Characterization of an Associated Microfibril Protein through Recombinant DNA Techniques*

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The complete primary structure of a new extracellular protein associated with elastic fiber microfibrils was determined by recombinant DNA techniques. Antiserum to insoluble bovine ocular zonule protein was used to screen a λgt11 cDNA expression library constructed from whole chick embryo poly(A)* RNA. The cDNAs encoding immunoreactive fusion polypeptides were then used to rescreen the library by plaque hybridization. Nucleotide sequencing of overlapping cDNAs revealed an open translation reading frame of 1326 bases beginning at an initiation start sequence and ending at a stop codon. The contiguous cDNA sequence contains a 3'-untranslated region of 583 bases with a possible polyadenylation site 16 bases upstream from the poly(A) tail. Primer extension of chick aortic mRNA taken together with the sequence data, reveals a 5'-untranslated region of 95 bases extending upstream from the translation start site. Northern blot analyses indicated that the isolated cDNA hybridized with a 2.1-kilobase mRNA in preparations of whole chick embryo and chick embryonic aortic, heart, and muscle RNAs. The initial translation protein encoded by the cDNA is 53,932 kDa and possesses a hydrophilic amino acid composition with glutamic acid comprising 22% of the total amino acid residues. Antiserum was elicited to a synthetic peptide sequence (14 amino acids) encoded within the deduced protein primary structure. Western blots of extracted proteins from chick embryonic aortae cultured in the presence of 8-aminopropionitrile showed that the medullary and a mild salt extract contained an immunoreactive protein possessing an apparent molecular mass of 58,000 whereas harsh denaturants extracted a 32,000-kDa protein. Pulse-chase experiments using radiolabeled lysine showed that the newly synthesized 58,000-kDa protein was chased into a 32,000-kDa protein within a 2-24-h period. Immunoelectron microscopy of tissue sections from chick aortae, bovine nuchal ligament, and human ocular zonules showed that the peptide-elicited antibody localized specifically to ultrastructurally definable microfibril structures.

Mature elastic fibers, as defined by ultrastructural analysis, are composed of an amorphous core of elastin surrounded by a network of 8-12-nm tubular microfibrils (Low, 1961; Fahrenbach et al., 1966). Elastin is thought to be the component of the elastic fiber that imparts "elasticity" to a particular tissue (Partridge, 1962). The function of elastic fiber microfibril is postulated to be one of a template or scaffold onto which tropoelastin molecules are deposited and subsequently cross-linked (Ross, 1973; Cleary and Gibson, 1983).

While studies of elastin synthesis, gene structure, and expression have proceeded rapidly over the last decade, investigations into the structure, composition, and synthesis of elastic fiber microfibril protein(s) have progressed slowly. However, several recent reports have shed considerable light into the primary structure of two proteins that have been immunologically and ultrastructurally associated with extracellular microfibrils. Gibson et al. (1991) have determined the complete sequence of MAGP through cDNA analysis. The deduced amino acid sequence of MAGP reveals a protein of 21 kDa that possesses two unique domains of amino acid distribution. There is the amino-terminal region which contains high levels of glutamic, proline, and acidic amino acid residues and the carboxyl-terminal region which contains all of the cysteine residues and a preponderance of basic amino acid residues. These investigators also found that MAGP does not contain any consensus N-glycosylation sites and observed that the predicted molecular weight deduced from the nucleotide sequence was significantly lower than that determined by SDS-PAGE analysis of the nascent polypeptide chain. In addition to characterization of MAGP, two laboratory groups (Lee et al., 1991; Sakai et al., 1991) have detailed the partial primary structure of fibrillin, a large glycoprotein (350 kDa) found to be an integral component of microfibrils. The combined sequence data reveal some rather interesting features of the molecule in that it possesses regions of similar similarity to repeating sequences found in epidermal growth factor and TGF-β1-binding protein. Also, the carboxyl-terminal of fibrillin is enriched in basic amino acids with little potential for disulfide bond formation.

Although these latter reports provide significant data relative to the primary structure of proteins known to be associated with microfibrils, the specific role of these proteins in the formation of microfibrillar structures and association with elastin in elastic fiber microfibrils is still unknown. If, in fact, they represent the only proteins involved in elastic fiber formation is unclear. Currently, there are several other pro-

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‡ The abbreviations used are: MAGP, microfibril-associated glycoprotein; AMP, associated microfibril protein; DTT, dithiothreitol; kb, kilobase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
teins, in addition to fibrillin and MAGP, that have been reported to be major antigens within elastic fiber microfibrils. These include the enzyme lysyl oxidase (Serafini-Fraccassini et al., 1981), a 115-kDa glycoprotein (Bresson et al., 1983; Colombatti et al., 1985, 1987, 1988), and a 26-kDa microfibril-associated glycoprotein reported by Kobayashi et al. (1989). Although the association of the cross-linking enzyme lysyl oxidase with elastic fibers could be considered a necessary prerequisite to cross-linking of tropoelastin molecules, the unique function of the remainder of putative elastic fiber proteins has not been established. A number of other molecular weight proteins (polypeptides) have been extracted from elastic fiber-containing tissues (Gibson et al., 1989, 1991), but their possible relationship to other published microfibril proteins has not been established.

Determining the function of microfibril proteins in elastogenesis is complicated by the fact that ultrastructurally definable microfibrils exist in tissues extracellularly devoid of amorphous elastin. A number of different animal tissues have been shown to possess microfibrils containing common antigenic determinants and possessing structures similar to those associated with elastic microfibrils without containing any measurable elastic (Gibson and Cleary, 1983; Colombatti et al., 1985, 1987; Sakai et al., 1986; Streten et al., 1981, 1983; Streten and Gibson, 1988). These observations have raised the possibility that there is a major microfibril protein or a family of related microfibril proteins that are ubiquitous in connective tissues. Whether elastic fibers, i.e., those possessing elastin, possess an additional, unique microfibril component to direct the deposition of tropoelastin molecules is not known.

The objective of this study was to identify and characterize a protein intimately associated with elastic fiber microfibrils. Our approach was to by-pass the reported complexity and low yields found at the protein level and to focus our initial efforts on four bases upstream from the putative ATG start codon found in clone MF211A, was synthesized by the ε-cyanoethyl phosphoramidite method on a Du Pont Coder 300 (Pollock et al., 1980). The oligonucleotide was cleaved from the resin with NH$_4$OH and then purified by passage over a NEN-sorb column (Du Pont) followed by polyacrylamide gel electrophoresis and Sephadex G-25 chromatography (Pollock et al., 1990). The purified oligonucleotide (20 pmol) was 5' end-labeled with [γ-32P]ATP using 30 μCi of [γ-32P]ATP and 2 units of T4 polynucleotide kinase. The nucleotide kinase, in 20 μl of H$_2$O, was mixed with 20 μg of total RNA isolated from 11-day chick aorta. The mixture was heated at 80 °C for 5 min and annealed at 42 °C for 2 h. After this time, the solution was made 50 mM Tris HCl, pH 8.3, 75 mM KCl, 10 mM MgCl$_2$, 0.5 mM spermidine, 10 mM DTT, 1 mM of each dNTP, and 20 units of RNasin (Promega Biotech) and 30 units of avian myeloblastosis virus reverse transcriptase (Promega Biotech) were added to a final volume of 50 μl. The reaction mixture was incubated at 42 °C for 1 h after which time 10 μg of RNase was added and incubation continued for an additional 15 min at 24 °C. The reaction mixture was extracted with an equal volume of phenol/chloroform/isoamyl alcohol to precipitate hybrid (DNA/RNA) precipitated with 0.3 volumes of 9 M ammonium acetate and 2 volumes of ethanol for 18 h at -20 °C. After washing with 70% ethanol and drying in vacuo, samples were dissolved in 95% formamide, 20 mM EDTA, 0.05% phenol/chloroform/isoamyl alcohol, and heated at 90 °C for 3 min and analyzed on a 7 M urea, 8% polyacrylamide gel together with a set of single-stranded RNA standards (Bethesda Research Laboratories) ranging in size from 45 to 250 bases. The resultant gel was exposed to Kodak X-ray film using an intensifying screen (~70 °C). The size of the primer extension product was determined by size regression on a gel at a confidence level of 95% (Statview 512+, Brainpower Inc.).

**Northern Blot Analysis**—Total RNA was isolated from various chick embryonic tissues by the guanidinium isothiocyanate method (Foster et al., 1980). Poly (A)$^+$ RNA was isolated by affinity chromatography on an oligo(dT)-cellulose column (Maniatis et al., 1982). Poly(A)$^+$ RNA was fractionated on a Du Pont Coupler 2100 using t-butyloxycarbonyl chemistry on a single-stranded RNA standards (Bethesda Research Laboratories) ranging in size from 45 to 250 bases. The resultant gel was exposed to Kodak X-ray film using an intensifying screen (~70 °C). The size of the primer extension product was determined by size regression on a gel at a confidence level of 95% (Statview 512+, Brainpower Inc.).

**Antiserum Preparation**—Two separate antiserum preparations were used in our studies. The antiserum used for the initial screening of the cDNA expression library (CL4-22) was prepared against bovine ocular zonule protein that remained insoluble after consecutive extractions of acetone-dried ocular zonule with low salt, guanidine, and 0.05% xylene cyanol and heated at 90 °C for 5 min and analyzed on a 7 M urea, 8% polyacrylamide gel together with a set of single-stranded RNA standards (Bethesda Research Laboratories) ranging in size from 45 to 250 bases. The resultant gel was exposed to Kodak X-ray film using an intensifying screen (~70 °C). The size of the primer extension product was determined by size regression on a gel at a confidence level of 95% (Statview 512+, Brainpower Inc.).

**Sequence Analysis**—cDNA inserts directly or after digestion with PstI or PvuII were subcloned into either M13 mp18 (New England Biolabs) or λ sgscript SK+ (Stratagene) as we have previously reported (Foster et al., 1988; Pollock et al., 1990). Single- and double-stranded sequencing was performed by the dideoxy nu-
and then homogenized in neutral salt buffer (0.02 M phosphate, 0.5 M NaCl, 0.002 M EDTA, 0.005 M N-ethylmaleimide) with a Brinkman Polytron at high speed for 30 s. Diisopropylfluorophosphate was added to 1 \( \mu M \) and the homogenate extracted at 4 °C for 16 h with shaking. Following centrifugation (15,000 \( \times g \), 4 °C, 30 min), the supernatant was collected and stored at 4 °C, and the pellet was extracted with 6 M urea, 50 mM DTT at 4 °C for 16 h with shaking. The extraction mixture was centrifuged as described above and the supernatant collected, made 1 \( \mu M \) in diisopropylfluorophosphate, and stored at 4 °C.

*Organ Culture of Chick Embryonic Aortae—*Aortae were excised from 10-day chick embryos under sterile conditions and placed in Dulbecco’s modified Eagle’s medium (1.0 ml/0.1 mg tissue) containing 50 \( \mu g/ml \) β-aminopropionitrile, 100 units/ml penicillin, and 100 \( \mu g/ml \) streptomycin and incubated at 37 °C in a 5% CO₂ environment for 4 h. The medium was then removed and made 1 \( \mu M \) in diisopropylfluorophosphate, and the tissue was homogenized in neutral salt buffer (see above) with a Brinkman Polytron at high speed for 30 s. Diisopropylfluorophosphate was added to 1 \( \mu M \) and the homogenate extracted at 4 °C for 16 h with shaking. Following centrifugation (15,000 \( \times g \), 4 °C, 30 min), the supernatant was collected and stored at 4 °C and the pellet was extracted with 6 M urea, 50 mM DTT at 4 °C for 16 h with shaking.

For pulse-chase experiments, the aortae were incubated in medium for 4 h. The medium was then removed and made 1 \( \mu M \) in diisopropylfluorophosphate, and stored at 4 °C. For pulse-chase experiments, the aortae were incubated in medium (detailed above) containing 20 \( \mu g/ml \) of L-[4,5-\( ^3 \)H]lysine (87.5 Ci/mmol, Du Pont-New England Nuclear) for 2 h, the medium removed and replaced with fresh medium containing no isotope for 24 h, and then the aortae were rinsed and extracted directly with 8 M urea, 50 mM DTT at 4 °C for 16 h with shaking as described above.

**Western Blot Analysis—**Aliquots (40–60 µl) from urea/DTT extracts of bovine nuchal ligament, chick aorta, liver, and brain tissues, medium, neutral salt buffer, and urea/DTT extracts of the chick aortae organ cultures were analyzed by 10% SDS-PAGE. Proteins possessing similar solubility properties and molecular weight to a protein identified in bovine ocular zonules (Streeten and Gibson, 1988). Other immunoreactive proteins are also visible in the area of 55–60 and 20–25 kDa. The major immunoreactive protein seen in the chick aortic extract possesses an apparent molecular weight of 32,000–33,000.

Since antisera CL4–22 reacted with a chick aortic protein possessing similar solubility properties and molecular weight to a protein identified in bovine ocular zonule and nuchal ligament, we proceeded to use it to screen a chick embryo λgt11 cDNA expression library. Although not shown, we have also determined that antisera CL4–22 localizes to the microfibrillar structures of chick aortic elastic fibers (Horrigan, 1990).

Two plaques containing immunoreactive fusion polypeptides were detected among the 5 \( \times 10^9 \) plaques screened with preadsorbed CL4–22. These positive plaques were designated MF814 and MF211. Western blot analysis of MF814 and MF211 lysogens, using both antisera CL4–22 and β-galactosidase monoclonal antibody, confirmed the presence of immunoreactive fusion polypeptides. The fusion protein synthesized by MF814 was used to epitope select antibodies from the original bovine ocular zonule antisera (CL4–22). Immunoelectron microscopy revealed that epitope-selected antibodies bind to elastin-associated microfibrils in chick aortic sections whereas antibodies selected by wild type λgt11 did not show any reaction (Horrigan, 1990).

The enzyme restriction maps of MF814 and MF211 cDNAs revealed overlapping fragments (see Fig. 2). MF211, the largest of the cDNAs which extends further than MF814 in both 5' and 3' directions, was used to rescreen the library by plaque hybridization. Twelve positive plaques were found, only one of which (MF211A) contained sequences extending 5' to the cDNA sequences found in MF211. MF814 and MF211 possessed cDNA inserts of 805 and 1790 nucleotides, respectively. MF211A contained a cDNA insert of 645 nucleotides including 176 base pairs not found in MF211. The restriction maps and nucleotide sequencing strategy for cDNA characterizations are given in Fig. 2.

The nucleotide and deduced amino acid sequences of the

![Figure 1. Western blot analysis with zonal antisera. 6 M urea, 50 mM DTT extracts of bovine nuchal ligament and 10-day chick aorta were run on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with CL4-22 antisera. Lane 1, bovine nuchal ligament extract; lane 2, chick aorta extract.](image-url)
protein encoded by the overlapping cDNAs are provided in Fig. 3. The length of the contiguous cDNA sequence is 1928 nucleotides. A single, long open reading frame of 1326 bases initiates at an ATG, 40 nucleotides from the 5' end, and terminates at TAG. Although no termination codon precedes the putative initiation ATG codon, the nucleotide sequence surrounding the codon, i.e. GCCGCCACCATGT, agrees with the consensus sequence for initiation start sites with the exception of the 3' nucleotide (Kozak, 1987). The composite cDNA sequence contains a 3'-untranslated region of 563 nucleotides and a possible polyadenylation signal, ATTAAA, 16 nucleotides upstream from the poly(A) tail. This putative polyadenylation signal is not the conserved sequence found in most eucaryotic mRNAs, i.e. AATAAA, but is identical to the polyadenylation signal found in chick ovalbumin mRNA (Jung et al., 1980).

Identification of mRNA—In order to establish the size of the encoding mRNA and the length of the 5'-untranslated region, both Northern blot and primer extension analyses were performed. Fig. 4 provides a Northern blot analysis of poly(A)+ selected RNA isolated from 10-day whole chick embryo RNA (lane A), 10-day chick aortic RNA (lane B), and 14-day chick aortic RNA (lane C) using the cDNA insert of MF211. All RNA samples reveal hybridization to a single mRNA species of approximately 2.1 kb.

In order to obtain some insight into the expression of the 2.1-kb mRNA in other tissues of the chick embryo especially as it may correlate with tropoelastin mRNA levels, a survey of various issues was performed using both tropoelastin and MF211 cDNAs. Fig. 5 presents a quantitative analyses of the relative abundance of both transcripts in a selection of chick tissues. Aortic and heart tissues possess significant amounts of both tropoelastin and 2.1-kb transcripts, skeletal muscle, and liver possess low levels whereas brain tissue mRNA did not hybridize with either cDNA at this exposure time of the film (24 h).

Fig. 6 shows the results of primer extension of chick aortic RNA (11 day) using a synthetic oligodeoxynucleotide primer complementary to a sequence starting four nucleotides upstream from the putative ATG start codon. Complete addition of reactants resulted in a single extension product 91 bases in length (lane A). Control reactions included the omission of reverse transcriptase (lane B) and omission of chick RNA (lane C). We have repeated these experiments using unlabeled primer and radiolabeled nucleotide and found identical results (data not shown). These data illustrate that the 5'-untranslated region of the transcript is 95 bases in length and when taken together with the composite cDNA sequence, predicts a mRNA size of 1979 bases excluding the length of the poly(A) tail. The measured mRNA size, i.e. 2.1 kb (see Fig. 4) is approximately 120 bases longer than the predicted length determined by nucleotide sequencing and primer extension. Assuming that the mRNA possesses a poly(A) tail of normal length, i.e. 100-200 nucleotides, the experimentally determined size of the mRNA by Northern blot analysis is in close agreement with data generated from nucleotide sequencing and primer extension. Also, the single primer extension product seen in Fig. 6 and verified by an alternate radiolabeling technique, signifies that a single transcription start site is operational for generation of transcript in the 11-day embryonic chick aorta.

Protein Sequence Encoded—The open reading frame of the composite cDNA encodes a protein of 442 amino acids, giving a calculated molecular weight of 53,932 (see Fig. 3). A search of the GENEMBL data banks using six different potential reading frame configurations, i.e. both DNA strands with frameshifts for both strands, did not reveal any significant similarity (>27%) to any reported, encoded protein primary structures. The deduced amino acid composition is composed of 46% acidic and basic amino acid residues, with a particularly high amount of glutamic acid (22%). This high amount of negative charge from glutamic acid residues is balanced by an equally high percentage of positive charge originating from arginine (12.6%) and lysine (10.1%) residues. A hydropathy plot (Kyte and Doolittle, 1982) of the primary structure is provided in Fig. 7A. Most secreted proteins contain a signal peptide in their NH2-terminal sequence for vectorial transport across the rough endoplasmic membrane. Even though the NH2-terminal region of the encoded 54-kDa protein exhibits positive hydropathy values, the actual amino acid sequence does not contain a classically defined signal peptide sequence since it lacks a grouping of hydrophobic amino acids (von Heijne, 1985). On the other hand, the peptide sequence within the NH2-terminal region is similar to sequences shown to act as functional signal sequences (Kaiser et al., 1987).

The linear distribution of acidic and basic amino acid residues is provided in Fig. 7B. The positive and negative charge distribution along the length of the protein is not uniform but is segregated into specific regions to create a polarized molecule. It is interesting to note that the COOH-terminal region possesses a concentration of basic amino acids since this segregation of charge is also found in fibrillin (Lee et al., 1991; Sukai et al., 1991) and MAGP (Gibson et al., 1991). The deduced amino acid sequence does not contain any N-linked glycosylation sites as defined by Asn-X-(Ser/Thr) sequences. This does not exclude the possibility that the
FIG. 3. Nucleotide and deduced amino acid sequence of the overlapping cDNAs. The complete nucleotide sequence and deduced amino acid sequence of the contiguous cDNA sequences are provided. Numbers to the left refer to the nucleotide sequence. Numbers to the right refer to the amino acid sequence. A synthetic peptide was made corresponding to the underlined amino acid sequences.

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protein is glycosylated, but does indicate that any carbohydrate addition would be O-linked.

The deduced amino acid sequence of the 54-kDa protein differs from those reported for MAGP (Gibson et al., 1991), fibrillin (Lee et al., 1991; Sakai et al., 1991), and the enzyme lysyl oxidase (Trackman et al., 1990). This finding is not surprising since all of these associated microfibril proteins are encoded by mRNAs that differ significantly in size from one another and with the 2.1-kb transcript that we have identified.

**Immunoreactivity of Antiserum Directed against a Deduced Peptide Sequence**—In order to verify that the protein encoded by the characterized cDNA is a component of extracellular microfibrils, a synthetic peptide (described under "Experimental Procedures") corresponding to an antigenic region of the deduced protein was used to elicit antiserum. The peptide sequence synthesized was used to search the GENEMBL data banks independently of the total protein sequence and no significant similarity (<21%) was found to any reported sequences. The resultant peptide antiserum was used to define the molecular size of immunoreactive protein in several chick tissues and chick aortae organ culture extracts and to localize the immunoreactive protein in the extracellular matrices of several tissues known to contain microfibrils. Fig. 8 provides a Western blot analysis of tissue extracts obtained from 10-day chick embryo brain (lane A) and liver (lane B) and aortae (lane C) using denaturing conditions (6 M urea, 50 mM DTT). The only tissue that contains detectable amounts of an immunoreactive 32-kDa protein is the aorta (lane C). This finding is particularly important since it demonstrates that the antiserum elicited to a synthetic peptide encoded within the 54-kDa protein reacts with the same size antigen (32 kDa) as that of bovine ocular zonules antiserum (CL4-22) as shown in Fig. 1.

Since the 32-kDa protein was identified only after harsh extraction of tissues, aortic organ cultures were used in an attempt to identify newly synthesized, putatively "less insoluble" immunoreactive proteins. Fig. 9 provides a Western blot analysis of immunoreactive proteins found in the medium.
Fig. 4. Northern blot analysis. Two-μg aliquots of poly(A+) RNA were separated on a formaldehyde-agarose gel, transferred to Nytran, and hybridized with radiolabeled cDNA insert from MF211. Lane A, 10-day whole chick embryo RNA; lane B, 10-day chick aortic RNA; lane C, 14-day chick aortic RNA.

Fig. 5. Quantitation of tropoelastin and 2.1-kb mRNAs in chick embryonic tissues. Aortic, brain, heart, liver, and skeletal muscle tissues were removed from 10-day embryos, RNA isolated, and poly(A)+ RNA selected as detailed in the text. Northern blot analysis using 2 μg of poly(A)+ was performed using 32P-labeled tropoelastin and MF211 cDNAs. Following autoradiography of the blots, the amounts of tropoelastin and MF211 mRNAs were quantified by laser densitometry. Optical density units are the integrated densities/2 μg of RNA. Values represent the average from triplicate analyses ± standard deviation. * designates that mRNA was not detectable.

(lane A), a neutral salt buffer extract (lane B), and a 6 M urea/DTT extract (lane C) of chick embryonic aortae cultured in the presence of an inhibitor of lysyl oxidase, i.e. β-aminopropionitrile, for 24 h. The medium (lane A) and neutral salt buffer extract (lane B) contain a major immunoreactive protein of 58 kDa whereas the urea extract (lane C) contains one major immunoreactive protein of 32 kDa. In order to determine if the 32-kDa protein was a degradation product of the 58-kDa protein, a pulse-chase experiment was performed. Aortae were incubated with [3H]lysine for 2 h, the medium replaced with fresh medium, and incubation continued for 24 h. Aortae were extracted directly with 6 M urea/DTT at 2 and 24 h, aliquots analyzed by Western blots, and the amount of radioactivity determined in the 58- and 32-kDa immunoreactive bands (Fig. 10). At 2 h, only the 58-kDa protein was

Fig. 6. Primer extension analysis 54-kDa mRNA. A radiolabeled oligodeoxynucleotide (see text for details) was annealed to 20 μg of RNA isolated from 11-day chick embryonic aortae. The primer was extended with reverse transcriptase and the products separated on an 8% denaturing polyacrylamide gel (lane A). Controls were performed without the addition of reverse transcriptase (lane B) and without the addition of RNA (lane C). The size of the primer extension product was determined by simple regression analysis using the mobility versus the log of the RNA sizes at a confidence level of 95% (Statview 512+, Brainpower Inc.). Correlation between mobility and size was 0.998.
Associated Microfibril Protein

FIG. 8. Western blot analysis with antibody made to synthetic peptide. 6 M urea, 50 mM DTT extracts of 10-day chick tissues were run on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with affinity purified anti-peptide antibody (see Fig. 3 for peptide sequence). Lane A, brain; lane B, liver; and lane C, aorta.

FIG. 9. Western blot analysis of chick embryonic aortic organ cultures. Medium and extracts of 10-day embryonic chick aortae were separated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with affinity purified anti-peptide antibody. Lane A, organ culture medium; lane B, neutral salt buffer extract; lane C, 6 M urea with 50 mM DTT extract; molecular weights of standard proteins are indicated. The apparent molecular weights of the immunoreactive proteins were determined by simple regression analysis using the mobility versus the log of the standard molecular weights at a confidence level of 95% (Statview 512+, Brainpower Inc.).

Radiolabeled. After a chase period of 24 h, 62% of the total radioactivity initially present in the 58-kDa protein was found in the 32-kDa protein while 9% remained in the 58-kDa protein. It should be noted that the antiserum used for identification of immunoreactive proteins was elicited to a peptide sequence located within amino acid residues 268–281 (see Fig. 3). Because the 32-kDa protein possesses this peptide it must represent the COOH-terminal half of the protein which contains a clustering of lysine residues. This concentration of lysine residues within the COOH terminus would explain why more than half of the radioactivity was recovered in the 32-kDa protein. These results demonstrate that the 58-kDa protein initially extracted from the aortic organ culture can be chased into a 32-kDa polypeptide strongly suggesting a precursor-product relationship.

The results obtained from these latter experiments reveal a discrepancy between the projected (Fig. 3) and determined (Fig. 9) molecular weight of the deduced protein sequence. Specifically, the cDNA sequence predicts a protein of 54 kDa and the determined molecular mass is 58,000. This difference between molecular weights determined by actual calculations from the primary structure and mobility on SDS gels is not uncommon and most likely reflects anomalies inherent in SDS-PAGE. A pertinent example of this discrepancy in molecular weights as determined by SDS-PAGE is the recent publication of Gibson et al. (1991) where they reported an 11 kDa difference between the deduced molecular mass of MAGP (20 kDa) and the molecular mass of the nascent chain determined by SDS-PAGE (31 kDa).

Fig. 11 provides immunoelectron micrographs showing the localization of peptide antiserum in 10-day embryonic chick aortae (Fig. 11, A and B), 1-day calf nuchal ligament (C), and 46-year human zonular ligament (D). In all of the tissues examined, the elicited peptide antibody localizes specifically to microfibrillar structures thereby confirming that the encoded peptide sequence is present as an antigen in ultrastructurally definable microfibrils.

DISCUSSION

We have determined the complete primary sequence of a unique protein associated with connective tissue microfibrils that we are referring to as “associated microfibril protein,” (AMP). The deduced primary sequence does not conform to any of the reported microfibril proteins in either amino acid sequence, composition or molecular size. The MF211 cDNA
Associated Microfibril Protein

FIG. 11. Immunolocalization of antibody elicited to a synthetic peptide. Immunolocalization of antibody elicited to a synthetic peptide. Ultrathin sections were pretreated with 6 M urea with 50 mM DTT. A, 10-day embryonic chick aorta. Two elastic fibers (EL) show gold-labeling of their surrounding microfibrils (×58,750). B, 10-day embryonic chick aorta. Bundles of elastic fiber microfibrils exhibit gold-labeling (×58,750). C, 1-day calf nuchal ligament. Microfibrillar bundles within a large immature elastic fiber, labeled with gold particles (×58,750). D, 46-year old human ocular zonule. Microfibril (ZN) inserting on the anterior lens capsule (CP) show diffuse gold-labeling. Capsule is unlabeled. (×58,700)

used to detect the AMP mRNA in a number of chick tissues hybridizes with only a 2.1-kb transcript further confirming the uniqueness of protein and nucleotide sequences reported. The association of AMP protein with extracellular microfibrils is established by immunoelectron microscopy.

Historically, the extreme insolubility of elastic fiber microfibrillar protein(s) and lack of sufficient soluble material for biochemical analyses have been attributed largely to the formation of disulfide cross-links. Although microfibril-associated proteins have been reported to be glycosylated, the hydrophilic character of carbohydrate units would not explain the chemical basis for extreme insolubility. Since tropoelastin contains only 2 cysteine residues in the COOH-terminal portion of the molecule, it is doubtful that tropoelastin molecules and elastic fiber-associated microfibril proteins are linked extensively via disulfide bond formation. Alternatively, the high content of cysteine residues reported within some microfibril-associated proteins could signify an intramolecular self-assembly structure dependent upon cystine formation. It is obvious from the primary sequence of AMP that the formation of disulfide cross-links cannot play a major role in determining insolubility, especially since the COOH-terminal region of the molecule lacks any cysteine residues and necessitates very harsh conditions for extraction. Therefore, in our experimental design to identify a soluble precursor protein from chick aorta we chose to use an in vitro system wherein we could examine an initially synthesized protein rather than examining a mature, perhaps processed, extracellular matrix component. Previously, we had attempted to extract frozen chick aortae with a spectrum of buffers and were able to detect only a 32-kDa protein. The organ culture system did allow us to identify an immunoreactive protein that possessed an apparent molecular weight close to that predicted by nucleotide sequence analysis.

We hypothesize that the 58-kDa AMP (using the apparent molecular determined by SDS-PAGE) is post-translationally cleaved to a 32-kDa fragment that becomes an integral component of extracellular elastic fibers. Our hypothesis is based on pulse-chase experiments in which we were able to establish a precursor-product relationship between newly synthesized 58- and 32-kDa proteins in organ cultures. Our results do not exclude the possibility that the remaining segment of the encoded protein, i.e. a predicted 27-kDa species from the NH₂-terminal region of the molecule, is also incorporated into the insolubilized matrix.

The fact that we have successfully used an inhibitor of lysyl oxidase in these studies to illustrate a possible precursor-product relationship in microfibril protein assembly suggests two possible mechanisms that account for microfibril insolubility. One explanation is that the microfibril proteins existing within elastic fibers may be rendered insoluble because they are trapped within a highly cross-linked elastin network. However, this hypothesis does not explain the apparent insolubility of microfibril-associated protein in tissues lacking elastin. A second explanation is that one or more of the microfibrillar proteins are substrates for lysyl oxidase and
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