Elastic fibers consist primarily of an amorphous elastin core associated with microfibrils, 10–12 nm in diameter, containing fibrillins and microfibril-associated glycoproteins (MAGPs). To investigate the interaction of MAGP-1 with tropoelastin and fibrillin-1, we expressed human MAGP-1 as a T7-tag fusion protein in Escherichia coli. Refolding of the purified protein produced a soluble form of MAGP-1 that displayed saturable binding to tropoelastin. Fragments of tropoelastin corresponding to the N-terminal, C-terminal, and central regions of the molecule were used to characterize the MAGP-1 binding site. Cleavage of tropoelastin with kallikrein, which cleaves after Arg in the central region of the molecule, disrupted the interaction, suggesting that the separated N- and C-terminal fragments were insufficient to determine MAGP-1 binding to intact tropoelastin. In addition, no evidence of an interaction was observed between MAGP-1 and a tropoelastin construct consisting of domains 17–27 that brackets the kallikrein cleavage site, suggesting a complex mechanism of interaction between the two molecules. Binding of MAGP-1 was also tested with overlapping recombinant fibrillin-1 fragments. MAGP-1 bound to a region at the N terminus of fibrillin-1 in a calcium-dependent manner. In summary, these results suggest a model for the interaction of elastin with the microfibrillar scaffold.

Elastic fibers are composed of an amorphous core, consisting mainly of elastin, surrounded by 10–12 nm microfibrils composed of fibrillins, MAGPs and several other components. During elastic fiber synthesis, the microfibrils appear before the amorphous core and are believed to act as a scaffold for the deposition of tropoelastin. The incorporation of tropoelastin into the nascent elastic fiber is likely to depend on its interactions with microfibrillar proteins and its ability to self-associate through the process of coacervation. Self-association through coacervation involves the hydrophobic domains of the tropoelastin molecule (1, 2). The C-terminal domain, which contains the only two cysteine residues of tropoelastin has been proposed as the site of interaction with the microfibrillar components through the formation of a basic, intramolecular disulfide-bonded loop (3). Antibodies directed against this region disrupt fiber formation in vitro (4), while in lamb ductus arteriosus, loss of the C terminus in a 52-kDa proteolytically derived tropoelastin product prevents incorporation into the fiber (5). Microfibrils appear ultrastructurally after rotary shadowing as beaded filaments with a periodicity of 50–55 nm (6). The main structural protein of the microfibrils, fibrillin-1, is periodically arranged, with the N and C termini in or close to the beads (7). Another component of microfibrils, originally identified in nuchal ligaments, is a 31-kDa glycoprotein termed MAGP-1 (8). MAGP-1 has been localized by immunogold-labeling to the beaded structures of the microfibrils (9). Both structural components, fibrillin-1 and MAGP-1, are covalently bound to the microfibrils by disulfide linkages (8, 10, 11) and transglutaminase-derived cross-links (12, 13). The structure and functions of the microfibrils depend on calcium. The conformation of entire microfibrils (14) or of the fibrillin-1 polypeptide (15) for example is determined by bound calcium in addition to functional roles of calcium in interactions between microfibrillar components (16).

The interactive repertoire of microfibrillar and elastic fiber components determines the development and integrity of these functional entities in various tissues. Toward this end it has been documented that MAGP-1 can bind to the C-terminal portion of tropoelastin (4, 12). A region near the N terminus of fibrillin-1, which encompasses the proline-rich region, downstream epidermal growth factor EGF-like domains and the second 8-Cys domain, has been shown to recognize tropoelastin (17). The analogous region of fibrillin-2 also binds tropoelastin, suggesting a role for the fibrillins in aligning tropoelastin molecules prior to cross-linking (17). Furthermore, indications for interaction between fibrillin and MAGP-1 were obtained by co-immunoprecipitation of these two microfibrillar proteins from the culture medium of fetal chondrocytes (18).

In this study, we further investigated the interactions of MAGP-1 with tropoelastin and fibrillin-1. To facilitate these studies, we expressed human MAGP-1 as a T7-tag fusion in Escherichia coli. Refolding of the purified protein produced a soluble form of MAGP-1 that was used to define binding epitopes on tropoelastin and fibrillin-1. These results suggest a model for the interaction of elastin with the microfibrillar scaffold of the elastic fiber.
The 911-bp PCR product was purified by agarose gel electrophoresis and ligated to the plasmid pET-MAGP in pET-MAGP corresponded to the cDNA sequence summarized in Fig. 1B. The thermal cycler program used for the Amplification of the plasmid pET-MAGP. DNA sequencing was used to confirm that the MAGP-1 sequence was amplified. DNA sequencing described previously (Ref. 19, data not shown). Two mismatches were detected compared with the published human MAGP-1 gene sequence (19). One was a C to T transition at position 432 of the coding sequence. Both of these mismatches were silent mutations at the third positions of the codons involved, and thus had no effect on the amino acid sequence of the resulting protein.

Expression and Purification of T7-MAGP—100 ml of 2YT medium containing 50 µg/ml ampicillin was inoculated with an overnight culture of E. coli BL21(DE3) cells containing pET-MAGP and grown at 37 °C until A600 was ~0.8. IPTG was then added to 0.4 mM final concentration. After 2 h, the induced culture was harvested and resuspended in 10 ml of lysis buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml lysozyme). The mixture was immediately divided into 5-ml aliquots and frozen in liquid nitrogen for 5 min. After thawing, the freeze/thaw cycle was repeated 2–3 times. The mixture was added to 10 ml of denaturing buffer (50 mM Tris, pH 8.0, 0.6 M guanidine hydrochloride, 25 mM dithiothreitol). Residual insoluble material in the denaturing buffer solution was removed by centrifugation, and the soluble T7-MAGP remaining in the supernatant was allowed to refold by dialysis against 50 mM Tris, pH 8.0.

Recombinant Elastin and Antibodies—Recombinant human tropoelastin (SHEL) and a deletion construct lacking exon 26A (SHEL26A) were prepared as described previously (21, 22). Kallikrein digestion of tropoelastin has been described previously (23) and results in the cleavage of tropoelastin after Arg221, at the junction of domains 25 and 26. Anti T7-tag antibody (Novagen) was used as described by the manufacturer. The region of the SHEL gene corresponding to exons 17–27 of tropoelastin was amplified using the primers 5'-CCATGGGGCGTAG-3' and 5'-GGATCCCAGGGCCAGTATGAC-3' (5 °C for 3 min then cooled to 72 °C). The thermal cycler program used for the Amplification and purification of recombinant fibrillin-1 polypeptides rF20, rF23, rF31, and rF38 have been described in detail previously (7, 11, 16, 26). Recombinant fibrillin-1 polypeptides used in this study are summarized in Fig. 1B.

N-terminal Sequencing and Mass Spectrometry—N-terminal sequence was carried out using an Applied Biosystems Procise Sequencer by the Biomolecular Resource Facility at the Australian National University. MALDI-TOF mass spectrometry was carried out on a Perseptive Voyager DE STR spectrometer by the Biomolecular Mass Spectrometry Unit, University of New South Wales.

Protein Interaction Studies—Protein interactions were examined using the ligand overlay blotting technique described previously (20) with some modifications. Proteins were separated by SDS-PAGE (10% w/v acrylamide) and transferred to PVDF membranes (Gelman) in transfer buffer (12 mM Tris, 96 mM glycine, pH 8.3, 20% methanol, 0.1% SDS) using a Hoefer Transblot apparatus. Detects were prepared by spotting 2 µl of protein solution (typically 0.5–1.0 mg/ml) directly onto nitrocellulose membranes (Gelman Sciences). After drying, membranes were blocked by incubating in 3% nonfat milk/phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl) for 30 min at room temperature. After rinsing 3 times in PBS, membranes were incubated at 4 °C for 16 h in a 10-µg/ml solution of T7-MAGP prepared in PBS containing 0.05% nonfat milk. Membranes were rinsed 3 more times and then incubated with anti-T7 tag antibody (Novagen, 1:5000). Blots were developed in 100 mM Tris, pH 7.5 containing 0.8 mg/ml diaminobenzidine (Sigma), 0.01% H2O2, and 0.4 mg/ml NiCl2 until bands were clearly visible. Levels of T7-MAGP binding on dot blots were quantified by digital scanning and ImageQuant software (version 4.2a, Molecular Dynamics).

Ligand overlay blots used to examine the interaction of T7-MAGP with the fibrillin-1 fragments were carried out similarly, except that Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl) replaced the PBS, and the 10-µg/ml solution of T7-MAGP contained 2 mM CaCl2. For solid phase binding assays 6.5–10 µg/ml bovine MAGP-1 in TBS was spotted onto multwell plates (Nunc MaxiSorp, 96 wells) for 16 h at 4 °C. After washing with TBS containing 2 mM CaCl2 and 0.05% (v/v) Tween 20 (wash buffer), the nonspecific binding sites were blocked with 5% (w/v) nonfat dry milk in TBS containing 2 mM CaCl2 (binding buffer) for 1 h at room temperature. The MAGP-1-coated wells were incubated with serial dilutions of recombinant fibrillin-1 subdomains for 2 h at room temperature, washed three times, and incubated with specific antiserum against the fibrillin-1 subdomains (diluted 1:250 in binding buffer) for 1 h. After three washes, the wells were incubated with a peroxidase conjugate of a goat anti-rabbit immunoglobulin (Bio-Rad, 1:800) for 1 h and washed again. Bound ligands were detected by a color reaction for 3–4 min with 1 mg/ml 5-aminosalicylic acid (Sigma) in 20 mM phosphate buffer, pH 6.8, containing 0.05% H2O2 (100 µl of solution per well), which was terminated by the addition of 2 N NaOH (100 µl per well). Color yields were determined at 490 nm.

RESULTS

Characterization of T7-MAGP—T7-MAGP expression in bacterial cultures was examined by SDS-PAGE, which revealed expression of a protein of an apparent molecular mass of 32 kDa.
kDa (Fig. 2). This differed from the 20 kDa predicted from the amino acid sequence. N-terminal sequencing and mass spectrometry were used to confirm the identity of the purified protein. The N-terminal sequence of the isolated protein (ASMTGGQQ) was identical to the sequence expected for T7-MAGP assuming the removal of the N-terminal methionine by endogenous \textit{E. coli} methionine aminopeptidase. By MALDI-TOF analysis, the mass of T7-MAGP was found to be 20,281 \pm 81 Da, which correlated well with the expected mass of 20,212 Da based on the amino acid sequence. This result confirmed that its performance in SDS-PAGE analysis was due to aberrant migration as described previously for the natural protein (27).

**T7-MAGP Binding to Tropoelastin**—Total \textit{E. coli} cell lysates containing the overexpressed tropoelastin isoform SHEL\textsubscript{26A} were examined for the ability of the bacterially expressed T7-MAGP to bind to tropoelastin using a ligand blot assay (Fig. 3). T7-MAGP bound specifically to SHEL\textsubscript{26A}. The specificity of T7-MAGP for SHEL\textsubscript{26A} was demonstrated by its lack of affinity for any of the \textit{E. coli} host cell proteins (Fig. 3) and by its lack of binding to bovine serum albumin compared with equivalent amounts of tropoelastin (data not shown). Control experiments in which MAGP-1 was omitted from the overnight incubation confirmed that the T7-tag antibodies did not crossreact with tropoelastin (data not shown).

**Binding of T7-MAGP to Kallikrein-digested Tropoelastin**—Human plasma kallikrein and porcine pancreatic kallikrein have previously been shown to cleave tropoelastin after Arg\textsubscript{515} at the end of domain 25, resulting in an N-terminal fragment of \textasciitilde45 kDa and a C-terminal fragment of \textasciitilde22 kDa (Fig. 1 and Ref. 23). N-terminal sequencing and mass spectrometry data confirmed that the C-terminal fragment resulting from these digests is intact and would be expected to behave similarly to the corresponding region of the full-length molecule (data not shown; Ref. 23). Neither of the proteolytically derived tropoelastin fragments showed binding to T7-MAGP at levels comparable to or approaching those seen for the intact tropoelastin (Fig. 4a). In many cases, the only band to appear in the kallikrein digest lane of the ligand overlay blots corresponded to trace amounts of intact tropoelastin that had not been completely digested. Solid phase assays of the C-terminal fragment, isolated as described previously (23), showed that the lack of T7-MAGP binding to this fragment in the ligand

![Fig. 1. Schematic representations of recombinant tropoelastin and fibrillin-1 constructs. a, SHEL17–27 spans the region of tropoelastin between domains 17 and 27. b, Fibrillin-1 and deletion constructs.](image)

![Fig. 2. Purification of T7-MAGP from inclusion bodies. The insoluble fractions of cells expressing T7-MAGP were washed in buffer containing 6 M guanidine hydrochloride. T7-MAGP was then refolded by dialysis into 50 mM Tris, pH 8.0. Lane 1, protein composition of an uninduced culture; lane 2, protein composition of an IPTG-induced culture; lane 3, soluble, refolded T7-MAGP. The positions of globular marker proteins are indicated in kDa and the position of T7-MAGP is indicated by an arrow.](image)
overlay assays was not due to the denaturing effect of SDS during electrophoresis. This was under conditions where full-length tropoelastin clearly bound T7-MAGP (data not shown). These results suggested that either the major MAGP-1 binding site in tropoelastin is cleaved by kallikrein or that kallikrein digestion disrupts a conformational requirement for T7-MAGP binding.

**Binding of T7-MAGP to SHEL17–27**—The recombinant tropoelastin polypeptide SHEL17–27 was designed to test whether MAGP-1 could bind to the central region of tropoelastin sequence encompassing the domain 25/26 kallikrein site. SHEL17–27 extends from the beginning of domain 17 to the end of domain 27, with an intact domain 25/26 region. Ligand overlay assays showed no evidence for an interaction with T7-MAGP, suggesting that this part of tropoelastin was insufficient for binding to MAGP-1 (Fig. 4b). Combined with the kallikrein digestion data presented above, the results indicate that binding of MAGP-1 to tropoelastin in this system cannot be explained by the presence of a single defined MAGP-1 binding site on tropoelastin. Instead, the lack of binding to kallikrein digestion products comprising the N terminus to domain 25 and domain 26 to the C terminus, and to the fragment comprising domains 17–27 (SHEL17–27), reveals a complex interaction between MAGP-1 and the intact tropoelastin molecule.

**Binding of MAGP-1 to Fibrillin**—In ligand overlay blots with fibrillin-1 fragments, T7-MAGP showed saturable binding to immobilized fibrillin-1 fragment rF16 (diamonds) but not to fragments rF20 (squares) or rF6H (triangles). b, comparison of T7-MAGP binding to a range of fibrillin-1 fragments. Dot blots with 10 µg of each immobilized fibrillin-1 fragment were used in ligand overlay assays with soluble T7-MAGP. All assays were normalized to the value obtained for the rF16 sample and expressed as percentages. Error bars indicate S.D.
was coated to the plastic surface of multiwell plates at 6.5 µg/ml and tropoelastin (lane 2) were used in ligand overlay assays in PVDF membranes with immobilized fibrillin-1 fragment rF23 and bovine MAGP-1 mains to immobilized bovine MAGP-1.

Authentic bovine MAGP-1 was produced to study the interactions with these elastic fiber components. Previous forms of recombinant MAGP-1 had been produced in eukaryotic expression systems, which were chosen because the naturally occurring protein is likely to undergo post-translational modifications such as O-linked glycosylation (8) and sulfation of tyrosine residues (28). The formation of specific intrachain disulfide bonds may also affect the ability of MAGP-1 to function. We sought to closely base a refolding protocol on that described for the natural protein. It was previously shown that MAGP-1 extracted from bovine tissue could be denatured, reduced, and then refolded so that its ability to bind type VI collagen was restored (20). The type VI collagen binding site was localized to a region at the N terminus of MAGP-1 that lacks cysteine residues. As the binding site for tropoelastin on MAGP-1 overlaps the type VI collagen binding site (20), we considered that a similar refolding procedure should be applicable to bacterially expressed MAGP-1. In this study, we have developed a recombinant MAGP-1 that demonstrates binding to tropoelastin. The T7-MAGP produced in this manner specifically and saturably bound tropoelastin in E. coli lysates but not E. coli proteins. The ability of T7-MAGP to bind tropoelastin, even in the absence of glycosylation and sulfation, reveals that these post-translational modifications are not required for its interaction with tropoelastin.

Ligand overlay assays of recombinant MAGP-1 with fragments produced by kallikrein digestion of tropoelastin suggested that the boundary between domains 25 and 26 of tropoelastin might be involved in the interaction with MAGP-1. This region is in an exposed position on tropoelastin, and likely to be available to interact with other proteins (23). The C-terminal fragment resulting from kallikrein digestion has previously been shown to have a C terminus identical to that found in the full-length protein (23). Since full-length tropoelastin and the truncated forms were treated in precisely the same manner during SDS-PAGE and blotting, the lack of binding to the C-terminal fragment was due neither to a loss of residues at this end of the molecule nor to denaturation during electrophoresis. In other studies, antibodies against the C-terminal end of tropoelastin reduced the binding of MAGP-1 to tropoelastin in a solid phase assay (12) or abolished the assembly of elastic fibers in cell culture models (4, 12). Based on this evidence, the binding site for MAGP-1 had been assigned to the C-terminal end of tropoelastin. It is possible that in those studies, binding of bulky antibodies to the C terminus of tropoelastin may have sterically interfered with the interaction between MAGP-1 and domains 25/26 of tropoelastin. Antibodies typically have a molecular mass of 150 kDa, in contrast to the smaller tropoelastin (60 kDa). Another explanation would be the requirement for both the C terminus and the regions between domains 25 and 26 for a fully functional MAGP-1 binding site on tropoelastin. This explanation would be consistent with our observation that T7-MAGP binding to tropoelastin is completely abolished by proteolytic cleavage between domains 25 and 26 combined with the absence of binding activity of SHEL17–27, which spans the region of tropoelastin cleaved by kallikrein. The fact that in solid phase binding assays with MAGP-1 and tropoelastin the interaction could be maximally reduced with antibodies against the C-terminal end of tropoelastin by about 50% (12) further supports the existence of other regions important for the binding site.

No interaction between MAGP-1 and fibrillin was detected in earlier work involving reduced fibrillin-1 from bovine ligamentum nuchae (12). Recently, however, the first pieces of evidence for binding between MAGP-1 and fibrillin emerged in co-immunoprecipitation studies in a cell culture system (18). In our work, we directly tested binding of T7-MAGP to recombinant fragments of fibrillin-1, which were produced as soluble proteins through eukaryotic expression (7, 11, 16, 26). T7-MAGP bound saturably to fibrillin-1 fragment rF16 (N-terminal portion), but not to fragments rF20 (central part) or rF6H (C-terminal portion). Comparison of the structures of rF16 and rF20 suggested that T7-MAGP was likely to bind to a region within the first nine domains of fibrillin-1 (see Fig. 1b). This was confirmed by the calcium-dependent binding of T7-MAGP.

FIG. 6. Calcium dependence of the T7-MAGP/fibrillin-1 interaction. PVDF membranes with immobilized fibrillin-1 fragment rF23 (lane 1) and tropoelastin (lane 2) were used in ligand overlay assays in the presence or absence of 2 mM CaCl₂. Note that binding of T7-MAGP to rF23 is calcium-dependent, while binding to tropoelastin is not dependent on calcium.

FIG. 7. Solid phase binding assay of soluble fibrillin-1 subdomains to immobilized bovine MAGP-1. Authentic bovine MAGP-1 was coated to the plastic surface of multiwell plates at 6.5 µg/ml and incubated with serial dilutions of fibrillin-1 subdomains rF16 (squares), rF23 (triangles), rF20 (circles), and rF6H (inverted triangles) in concentrations as indicated. Non-specific binding to noncoated wells was subtracted from the binding profiles. The relative positions of the subdomains within the fibrillin-1 molecule are shown schematically in Fig. 1. A binding test with a coating concentration of 10 µg/ml MAGP-1 and otherwise identical conditions produced very similar results.

and rF23 bound strongly to immobilized MAGP-1, while fragments rF20 and rF6H did not show binding activity. These data clearly demonstrate that (i) recombinant fibrillin-1 interacts with authentic MAGP-1 (ii) binding activity is also maintained when MAGP-1 is used as an immobilized ligand, and (iii) the fibrillin-1/MAGP-1 interaction site is conserved between species (human fibrillin-1, bovine MAGP-1).

DISCUSSION

This study was designed to shed light on the mechanism through which the amorphous elastin core of the elastic fiber is linked to microfibrils. MAGP-1 is known to bind tropoelastin, and recent immunoprecipitation studies indicated the possibility of binding to fibrillin (18). Recombinant human T7-MAGP was produced to study the interactions with these elastic fiber components. Previous forms of recombinant MAGP-1 had been produced in eukaryotic expression systems, which were chosen because the naturally occurring protein is likely to undergo post-translational modifications such as O-linked glycosylation (8) and sulfation of tyrosine residues (28). The formation of specific intrachain disulfide bonds may also affect the ability of MAGP-1 to function. We sought to closely base a refolding
to rF23 comprising these nine domains plus one additional C-terminal domain. Fragments rF31 and rF38 did not bind T7-MAGP. These two polypeptides span the length of rF23, except for the unique N-terminal domain and first EGF-like domain of fibrillin-1. The lack of binding to rF31 and rF38 suggests that T7-MAGP bound either within the first N-terminal two domains of fibrillin-1, and/or that the first two calcium-binding EGF-like (cbEGF) domains were involved in the interaction. Although the data presented do not eliminate the possibility that the unique N-terminal domain could be involved in binding MAGP-1, the calcium-dependent nature of the interaction favors a model for the involvement of the cbEGF domains 1 and 2. Calcium binding to T7-MAGP is unlikely since the secondary structure of T7-MAGP was not influenced by the presence of calcium (data not shown). The binding of MAGP-1 to the N-terminal region of fibrillin-1 is consistent with the (i) proposed arrangement of fibrillin molecules in which the N termini are located near the beaded structures of the beaded filaments (7) and (ii) localization of MAGP-1 to the beads (9). Similar binding properties to the N-terminal region of fibrillin-1 were observed for fibulin-2, which displayed calcium-dependent binding to fibrillin-1 fragments rF11 (similar to rF16) and rF23, but not to rF20 (16). The authors concluded that fibulin-2 bound to the first two cbEGF modules of fibrillin-1. These results raise the question of whether the binding sites for MAGP-1 and fibulin-2 are similar or separate epitopes and whether these two proteins compete for binding to fibrillin-1, which may represent important mechanisms in the development and/or homeostasis of microfibrils.

During the development of microfibrils and elastic fibers, fibrillins, MAGP-1 and elastin probably interact in complex multivalent patterns. Since microfibrils appear before the amorphous core of elastin, in initial steps it may be that the interaction of MAGP-1 with the N-terminal region of fibrillin-1 is required for the assembly process of microfibrils. Interactions of newly synthesized tropoelastin with N-terminal regions of fibrillin-1 or fibrillin-2 in microfibrils may then be necessary to align tropoelastin for cross-linking (17). Binding of MAGP-1 to tropoelastin may serve to stabilize interactions between microfibrils and the elastin core at more mature stages.

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