Biol.* **103,** 2499–2509
30. Dietz, H. C., and Pyeritz, R. E. (1995) *Hum. Mol. Genet.* **4,** 1799–1809
31. Putnam, E. A., Zhang, H., Ramirez, F., and Milewicz, D. M. (1995) *Nat. Genet.* **11,** 456–458
32. Mayer, U., Pöschl, E., Gerecke, D. R., Wagman, D. W., Burgeson, R. E., and Timpl, R. (1995) *FEBS Lett.* **365,** 129–132
33. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
34. Morris, N. P., Keene, D. R., Glanville, R. W., Bentz, H., and Burgeson, R. E. (1986) *J. Biol. Chem.* **261,** 5638–5644
35. Laemmli, U. K. (1970) *Nature* **227,** 680–685
36. Bulaj, G., and Otlewski, J. (1995) *J. Mol. Biol.* **247,** 701–716
37. Sakai, L. Y., Keene, D. R., Glanville, R. W., and Bächinger, H. P. (1991) *J. Biol. Chem.* **266,** 14763–14770
38. Selander-Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J., and Drakenberg, T. (1992) *J. Biol. Chem.* **267,** 19642–19649
39. Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G., and Stuart, D. (1995) *Cell* **82,** 131–141
40. Knott, V., Downing, D. K., Cardy, C. M., and Handford, P. (1996) *J. Mol. Biol.* **255,** 22–27
41. Wu, Y.-S., Bevilacqua, V. L. H., and Berg, J. M. (1995) *Chem. Biol.* **2,** 91–97
42. Raghunath, M., Kielty, C. M., Kainulainen, K., Child, A., Peltonen, L., and Steinmann, B. (1994) *Biochem. J.* **302,** 889–896
43. Downing, A. K., Knott, V., Werner, J. M., Cardy, C. M., Campbell, I. D., and Handford, P. A. (1996) *Cell* **85,** 597–605
44. Kielty, C. M., and Shuttleworth, C. A. (1993) *FEBS Lett.* **336,** 323–326
45. Kielty, C. M., Woolley, D. E., Whittaker, S. P., and Shuttleworth, C. A. (1994) *FEBS Lett.* **351,** 85–89
46. Giannelli, F., Green, P. M., Sommer, S. S., Poon, M. C., Ludwig, M., Schwaab, R., Reitsma, P. H., Goossens, M., Yoshioka, A., and Brownlee, G. G. (1996) *Nucleic Acids Res.* **24,** 103–118
47. Gandrille, S., Borgel, D., Eschwege-Gufflet, V., Aillaud, M., Dreyfus, M., Matheron, C., Gaussem, P., Abgrall, J. F., Jude, B., Sie, P., Toulon, P., and Aiach, M. (1995) *Blood* **85,** 130–138
48. Hewett, D. R., Lynch, J. R., Smith, R., and Sykes, B. C. (1993) *Hum. Mol. Genet.* **2,** 475–477
49. Kainulainen, K., Karttunen, L., Puhakka, L., Sakai, L., and Peltonen, L. (1994) *Nat. Genet.* **6,** 64–69
50. Superti-Furga, A., Raghunath, M., and Willems, P. J. (1992) *J. Med. Genet.* **29,** 875–878
51. Godfrey, M., Raghunath, M., Cisler, J., Bevins, C. L., DePaepe, A., Di Rocco, M., Gregoritch, J., Imaizumi, K., Kaplan, P., Kuroki, Y., Silberbach, M., Superti-Furga, A., Van Thienen, M.-N., Vetter, U., and Steinmann, B. (1995) *Am. J. Pathol.* **146,** 1414–1421
52. Wang, M., Price, C., Han, J., Cisler, J., Imaizumi, K., Van Thienen, M.-N., DePaepe, A., and Godfrey, M. (1995) *Hum. Mol. Genet.* **4,** 607–613
53. Putnam, E. A., Cho, M., Zinn, A. B., Towbin, J. A., Byers, P. H., and Milewicz, D. M. (1996) *Am. J. Med. Genet.* **62,** 233–242

Calcium Stabilizes Fibrillin-1 against Proteolytic Degradation

Dieter P. Reinhardt, Robert N. Ono and Lynn Y. Sakai

J. Biol. Chem. 1997, 272:1231-1236.
doi: 10.1074/jbc.272.2.1231