Further Characterization of Proteins Associated with Elastic Fiber Microfibrils Including the Molecular Cloning of MAGP-2 (MP25)*

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Mark A. Gibson‡§, George Hatzinikolas‡, Jaliya S. Kumaratilake‡, Lawrence B. Sandberg†, Jillian K. Nicholl¶, Grant R. Sutherland, and Edward G. Cleary†

From the ‡Department of Pathology, University of Adelaide, Adelaide, South Australia 5005, Australia ¶Department of Cytogenetics and Molecular Genetics, Women's and Children's Hospital, Adelaide, South Australia, 5006 Australia, and §Petris Memorial Veterans Hospital, Loma Linda, California 92367

Together with the 31-kDa microfibrill-associated glycoprotein (MAGP), four polypeptides designated MP340 (340 kDa), MP78 (78 kDa), MP70 (70 kDa), and MP25 (25 kDa) have previously been identified in tissue extracts designed specifically to solubilize the microfibrillar component of elastic fibers. In the present study, both MP78 and MP70 were shown to be forms of a protein which is closely related to the human protein βig-h3, and MP340 was confirmed to be the bovine form of fibrillin-1. Peptide sequences from MP25 proved to be unique, and affinity-purified anti-MP25 antibodies were shown, by immunofluorescence and immunoelectron microscopy, to localize specifically to the elastin-associated microfibrils. This confirmed that MP25 was a distinct component of these structures. Expression screening of nuchal ligament cDNA libraries yielded a cDNA, cM10A (770 base pairs) which encodes amino acid sequences matching those of the MP25 peptides. Further library screening with cM10A identified cDNAs which encode the complete primary structures of bovine and human MP25. Bovine and human MP25 were found to be around 80% homologous and contain 170 and 173 amino acids, respectively. Data base searches revealed that MP25 had significant similarity of structure only with MAGP, indicating that the two proteins form a new family of microfibrillar proteins. In acknowledgment, MP25 has been formally renamed MAGP-2, and MAGP is referred to as MAGP-1. The close similarity between the two proteins (57%) is confined to a central region of 60 amino acids where there is precise alignment of 7 cysteine residues. Elsewhere the MAGP-2 molecule is rich in serine and threonine residues and contains an RGD motif. MAGP-2 lacks the proline-, glutamine-, and tyrosine-rich sequences and a hydrophobic carboxyl terminus, characteristic of MAGP-1. These structural differences suggest that MAGP-2 has some functions which are distinct from those of MAGP-1. The locus of the human MAGP-2 gene was identified on chromosome 12 in the region of 12p12.3–12p13.1.

Considerable interest has centered recently on microfibrils, 10–12 nm in diameter, which occur in the extracellular matrix of a diverse range of tissues. Parallel arrays of 12-nm microfibrils are found as components of elastic fibers in tissues such as arteries, lung, and some ligaments. These bundles of microfibrils appear to act as templates for the deposition of tropoelastin during elastin formation. Morphologically indistinguishable microfibrils also occur as elastin-free bundles in tissues, such as the ciliary zonule of the eye, periodontal ligament, skeletal muscle, and kidney, where they serve an anchoring function. In other tissues, such as skin, some microfibrillar bundles become associated with elastin, whereas others remain elastin-free. The reasons for these tissue differences are unclear, but they may be due, at least in part, to variations in the molecular composition of the microfibrils and associated proteins (1–5). Several proteins have been identified which appear to be structural components of both elastin-associated and elastin-free microfibrils. These include the 350-kDa glycoproteins fibrillin-1 and fibrillin-2 and the 31-kDa, elastin-binding protein, MAGP3 (6–12). Fibrillin-1 and fibrillin-2 have been linked to the congenital connective tissue disorders Marfan syndrome and congenital contractual arachnodactyly, respectively (13–15). Both disorders are characterized by major skeletal and ocular defects, but cardiovascular problems associated with Marfan syndrome are usually lacking in patients with congenital contractual arachnodactyly (16). This pointed to developmental and tissue differences in the expression of the two fibrillins, and this has been confirmed by recent studies (11, 12, 17). In some tissues, such as elastic ear cartilage, fibrillin-1 and fibrillin-2 were found by immunohistochemistry and in situ hybridization to have very different distribution patterns (12). The findings led these authors to propose that fibrillin-1-containing microfibrils provide long-term force-bearing structural support whereas fibrillin-2-containing microfibrils regulate the early process of elastic fiber assembly. However, there appears to be extensive overlap of expression of the two proteins during the development of a number of tissues (11, 12, 17). This suggests that variations in other microfibrill-associated proteins may also be important for tissue-specific functions of the different groups of 12-nm microfibrils.

We have previously reported the preliminary characterization of the major proteins extracted from the developing elastic tissue, fetal bovine nuchal ligament, using a reductive saline treatment. This reductive saline treatment, when preceded by exhaustive extraction with the chaotropes guanidinium chloride, is relatively specific for the solubilization of microfibrillar

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) Bos taurus U37282, Homo sapiens U37283.

† To whom correspondence should be addressed: Dept. of Pathology, University of Adelaide, South Australia, 5005. Tel.: 61-8-303-5385; Fax: 61-8-303-4408; E-mail: mgibson@medicine.adelaide.edu.au.

‡ The abbreviations used are: MAGP, microfibrill-associated glycoprotein; DIG, digoxigenin; MFAP, microfibrill-associated protein; MP, microfibrillar protein; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; kb, kilobase pair(s).
proteins which otherwise would remain insoluble due to extensive intermolecular disulfide bonding (18). In addition to MAGP (31 kDa), which we had previously characterized (7, 19), this treatment extracted four polypeptides with apparent molecular masses of 340, 78, 70, and 25 kDa which were named MP340, MP78, MP70, and MP25, respectively. In the present report we describe the further characterization of these species. MP340 was identified as bovine fibrillin-1 by peptide sequencing. MP78, MP70, and MP25 were shown by peptide mapping to be isoforms of a single protein. Sequencing of the peptides indicated that the protein is closely related to the recently cloned but poorly characterized human protein, βig-h3 (20). Peptide sequencing of the 25-kDa species (MP25) indicated that it is a distinct protein which we have now cloned. Herein we report that MP25 immunolocalizes specifically to elastin-associated microfibrils in developing elastic tissue and that the protein has structural similarities to MAGP. Thus we have renamed MP25 as MAGP-2 and now refer to MAGP as MAGP-1.

EXPERIMENTAL PROCEDURES

Purification of Microfibrillar Proteins—Microfibrillar proteins were purified as described previously from reductive saline extracts of nuchal ligament tissue from fetal calves (approximately 210 days of age) using a combination of DEAE ion-exchange and gel permeation chromatography (18). Since chromatographic separation of MP78 and MP70 proved difficult, each polypeptide was obtained in pure form by electrophoresis of a purified mixture of the two proteins on high resolution 4% agarose gels (Proliase system, FMC BioProducts, Rockland, ME). Coomassie Blue staining was used to identify the appropriate protein bands which were then excised from the gel. The excised pieces of agarose were melted at 70°C in 9 volumes of 50 mM Tris buffer, pH 8.0, containing 1% SDS and 1 mM EDTA. The agarose was allowed to reseal on ice and then it was shattered by freezing to –70°C. After thawing, the agarose was removed from the protein, which remained in solution, by centrifugation at 5,000 × g for 20 min at 4°C. The protein was recovered by precipitation with 10 volumes of ice-cold acetone containing 1 mM HCl, and then it was redissolved either in 70% formic acid for cyanogen bromide digestion or in 4 M urea, 40 mM Tris, pH 8.4, for digestion with endoproteinase Lys-C.

Preparation and Sequencing of Peptides—Purified proteins were digested with cyanogen bromide or endoproteinase Lys-C as described previously (8, 18). In some instances the digest was analyzed by SDS-PAGE on a 15% gel followed by Western blotting onto poly(vinylidene fluoride) membrane (Bio-Rad). The membrane was stained with Coomassie Blue. Prominent peptide bands were excised and sequenced directly (21). In other instances, peptides were separated by a high performance liquid chromatography system (Waters, Milford, MA). Digests were applied to a PRP-1 reverse phase column (150 × 4.1 mm) (Hamilton, Reno, NA) and eluted with a 55-ml gradient of 0–50% acetonitrile in 0.05% trifluoroacetic acid at 1 ml/min. Peptide peaks were identified at 214 nm and prepared for sequencing on an Applied Biosystems gas-phase sequenator model 473 as described previously (8). Repetitive yields averaged at 96%.

Antibody Characterization, Immunofluorescence, and Immunoelectron Microscopy—Antibodies to highly purified MAGP-2 were raised in rabbits, and their specificity was confirmed by immunoblotting against MAGP-2 and other microfibrillar proteins as described previously (18). For immunohistochernistry the antibodies were affinity-purified on human MAGP-2 cDNA clone, cH61, was nick-translated with biotin-14-dATP and was hybridized in situ to human placental cDNA library in axt 11 (Clontech Ltd., Palo Alto, CA). The methodology for the above procedures has previously been described in detail (8). The BLAST network service at the National Center for Biotechnology Information (E-mail: blast@ncbi.nlm.nih.gov) was used for database searches.

Isolation of RNA and Northern Blotting—Nuchal ligament tissue was dissected from a 210-day-old fetal calf, within 1 h of maternal death, and immediately frozen in liquid nitrogen. Total RNA was purified by guanidine isothiocyanate extraction followed by CsCl density gradient centrifugation and poly(A) RNA was selected by chromatography on oligo(dT)-cellulose using standard methods (24). Northern blotting with poly(A) RNA was conducted with antisense DIG-RNA transcripts of MAGP-1 cDNA clone cM32 and MAGP-2 cDNA clone cM10A, using previously described methods (8).

Fluorescence in Situ Hybridization to Human Chromosomes—A human MAGP-2 cDNA clone, cH61, was nick-translated with biotin-14-dATP and was hybridized in situ (at a concentration of 15 ng/ml) to metaphase preparations from two normal male subjects. Signal was detected using a fluorescence in situ hybridization technique as described previously (25), except chromosomes were stained before analysis with both propidium iodide, as a counterstain, and 4,6-diamidino-2-phenylindole-2HCl for chromosome identification. Metaphase preparations were photographed, and the images were computer-enhanced for clarity.

RESULTS

Identification of MP78 and MP70 with βig-h3—The separation of MP78 and MP70 proved difficult using chromatographic methods. However, purification of each protein was readily achieved using preparative electrophoresis in agarose gels (Fig. 1A). Peptide mapping of CNBr digests revealed almost identical patterns for both MP78 and MP70, indicating that they are isoforms of the same protein (Fig. 1B). Sequence analysis of five of these peptides revealed strong similarities with sequences found in human βig-h3 (Fig. 2A), suggesting that MP78 and MP70 were forms of bovine βig-h3.
Identification of MP340 with Fibrillin-1—

Digestion of purified MP340 with CNBr and endoproteinase Lys-C yielded a complex mixture of peptides. This made purification of individual peptides difficult. Nevertheless, over 10 peptides were obtained with sufficient purity for sequencing. Each exhibited a strong similarity to an amino acid sequence from human fibrillin-1. An example is shown in Fig. 2B. No peptides with sequences specific for fibrillin-2 were found. The result suggests that MP340 is the bovine form of fibrillin-1.

Identification of MAGP-2 (MP25) as a Structurally and Immunologically Distinct Protein—

Several peptides obtained from CNBr and Lys-C digests of bovine MAGP-2 (MP25) were sequenced, and each was shown to be distinct from any known protein. Polyclonal antibodies raised in rabbits to purified MAGP-2 showed no cross-reactivity on Western blots (Fig. 3) with the other major components of reductive saline extracts, i.e. MAGP-1, MP340 (fibrillin-1), or MP78/70 (b\(\text{ig-h3}\)). The anti-MAGP-2 antibodies were further purified by affinity chromatography on MAGP-2 that had been electrophoresed on SDS-PAGE and transferred to nylon. These antibodies were used for the cloning and immunolocalization studies described below.

Immunolocalization of MAGP-2 to Elastic Fiber Microfibrils—Using the immunofluorescence technique, affinity-purified anti-MAGP-2 antibodies localized strongly and specifically to elastic fibers of developing nuchal ligament (Fig. 4A). No staining of other structures such as collagen fibers or cell surfaces was detected. This staining pattern was indistinguishable from similar sections stained with anti-tropoelastin antibodies (Fig. 4B). Control sections incubated with IgG from preimmune rabbit serum or diluted preimmune serum showed no staining (not shown). Electron microscopy with the immunogold technique was used to determine the ultrastructural location of the MAGP-2 to the elastic fiber (Fig. 5A). Fig. 5A shows a field containing regions of two elastic fibers separated by a thin cellular process. The left a microfibril-rich region is evident, whereas on the right an extensive region of "amorphous" elastin can be seen. As visualized by protein A-gold beads, the anti-MAGP-2 antibodies localized only to the 12-nm microfibrils, seen both as a mantle surrounding each elastic fiber and as electron-dense material entrapped within the amorphous core of elastic fibers. No gold particles were located on the amorphous elastin of the fibers or on other structures of the surrounding matrix. In control sections stained with pre-

![Fig. 2. Comparison of amino acid sequences. A, amino acid sequences encoded in human \(\text{b}\(\text{ig-h3}\) cDNA (20) (above) compared with sequences from MP78 and MP70 peptides (below). B, a unique amino acid sequence encoded in human fibrillin-1 cDNA (10) (above) compared with a sequence from a MP340 peptide (below). The location of each sequence within the molecule is indicated by the residue numbers shown above. Vertical lines indicate identity within the sequences. X denotes an amino acid assignment which could not be clearly designated.

![Fig. 3. Immunoblotting of microfibrillar proteins with affinity-purified anti-MAGP-2 antibodies. Panel A, a typical reductive saline extract of fetal nuchal ligament analyzed by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Panel B, a similarly stained 12% gel of the major proteins purified from such extracts. Panel C, a matching immunoblot stained with anti-MAGP-2 antibodies. Lane 1, MP340 (fibrillin-1); lane 2, MP78/70 (\(\text{b}\(\text{ig-h3}\)); lane 3, MAGP-1; and lane 4, MAGP-2 (MP25).]

![Fig. 4. Immunofluorescence localization of anti-MAGP-2 antibodies to elastic fibers. Unfixed sections of nuchal ligament from a 210-day-old fetal calf were stained with anti-MAGP-2 antibodies (Panel A) and anti-tropoelastin antibodies (Panel B) as described in the text. Magnification, \(\times\) 170]
immune rabbit serum, no localization of gold particles to the elastic fibers was observed (Fig. 5B). In contrast to the anti-MAGP-2 antibodies, the anti-tropoelastin antibodies localized to the amorphous core of the fibers (Fig. 5C). Thus it is evident that MAGP-2 is specifically associated with the elastin-associated microfibrils but not with the elastin core of the elastic fiber.

Identification of cDNA Clones for MAGP-2—To obtain cDNAs for MAGP-2, affinity-purified antibodies were used to screen two nuchal ligament cDNA libraries. Several immunoreactive clones were obtained and partially sequenced. One clone, cM10A (770 bp) with an extensive open reading frame, encoded amino acid sequences which matched several MAGP-2 peptide sequences, indicating that it was an authentic MAGP-2 cDNA. Clone cM10A was used to rescreen the libraries and several additional clones were isolated. One clone, c5 (903 bp) encoded the entire coding sequence for the protein. Northern blotting of nuchal ligament mRNA with clone cM10A as the probe revealed a single mRNA species of similar size to the 1.1-kb MAGP-1 mRNA (Fig. 6). Allowing for a more extensive poly(A^+) tail on the mRNA than the cDNA, this result indicated that the full-length bovine MAGP-2 cDNA was about 900-1000 bp in length. This is consistent with the sequence deduced from MAGP-2 cDNA clones c5 and cM10A. The bovine cDNA sequence is 907 bp in length containing a 510-bp coding region, a short 5'-untranslated region, and an extensive 3'-noncoding region containing two putative polyadenylation signals and a short poly(A^+) tail (Fig. 7). Clone cM10A was also used as a probe to identify several cDNAs for human MAGP-2 in a placental cDNA library. The largest of these, clone cH61, was 911 bp long and contained an open reading frame of 519 bp as well as extensive 5'- and 3'-untranslated regions of 186 and 206 bp, respectively (Fig. 7). The coding regions of human and bovine MAGP-2 cDNA were found to be 83% homologous. From the alignment of the bovine and human MAGP-2 cDNAs described here it seems likely that some additional 5'-untranslated sequence exists in the bovine mRNA and that additional 3'-untranslated sequence occurs in the human mRNA.

Amino Acid Sequence for MAGP-2—The 170-amino acid sequence encoded by the bovine cDNAs shows excellent homology with the four sequences obtained from MAGP-2 peptides (Fig. 8). The encoded protein contains a putative signal peptide of
predominantly hydrophobic amino acids at the amino terminus. The "(-3,-1)" rule (26) predicts that cleavage occurs after serine 18, resulting in serine 19 forming the aminoterminus of the secreted protein. Cleavage at this point would yield a mature polypeptide of 152 amino acids. The protein sequence encoded by human MAGP-2 cDNA, where it differs from the bovine sequence, is also shown in Fig. 8. Overall the protein sequences were found to have a degree of homology of 78%. Most of the differences were due to conserved amino acid substitutions although one repeat insertion of three amino acids (VLA) was found in the human MAGP-2, making it slightly larger than bovine MAGP-2.

Searches of the GenBank DNA and Swiss protein data bases revealed that MAGP-2 has little similarity to other known proteins with the exception of MAGP-1. Like MAGP-1, MAGP-2 was found to be a highly hydrophilic protein containing two distinct domains, an acidic cysteine-free amino-terminal half and a basic, cysteine-rich carboxyl-terminal half (Fig. 9A). However, there are major structural differences between the proteins. The amino-terminal domain of MAGP-2 is rich in serine and threonine residues and lacks the proline-, glutamine-, and tyrosine-rich sequences found in MAGP-1. This domain of MAGP-2 also contains a RGD putative integrin-binding motif and a consensus sequence for N-glycosylation. Both motifs are lacking in the MAGP molecule. The carboxyl-terminal domain of MAGP-2 contains 8 cysteine residues in contrast to the 13 found in MAGP, and MAGP-2 lacks a hydrophobic region at the extreme C terminus. Overall MAGP-2 has a relatively neutral isoelectric point (pI 6.6), whereas MAGP-1 is highly acidic (pI 4.7). Close sequence similarity (57%) is confined to a 60-amino acid region in the center of the two proteins (Fig. 9B). This region contains the first 7 cysteines of MAGP-2 and the first 8 cysteines of MAGP-1. All of these residues in MAGP-2 precisely align with cysteines in MAGP-1, in which cysteine 3 is the unmatched residue. The distance between each cysteine is precisely conserved between the two proteins. The consensus sequence between the proteins is also shown. Note that two MAGP-2 peptide sequences match those encoded by MAGP-2 cDNA in this region and that they are clearly distinct from the corresponding sequences of MAGP-1.

It is interesting that this central region of structural similarity between MAGP-2 and MAGP-1 corresponds precisely, in the bovine and human MAGP-1 genes, with exons 7 and 8 and FIG. 6. Identification of MAGP-2 mRNA. Poly(A) RNA from developing nuchal ligament was electrophoresed on a 1% agarose gel (1 µg lane), Northern blotted, and hybridized with DIG-labeled RNA transcripts of MAGP-1 cDNA clone cM32 (lane 1) and MAGP-2 cDNA clone cM10A (lane 2). The size of bovine MAGP-1 mRNA has previously been estimated as 1.1 kb (8), and this is indicated by the arrow.

FIG. 7. Restriction map of cDNA clones for bovine and human MAGP-2. The diagram shows the bovine MAGP-2 cDNA clone cM10A, identified by expression screening, compared with subsequently isolated, larger cDNAs encoding the entire amino acid sequences of bovine (c5) and human MAGP-2 (cH61). Coding regions are shown in black and their lengths are indicated. Noncoding regions are shown in white. Polyadenylation signals (PAS) are marked.

FIG. 8. Comparison of bovine and human MAGP-2 amino acid sequences deduced from cDNAs. The nucleotide sequence of bovine MAGP-2 cDNA clone c5 is shown above the deduced amino acid sequence of bovine MAGP-2. The 5' end of clone cM10A is indicated. Peptide sequences from digests of bovine MAGP-2 are shown in italics. The human MAGP-2 sequence, translated from cDNA clone cH61, is also shown where it differs from the bovine sequence. Note the insertion of the sequence VLA after amino acid number 60. Underlined in order are: (a) an RGD putative cell-binding sequence, (b) a potential site for N-glycosylation, and (c) two AATAAA signals for polyadenylation. MAGP-2 was found to be a highly hydrophilic protein containing two distinct domains, an acidic cysteine-free amino-terminal half and a basic, cysteine-rich carboxyl-terminal half (Fig. 9A). However, there are major structural differences between the proteins. The amino-terminal domain of MAGP-2 is rich in serine and threonine residues and lacks the proline-, glutamine-, and tyrosine-rich sequences found in MAGP-1. This domain of MAGP-2 also contains a RGD putative integrin-binding motif and a consensus sequence for N-glycosylation. Both motifs are lacking in the MAGP molecule. The carboxyl-terminal domain of MAGP-2 contains 8 cysteine residues in contrast to the 13 found in MAGP, and MAGP-2 lacks a hydrophobic region at the extreme C terminus. Overall MAGP-2 has a relatively neutral isoelectric point (pI 6.6), whereas MAGP-1 is highly acidic (pI 4.7). Close sequence similarity (57%) is confined to a 60-amino acid region in the center of the two proteins (Fig. 9B). This region contains the first 7 cysteines of MAGP-2 and the first 8 cysteines of MAGP-1. All of these residues in MAGP-2 precisely align with cysteines in MAGP-1, in which cysteine 3 is the unmatched residue. The distance between each cysteine is precisely conserved between the two proteins. The consensus sequence between the proteins is also shown. Note that two MAGP-2 peptide sequences match those encoded by MAGP-2 cDNA in this region and that they are clearly distinct from the corresponding sequences of MAGP-1.
The predicted size of the mature MAGP-2 polypeptide encoded by the cDNA is around 17 kDa for both the bovine and human forms of the protein. This is somewhat smaller than the apparent molecular mass of 25 kDa observed on SDS-PAGE. The size discrepancy may be due to glycosylation of the protein which has a consensus sequence for N-linked carbohydrate attachment. However, it should be noted that MAGP-1, with an actual molecular mass calculated as 20 kDa, migrates on gels as a 31-kDa species even in the absence of carbohydrate side chains (8). This anomalous migration appears to be a function of the primary structure of MAGP-1 and a similar effect may explain the difference in the observed and calculated sizes of MAGP-2. Since MAGP-2 contains eight cysteine residues it is possible that these all pair to form intramolecular disulfide bonds. However, there is evidence that all of these residues are not solely involved in intramolecular linkages. MAGP-2, like fibrillin-1 and MAGP-1, is resistant to extraction from tissue homogenates with the strong chaotrop, 6 M guanidinium chloride, but it is readily solubilized in saline if a reducing agent is included to disrupt disulfide linkages (18). This indicates that MAGP-2 forms intermolecular disulfide bonds either with other MAGP-2 molecules or with other components of the microfibrils and thus has a structural role in association with these entities.

Human Chromosome Localization—Twenty-seven metaphases from a normal male subject were examined for fluorescent signal. Twenty-six of these metaphases showed signal on one or both chromosomes of chromosome 12 in the region 12p12.3–12p13, where 46% of the signal was at 12p12.3, 24% was at 12p13.1, and 30% was between these bands. This indicates that the MAGP-2 gene is located close to the interface of the two bands (Fig. 10). There was a total of 10 nonspecific background dots observed in the 27 metaphases. A similar result was obtained with metaphases from a second normal male subject (data not shown).

**DISCUSSION**

It is long established that the elastin-associated microfibrils are distinct in composition from elastin itself on the basis of major differences in amino acid composition, glycosylation, and susceptibility to digestion with different proteases (1). However, it is only recently that individual glycoproteins and proteins have been identified with the microfibrils by immunoelectron microscopy. Progress has been further hampered by observations that a number of serum components including serum amyloid P, fibronectin, and vitronectin appear to become increasingly bound to the microfibrils with tissue aging (2). However, an increasing number of proteins and glycoproteins have been immunolocalized to these structures in developing tissues. Many of these candidate microfibrillar proteins have been cloned and sequenced. These include: fibrillins 1 and 2 (350 kDa); MAGP, also recently described as MFAP2 (31 kDa); MFAP1 (57 kDa); MFAP3 (41 kDa); and the 36-kDa MFAP4 (8, 9, 11, 29–32). Unlike the fibrillins, MFAPs 1–4 do not exhibit structural similarities to each other.

It is now established that the major structural elements of the microfibrils are the fibrillins, which are large rodlike glycoproteins. Evidence suggests that they are arranged as parallel bundles of 6–8 molecules joined end to end in a head to tail manner (33). It is not yet clear if fibrillin-1 and fibrillin-2 exclusively form distinct microfibrils or if they coexist within the same microfibril in some instances (11, 12). Rotary shadowing of microfibrils has revealed a "beads on a string" morphology for these structures (33, 34). The interbead regions appear to correspond to fibrillin bundles but the composition of the beads is less clear. MAGP-1 is a small acidic, elastin-binding protein which is associated with these beadlike structures where it forms disulfide and possibly transglutaminase cross-links (7, 8, 35). Thus MAGP-1 may play roles in stabili-
ization of the end to end and/or lateral aggregation of fibrillin bundles within the microfibril and also in the binding and alignment of the elastin precursor, tropoelastin, to the microfibrils during elastic fiber development.

We have previously described the identification of four polypeptides, which we provisionally named MP340, MP78, MP70, and MP25 (now referred to as MAGP-2), that were extracted in close association with MAGP-1 from the elastin-rich nuchal ligament of fetal calves. Affinity-purified antibodies to MP340 and a mixture of MP78 and MP70 were shown to localize to the 12-nm microfibrils in both elastic and nonelastic tissues, indicating that the proteins were associated with these structures.

In order to characterize further and possibly clone the above polypeptides, peptide digestions were made, and individual peptides were purified and sequenced. Several peptide sequences from MP340 were shown to match closely sequences found in fibrillin-1 (10). This confirmed earlier suggestions that MP340 is bovine fibrillin-1 (18). It is noteworthy that none of the peptides matched fibrillin-2 sequences (11) indicating that reductive saline extracts of nuchal ligament are substantially free of this form of fibrillin. This suggests either that the two fibrillins have significantly different solubility properties or that fibrillin-1 is the predominant form of fibrillin present at this stage of development in the nuchal ligament.

Peptide mapping strongly indicates that MP78 and MP70 are isoforms of the same protein. This may explain the difficulties encountered in attempts to separate them chromatographically. A close similarity was found between sequences of these peptides and the recently identified human protein βig-h3, inferring that MP78/70 is the bovine version of the same protein. βig-h3 is a novel 68-kDa protein recently discovered by differential screening of cDNAs made from A549 human lung adenocarcinoma cells in which the expression of the protein was stimulated by transforming growth factor-β1 (20). The predicted 683-amino acid sequence of secreted βig-h3 contains four regions of internal homology, 11 cysteine residues, and a RGD motif which may represent an integrin binding sequence (36). More recently, a recombinant form of the protein was shown to block the adhesion of several cell types to plastic culture dishes, leading the authors to suggest βig-h3 may be an extracellular matrix protein (37). This anti-adhesion effect was not mediated by the RGD motif as the carboxy-terminal region containing the RGD sequence was found to be absent from the recombinant protein. The evidence suggested that the 68-kDa recombinant protein had been processed from a larger form of βig-h3 which has a predicted molecular weight of 76,000. It seems likely that MP78 represents the larger form and MP70 relates to the processed form of bovine βig-h3. Interestingly, in an independent study of human eye tissue by Escribano et al. (38), βig-h3 was found to be expressed by two cell lines derived from ciliary epithelium. Ciliary epithelium is the tissue which attaches the microfibril-rich ciliary zonule to the ciliary body and thus it is possible that the epithelial cells synthesize βig-h3 for incorporation into the zonular microfibrils. In the same study βig-h3 was also found, by immunofluorescence, to be present on the extracellular surface of corneal epithelial cells (38). Our own studies indicate that βig-h3 (MP78/70) can become tightly bound to the extracellular matrix, where it appears to be associated with the microfibrillar proteins, fibrillin-1 and MAGP-1. Therefore, it seems that βig-h3 may be associated both with microfibrils and with the cell surface. This behavior is reminiscent of that of the 67-kDa elastin-binding protein, also identified in zonular extracts (39), which is considered to direct the secretion of elastin from the endoplasmic reticulum to elastic fiber assembly sites at the cell surface (40, 41). The primary structure of the 67-kDa elastin-binding protein is still uncertain, although recent evidence suggests that it may be an alternatively spliced form of β-galactosidase (42). There is an intriguing possibility that βig-h3(MP78/70) may be related structurally and/or functionally to the 67-kDa elastin-binding protein.

It is interesting that the human gene for βig-h3 is located on chromosome 5 at band q31 (37) which is close to the loci of the fibrillin-2 gene (5q21–31) (14) and the gene for another 41-kDa microfibrillar-associated protein known as MFAP3 (5q32–33.2) (31). The loci of fibrillin-1 and MFAP-1 genes are also close together on chromosome 15 (14, 30, 43). It is possible that this gene clustering serves some purpose in the coordination of gene expression of microfibrillar proteins. However, it should be noted that the genes for several collagens and fibronectin, which are not necessarily coordinately expressed with each other, are located in close proximity on chromosome 2 (44).

Analysis of peptide sequences from MAGP-2 indicated that the protein was distinct from all known proteins. Affinity-purified anti-MAGP-2 antibodies immunolocalized strongly to the elastin-associated microfibrils but showed no cross-reactivity with fibrillin, βig-h3, or MAGP-1. This confirmed that MAGP-2 was also a discrete microfibrillar component. Molecular cloning was used to obtain the entire coding sequences of its bovine and human genes. Interestingly, data base searches revealed little sequence similarity to other known proteins with the exception of MAGP-1 (8). MAGP-1 and MAGP-2 were found to have an extensive region of sequence resemblance, and thus they appear to represent a new family of microfibril-associated proteins. In acknowledgment of their structural similarity, we support and retain the names MAGP-1 and MAGP-2 in preference to MFAP-2 and MFAP5. Neither protein has a structural relationship to MFAP-1, MFAP-3, or MFAP-4.

While the close resemblance of the central regions of MAGP-2 and MAGP-1 suggests that the two molecules may share some activities, the structural diversity of other regions indicates that the proteins may also have distinct functions. MAGP-2 lacks tyrosine-rich and glutamine-rich motifs in the amino-terminal region and a hydrophobic carboxy-terminal region, found in MAGP-1 (8). These domains may be important for the known modifications and interactions of MAGP-1 which include tyrosine sulfation, transglutamine cross-linking, and tropoelastin binding (35). It will be interesting to establish if any of these properties are shared with MAGP-2. Unlike MAGP-2, MAGP-1 contains an odd number of cysteine residues, and the evidence presented here suggests that cysteine 3 is the unpaired cysteine. This residue is likely to confer on MAGP-1 some additional intermolecular disulfide bonding properties. In contrast, the presence of a conserved RGD motif in a hydrophilic sequence of MAGP-2 suggests that it may have integrin-binding properties, lacking in MAGP-1, and thus may play an additional role in the interaction of the microfibrils with the cell surface.

Preliminary studies on the distribution of MAGP-2 during tissue development indicate that the protein is associated with elastin-associated microfibrils, and with elastin-free microfibrils in a number of tissues. However, MAGP-2 appears to have a more restricted tissue distribution than MAGP-1 and fibrillin-1 in late-fetal and adult tissues. For instance, our studies suggest that MAGP-2 is absent from the microfibrils of the ciliary zonule and the connective tissue around kidney tubules. Also MAGP-2 gene expression appears to be more closely linked than that of MAGP-1 to the switching on of the elastin gene in developing nuchal ligament.

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that MAGP-2 may be involved in developmental and tissue-specific modulation of microfibril function. Interestingly, a recent study has shown that, during murine embryogenesis, fibrillin-2 has a more restricted profile of expression than fibrillin-1 in terms of both developmental stages and tissue distribution (12). Fibrillin-2 generally appeared earlier and accumulated for a shorter period than fibrillin-1. Of particular note are the findings that the expression of fibrillin-2 is very low or absent from the ciliary zonule and that, in kidney, it is restricted to the glomerulus (12). This indicates that there are similarities in the tissue distributions of MAGP-2 and fibrillin-2 and raises the possibility that MAGP-2 is specifically associated with fibrillin-2-containing microfibrils. To test this idea we are conducting a more thorough study of MAGP-2 expression patterns. In addition, further structural characterization of MAGP-2 and its human gene, which we have located on chromosome 12 at 12p12.3–12p13.1, is in progress.

In conclusion, molecular cloning and peptide sequencing techniques have been instrumental in determining the structural inter-relationships of five polypeptides which we have identified with the microfibrillar component of elastic fibers. The polypeptides are derived from four distinct proteins, two of which are structurally related to each other. A major challenge is ahead to determine how these microfibril-associated proteins interact with one another and with other proteins. This in turn will lead to a better understanding of the function of microfibrils, in elastic and nonelastic tissues, including the role of these structures in the complex process of elastic fiber assembly.

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