The primary structure of human Elastin microfibril interface-located protein (EMILIN), an elastic fiber-associated glycoprotein, consists of a globular C1q domain (gC1q) at the C terminus, a short collagenous stalk, a long region with a high potential for forming coiled-coil α helices, and a cysteine-rich N-terminal sequence. It is not known whether the EMILIN gC1q domain is involved in the assembly process and in the supramolecular organization as shown for the similar domain of collagen X. By employing the yeast two-hybrid system the EMILIN gC1q domains interacted with themselves, proving for the first time that this interaction occurs in vivo. The gC1q domain formed oligomers running as trimers in native gels that were less stable than the comparable trimers of the collagen X gC1q domain since they did not withstand heating. The collagenous domain was trypsin-resistant and migrated at a size corresponding to a triple helix under native conditions. In reducing agaro gels, EMILIN also migrated as a trimer, whereas under non-reducing conditions it formed polymers of many millions of daltons. A truncated fragment lacking gC1q and collagenous domains assembled to a much lesser extent, thus deducing that the C-terminal domain(s) are essential for the formation of trimers that finally assemble into large EMILIN multimers.

Elastin microfibril interface-located protein (EMILIN) belongs to the C1q/TNF superfamily of proteins (1) and is a considerable component of the elastic fiber system. Elastic fibers confer the properties of resiliency and elastic recoil to connective tissues and are secreted in the extracellular matrix (ECM) of many tissues in various forms. In the elastic ligaments they are deposited as solid branching and unbranching fine and thick rod-like fibers, in the blood vessels as concentric sheets of lamellae, in the elastic cartilages as a three-dimensional meshwork of fine fibrils, and in skin and lung as a combination of these (2). As previously studied in the chick model, the major characteristics of EMILIN, first extracted by means of denaturing and reducing agents, are as follows. It is preferentially extracted from tissues using buffers containing guanidine HCl and reducing agents; it forms a fibrillar network in the ECM of in vitro grown smooth muscle cells and fibroblasts and in the ECM of several tissues including blood vessels, skin, heart, lung, kidney, and cornea; it codistributes with elastin in most sites (3–7); it is a component of elastic fibers being mainly localized at the interface between amorphous elastin and the surrounding microfibrils (8); finally and more important for the functional significance of EMILIN, the process of in vitro elastin deposition was perturbed by the addition of anti-EMILIN antibodies to the culture medium suggesting that this protein plays a leading role during the elastic fiber formation process (8).

Recently, the primary structure of the human EMILIN has been elucidated (1). The mature form of this multimodular protein consists of 996 amino acids and differs from all other elastin-associated proteins; the domain organization includes a C1q-like globular domain (gC1q) at the C terminus, a short uninterrupted collagenous stalk, containing two cystein units, and at least four heptad repeats with a high potential for forming coiled-coil α helices. At the N terminus, a cysteine-rich sequence (EMI domain) is homologous to a region of multimerin (9) (a diagram showing the EMILIN structure is shown in Fig. 1). The gC1q domain is shared with several other ECM constituents including type VIII and type X collagens in which it represents the equivalent of the C-propeptide of fibrillar collagens, and it is believed to play a similar function. While type X collagen is specifically expressed in hypertrophic cartilage (10), type VIII collagen is found both in the subendothelium and in the elastin-rich tunic media (11, 12) of blood vessels where it is thought to be a crucial element of the structure. Thus, like EMILIN, type VIII collagen is a significant component of the various proteins associated with elastin-rich tissue. Unlike the fibrillar procollagens, the C-propeptides (i.e. the gC1q domains) of type VIII and X collagens are not cleaved following secretion into the ECM but are thought to play a similar role in the initial chain selection and trimerization of the triple helix and in the assembly of the individual triple-helical monomers into higher order structures (13). Furthermore, all mutations detected in Schmid type metaphaseyel chondrodysplasia have been located in the gC1q domain and have been proposed to affect the initial stages of type X collagen folding and assembly (14, 15). Supportive evidence was later provided by rotary shadowing data; the gC1q do-
domains, which are highly hydrophobic, appeared to exert a primary role in the aggregation of type X collagen molecules (16).

Currently the oligomerization state of EMILIN and the exact nature of the forces stabilizing EMILIN assemblies have not been identified. Preliminary data from SDS-polyacrylamide gel electrophoresis run under nonreducing conditions revealed that intracellularly EMILIN behaved as a monomeric protein. Once secreted from the cell it underwent intermolecular cross-linking by disulfide bonds giving rise to high molecular weight aggregates (7). This suggested that S–S bond formation might be a contributing factor in multimer organization (7). To elucidate the putative oligomerization of EMILIN, we investigated the self-association of the gC1q domain and of the entire molecule and found that formation of EMILIN trimers depends on the gC1q domain and that further multimerization is apparently mediated solely by S-S bonds.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids for the Two-hybrid System—**To study the EMILIN intermolecular interactions, several constructs encoding LexA fusion proteins containing the C-terminal domains (see below) were generated by polymerase chain reaction (PCR) employing 1 ng of EMILIN-pGEM-T as a template (1), 10 pM each primer (see below), 2.5 units of Taq polymerase (Promega Corp.), and 0.2 mM each of the four deoxynucleotide triphosphates (Pharmacia Ultrapure, Amersham Pharmacia Biotech). The primers used were the following: sense 5'-GAATTCCGCCATGCCCCAGCTTAGA-3' and antisense 5'-CCCATGGCTACGCGTGTTCAAGCTCTGG-3' for the construct yA which includes the gC1q, the collagenous (COL), and the leucine zipper (LZ) domains, from position 2152–3051 of the published sequence (GenBankTM Data base, accession number AF088916); the sense 5'-GG-GAATTGCATGCCCCCTGCCCACCATG-3' in combination with the previous antisense primer used for the construct yA for the construct yB including the gC1q domain only. The underlined bases correspond to appended EcoRI and XhoI restriction enzyme recognition sites plus two additional protective nucleotides. The amplification was performed for 25 cycles with a temperature profile of 1 min at 95 °C, 1 min at 58 °C, and 45 s at 72 °C. The PCR fragments, digested with the appropriate restriction enzymes, were purified with High Pure Cartridges (Roche Molecular Biochemicals), ligated overnight in both pLexA and pB42AD vectors (CLONTECH) expressing the binding and the activation domains, respectively, and transformed in Escherichia coli competent DH5α strain. Ampicillin-resistant colonies were screened for the presence of the inserts by restriction analysis. The nucleotide sequence of candidate constructs were determined, and the selected plasmids were used in the yeast transformation assay.

**Yeast Transformation and Selection of Interactors—**The Saccharomyces cerevisiae strains EGY48 [p8op-lacZ] (MATα, his3, trp1, ura3, LexAop(x6)-LEU2) carrying the reporter plasmid p8op-LacZ and YMA271 (MATα, ura3-52, his3-200, lys2-801, ade2-101, ade5, trp1-901, leu2-3, 112, tyr1-501, gal4-d512, gal80-D538, ade5-hisG) were used for the present assays. Yeast cultures were grown at 30 °C in either YPD (1% yeast extract, 2% peptone, and 2% glucose) or SD minimal medium (0, 5% yeast nitrogen base without amino acids, 2% glucose, and 1% desired amino acid dropout solution). Growth and manipulation of yeast strains was carried out using the procedures described in the Matchmaker Two-hybrid system user manual (CLONTECH). The hybrid constructs (pLexA-yA or -yB and pB42AD-yA or -yB) were sequentially transformed into the EGY48 p8opLacZ yeast reporter strain by the PEG/lithium acetate transformation procedure, using the YEASTMAKER™ yeast transformation system kit (CLONTECH). Transformants were allowed to grow at 30 °C usually for 2–3 days in SD-his-trp-ura medium and then in SD/GalRaftar/his−/leu−/trp−/ura-X-gal selective medium. The Leu+, LacZ+, positive clones were subjected to a mating assay to confirm the protein-protein interaction. Briefly, the plasmids containing the fusion proteins were individually transfected in either EGY48 [p8op-lacZ] or YMA271 yeast strains. The two transformed strains were simultaneously incubated in YPD medium at 30 °C for 18 h, and an aliquot was plated onto SD/- His/-Trp/- Ura plates. After incubation at 30 °C for 3 days, the diploid cells forming visible colonies were picked up and streaked on SD/GalRaftar/his−/leu−/trp−/ura-X-Gal-containing plates. Blue colonies usually appeared between 24 and 120 h. As a positive control we used the p53 protein fused to the LexA binding domain and the SV40 large T cell antigen as a prey. Negative controls consisted of EGY48 transformed with one of the following constructs: pLexA-yA or -yB; pLexA-lam; and pB42 AD-yA or -yB.

**Production of Recombinant Prokaryotic Domains of Human EMILIN—**The sequence corresponding to the C-terminal domain of EMILIN (gC1q construct pB) was amplified from the EMILIN-pGEM-T template (see above) with the same primers used for generating yB, in
which the 5'-ends were replaced with the BamHI and KpnI recognition sites for sense and antisense primers, respectively. It was then ligated in frame in the Hisa-tagged pQE-30 expression vector (Qiagen GmbH) and transformed into BL21 cells. Positive clones were selected, and the cloned fragment was sequenced in both directions to check for errors generated during PCR. Five hundred ml of liquid culture grown on LB medium OD A$_{600_{	ext{nm}}}$ was induced with 2 mM isopropyl-1-thio-$eta$-D-galactopyranoside for 3 h at 37 °C. The culture was then centrifuged at 4,000 × g for 20 min, and the cell pellet was resuspended in sonication buffer (50 mM sodium phosphate, pH 8.0, 0.3 mM NaCl at 5 volumes/g of wet weight. The samples were frozen in a dry ice/ethanol bath, thawed in cold water, and sonicated on ice (1 min bursts/1 min cooling/2–300 watts), and cell breakage was monitored by measuring the release of nucleic acids at A$_{260_{	ext{nm}}}$.

The cell lysate was centrifuged at 10,000 × g for 20 min; the supernatant was collected, and purification of the different domains was performed by affinity chromatography on nickel-nitrilotriacetic acid resin (Qiagen GmbH) under native conditions. The recombinant protein was eluted from the affinity column in sonication buffer, pH 6.0, containing 10% glycerol, and 0.2 M imidazole. After dialysis against 25 mM Na$_2$PO$_4$, 150 mM NaCl, pH 6.0, the native status of the polypeptide was checked by CD spectra analysis.

**Protein Expression in 293-EBNA Cells**—In order to express high amounts of distinct EMILIN constructs, the eukaryotic expression plasmid pCEP-Pu/AC7 containing the BM-40 signal peptide (17) was employed. The cloned fragments included the following: 1) the entire coding sequence of the putative mature EMILIN obtained by ligating a 5′-PCR-amplified fragment (from position 64 to 2268) to a 3′ fragment (from position 2266 to 3051) (EMILIN); 2) a frameshifted construct extending from position 64 and including only the first and half of the second LZ bearing a stop codon at position 2426 (EMILIN-T); 3) the aMA construct that includes the leucine zipper domains, the collagenous domain, and the gC1q-like domain (LEU-COL-gC1q) (from position 2152 to 3051); 4) the mC construct including the collagenous and the gC1q domains (COL-gC1q) only (from position 2440 to 3051). The PCR reactions for EMILIN and EMILIN-T constructs were performed as described previously (1) employing the sense primer, 5′-CTAGCTAGC-GGG CTCCTGGGGCTGGAGGAC-3′, in combination with the same antisense primer used for construct yA except for bearing a NotI site. The underlined bases correspond to the NotI recognition site plus one appended nucleotide. The same restriction sites were substituted in the oligonucleotides employed for priming the other two constructs. All amplified fragments were sequenced in both directions. The human embryonic kidney cell line 293-EBNA, constitutively expressing the EBNA-1 protein from Epstein-Barr virus (Stratagene), was transfected by electroporation with the EMILIN constructs. Just before the transfection, a half-million cells were collected and resuspended in serum-free culture medium (Dulbecco’s modified Eagle’s medium) containing 25 mM NaCl and incubated for 5 min in the presence of 10 μg of DNA. The cells were electroporated and plated in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum. 24 h later 500 ng/ml puromycin were added to the medium for the selection. Puromycin-resistant cells were selected and assayed for recombinant protein expression by precipitating the spent (10 min at 13,000 rpm) serum-free culture medium with 50% (w/v) trichloroacetic acid and 1% Triton X-100 and analyzing the precipitate by SDS-PAGE. To detect the production of COL-gC1q (mC) and LZ-COL-gC1q (mA) polypeptides, which were produced in lower amounts, the precipitates were separated by SDS-PAGE and immunoblotted with a polyclonal antibody against recombinant EMILIN B, comparison between the human recombinant EMILIN produced by stably transfected 293-EBNA cells and tissue EMILIN. Lane 1, EMILIN was collected from serum-free culture medium; lane 2, a human placenta was extracted in 6 M guanidine HCl. Aliquots of EMILIN and of placenta extract were separated by a 6% SDS-PAGE under reducing conditions and immunoblotted as in A.

against human EMILIN was obtained by subcutaneous immunization with 150 μg of purified recombinant protein. Before use the antiserum was absorbed onto a CNBr-activated Sepharose resin saturated with mock-transfected cell proteins. The antiserum was assayed for the reactivity against recombinant EMILIN (titer around 1:10,000) and against its recombinant domains by an enzyme-linked immunosorbent assay. The serum titer against unrelated proteins (COL VI, fibronectin, vitronectin, and elastin) was more than 100-fold lower. This antiserum stained the ECM of several human tissues including vessels, kidney, and subcutaneous tissue. For immunoblotting the various recombinant proteins and a reduced 6 % guanidine HCl human placenta extract were resolved by SDS-PAGE and electrophoretically transferred to nylon filters (Amersham Pharmacia Biotech). Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies and an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech) and were shown to recognize the recombinant EMILIN polypeptides (Fig. 2A) as well as genuine tissue EMILIN (Fig. 2B). This latter migrated slightly less than EMILIN secreted by transfected 293-EBNA cells.

**Determination of the Native Molecular Size by Electrophoresis under Non-denaturing Conditions**—“Native” electrophoresis in the absence of SDS or urea represents a useful technique for determining the native size and subunit structure of proteins; under these conditions mobility depends on size, shape, and intrinsic charge of the protein. Proteins of standard molecular size (non-denatured protein molecular weight marker kit, Sigma) and purified recombinant polypeptide samples were

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**Fig. 2. Relative migration on SDS-PAGE of EMILIN recombinant polypeptides and of genuine EMILIN protein from human placenta.** A, recombinant polypeptides of human EMILIN produced by the stably transfected 293-EBNA cells. Polypeptides collected from serum-free culture medium of 293-EBNA cells transfected with cDNAs for EMILIN (lane 1), EMILIN-T (lane 2), LZ-COL-gC1q (mA) (lane 3), COL-gC1q (mC) (lane 4), and gC1q (mB) (lane 5) were separated by a 10% SDS-PAGE under reducing conditions and immunoblotted with a polyclonal antibody against recombinant EMILIN B, comparison between the human recombinant EMILIN produced by stably transfected 293-EBNA cells and tissue EMILIN. Lane 1, EMILIN was collected from serum-free culture medium; lane 2, a human placenta was extracted in 6 M guanidine HCl. Aliquots of EMILIN and of placenta extract were separated by a 6% SDS-PAGE under reducing conditions and immunoblotted as in A.
EMILIN Self-assembly

Separated in a continuous electrophoresis system at pH 8.2, under non-denaturing conditions in 10, 12, and 15% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R-250. The molecular size of a given protein is estimated in this system indirectly by the following method. The electrophoretic mobility ($R_e$) of each protein relative to the tracking dye is determined on a set of gels of various polyacrylamide concentrations; $100 \times \log R_e \times 100$ is plotted against the percent gel concentration for each protein, and the slope of such a plot is the retardation coefficient ($K_p$). From these various plots, individual slopes ($K_p$) are determined for each protein, and the logarithm of the negative slope is plotted against the logarithm of the molecular size of each protein. This then produces a linear plot from which the molecular size of an unknown protein may be determined (18).

Enzyme Digestion—For trypsin digestion the LZ-COL-gC1q (mA) polypeptide was dialyzed against phosphate-buffered saline and incubated with 0.2 mg/ml trypsin for 16 h at 4 °C. At the end of the enzyme treatment, the digested proteins were precipitated with 10% (w/v) trichloroacetic acid and analyzed by gel electrophoresis on 15% (w/v) polyacrylamide gel under reducing and non-denaturing conditions.

Thermal Denaturation—Aliquots of purified gC1q (pA) polypeptide were resuspended in SDS sample buffer and placed in a DNA gradient thermal cycler block (Robocycler, Stratagene). A stepwise temperature gradient was set up, and samples were subjected to a range of temperatures from 52 to 68 °C. At the end of the incubation, the samples were removed and resolved in SDS-PAGE.

Agarose Gels—Samples in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% (w/v) SDS were analyzed by agarose gel electrophoresis under both reducing and non-reducing conditions using 0.8% (w/v) high melting agarose (Seakem, FMC Bioproducts, Rockland, ME) in the stacking gel and 1.4% (w/v) low melting agarose (Seakem) in the separating gel. vWF and fibronectin from human plasma obtained from healthy donors after informed consent were purified by gel filtration chromatography on Sephacryl CL-4B columns (19) and by gelatin-Sepharose affinity chromatography (20), respectively. Murine IgM was purified by sequential precipitation with caprylic acid and ammonium sulfate (21) from 8M urea prior to loading on the gel. The gC1q polypeptide was analyzed using the Menendez-Arias program (23).

Circular Dichroism Spectroscopy—The gC1q (pA) polypeptide was processed for CD spectroscopy analysis. A Jasco J-600 CD/ORD spectrophotometer interfaced to an Olidata computer for data collection was used for all the measurements. Calibration of the instrument was performed with d-(+)-10-camphorsulfonic acid at 290 nm. Standard conditions were 25 mM NaPO$_4$, 150 mM NaCl, pH 6.0, using a 0.2-cm path length cuvette and with a water bath at 10 °C. Then the spectra were analyzed using the Menendez-Arias program (23).

RESULTS

In Vivo Self-interaction of the C-terminal (gC1q) Domain of EMILIN—Two distinct constructs corresponding to the C-terminal regions of EMILIN, construct aZ (LZ-COL-gC1q) and construct bY (gC1q), were fused with both the DNA binding domain and with the activation domain of LexA. The hybrid constructs were sequentially transformed in the EGY48 yeast strain (popLacZ) to check for their ability to self-activate the reporter genes LEU2 and lacZ and to enter the nucleus. The constructs were then transformed in different combinations (yaYaYb, ybYbY and yaYaYb) in the same yeast strain previously transfected with popLacZ. The interactions were assayed by plating transfectants on SD/Leu$, His$-, Trp$-, Ura and on SD/Leu$, His$, Trp$, Ura, Gal/Raf, X-gal agar plates. The growth of the yeast transfectants in the incomplete medium and the β-galactosidase activity were estimated. The EMILIN domains were shown to interact with each other in all combinations, and growth in the incomplete medium, and induced β-galactosidase activity to an extent comparable to that observed for the positive control (see Table I). Yeast cells transfected with both ya and yb constructs and the partner vector without any insert did not grow in the selective medium indicating that the activation of the reporter genes was due to the interaction of the EMILIN domains (data not shown). The binding was further verified by yeast mating, a system complimentary to the cotransfection assay (24) (Table I). It is not clear if the leucine zipper domain enhances the interaction; however, for the first time we demonstrate that the gC1q domain, which in these experiments is the essential element for the binding, is able to self-assemble in vivo.

Assembly of the C-terminal gC1q Domain—The above experiments provided the initial information to conclude that the gC1q domain of EMILIN can interact with itself. However, the two-hybrid system is not informative as to whether this domain can form homotrimers. Thus, to investigate also whether the gC1q domain of EMILIN, in analogy with those of several of the other members of the C1q/TNF superfamily such as type X (25–27) and type VIII (28, 29) collagens, the complement C1q component (30, 31), and ACRP30 (32), can form trimers, the gC1q domain of EMILIN can interact with itself. However, the experiments provided the initial information to conclude that the gC1q domain of EMILIN can interact with itself. However, the two-hybrid system is not informative as to whether this domain can form homotrimers. Thus, to investigate also whether the gC1q domain of EMILIN, in analogy with those of several of the other members of the C1q/TNF superfamily such as type X (25–27) and type VIII (28, 29) collagens, the complement C1q component (30, 31), and ACRP30 (32), can form trimers, the gC1q (pB) polypeptide of EMILIN was purified from bacterial lysates by affinity chromatography on nickel-nitrilotriacetic acid resin and separated in PAGE under non-denaturing conditions. The CD spectrum of the polypeptide showed that the secondary structure was preserved and that it had a native conformation (data not shown). Systematic examination of electrophoresis in gels of different porosity (10, 12, and 15% acrylamide concentrations) showed that the apparent molecular size of the native gC1q (pB) band compared with the migration of four molecular weight standards (albumin, egg albumin, carbonic anhydrase, and α-lactalbumin) corresponded to that of a trimer (Fig. 3B). Under the same running conditions the presence of 5% 2-β-mercaptoethanol was ineffective (data not shown) suggesting that disulfide bonds are not contributing to the stability of the trimer under native conditions.

Next, the resistance of the EMILIN gC1q (pB) polypeptide to denaturing agents was compared with the resistance of the equivalent gC1q domain of type X collagen. It is thought that strong non-covalent interactions within the gC1q domains of type X (27) and likely of type VIII (29) collagens are responsible for oligomer stability under denaturing conditions. In order to investigate the stability of the trimers formed in this system, a solution of EMILIN or type X collagen was denatured by boiling for 20 min in 2% SDS or for 5 min in the presence of 2% SDS and 8 M urea prior to loading on the gel. The gC1q polypeptide trimers of type X collagen were not affected by overheating in presence of the detergent. When the sample was treated with 8 M urea most of the type X collagen gC1q polypeptide migrated as a monomer and only a small fraction with a size compatible with a dimer. On the contrary, the EMILIN gC1q

| Table I | Interactions of different EMILIN constructs assessed by the two-hybrid system |
|---------|-----------------------------------------------|
| DNA-binding domain | Activation domain | Cotransformation | Mating |
| Clq | Clq | ++ | ++ |
| Clq | Clq | + | + |
| Clq | Clq | + | + |
| Clq | Clq | ++ | ++ |

Constrains ya and yb were fused with the DNA binding domains and the activation domains of LexA and cotransformed in different combinations (ya/Ya; yb/Yb, ya/Yb). β-Galactosidase activity was revealed by plating transformants on X-gal containing agar and observing growth of blue colonies in comparison with that of positive and negative controls. The interaction was confirmed by using an alternative system, yeast mating; for transforming the EGY48 strain with one construct and the complementary mating YM4771 strain with the other construct. Plus signs refer to the high (+ + +), medium (+ +), and low (+) intensity of the blue color.
polypeptide did not withstand boiling in SDS, and all the polypeptide migrated as a monomer (Fig. 4, lane 3). When the sample was treated with 8 M urea an additional minor faster migrating band appeared, whose intensity varied considerably in different experiments and the nature of which was not further investigated. Similar results were obtained with a eukaryotically produced EMILIN gC1q (pB) polypeptide (data not shown).

The finding that the EMILIN gC1q (pB) polypeptide lost its trimeric structure upon heating prompted a more thorough investigation of its thermostability. Native trimers were incubated at different temperatures (38, 52, 56, 58, 60, 62, 64, and 68 °C) for 5 min in a sample buffer containing 2% SDS before analysis by SDS-PAGE. The trimers were totally resistant to thermal denaturation up to 54 °C (Fig. 5). At 58 °C, about 50% of the domain still remained as trimer, whereas at 64 °C the trimers were completely separated into monomers. The migration of the EMILIN gC1q monomer on SDS-PAGE was similar to that predicted (about 17 kDa) from its amino acid sequence; however, the mobility of the trimer was much faster than expected with a molecular mass of about 32 kDa, suggesting a very compact conformation also in the presence of 2% SDS. Finally, disulfide bonds were not involved in the thermal stability since the same migration pattern was detected also in the presence of 5% 2-mercaptoethanol (data not shown).

Trypsin Digestion—Trypsin digestion is considered a reliable method to study the structure of proteins. The enzyme cleaves polypeptide bonds after arginine and lysine residues, thereby providing information about the secondary and tertiary structure of the protein. The results of trypsin digestion can be used to infer the presence of disulfide bonds, the location of charged residues, and the overall conformation of the protein. In the context of the EMILIN gC1q polypeptide, trypsin digestion experiments were performed to confirm the absence of disulfide bonds and to gain insights into the protein’s structure.
assay to check for the formation of stable triple helical molecules (33), and this conclusion is based on the presumption that unfolded or incompletely folded collagen helices are sensitive to proteolytic digestion. In order to investigate the stability of the triple helix of EMILIN, 293-EBNA cells transfected with the COL-gC1q (mC) or the LZ-COL-gC1q (mA) constructs were grown in 50 μg/ml ascorbate. The conditioned medium was digested overnight and analyzed on a 12% polyacrylamide gel under native conditions to preserve the triple helices. The digestions of the COL-gC1q (mC) polypeptide did not result in reproducible results, one reason being the very low level of expression of this construct. However, consistent results were obtained with the LZ-COL-gC1q (mA) polypeptide. Compared with the mobility of standard proteins (Fig. 6, lanes 3–6), the migration of the LZ-COL-gC1q (mA) polypeptide suggested that it also assembled to form a trimer (Fig. 6, lane 1), as expected from the behavior of the gC1q (pB) polypeptide (see Fig. 3). The trypsin digestion resulted in a polypeptide with a much faster mobility (Fig. 6, lane 2) compatible with the mobility of a trypsin-resistant triple helix formed by the 17 GXY triplets of the COL domain. In SDS-PAGE performed under denaturing conditions, the digested product migrated with the dye front indicating that the triple helix had been denatured (data not shown).

**Multimer Formation by Recombinant EMILIN**—It has been reported (3) that EMILIN can be extracted from tissues only if reducing agents are included in buffers containing 6 M guanidine HCl. Further indication that this protein associated to form higher order structures was obtained in biosynthetic studies *in vitro* in which it was demonstrated that soon after secretion EMILIN monomers assembled to form huge aggregates unable to enter SDS-polyacrylamide gels (7). By having established in the present study that EMILIN undergoes a spontaneous trimerization step that initiates at the C-terminal gC1q domain, as was previously demonstrated to occur for type X collagen (14, 15), the modalities of further EMILIN multimerization were then investigated.

First, we analyzed whether the gC1q domain, by facilitating the trimer formation, was essential for the multimerization of EMILIN. For this purpose a full-sized EMILIN and a shorter EMILIN polypeptide (EMILIN-T), lacking about half of the LZ, the COL, and the gC1q domains, were collected from 293-EBNA cells and were then resolved under both reducing and non-reducing conditions using an agarose gel system (Fig. 7). As molecular size standards non-reduced laminin-1 (about 900 kDa) and fibronectin (about 550 kDa) were used. Under non-reducing conditions laminin-1 (lane 4), fibronectin (lane 3), and EMILIN-T (lane 2) behaved as relatively fast-migrating components, whereas EMILIN (lane 3) migration was considerably retarded. The high molecular weight aggregates were apparently due to intermolecular S–S bonds, since under reducing conditions EMILIN (lane 5) migrated slightly above the fibronectin monomer (lane 7) suggesting that the smallest EMILIN multimer under reducing conditions is very likely a trimer.

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**Fig. 4. Resistance of gC1q trimers of EMILIN and of collagen type X against denaturing agents.** Samples of gC1q (pB) and type X gC1q were incubated first for 15 min at 20 °C in a buffer containing 10 mM Tris, pH 6.8, 2% SDS, 5% glycerol, and 0.005% bromphenol blue in the absence (lanes 1 and 3) or in the presence of 8 M urea (lanes 2 and 4) and then boiled for 5 (lanes 2 and 4) or 20 min (lanes 1 and 3) and loaded onto a 12% SDS-PAGE. In contrast to the gC1q trimer of collagen X that is stable in the presence of SDS and dissociates only partially to monomers and dimers in the presence of SDS and 8 M urea, the gC1q trimer of EMILIN is completely dissociated into monomers in SDS; the addition of 8 M urea results in the separation of the EMILIN gC1q monomer in two closely spaced bands. Recombinant human type X gC1q was purified from the supernatant of 293 cells transformed with a cDNA fragment coding for the entire gC1q (27).

**Fig. 5. Thermostability of the gC1q domain of EMILIN.** A native gC1q trimer (pB) in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, and 0.1% bromphenol blue) was subjected to heating at the indicated temperatures for 5 min under non-reducing conditions before loading to a 12% SDS-PAGE.
(about 500 kDa). Under non-reducing conditions EMILIN-T migrated as a broad band indicating that some intermolecular S–S bonds might participate in the formation of minor aggregates, if compared with the intact form. Whereas most of the cysteine residues are very closely spaced and paired, a cysteine present in the partial epidermal growth factor-like motif is unpaired and could be involved in these intermolecular bonds.

To have a better indication of the sizes of the EMILIN multimers, EMILIN was freshly collected from 293-EBNA cells and resolved under non-reducing conditions in agarose gels. As a control for the migration we employed the plasma vWF which forms multimers of several million daltons (34). EMILIN migrated as two major closely spaced multimers, one of which migrated more slowly than the largest vWF multimer (Fig. 8). Combined with the fact that EMILIN-T, which is lacking the gC1q domain, is incapable of forming massive multimers, these data suggest that the gC1q domain is essential for the initial steps of the trimer formation. Following the assembling in trimers, EMILIN multimerizes to form large aggregates through disulfide bonds.

**DISCUSSION**

The experiments reported clearly show that EMILIN pro- torchers associate to form a homotrimer through a relatively stable interaction of their C-terminal gC1q domain. This allows the nucleation of the triple helix and then a further quaternary assembly to higher order polymers via intermolecular disulfide bonds. This type of supramolecular organization is unique among the constituents and the associated components of the elastic fiber.

Initial evidence for EMILIN self-association was obtained from the application of the two-hybrid system to the analysis of
the gC1q domain. Although one of the major applications of the two-hybrid system has been the screening of cDNA libraries to find clones encoding proteins that bind specific nuclear or cytoplasmic target protein, the same methodology is also applicable for testing a supposed interaction between protein domains or amino acids stretches in vivo. Several combinations of proteins have been successfully studied, but in very few instances have ECM proteins been analyzed. Notable are the thrombospondin type I repeat that showed self-interaction (35) and the C-terminal domain of type IV collagen identified with the use of the N-terminal domain of type VI collagen as a bait (36). In this work for the first time, the self-interaction of a gC1q domain was demonstrated to occur employing an in vivo technology. The self-assembly of the EMILIN gC1q domain was verified by two complementary systems such as yeast mating and cotransformation, and although not quantitative they both strongly suggested a specific binding.

The finding that both bacterial and eukaryotic cell expression systems provided a native gC1q domain that formed trimers in analogy with several other members of the TNF/C1q superfamily of proteins, namely C1q (30, 31), collagens type VIII (28, 29) and type X (26, 27), ACRP30 (37), and multimerin (9), indicated that also the smallest EMILIN protomer is a trimer. The chain composition of these different superfamily members varies from heterotrimers formed of three distinct chains as in C1q to homotrimers as in collagen types VIII and X (39–41), ACRP30 (42), multimerin (43), and EMILIN (Fig. 9). Whereas the only multimers detected for EMILIN gC1q domain are represented by trimers, larger multimers of gC1q domains have been reported recently to occur with collagen type X; in this case the denatured chimeric domain refolded on solid state migrated as three separated forms, the largest of which corresponded to an hexamer (44). Higher order multimerization could also be seen in solution upon addition of crosslinkers, and these data are in accord with the electron microscopic observations that collagen type X forms a multimeric hexagonal lattice (45).

Furthermore, the strength of the self-association was quite variable among the different gC1q homotrimers as well; collagen type X (25–27) and multimerin (43) were able to withstand even very strong denaturing conditions, the latter still being largely a trimer at 90–100 °C in the presence of 2% SDS, whereas ACRP30 was not stable under standard SDS-PAGE conditions (42). The thermal stability of the EMILIN gC1q homotrimer was lower compared with that of the collagen type X gC1q domain and resembled ACRP30 since under standard SDS-PAGE conditions it migrated as a monomer even in the absence of 8 M urea. Finally, the single cysteine residue per subunit of EMILIN gC1q domain is apparently not utilized to form intermolecular disulfide bridges. We have extensively investigated under both native and mild denaturing conditions whether any evidence of dimers could be demonstrated, but no such evidence was ever obtained even using sensitive immunoblotting analysis.3 The likelihood that a dynamic equilibrium of reversible S–S bonds formed between the unpaired cysteine of the three gC1q domains, as has been shown to occur in other proteins systems (46), is very unlikely based on the model of the

\[ \text{EMILIN Self-assembly} \]

\[ \text{-Type VIII collagens-} \]
\[ \text{-Type X collagen-} \]
\[ \text{-ACRP-} \]
\[ \text{-EMILIN-} \]
\[ \text{-Multimerin-} \]
\[ \text{-Heterotrimers-} \]
\[ \text{-Three chains-} \]
\[ \text{-Collagen stalks-} \]
\[ \text{-Homotrimers-} \]
\[ \text{-Collagen stalks-} \]
\[ \text{-Homotrimers-} \]

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3 G. Mungiguerra, R. Doliana, and A. Colombatti, unpublished results.
ACRP gC1q domain (32); in the EMILIN domain this cysteine should not be at the interface between the monomers but would be located on the external surface of the trimer and thus too far apart to be able to form disulfide bonds with the equivalent residues in the other two monomers.

A number of hydrophobic residues, shared among the different gC1q domains, have been identified either directly on the crystal of the ACRP gC1q domain (32) or by molecular modeling on the gC1q domain of collagen type X (47) as being part of the hydrophobic contacts that produce the close packing between the monomers. In type X a group of substituted residues causing metaphysal chondrodysplasia type Schmid are localized in the area that forms the polar and hydrogen-bonded region within the trimer tunnel. The mutant polypeptides still retain the ability to trimerize, but these mutations cause a lower thermal stability of the mutant compared with wild-type trimers (47–50). The EMILIN gC1q sequence contains the same or very similar residues as those present in the wild-type collagen type X gC1q domain; thus the lower thermal stability detected as compared with that of collagen type X should be attributed to the partly different neighboring residues or to still unknown and more subtle differences. Based on the model of the ACRP30 gC1q domain crystal structure, this trimer is composed of three 10 β-strand jellyroll motif subunits that trimerize to produce a tight association contributed by the loss of solvent-accessible surface with several hydrophobic contacts provided by conserved residues (32). The molecular modeling of the collagen type X gC1q domain, which contains additional hydrophobic residues, inferred that the total buried solvent-accessible surface on trimerization is even larger for this polypeptide if compared with that of the ACRP30 (47) providing also a possible structural explanation for its unusual thermal stability (26, 47). The relatively lower thermal stability of the EMILIN gC1q domain (T_{m} at about 58 °C) compared with that of the collagen type X domain suggests that the size of the hydrophobic surface of the EMILIN domain could resemble that of the ACRP30. In addition, the contribution of the COL and of the LZ domains should not be underestimated; the formation of the collagenous triple helix especially is likely to provide additional strength to the trimers. Preliminary quantitative data employing the two-hybrid system technology indicate that the presence of the COL and LZ domains confers a stronger interaction.4 It is conceivable that the formation of the homotrimer of the EMILIN gC1q domains acts as a nucleation process in a C- to N-terminal direction to give rise to a triple helix; the trypsin resistance of the 17 collagenous triplets of EMILIN, isolated from cells grown in the presence of ascorbate, indicate that the COL domain is functionally stable and should form a straight collagen rod of about 14 nm.

Several of the gC1q domain-containing molecules assemble to quaternary structures composed of multimers of 9–12 polypeptides, as in ACRP (37), 18 polypeptides, as in collagen type X (45) and likely also VIII (51), or well above 24 polypeptides, as in multimelin (9). The sizes of the EMILIN multimers are equal to or larger than the multimers of vWF, and in fact they can reach sizes of several millions of daltons corresponding to complexes larger than several dozens of polypeptides. Nevertheless, native EMILIN has never been isolated from tissues, and it has yet to be demonstrated that the large assemblies secreted by transfected 293-EBNA cells are also found in vivo. The critical importance of the gC1q domain in EMILIN assembly has been highlighted by the agarose gel data. The lack of this domain in the frameshift variant EMILIN-T conceivably compromised the initial association of the three polypeptides to form the stable homotrimer, thus preventing to a large extent the further formation of S–S-bonded multimers. However, some residual capacity to associate via disulfide bridges is still present since under non-reducing conditions EMILIN-T migrated between fibronectin (about 500 kDa) and laminin (about 900 kDa).

How are the EMILIN multimers interacting with the elastin core and/or with the microfibrils? It has been recently reported that the elastin-binding protein, MAGP-1, bound both native and pepsin-treated forms of type VI collagen (52). The major MAGP-1-binding site was in the triple-helical region of the α3(VI) chain, and this interaction was inhibited by tropoelastin, suggesting that the binding sites for tropoelastin and type VI collagen may be in the same domain of MAGP-1. Thus MAGP-1 may mediate a molecular interaction between type VI collagen microfibrils and fibrillin-containing microfibrils. Whether EMILIN and more specifically its COL domain might serve the same function is a matter to be investigated but represents a reasonable hypothesis.

Finally, and given the similarity in the early assembly steps between EMILIN and collagen types VIII and X and the presence of a functional triple helix in EMILIN, the question arises whether EMILIN might be considered a member of the collagen family. Although part of the ECM is like most collagens, only 5% of the EMILIN sequence is collagenic, much less than the percentage present in the most unusual collagen known to date, type VI, in which altogether about 20% of the sequence is collagenous (53). Hence, it would seem more appropriate to consider EMILIN as a multimodular protein with a very short collagenous domain apparently devoid of the capability to form by itself supramolecular structures.

Acknowledgments—We thank Drs. Klaus von der Mark, Friedrich Alexander University, Erlangen, Germany, for providing recombinant gC1q-like polypeptide domain of collagen type X; Neil Smyth, Institute for Biochemistry, University of Cologne, Cologne, Germany, for providing recombinant pCEP-Pu/AC7 vector; and Gianluca Tell, University of Udine, Udine, Italy, for performing the CD spectra analysis.

REFERENCES

1. Doliana, R., Menghi, M., Bucchiotti, F., Giacomello, R., Deutzmann, R., Volpin, D., Bressan, G. M., and Colombatti A. (1999) J. Biol. Chem. 274, 16773–16781
2. Cleary, E. G., and Gibson, M. A. (1996) in Extracellular Matrix (Comper, W. D., ed) Vol. 2, pp. 95–140, Harwood Academic Publishers, Amsterdam
3. Bressan, G. M., Castellani, I., Colombatti, A., and Volpin, D. (1983) J. Biol. Chem. 258, 13262–13267
4. Colombatti, A., Bressan, G. M., Castellani, I., and Volpin, D. (1985) J. Cell Biol. 100, 18–26
5. Paulauskas, A., Bressan, G. M., Volpin, D., and Castellani, I. (1985) Collagen Relat. Res. 5, 181–191
6. Colombatti, A., Poletti, A., Bressan, G. M., Carbone, A., and Volpin, D. (1987) Collagen Relat. Res. 7, 259–275
7. Colombatti, A., Bonaldo, P., Volpin, D., and Bressan, G. M. (1988) J. Biol. Chem. 263, 17534–17540
8. Bressan, G. M., Daga-Gordini, D., Colombatti, A., Castellani, I., Marigo, V., and Volpin, D. (1993) J. Cell Biol. 121, 201–212
9. Hayward, C. P. M., Warkentin, T. E., Horsewood, P., and Kelton, J. G. (1991) Blood 77, 2556–2560
10. Schmid, T. M., and Linsenmayer, T. F. (1985) Dev. Biol. 107, 373–381
11. Sage, H., Pritzl, P., and Bornstein, P. (1986) Biochemistry 19, 5747–5755
12. Kettleberger, R., Davis, P. F., and Greenhill, N. S. (1989) Biochem. Biophys. Res. Commun. 159, 414–419
13. Barber, R. E., and Kwan, A. P. (1996) Biochem. J. 320, 479–485
14. Brass, A., Kadler, K. E., Thomas, J. T., Grant, M. E., and Boot-Handford, R. P. (1992) FEBS Lett. 303, 126–128
15. Bulleid, N. J., Dalley, J. A., and Lees, J. F. (1997) EMBO J. 16, 6694–6701
16. Schmid, T. M., and Linsenmayer, T. F. (1984) J. Biol. Chem. 259, 9504–9509
17. Kohfeldt, E., Maurer, P., Vannahme, C., and Timpl, R. (1997) FEBS Lett. 414, 557–561
18. Hedrick, J. L., and Smith, A. L. (1968) Arch. Biochem. Biophys. 126, 155–164
19. De Marco, L., Girolami, A., Zimmerman, T. S., Ruggeri, Z. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7424–7428
20. Vuento, M., and Vaheri, A. (1979) Biochem. J. 183, 331–337
21. Peroza, F., Carbone, R., Ferrone, S., and Dammacco, F. (1990) J. Immunol. Methods 125, 9–16
22. Paulauskas, A., Aumaile, M., Deutzmann, R., Timpl, R., Beck, K., and Engel, J. (1987) Eur. J. Biochem. 166, 11–19
23. Menendez-Arias, L., Gomez-Gutierrez, J., Garcia-Fernandez, M., Garcia-Tejedor, A., and Moran, F. (1989) Comput. Appl. Biosci. 4, 479–482

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4 S. Bot, R. Doliana, and A. Colombatti, unpublished results.
