MAGP-2 Has Multiple Binding Regions on Fibrillins and Has Covalent Periodic Association with Fibrillin-containing Microfibrils*

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The interactions of microfibril-associated glycoprotein (MAGP)-2 have been investigated with fibrillins and fibrillin-containing microfibrils. Solid phase binding assays were conducted with recombinant fragments covering fibrillin-1 and most of fibrillin-2. MAGP-2, and its structure relative MAGP-1, were found to bind two fragments spanning the N-terminal half of fibrillin-1 and an N-terminal fragment of fibrillin-2. Blocking experiments indicated that MAGP-2 had a binding site(s) close to the N terminus of the fibrillin-1 molecule that was distinct from that for MAGP-1 and an additional, more central binding site(s) that may be shared by the two MAGPs. Immunogold labeling of developing nuchal ligament showed that MAGP-2 had regular covalent and periodic (about 56 nm) association with fibrillin-containing microfibrils of elastic fibers in this tissue. Further analysis of isolated microfibrils indicated that MAGP-2 was attached at two points along the microfibril substructure, “site 1” on the “beads” and “site 2” at the “shoulder” of the interbead region close to where the two “arms” fuse. In contrast, MAGP-1 was located only on the beads. Comparison of the MAGP-2 binding data with known fibrillin epitope maps of the microfibrils showed that site 1 correlated with the N-terminal MAGP-2 binding region, and site 2 correlated with the second, more central, MAGP-2 binding region on the fibrillin-1 molecule. Of particular note, immunolabeling at site 2 was markedly decreased, relative to that at site 1, on extended microfibrils with bead-to-bead periods over 90 nm, suggesting that site 2 may move toward the beads when the microfibril is stretched. The study points to MAGP-2 being an integral component of some populations of fibrillin-containing microfibrils. Moreover, the identification of multiple MAGP-binding sequences on fibrillins supports the concept that MAGPs may function as molecular cross-linkers, stabilizing fibrillin monomers in folded conformation within or between the microfibrils, and thus MAGPs may be implicated in the modulation of the elasticity of these structures.

MAGP-1 and MAGP-2 represent a two-member family of structurally related glycoproteins that have specific association with fibrillin-containing microfibrils in tissues (1, 2). Fibrillin-containing microfibrils, in association with elastin, comprise elastic fibers that are major structural components of elastic tissues such as lung, arteries, and elastic ligaments. The microfibrils also occur as elastin-free bundles in the extracellular matrix of a diverse range of tissues including ocular zonule, skeletal muscle, skin, and kidney (3–6). As their name suggests, the major structural components of these microfibrils are the fibrillins that are large and rod-like 350-kDa glycoproteins (6–8). Two fibrillins, fibrillin-1 and -2, have been extensively characterized (9–11), and a third fibrillin, fibrillin-3, has been reported recently to occur in humans but not in rodents (12). Fibrillin-1 and -2 have been shown to interact with one another and to overlap spatially (13, 14). The fibrillin molecules appear to be arranged within the microfibrils as parallel bundles of 4–8 molecules joined in series in a head-to-tail manner. However, the precise organization of the fibrillin monomers remains to be elucidated, and several models for the molecular architecture of the microfibrils have been proposed (15–18). Recent evidence suggests that fibrillins 1 and 2 can form separate microfibrils but that the proteins can also occur within the same microfibril (14). Mutations in the genes for fibrillin-1 and fibrillin-2 have been linked to the genetic disorders Marfan syndrome and congenital contractual arachnodactyly, respectively (Online Mendelian Inheritance in Man numbers 154700 and 121050). Depletion of normal fibrillin-containing microfibrils in Marfan patients can cause progressive weakness in the aortic wall leading to aneurysms and premature death from rupture of the vessel. The condition is also characterized by skeletal, ocular, and lung defects. A recent study has shown that the microfibrils can act as tissue stores for latent TGF-β1 complexed with latent TGF-β-binding proteins (19). It is possible that some manifestations of Marfan syndrome such as lung abnormalities and long bone overgrowth may be linked to inappropriate TGF-β activation due to depletion of the microfibrils in these tissues (20).

In addition to latent TGF-β-binding proteins (21–24), a number of other macromolecules have been identified in association with fibrillin-containing microfibrils. These include fibulins (25–27), emilins (28), decorin (29), biglycan (30), versican (55), microfibril-associated proteins (32–34), and MAGPs (1, 2, 5, 35, 36). The evidence indicates that most of these molecules can be associated with the microfibrils in an intermittent manner, suggesting that they are not integral components of these structures. However, there is strong biochemical and immunoelectron microscopic evidence that MAGP-1 is covalently and periodically located in the “beads” of the beaded filament substructure of fibrillin-1-containing microfibrils in most if not all tissues.
tissues, pointing to an integral structural role for the glycoprotein (35–37). MAGP-1 has been shown to self-aggregate and bind in vitro to the N-terminal region of fibrillin-1 and to the elastin precursor, tropoelastin (38–40). Thus it has been proposed that MAGP-1 may stabilize the head-to-tail interaction of fibrillin monomers within the beads (37) and/or act as an elastin-binding protein on the surface of the microfibril (41). MAGP-2 also interacts with collagen VI, and thus, may function as a linker molecule between the fibrillin-containing microfibrils and adjacent collagen VI microfibrils (39).

MAGP-2 is the second member of the MAGP family and shares a characteristic central cysteine-rich motif with MAGP-1 that is considered to be important for binding to the fibrillin-containing microfibrils (1, 42, 43). MAGP-2 exhibits more restricted tissue and developmental patterns of distribution suggesting that it has a more specialized role in microfibril biology than MAGP-1 (36). MAGP-2 has been reported to bind the C-terminal regions of fibrillins 1 and 2 via the central cysteine-rich motif (43). MAGP-2 lacks the tropoelastin and collagen VI-binding sequences of MAGP-1, but in its place it contains an active RGD motif that interacts with several cell types via αβ3 integrin (44). It has been proposed that MAGP-2 may function in microfibril and elastic fiber assembly and in the interaction of microfibrils with cell surfaces at specific stages of tissue development (43, 44).

Previously, it has been unclear if MAGP-2 occurs as a monomer of, or is associated intermittently with, the populations of microfibrils in the tissues where it is found. In the present study we show that MAGP-2 is covalently and periodically located along the fibrillin-containing microfibrils of the developing nuchal ligament, strongly suggesting that the protein is an integral component of most, if not all, of the microfibrils in this elastic fiber-rich tissue. In addition, we have identified MAGP-2 binding regions in the N-terminal part of the fibrillin-1 and -2 molecules. The evidence indicates that MAGP-2 is associated with the microfibrils at two points on its substructure, on the “head” and at the “shoulder” of the interbead region. The findings suggest that MAGP-2 may be involved in the stabilization of folded fibrillin monomers within the microfibril by forming interdomain and/or intermonomer connections.

MATERIALS AND METHODS

Purification of MAGPs and Recombinant Fibrillin Fragments—MAGP-1 and MAGP-2 were purified from nuchal ligament tissue as described previously (39). Full-length human cDNAs for fibrillin-1, corresponding to bases 122–8923 of the cDNA sequence (GenBank™ accession number NM_001999.2), and fibrillin-2, corresponding to bases 135–8304 (GenBank™ accession number NM_019999.2), were obtained by reverse transcription of human placental RNA using Superscript II reverse transcriptase (Invitrogen) followed by PCR with Pfu turbo DNA polymerase (Stratagene, La Jolla, CA) using methods described previously (46). For fibrillin-1 cDNA, the sense primer was 5′-CCGCTGCAGCATGCTGCGAGGCC-3′ and the antisense primer was 5′-GATGATTCTGATT-3′. For fibrillin-2 cDNA, the sense primer was 5′-ACAGGTTCCGTCGCTACA-3′ and the antisense primer was 5′-GAGTGCAAAATCAACGGC-3′. An 18-nucleotide sequence encoding a His6 tag was introduced into the fibrillin-1 and -2 cDNA sequences by the extension technique was then used to attach the sequence encoding the signal peptide, and in addition, Fib-1 (H)NT encoded an N-terminal His6 tag, following their purification from the recombinant full-length templates.

Each fragment was cloned into pGEM T-easy, as above, and then subcloned into the NotI restriction site of the mammalian expression vector pCEP4 (Invitrogen). Individual clones were fully sequenced to check for errors. The selected constructs were stably transfected into 293-EBNA cells, and recombinant protein fragments were purified from the culture medium using nickel-affinity chromatography with methods described previously (46).

Antibodies—Polyclonal antibodies to MAGP-1 (R18), MAGP-2 peptide SETEDDPNLVNDPDET (R49p), and monoclonal anti-MAGP-1 antibody (11B) have been described previously (36, 37, 45). Monoclonal anti-fibrillin-2 antibody, 16E12, was made following standard protocols by using as antigen a synthetic peptide with the sequence GGGFAP5SGNNGYGGPG specific for the glycine-rich region of fibrillin-2, coupled to diphtheria toxoid. The specificity of 16E12 was confirmed by enzyme-linked immunosorbent assay and Western blotting against the recombinant fragments of fibrillins-1 and -2 (data not shown). Anti-fibrillin-1 monoclonal antibodies 26 and 11C1.3 were purchased from Chemicon International (Temecula, CA). Anti-His, monoclonal antibodies were purchased from Qiagen (Valencia, CA).

Solid Phase Binding Assays—Solid phase binding assays were conducted as described previously (39). Briefly, a test protein (MAGP or recombinant fibrillin fragment) was coated onto the wells of a microtiter plate (Nunc Maxisorb) in TBS and incubated overnight at 4 °C. Control wells were incubated with BSA. After rinsing and blocking with 3% skimmed milk, the wells were incubated with a second test protein in liquid phase for 3 h at 37 °C in TBS containing 5 mM CaCl2. After further rinsing, binding was detected with specific primary antibody followed by the appropriate secondary conjugate, with 5-bromo-4-chloro-3-indolyl- methylbenzidine substrate (Sigma). Anti-His, monoclonal antibody was used to detect binding of His6-tagged recombinant fibrillin fragments in the liquid phase, except that antibodies 26 and 16E12 were used to detect binding of fragments Fib-1 (H)NT and Fib-2 (NT)H, respectively. Polyclonal anti-MAGP-1 antibody R18 was used to detect MAGP-1 binding.

Affinity Blotting—Ligand blotting was conducted using a method described previously (39). Briefly, recombinant fibrillin-1 fragments (1 μg/lane) were subjected to SDS-PAGE on a 6.5% gel under nonreducing conditions and transferred to polyvinylidene difluoride membrane. The membrane was rinsed in TBS containing 5 mM CaCl2 for 1 h and blocked with 10% skimmed milk in the same buffer for 1 h. The individual blots were then incubated with or without added MAGP-2 (5 μg/ml) for 2 h at room temperature in TBS containing 0.05% skimmed milk and 5 mM CaCl2. After rinsing, specific binding was detected using anti-MAGP-2 antibody (1:100 dilution in TBS plus 2% skimmed milk) followed by goat anti-rabbit alkaline phosphatase conjugate conjugate (Bio-Rad) as the secondary antibody and nitro blue tetrazolium and bromochloroindolyl phosphate as substrates.

Isolation of Microfibrils—Fibrillin-containing microfibrils were purified using a method based on that of Kiely et al. (49). Briefly, nuchal ligament tissue (6 g) from a 230-day-old fetal calf was finely diced with a razor blade, rinsed several times in TBS, and resuspended in 18 ml of TBS containing 0.5 mM EDTA (pH 7.4). The sample was adjusted to an initial density of 1.35 g/ml with CsCl and centrifuged at 100,000 × g for 20 min. The supernatant was chromatographed in three batches on a column of Sepharose CL-6B with 50 mM Tris-HCl, pH 7.4, containing 0.4 M NaCl, 10 mM CaCl2, 2 mM MgCl2, and 1 mM phenylmethylsulfonyl fluoride. The suspension was incubated with 1.8 μg of highly purified type VII collagenase (specific activity, 1980 units/mg) (Sigma) for 8 h at 37 °C and centrifuged at 10,000 × g for 20 min. The supernatant was chromatographed in three batches of hydrolysis of hyaluronan in Tris/NaCl buffer (composition as above but lacking CaCl2). Flow rate was 30 ml/h, and 3-ml fractions were collected. Fractions containing fibrillin-containing microfibrils were identified by direct dot blotting on Zetaprobe-plus nylon membrane (Bio-Rad) with antibody 26. The fibrillin-containing fractions (void volume peaks) from the three runs were combined and treated with a 1:60 mixture of hyaluronidase and chondroitinase ABC in Tris/NaCl buffer (composition as above but lacking CaCl2) at 4 °C. Samples of this digest were then analyzed by CsCl density gradient centrifugation in Tris/NaCl buffer (native conditions) or in Tris/NaCl buffer containing 6 μM GdnHCl (denaturing conditions). Each sample was adjusted to an initial density of 1.35 g/ml with CsCl and
then ultracentrifuged in a 70.1 Ti head (Beckman) at 30,000 rpm (62,000 × g) at 15 °C for 72 h. Each gradient was divided into 24 fractions, and those containing fibrillin-1 were identified by dot blotting and combined. Dot blotting for MAGP-2 was also conducted.

**Immunelectron Microscopy**—For post-embedding immunolabeling, the nuchal ligament was dissected from a 230-day-old fetal calf and diced into 1-mm³ blocks. In some instances, tissue pieces were treated with 8 % GdnHCl buffered with 50 mM Tris, pH 7.4, for 6 h, rinsed with TBS, and incubated with chondroitinase ABC for 18 h at 4 °C. The blocks were washed in TBS for 20 min, fixed in 0.1 M sodium cacodylate, pH 7.2, containing 4 % paraformaldehyde and 0.5 % glutaraldehyde for 2 h, extensively rinsed with 0.175 % sodium cacodylate, pH 7.2, and, finally, dehydrated and embedded in LRWhite resin. Sections ~75 nm thick were cut, recovered on 200 mesh collodion-coated nickel grids, and incubated with 0.01 % glycine for 30 min to block free aldehyde groups. After further blocking with TBS, 2 % ovalbumin for 30 min, the sections were incubated at 4 °C for 18 h with antibody (20 lg/ml) in TBS/ovalbumin. The sections were then rinsed five times in TBS/ovalbumin and incubated with goat anti-rabbit IgG antibodies conjugated with 10-nm gold particles diluted according to the manufacturer's instructions (Aurion, Wageningen, The Netherlands). The sections were extensively washed with TBS/ovalbumin followed by deionized water and then stained with alcoholic uranyl acetate and lead citrate for examination at 80 kV in a Philips CM100 transmission electron microscope.

For pre-embedding immunolabeling, the freshly diced nuchal ligament was washed four times in TBS over 2 h and then incubated with primary antibody (100 lg/ml) in TBS/ovalbumin for 4 h. The pieces were extensively washed with TBS/ovalbumin and incubated for 18 h with either gold-conjugated secondary antibody or goat anti-rabbit IgG antibodies in TBS/ovalbumin. Following extensive rinsing with TBS followed by 0.175 % cacodylate buffer, pH 7.2, the tissue was fixed for 2 h in 0.1 M cacodylate buffer, pH 7.2, containing 0.5 % paraformaldehyde and 1 % glutaraldehyde, rinsed, and post-fixed in 1 % OsO₄ for 1 h. The tissue then was dehydrated and embedded in epoxy resin, and sections were cut, stained, and examined as described for post-embedding labeling.

For immunolabeling and negative staining, microfibrils isolated on density gradients under nondenaturing conditions were suspended into a 400-mesh Formvar-coated nickel grid, rinsed with water, and incubated for 20 min with affinity-purified antibody to MAGP-2 (R49p) or MAGP-1 (R18) in TBS/ovalbumin plus 0.05 % Tween 20. To demonstrate saturation binding to the microfibrils, the primary antibody was used at a concentration of 100 lg/ml. For detailed mapping of antibody binding, a lower concentration of 20 lg/ml was used. Control grids were incubated with a matched concentration of “preimmune” rabbit IgG. All grids were washed four times with TBS/ovalbumin/Tween and incubated for 20 min with goat anti-rabbit IgG antibody conjugated with 10-nm gold particles. The grids were washed three times with TBS/ovalbumin/Tween followed by deionized water and then negatively stained with 1 % aqueous uranyl acetate, pH 4.5, for 45 s. Grids were air-dried and observed at 80 kV in a Philips CM100 transmission electron microscope.

For the dual labeling experiments, grids were incubated simultaneously with a rabbit polyclonal antibody R49p to MAGP-2 (20 mg/ml) and a mouse monoclonal antibody, either 11B to MAGP-1 (ascites diluted 1:20) or 11C1.3 to fibrillin-1 (ascites diluted 1:100). Controls grids were incubated with a mixture of preimmune rabbit IgG and diluted mouse ascites containing a monoclonal antibody to the parasite Giardia lamblia (37). All grids were then incubated with a mixture of the anti-rabbit IgG 10-nm gold conjugate and anti-mouse IgG + IgM 5-nm gold conjugate (Aurion), washed, and stained as above.

**RESULTS**

**Identification of Novel MAGP-2 Binding Regions on Fibrillins 1 and 2**—To investigate the molecular interactions of MAGPs with fibrillins, a series of recombinant His₆-tagged fragments of fibrillins 1 and 2 were expressed in human 293 EBNA cells (Fig. 1A) and purified by nickel-affinity chromatography to give single bands of correct size on SDS-PAGE (Fig. 1B). The yields varied, depending on the fragment, from 0.1 to 1 lg of protein/ml of medium. In solid phase binding assays, the specific interaction of MAGP-2 was identified with the two fragments from the N-terminal half of fibrillin-1 (Fib-1 (H)NT and Fib-1 (H)N) and the N-terminal region (Fib-2 NT/H)) of fibrillin-2 (Fig. 2A). Binding of MAGP-2 to fragment Fib-1 (H)NT was also detected by using the affinity blotting technique. However, MAGP-2 binding to the N-terminal fragment Fib-1 (H)NT was not detected by this method, suggesting that the binding site on this fragment was irreversibly labile to treatment with SDS detergent (Fig. 2B). These findings suggest that MAGP-2 has distinct binding activities to Fib-1 (H)NT and Fib-1 N(H). No MAGP-2 binding was detected to the two fragments encompassing the C-terminal half of the fibrillin-1, Fib-1 C(H) and Fib-1 (H)CT, or to fibrillin-2 fragment Fib-2 C(H). Most interesting, MAGP-1 was also found to bind to the same fibrillin fragments as MAGP-2 (Fig. 3). However, in contrast to MAGP-2, MAGP-1 binding to fragment Fib-1 (H)NT was also detected by affinity blotting (not shown). This suggested that the MAGPs might have distinct binding sites on fragment Fib-1 (H)NT or have differing conformational requirements for binding to this fragment. To determine whether MAGP-1 and MAGP-2 had distinct binding sites on fibrillins, blocking experiments were performed (Fig. 4). MAGP-1 was found to block binding of MAGP-2 to fragment Fib-1 (H)NT but not to fragments Fib-1 (H)NT and Fib-2 NT/H). Because the MAGPs do not directly bind to each other (data not shown), these findings indicate that MAGP-2 has a binding site(s) distinct from that of MAGP-1 on the N-terminal fragment of fibrillin-1, Fib-1 (H)NT, but that the two MAGPs may share the same site on fragment Fib-1 N(H). In addition, MAGP-2 had at least one binding site in the N-terminal half of fibrillin-2, which was not shared with MAGP-1.

**MAGP-2 Has Regular Covalent and Periodic Association with Fibrillin-containing Microfibrils in Developing Nuchal Ligament**—By using the post-embedding immunogold labeling.
technique with affinity-purified anti-(MAGP-2 peptide) antibody, MAGP-2 was localized specifically to the fibrillin-containing, elastin-associated microfibrils of the developing nuchal ligament (Fig. 5). Immunogold binding was significantly enhanced by sequential pretreatment of the unfixed tissue with chondroitinase ABC and 6M GdnHCl to enhance exposure of the reactive epitopes. However, no periodic labeling pattern was discernible by using this technique. It is noteworthy that the MAGP-2 was not removed from the microfibrils by the chaotropic GdnHCl treatment, consistent with MAGP-2 being attached to the structures by covalent disulfide bonding.

Subsequently, pre-embedding labeling techniques were used to investigate the pattern of MAGP-2 attachment to the microfibrils (Fig. 6). By using this method with primary anti-MAGP-2 antibody only, little discernible antibody binding to the microfibrils was evident (Fig. 6A). Immunogold labeling proved to be impracticable due to inadequate penetration of the tissue by the gold particles (not shown). However, precise 56 nm periodic labeling along the ligament microfibrillar bundles was observed when the visualization of anti-MAGP-2 antibody binding was enhanced by subsequent treatment with anti-rabbit IgG secondary antibody (Fig. 6, B and C). No periodic labeling of the control tissue, the ciliary zonule of the eye, was observed (Fig. 6D). The zonule consists predominantly of fibrillin-containing microfibrils but lacks MAGP-2 (36). Nuchal ligament tissue was also immunolabeled for MAGP-1. In this instance, periodic staining of the microfibrils was evident with primary antibody alone (Fig. 6E). However, the signal was greatly enhanced after subsequent treatment with secondary antibody to give a labeling pattern similar to that for MAGP-2 (Fig. 6F). No labeling of the microfibrils was observed in control tissue treated with preimmune rabbit serum in place of primary antibody (Fig. 6G). The widespread periodic labeling of microfibrillar bundles for MAGP-2 and MAGP-1 indicates that both glycoproteins are associated.
with each periodic subunit of most, if not all, of the fibrillin-containing microfibrils in developing nuchal ligament. The results strongly support the concept that both MAGPs are integral components of these structures.

**MAGP-2 Co-purifies with Fibrillin-containing Microfibrils**—Fibrillin-containing microfibrils were purified to define more precisely the location of MAGP-2 within the bead-to-bead period of the microfibrils. A collagenase-digested extract of nuchal ligament tissue was analyzed by gel permeation chromatography on Sepharose CL-2B, and fractions containing fibrillin-1 and MAGP-2 were identified by dot blotting. Fibrillin-containing microfibrils were purified to define more precisely the location of MAGP-2 within the bead-to-bead period of the microfibrils. The results indicate that the MAGP-2 was covalently bound to the isolated fibrillin-containing microfibrils.

**MAGP-2 Is Attached at Two Distinct Sites on Fibrillin-containing Microfibrils**—To determine more precisely the location of MAGP-2 within the fibrillar substructure, purified microfibrils were sprayed onto grids, immunogold labeled with anti-MAGP-2, and then visualized by negative staining (Fig. 5A). Strong labeling for MAGP-2 was evident on most of the periods along the isolated microfibrils when the antibody was used under conditions close to saturation (100 μg/ml) (Fig. 8A). In controls treated with preimmune rabbit IgG followed by immuno-gold conjugate, no gold labeling was observed (Fig. 8B). However, at this high level of labeling, it was difficult to determine the precise binding site for each gold particle on the microfibril substructure, and more dilute antibody (20 μg/ml) was used for the mapping experiments described below. The microfibrils also labeled strongly with anti-MAGP-1 antibodies (Fig. 8C). Dual immunogold labeling of the microfibrils for MAGP-2 (10 nm gold) and MAGP-1 (5 nm gold) confirmed that both MAGPs were present in the same microfibrils (Fig. 8D). Control grids incubated with preimmune rabbit IgG and a monoclonal antibody to *G. lamblia* showed no binding of the 5- or 10-nm gold particles to the microfibrils (data not shown). The negative staining process allowed the bead-to-bead substructure, including the two “arms” of the shoulder regions (16), to be discerned for a significant number of the periods along each microfibril (Fig 8E). Because the arms of the interbead region occur to one side of the bead only, the orientation of individual microfibrils could be determined. The point of attachment of each immunogold particle was elucidated, with respect to the periodic substructure, on a large number of microfibrils labeled for MAGP-2 and MAGP-1. Of particular note, in unstretched microfibrils (<60 nm period), the immunogold for MAGP-2 predominantly bound to the interbead region close to the shoulder where the two arms appear to fuse. An example is shown in Fig. 8E, a. However, in microfibrils of increased periodicity, binding to the beads also became evident (Fig. 8E, b–d). In highly extended microfibrils (<100 nm periods) only the beads were labeled (Fig. 8F). Dual labeling of unstretched microfibrils confirmed that the inter-bead MAGP-2-binding site was close to the epitope recognized by anti-fibrillin-1 monoclonal antibody 1C13, which has been located at the shoulder of the interbead region (16) (Fig. 8G).

The location of each immunogold particle was measured as a percentage of the bead-to-bead period from the front of the bead (Fig. 9). The total number of microfibril-bound gold particles measured was 225 for MAGP-2 and 43 for the MAGP-1 control. As indicated in Fig. 8, the immunogold labeled the microfibrils for MAGP-2 at two points along the microfibrils, at the center of the bead, named site 1, and at a point on the shoulder region close to where the arms fuse, named site 2 (Fig. 9A). The average location for site 1 was determined as 11.4 ± 3.3% and for site 2 as 36.15 ± 6.7% of the bead-to-bead period. In contrast, labeling for MAGP-1 occurred only on the beads with an average location of 10.09 ± 9% of the period (Fig. 9B). Analysis of the immunogold binding for MAGP-2 as a function of periodicity from three independent labeling experiments (Fig. 10) confirmed that the proportion of label on the beads (site 1, defined as 1–19% of the period) increased as the period increased such that binding at the shoulder of the interbead region (site 2, defined as 25–50% of the period) was scarce on microfibrils with periods in excess of 110 nm (Fig. 10).

**DISCUSSION**

The molecular composition and organization of the fibrillin-containing microfibrils have been the subjects of intense re-

![Image](http://www.jbc.org/)

**Fig. 5. MAGP-2 is covalently associated with fibrillin-containing microfibrils.** Post-embedding immunogold labeling of 230-day-old fetal bovine nuchal ligament with affinity-purified anti-MAGP-2 peptide antibody (20 μg/ml) (A and B) or IgG from normal rabbit serum as controls (C and D). Tissue received no pretreatment (A and C) or was preincubated sequentially with chondroitinase ABC and 6 M GdnHCl prior to fixing and embedding (B and D). Note the strong specific immunogold labeling of the microfibrillar component of developing elastic fibers even after extraction with GdnHCl (B). Bar, 500 nm.

brils were the only discernible structures present (data not shown). Thus the results indicate that the MAGP-2 was covalently bound to the isolated fibrillin-containing microfibrils.

**MAGP-2 Interactions with Fibrillins**

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**DISCUSSION**

The molecular composition and organization of the fibrillin-containing microfibrils have been the subjects of intense re-
search in recent years. In addition to fibrillins, a wide array of macromolecules have been described as having functional association with these structures (2). In most instances it remains uncertain if these adjunct molecules are integrated into the architecture of the microfibrils or if they are attached to the surface. To be an integral component, it is expected that a molecule would, like fibrillins, be a regular component of each or most of the bead-to-bead repeating units of a particular population of microfibrils and thus yield a periodic labeling pattern, corresponding to the repeating unit. To date, the only nonfibrillin molecule to have been demonstrated to show periodic immunolabeling of fibrillin-containing microfibrils in tissues is MAGP-1 (37). This finding, and the widespread covalent association of MAGP-1 with fibrillin-1, points to MAGP-1 being an integral, although not necessarily essential, structural component of fibrillin-1-containing microfibrils.

In the present study we have shown that MAGP-2 also has covalent periodic association with the fibrillin-containing microfibrils in developing elastic fibers of the elastic nuchal ligament. This was visualized as a regular cross-banding pattern corresponding to most of the periods within the microfibrillar bundles and was comparable with the staining pattern obtained for MAGP-1 (37). This finding, and the widespread covalent association of MAGP-1 with fibrillin-1, points to MAGP-1 being an integral, although not necessarily essential, structural component of fibrillin-1-containing microfibrils.

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The banding patterns for both MAGPs on elastic fiber microfibrils were not as complete as that observed previously for MAGP-1 in ciliary zonule (37), suggesting that antibody accessibility was more difficult in the denser ligament matrix. In contrast to MAGP-1, the identification of periodic MAGP-2 association with the tissue microfibrils required enhancement with secondary antibody. This suggested that either only a small number of MAGP-2 molecules may be present in each period or that the protein may not be readily accessible to the antibody due to it being buried within the microfibril and/or being masked by other proteins associated with the microfibrils in tissues. The observation that the anti-MAGP-2 antibody strongly labeled individual microfibrils, following their purification by CsCl density gradient ultracentrifugation, supports the latter alternative. Purification by ultracentrifugation has been shown to strip from the microfibrils noncovalently bound proteins such as latent TGF-β binding proteins (49), which appear to be attached via the N-terminal region of fibrillins (19). Thus the treatment is likely to enhance antibody access to these structures.

Isolated microfibrils were used to identify more precisely the MAGP-2 attachment site(s) on these structures. Immunolabeling for MAGP-2 was detected at two regions along the isolated microfibrils, at site 1 on the beads and at site 2 on the interbead region, close to the epitope for fibrillin-1 monoclonal antibody 11C1.3, corresponding to the shoulder region where the two arms converge, following the morphological model of microfibril substructure described by Baldock et al. (16). In contrast, immunolabeling for MAGP-1 was detected only on the beads, consistent with the study of Henderson et al. (37). The failure to detect a double MAGP-2 periodicity along microfibrils in tissues may be due to the broad period obtained using the two-step labeling technique. However, a more likely explanation is that MAGP-2 at site 1 is only made accessible to immunodetection following purification of individual microfibrils by CsCl density gradient centrifugation. Even with isolated microfibrils, site 1 only becomes readily detectable when the microfibrils are in an extended conformation, with periodicities greater than 70 nm (see Fig. 10).
The above findings suggested that MAGP-2 has at least two binding regions on fibrillin molecules. Only one MAGP-2 binding region has been reported previously (43), at the C-terminal end of fibrillins 1 and 2. However, in the present study, MAGP-2 was found to bind in solid phase in in vitro assays with two fibrillin-1 fragments, Fib-1 (H)NT and Fib-1 N(H), which encompass the N-terminal half of the fibrillin-1 molecule. The MAGP-2 appeared to be attaching at distinct sites on the two fragments, based on differential binding on overlay blots and competitive blocking with MAGP-2. MAGP-2 also bound strongly to the N-terminal fragment of fibrillin-2, Fib-2 NT(H), which corresponds to the region on fibrillin-1 encompassed by Fib-1 (H)NT and the N-terminal half of Fib-1 N(H). It is noteworthy that no MAGP-2 binding was detected to the C-terminal fragment of fibrillin-1 which contains the attachment region identified using a yeast two-hybrid system by Penner et al. (43). This finding may reflect differences in the methods used to detect the interaction. The yeast two-hybrid system relies on folding and interaction of the proteins in the cytoplasm where there is risk of nonspecific interaction particularly between cysteine-rich proteins. Our own yeast two-hybrid study with MAGP-2 as bait for a random-primed cDNA library identified clones for numerous cysteine-rich proteins including two overlapping clones encoding central regions of the fibrillin-2 molecule. On the other hand, the recombinant fibrillin fragments used in the solid phase binding assay have undergone conformational scrutiny, which is particularly important for proteins containing disulfide bonds, and post-translational modification, in the endoplasmic reticulum prior to secretion (50–52). However, it may be that disulfide exchange between the two proteins prior to secretion is a normal requirement for the interaction of MAGP-2 with the C-terminal region of fibrillins and that the conditions for this are not reproduced in the solid phase binding assay.

Most interesting, MAGP-1 was also found to bind to the same three recombinant fibrillin fragments as MAGP-2. MAGP-1 binding to fragment Fib-1 (H)NT was anticipated because it contains the binding region identified previously by Jensen et al. (40). In addition, MAGP-1 has been reported very recently (53) to bind the third 8-Cys motif of fibrillin-2, and this may account for the binding of MAGP-1 to Fib-2 NT(H). However, MAGP-1 binding to Fib-1 N(H) represents a novel interaction. Blocking experiments indicated that MAGP-1 and MAGP-2 each have a distinct binding site on Fib-1 (H)NT, corresponding to the N-terminal region of the fibrillin-1. The two glycoproteins also bind to Fib-1 N(H) in a competitive manner, indicating that they share a second, more central binding site on the fibrillin-1 molecule. It is possible that this site is the third 8-cysteine motif recently identified in fibrillin-2 as a MAGP-1-binding site (53). Of particular note, Fib-1 N(H) contains the so-called “neonatal” region of the molecule, mutations in which are linked to a neonatally lethal form of Marfan syndrome (54). It is possible that disruption of MAGP binding in, or close to, this region contributes to the severe phenotype. The Fib-2 NT(H) fragment, corresponding to the N-terminal half of fibrillin-2, appears to contain distinct MAGP-1- and MAGP-2-binding sites, although it remains to be established if this region contains multiple MAGP-2-binding sites like the analogous region of fibrillin-1.

The two MAGP-2 binding regions identified here on recombinant fibrillin-1 fragments were correlated with the two MAGP-2 binding regions detected on the isolated microfibrils (Fig. 11). Also shown are the known locations of the epitopes for monoclonal anti-fibrillin-1 antibodies 26 (10), 11C1.3 (16), and 13A1 (31) on fibrillin-1 and on isolated microfibrils (16, 31). MAGP-2 binding to fragment Fib-1 (H)NT is consistent with binding site 1 on the bead region of the microfibril substructure and the location of the 26 epitopes. MAGP-2 binding to fragment Fib-1 N(H) correlates with binding site 2 on the shoulder of the interbead region and the location of the 11C1.3 epitope. The location of a third MAGP-2 binding region close to the C terminus of fibrillin-1 described by Penner et al. (43), but not detected in the present study, is also shown. Epitope mapping with 13A1 (31) would predict this binding site to be located within the bead. It should also be noted that the microfibrils of the nuchal ligament also exhibit periodic immunolabeling for fibrillin-2, and thus it is possible that MAGP-2 attachment to this fibrillin may contribute to the observed immunolabeling pattern.

The lack of immunostaining of the shoulder region for MAGP-1 remains unexplained because both MAGPs bind to fragment Fib-1 N(H) in vitro, apparently at a shared binding site. The recently identified binding site for MAGP-1 on the third 8-Cys motif of fibrillin-2 (41) is also predicted to be located on the shoulder region in the Baldock model (16). It may be that the folded conformation of the fibrillin molecules within the microfibril precludes the binding of MAGP-1 but not MAGP-2. The finding also raises the possibility that the covalent incorporation of each MAGP into the microfibril occurs at
distinct stages during assembly and folding of the fibrillin monomers into the nascent microfibril, under the direction of the synthesizing cell type.

Most interesting, immunolocalization of MAGP-2 to the shoulder of the interbead region declines as a function of the periodicity of the microfibrils, particularly on stretched microfibrils where the bead-to-bead periodicity exceeds 90 nm. Mapping of the microfibrils with anti-fibrillin-1 monoclonal antibodies suggests that the N-terminal half of the fibrillin monomers may be extensively folded in assembled microfibrils and that this part of the fibrillin molecule spans between the bead and the shoulder regions of the microfibril substructure (16). These authors have also presented a model of fibrillin alignment in the microfibrils that predicts the unfolding of fibrillin monomers when the microfibrils are stretched beyond 90 nm. Thus the loss of immunoreactivity for MAGP-2 from the shoulder region in stretched microfibrils may be due to conformational change in the fibrillin monomers such that the MAGP-2 becomes inaccessible to the antibody or that the MAGP-2 is no longer attached to the

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**Fig. 8. Immunogold localization of MAGP-2 on isolated microfibrils.** Microfibrils, purified on density gradients under nondenaturing conditions, were coated onto Formvar-coated grids and immunogold labeled and negatively stained as described under “Materials and Methods.” A, anti-MAGP-2 antibody R49p at high concentration (100 μg/ml). B, IgG from normal rabbit serum control (100 μg/ml). Note the very strong specific labeling of the microfibrils. In experiments to determine the location of the immunogold particles on the microfibrils, lower antibody concentrations (20 μg/ml) were used to allow optimal visualization of the microfibril substructure (C–F). C, anti-MAGP-1 antibody. D, dual labeling with anti-MAGP-1 monoclonal antibody 11B (5 nm gold) and anti-MAGP-2 antibody (10 nm gold). E, a, anti-MAGP-2 antibody; unstretched (<60 nm) microfibrils showing labeling at the shoulder of the interbead region. b–d, microfibrils of intermediate periodicity showing labeling of the shoulder and the beads. The positions of the beads and clearly visible arms are indicated in the duplicate images. F, anti-MAGP-2 labeling of a stretched (>100 nm) microfibril showing labeling of the beads only. G, dual labeling of unstretched microfibrils showing co-localization of anti-MAGP-2 antibody (10 nm gold) and anti-fibrillin-1 monoclonal antibody 11C1.3 (5 nm gold). Arrowheads in all images indicate the position of the beads. Bars, 100 (A–D and F) and 50 nm (E and G).
binding site. However, because MAGP-2 appears to be covalently bound to the shoulder region in isolated microfibrils, the latter scenario seems unlikely. A further explanation may be that as the microfibril is stretched, the fibrillin monomers become unfolded such that MAGP-2 at binding site 2 moves toward the beads and results in their increased immunolabeling observed on extended microfibrils.

The covalent periodic association of MAGPs with the microfibrils supports the concept that they can be integrated into the structure of fibrillin-containing microfibrils to modulate their function. In particular, the identification of multiple binding sites for each MAGP on the fibrillin-1 molecule suggests that MAGP-2 aggregates may be involved in the fusion of the two arms by cross-linking within the same microfibril and/or between microfibrils within a bundle. It is also an interesting possibility that MAGPs may cross-link domains of individual fibrillin molecules. Such cross-links may stabilize the folded fibrillin conformations considered necessary for microfibril assembly and function. Because both the N- and C-terminal regions of fibrillin-1 have now been shown to bind MAGP-2, the possibility also exists that the glycoprotein may stabilize the head-to-tail linkage of the fibrillin monomers within the beads of the microfibrils. The identification of a second MAGP-2 attachment site at the shoulder region of the microfibrils suggests that MAGP-2 aggregates may be involved in the fusion of the two arms by cross-linking two parallel bundles of fibrillin monomers at this point of the interbead substructure. The microfibrils are considered to have some capacity for elasticity (16, 17), although recent evidence indicates that they are relatively stiff structures (18). The extent of cross-linking of MAGPs within the microfibril may influence the degree of unfolding permitted to the fibrillin monomers and thus MAGPs may control microfibril extensibility. The loss of MAGP-2 immunoactivity at the shoulder region in extensively stretched microfibrils supports this idea. To gain further understanding of the functions of MAGPs, additional binding studies are required to map and characterize more precisely all of their attachment sites on fibrillins 1 and 2 and the reciprocal sites on MAGP-1 and MAGP-2.

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