

Chick Myotendinous Antigen. II. A Novel Extracellular Glycoprotein Complex Consisting of Large Disulfide-linked Subunits

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ABSTRACT This report describes the biochemical characterization of a novel extracellular matrix component, "myotendinous antigen," which appears early in chick limb morphogenesis at sites connecting developing muscle fibers, tendons, and bone (Chiquet, M., and D. Fambrough, 1984; *J. Cell Biol.*, 98:1926–1936). This extracellular matrix antigen is a major component of the secretory proteins released into the medium by fibroblast and muscle cultures; the soluble form is characterized here. This form of myotendinous antigen is a large glycoprotein complex consisting of several disulfide linked subunits (M_r ~150,000–240,000). The differently sized antigen subunits are related, since they yielded very similar proteolytic cleavage patterns. M1 antibody can bind to the denatured subunits. The antigen subunits, as well as a M_r ~80,000 pepsin-resistant antigenic domain derived from them, are resistant to bacterial collagenase. Despite possessing subunits similar in size to fibronectin, myotendinous antigen appears to be both structurally and antigenically unrelated to fibronectin or to other known extracellular matrix components. About seven times more M1 antigen per cell nucleus was released into the medium in fibroblast as compared to muscle cultures. In muscle conditioned medium, myotendinous antigen is noncovalently complexed to very high molecular weight material that could be heavily labeled by [^3H]glucosamine and [^{35}S]sulfate. This material is sensitive to chondroitinase ABC and hence appears to contain sulfated glycosaminoglycans. We speculate that myotendinous antigen might interact with proteoglycans on the surface of muscle fibers, thereby acting as a link to tendons.

Monoclonal antibody M1 recognizes an epitope on an extracellular matrix component that seems to be enriched in tendons, myotendinous junctions, and in the endomysium at the tips of skeletal muscle fibers (11). This "myotendinous antigen" is, by virtue of its distribution, a candidate for linking muscle fibers to tendon fascicles and bones, both in the developing and the adult locomotive system of vertebrates. A first step towards assigning a function to the antigen recognized by M1 antibody is its biochemical characterization, which is the subject of this paper. We were able to isolate the antigen, by immunoprecipitation or immunoadsorption, in a soluble form from conditioned media of cells metabolically labeled with radioactive amino acids or sugars. The isolated antigen was compared to other known extracellular matrix components.

Since a preparation of type V collagen (44) had been used

as an immunogen for generating hybridoma cell lines leading to the isolation of M1 antibody (11), we initially determined whether the antigen was, in fact, type V collagen or another collagenous protein (for reviews, see references 4, 5, 15). The subunits of the antigen precipitated by M1 antibody turned out to migrate on SDS acrylamide gels at similar rates as monomers of fibronectin, a major extracellular matrix protein (24). Although M1 antibody and antifibronectin antibodies yield different tissue staining patterns in immunofluorescence experiments (11), we examined the biochemical properties of the antigen and fibronectin by comparing their subunit structure, susceptibility to proteases, and affinity to denatured collagen (an important feature of fibronectin [24]). The results indicated that myotendinous antigen is a glycoprotein complex consisting of large disulfide-linked subunits (some of which might be sulfated) that appears to be noncollagenous,

and that differs from fibronectin and other characterized extracellular matrix components. The possibility will be discussed that myotendinous antigen might be a component of extracellular "microfibrils" that are predominant structures in myotendinous junctions.

We chose to study myotendinous antigen because of our interest in muscle-connective tissue cell interactions during morphogenesis (11). In this context, it was important to determine which cells synthesize the antigen. We report here that cultured chick fibroblasts release at least seven times more newly synthesized antigen into the medium per nucleus than myogenic cells. This is comparable to the differences found in fibronectin (18) and collagen (36) synthesis between these two cell types. The experiments also demonstrated that myotendinous antigen is a major extracellular matrix component released by certain cultured cells, and sometimes might have been confused with fibronectin due to the similarly sized subunits (see Discussion).

To be an integral part of an extracellular matrix, a component has to interact with other matrix molecules (see, for example, reference 24). We found that myotendinous antigen does not have a high affinity for fibronectin or collagen, but seems to bind to a large chondroitin sulfate proteoglycan synthesized by cultured muscle cells. On the basis of the data in this and the preceding paper (11), we propose that M1 antibody recognizes a major, apparently novel, extracellular matrix component that might be involved in the association of muscle fibers with tendon fibroblasts.

MATERIALS AND METHODS

Immunoreagents: Isolation of monoclonal antibody M1 has been described in detail in the preceding paper (11). Monoclonal antibody B3 which reacts with chick cellular and plasma fibronectin (18) was a kind gift of Dr. J. M. Gardner (Massachusetts Institute of Technology). A crude antiserum against chick type I collagen was generated by the following way. 14-d-old chick embryos were homogenized in 0.5 M acetic acid, pH 2.0, and digested with pepsin as described by von der Mark and von der Mark (44). NaCl was added to the cleared supernate to a final concentration of 0.7 M. The precipitate that formed was highly enriched for type I collagen as judged by SDS PAGE (not shown). After dialysis against 0.5 M acetic acid, the resulting collagen solution was neutralized before immunization of rabbits. The resulting antiserum precipitated polypeptides from medium of metabolically labeled cells which, after pepsin digestion, co-migrated with the α_1 (I) and α_2 (I) bands of a type I collagen standard on SDS PAGE gels (not shown). This antiserum might cross-react with other collagens (especially type III). However, its sole use was a positive control in experiments using collagenase or stimulating collagen synthesis (see below).

Goat anti-mouse Fab antiserum was prepared by Dr. P. Gearhart (Johns Hopkins Medical School); a 0–40% ammonium sulfate cut of it was used as a second antibody for immunoprecipitations (see below). Rabbit anti-mouse Fab (affinity-purified IgG) obtained from Dr. Gearhart was iodinated by the chloramine-T method (19) and used at 0.2 μ g/ml for immunoblotting. Goat anti-rabbit IgG antiserum was purchased from Cappel Laboratories (Cochranville, PA).

Metabolic Labeling of Cell Cultures: Cell suspensions from 11-d-old embryonic chick breast muscle and from skin were obtained as previously described (42). 5×10^5 myogenic cells and 2×10^5 skin fibroblasts, respectively, were plated onto gelatinized 60-mm tissue culture dishes (Falcon Labware, Oxnard, CA) and grown in Eagle's minimal essential medium (MEM [Gibco Laboratories, Grand Island, NY]) supplemented with 10% horse serum (Gibco Laboratories) and 2% embryo extract. In some experiments, myogenic cells were enriched by subculturing after treatment of 1-d-old myogenic cultures with cytochalasin B (43) or monoclonal antibody CSAT (32), which was a kind gift of Dr. A. F. Horwitz (University of Pennsylvania School of Medicine). Both these agents detach viable myogenic cells from the substrate, but have little effect on contaminating fibroblasts. In subcultures of detached myogenic cells, 85–90% of all nuclei were found in myotubes after 3 d, compared to 65–70% in a parallel standard culture. Cultures were labeled at 3 or 4 d with 2-ml culture medium containing one of the following radioactive precursors (Amer-

sham Corp., Arlington Heights, IL): [35 S]methionine (100 μ Ci/ml; using methionine-free MEM); [3 H]glycine and -proline (50 μ Ci/ml each); [3 H]glucosamine (100 μ Ci/ml); and [35 S]sulfate (500 μ Ci/ml, using sulfate-free MEM). In some experiments, L-ascorbic acid (100 μ g/ml; Baker Co., Inc., Sanford, ME) and β -aminopropionitrile (50 μ g/ml, Sigma Chemical Co., St. Louis, MO) were added to the medium from a freshly made up 100 \times stock solution at the time of diluting. Labeling times in different experiments are indicated in the figure legends. Collected conditioned media were normally used immediately, but could be stored at 4°C or –20°C for 2 wk without appreciable loss of M1 antigen and fibronectin.

Immunoprecipitations: In a typical experiment, 100- μ l samples of labeled conditioned media were diluted to 500 μ l with borate-buffered saline (BBS;¹ 50 mM sodium borate, 150 mM NaCl, pH 8.3) containing 2 mM phenylmethanesulfonyl fluoride, 5 mM *N*-ethylmaleimide, and 2 mM EDTA as protease inhibitors. Diluted samples were centrifuged (27,000 g, 15 min) and transferred to 1.5-ml Eppendorf tubes that had been coated with 0.1% bovine serum albumin in BBS. 10 μ g of monoclonal antibody were added, followed after 2 h by 40 μ l of goat anti-mouse Fab antiserum, respectively. After an incubation of 1 h at 20°C and overnight at 4°C, samples were underlayered with a 200- μ l cushion of 1 M sucrose, 2% Triton X-100 in BBS. Immunoprecipitates were centrifuged through the sucrose Triton cushion (10,000 g, 10 min) and the tubes washed as described by Caravatti et al. (6).

Enzyme Digestions: Conditioned media were digested with 0.2 mg/ml pepsin (Worthington Biochemicals, Freehold, NJ; 2950 U/mg) at pH 2.0 for 24 h as described by Sasse et al. (36). Pepsin was inactivated by neutralizing the medium sample to pH 8 and heating it for 2 min at 90°C. Immunoprecipitates were generally treated with enzymes by adding 30 μ l enzyme solution to a washed precipitate, incubating as indicated below, and stopping the reaction by the addition of 10 μ l 4 \times SDS sample buffer (27) and boiling for 5 min. Enzymes used were: bacterial collagenase (Advanced Biofactures, Lynbrook, NY; 2,400 U/mg), 60 U/ml in 50 mM Tris-HCl, 10 mM calcium acetate, pH 7.4, for 1 h at 37°C; α -chymotrypsin (Worthington Biochemicals; 68 U/mg), 5 μ g/ml in 20 mM Tris-HCl, pH 8.0, for 15 min at 20°C; and chondroitinase ABC (from *Proteus vulgaris*; Seikagaku Kogyo/Miles), 5 U/ml in 20 mM Tris-HCl, pH 8.0, for 4 h at 37°C. Controls were treated identically except that enzyme was omitted from the incubation buffer.

Isolation of Myotendinous Antigen and Cellular Fibronectin by Affinity Chromatography: Chick fibroblasts were grown for 3 d in MEM containing 8% horse serum and 6% chick serum, both of which had been depleted of fibronectin by affinity chromatography to gelatin-Sepharose (10). 950 ml conditioned medium were collected, and saturated ammonium sulfate was added to a final concentration of 40%. The precipitate was dissolved in 100 ml, 150 mM NaCl, 1 mM CaCl₂, 10 mM cyclohexylaminopropane sulfonic acid, pH 11.0 (CAPS-buffer, 46), and dialyzed against the same buffer. Gelatin (10 mg/g of gel [Difco Laboratories Inc., Detroit, MI]) and M1 antibody (2 mg/g of gel) were coupled to CNBr-activated Sepharose-4B (Pharmacia Fine Chemicals, Piscataway, NJ) as recommended by the manufacturer. The dialyzed ammonium sulfate precipitate of conditioned medium was neutralized with HCl and applied first to a column containing 2 ml gelatin-Sepharose, then to a column of 3 ml Sepharose coupled with M1 antibody. Both columns were washed extensively with BBS. The gelatin-Sepharose column was further washed with 0.8 M urea in BBS and bound material (i.e., fibronectin released by the fibroblasts) was eluted with 4 M urea in BBS. The M1 antibody-Sepharose column was washed with a mixed detergent buffer (0.3 M NaCl, 0.1% SDS, 0.05% Triton X-100, 10 mM Tris-HCl, pH 8.4) before elution of bound antigen with 0.5% deoxycholate, 0.1 M triethylamine, pH 11.0 (45).

SDS PAGE and Fluorography: To immunoprecipitates (which were suspended in about 20–30 μ l sucrose-containing buffer; see above), we added 25 μ l of 2 \times concentrated SDS sample buffer (27) with or without β -mercaptoethanol. Protein samples obtained from affinity columns were mixed with 4 \times concentrated SDS sample buffer. Both nonreduced and mercaptoethanol-reduced samples were heated at 100°C for 5 min and run on 5–15% acrylamide gradient slab gels with a stacking gel according to Laemmli (27). Routinely, the horse serum proteins fibronectin (M_r ~220,000), α_2 -macroglobulin (M_r ~170,000), transferrin (M_r ~77,000), IgG heavy chain (M_r ~50,000) and IgG light chains (M_r ~25,000) were used as molecular weight markers; these had been calibrated against a set of standard marker proteins (2). Molecular weight calibrations ($10^{-3} M_r$) are indicated at right of each figure; S marks the boundary between stacking and running gels. Gels were stained with Coomassie Brilliant Blue R (Sigma Chemical Co.) and/or impregnated with 2,5-diphenyl-oxazole (28), dried, and fluorographed on Kodak XAR film with preflashing. In some experiments, radioactive polypeptide bands were cut out from dried gels and incubated in 0.5 ml 1 N NaOH overnight at 60°C in a scintillation vial. The solution was neutralized with acetic acid, scintillation cocktail was added, and

¹ Abbreviations used in this paper: BBS, borate-buffered saline.

samples were counted in a Beckman LS7500 counter (Beckman Instruments, Inc., Palo Alto, CA).

Other Methods: Immunoblotting was performed as described by Towbin et al. (40). Briefly, proteins from unfixed SDS gels were electrophoretically transferred to nitrocellulose sheets (BA-85, Schleicher & Schuell, Inc., Keene, NH). Free protein binding sites on the nitrocellulose were blocked by incubation with Tris-buffer containing hemoglobin and bovine serum albumin, and the sheets were then incubated with a solution of monoclonal antibody (10 $\mu\text{g/ml}$) followed by ^{125}I -labeled rabbit anti-mouse Fab (0.2 $\mu\text{g/ml}$). The sheets were washed, dried, and exposed to x-ray film. Radiolabeled polypeptide bands were cut out from SDS PAGE gels and digested with V8 protease or α -chymotrypsin (Worthington Biochemicals) as described by Cleveland et al. (12).

Velocity sedimentation analysis of myotendinous antigen was performed by loading 200- μl samples of [^{35}S]methionine-labeled fibroblast conditioned medium on 5 ml sucrose gradients (5–20% sucrose in BBS, pH 8.4, containing 1% Triton X-100) which were centrifuged for 17 h at 32,000 rpm in a Ti 50.1 rotor, using a Beckman ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Fractions were processed for immunoprecipitation and SDS gel analysis as described in the legend to Fig. 7. Positions of the horse serum proteins, serum albumin (5S), IgG (7S), fibronectin (12S), and α_2 -macroglobulin (16S) within the gradients were determined by running consecutive fractions on SDS gels that were stained with Coomassie Blue.

RESULTS

Myotendinous Antigen Is an Extracellular Matrix Component Released into the Medium by Cultured Cells

It has been shown in the accompanying paper (see Fig. 7 in reference 11) that, upon incubation of living chick primary skeletal muscle cultures with monoclonal antibody M1, fuzzy material on the culture substrate and on cell surfaces was labeled. Extraction of cultures with Triton X-100 left behind a cell-free matrix containing the antigen as revealed by immunofluorescence staining (not shown). The “myotendinous antigen” recognized by M1 antibody (11) is thus accessible from the outside of cells and is not extracted by methods that solubilize cell membranes. In fact, we did not succeed in extracting appreciable amounts of the antigen from cell culture matrices or chick tissues in a form still recognized by the antibody. However, myotendinous antigen is also released by cultured cells into the medium in a soluble form that was analyzed in the experiments described in this paper.

When chick primary myogenic cultures were labeled with [^{35}S]methionine, [^3H]glycine/proline, or [^3H]glucosamine, a polypeptide with $M_r \sim 220,000$, and a doublet with $M_r \sim 200,000/190,000$ in SDS PAGE were consistently immunoprecipitated by M1 antibody from the conditioned medium (Fig. 1). In addition, minor diffuse bands of $M_r \sim 240,000$, 170,000, and 150,000 were often detected in variable yields (Fig. 1). Protease inhibitors included in the immunoprecipitation reaction did not diminish the apparent heterogeneity of myotendinous antigen; moreover, the pattern of precipitated polypeptides remained stable if the labeled media were stored in the cold for up to 2 wk (not shown). Myotendinous antigen isolated from fibroblast-conditioned medium showed a somewhat different polypeptide pattern, the $M_r \sim 220,000$ component being much more heavily labeled than all other bands (see below; Fig. 8). The different polypeptides precipitated by M1 antibody are closely related. When the $M_r \sim 220,000$ band, the $M_r \sim 200,000/190,000$ doublet, and the $M_r \sim 150,000\text{--}170,000$ region were cut out from radiolabeled gels and digested separately by V8 protease or chymotrypsin, they yielded very similar proteolytic cleavage patterns (Fig. 2). This argues against M1 antibody precipitating several different proteins that share an epitope or that are complexed

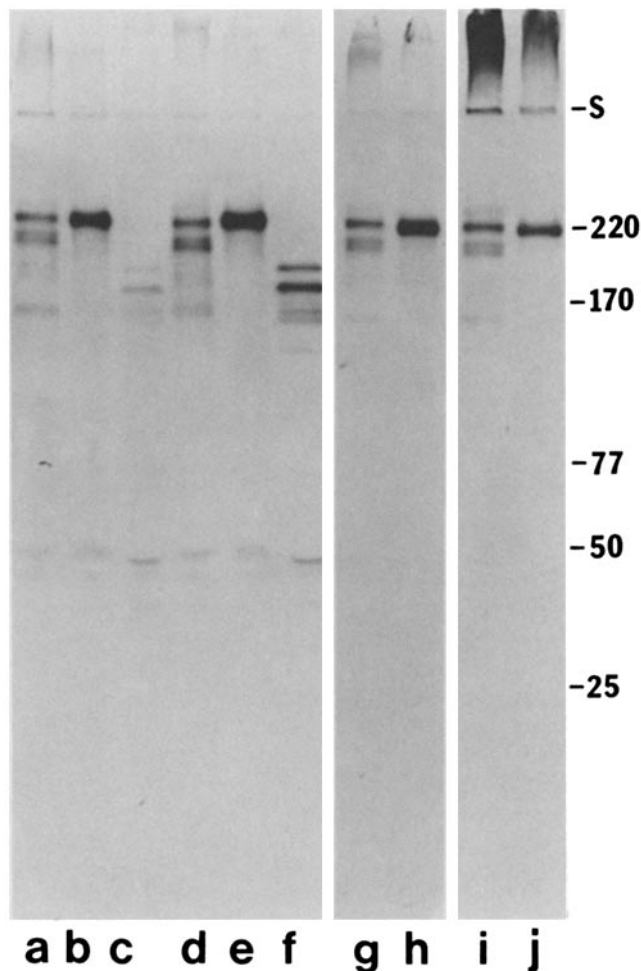
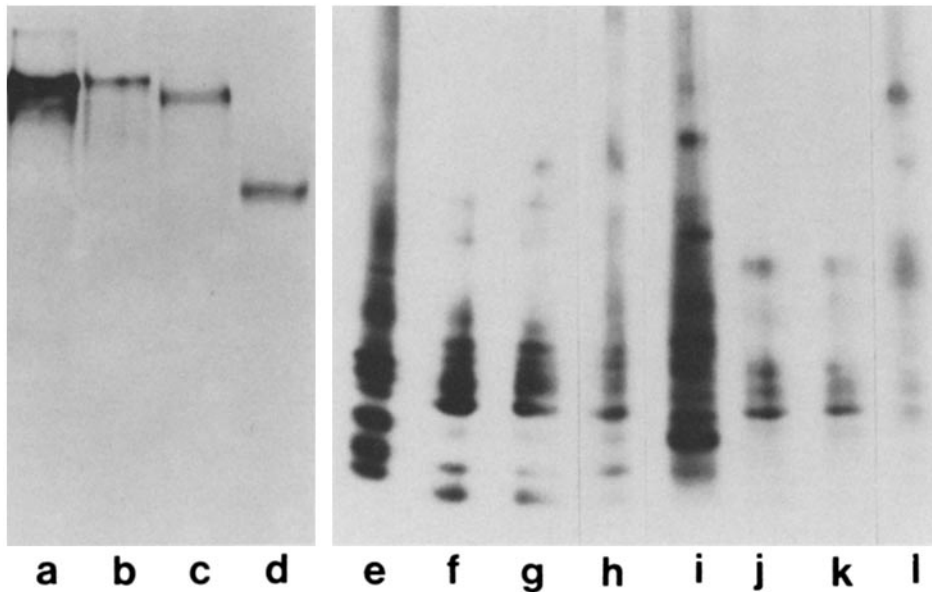


FIGURE 1 M1 antibody immunoprecipitations from radiolabeled chick muscle culture conditioned media. 3-d-old muscle cultures were incubated for 20 h with [^{35}S]methionine (a–c), [^{35}S]methionine in the presence of ascorbate and β -aminopropionitrile (d–f), [^3H]glycine and -proline (g and h), and [^3H]glucosamine (i and j), respectively. To 200- μl aliquots of the conditioned media, M1 antibody (a, d, g, and i), B3 antifibronectin antibody (b, e, h, and j), or anti-chick collagen antiserum (c and f) were added, followed by incubation with the corresponding second antibodies. Immunoprecipitates were washed, run on SDS PAGE gels, and the gels were fluorographed as described in Materials and Methods. Note that the pattern of polypeptides precipitated by M1 antibody is the same for all three labels and is not influenced by ascorbate and β -aminopropionitrile.

to each other in conditioned medium. The $M_r \sim 220,000$ fibronectin subunit, however, produced a different proteolytic cleavage pattern (Fig. 2) that excludes the possibility that M1 antibody recognizes a subset of fibronectin molecules (see below).

The possible origin of the apparent subunit heterogeneity of myotendinous antigen and the differences found in material isolated from fibroblast and muscle cultures, respectively, will be dealt with later (see Figs. 8 and 9). The glucosamine-labeled material precipitated by M1 antibody that runs in the stacking gel (Fig. 1) is not likely to be related to myotendinous antigen (see Fig. 5) and will be characterized further (see Fig. 9).

Fig. 1 makes it obvious that myotendinous antigen is a quite major component released by cultured cells into the medium. Under a wide variety of culture conditions, we found



20 $\mu\text{g}/\text{lane}$) or α -chymotrypsin (*i-l*; 20 $\mu\text{g}/\text{lane}$) on top of a new 10–15% acrylamide-SDS gel which was used to separate the cleavage products (12). A fluorograph of this gel is shown.

FIGURE 2 Differently migrating polypeptides precipitated by M1 antibody yield very similar proteolytic cleavage patterns which differ from fibronectin patterns. 1-ml aliquots of conditioned medium of chick fibroblasts labeled with [^{35}S]methionine was incubated with 100 μg B3 or M1 antibody followed by second antibody. The washed precipitates were run on an SDS PAGE gel which was dried without fixing and autoradiographed. The labeled fibronectin (*a, e, and i*) and the myotendinous antigen bands of $M_r \sim 220,000$ (*b, f, and j*), $M_r \sim 200,000/190,000$ (*c, g, and k*), and $M_r \sim 150,000-170,000$ (*d, h, and l*) were cut out separately from this gel and either rerun on a 5–15% acrylamide gel (*a-d*) or digested with V8 protease (*e-h*;

that radioactivity in the material precipitated by M1 antibody amounted to 20–100% as much radioactivity as found in fibronectin immunoprecipitated from the same amount of conditioned medium (not shown). The insolubility of myotendinous antigen so far precludes accurate measurements of its actual biosynthesis rate relative to total cellular protein synthesis.

Myotendinous Antigen Contains a Pepsin-resistant Domain, But Is Insensitive to Bacterial Collagenase

Since M1 antibody was derived from a hybridoma library for which pepsin-treated Type V collagen (44) was used as an immunogen (see our accompanying paper [11]), we investigated whether myotendinous antigen was related to this or any other known collagen types. Immunoprecipitated, the radiolabeled antigen was incubated with or without bacterial collagenase and then run in SDS PAGE. As shown in Fig. 3*b*, none of the polypeptides precipitated by M1 antibody was affected by this treatment. In the same experiment, labeled procollagens precipitated by a polyclonal anti-chick Type I collagen antiserum (Fig. 3*e*) were digested by the collagenase (Fig. 3*f*). By cutting out labeled bands from gels, it was determined that radioactivity in myotendinous antigen polypeptides decreased by 3%, but in procollagen bands by 52% under these conditions ($n = 3$). When conditioned medium of radiolabeled chick muscle cultures was digested with pepsin for 24 h before incubation with M1 antibody, a pepsin-resistant fragment of $M_r \sim 80,000$ was immunoprecipitated (Fig. 3*c*). Even with short pepsin treatments, no polypeptides of the size of Type V collagen chains were ever detected (not shown). The pepsin-resistant fragment of myotendinous antigen, isolated by immunoprecipitation, was further subjected to treatment with bacterial collagenase. As seen in Fig. 3*d*, this $M_r \sim 80,000$ peptide was, like the intact antigen subunits, not digested. In summary, at least one of the polypeptides precipitated by M1 antibody contains a large pepsin-resistant domain recognized by the antibody; since the polypeptides

are closely related (Fig. 2), this domain might be present in all of them. Unlike the major known collagens including Types IV and V (see Discussion), however, neither the native polypeptides nor the $M_r \sim 80,000$ pepsin-resistant fragment of myotendinous antigen are susceptible to bacterial collagenase.

Other lines of evidence suggest that the polypeptides precipitated by M1 antibody are not typical collagenous molecules. First, ascorbic acid and β -aminopropionitrile did not stimulate the release of M1 antigen polypeptides (Fig. 1*d*), whereas secretion of Type I procollagen chains was greatly enhanced under the same conditions (Fig. 1*f*; see e.g., references 4, 15). In a quantitative experiment, we found that, in the presence of these two agents, total protein synthesis increased by 11%, release of fibronectin and myotendinous antigen changed by +46% and –5%, respectively, whereas procollagen release was stimulated 11-fold ($n = 4$). Moreover, if compared to fibronectin, myotendinous antigen polypeptides were not preferentially labeled by [^3H]glycine/proline (Fig. 1*g*) as opposed to [^{35}S]methionine (Fig. 1*a*) as is the case for collagens (15). The ratio of [^3H]glycine/proline (as well as of [^3H]glucosamine) incorporated into the different antigen polypeptides was roughly the same as seen with [^{35}S]methionine, which makes it unlikely that the $M_r \sim 220,000$ band represents a “procollagen” precursor of smaller collagenous polypeptides.

Myotendinous Antigen Is Antigenically Different from Fibronectin

The partial colocalization of myotendinous antigen with fibronectin *in vivo* and *in vitro* (see the accompanying paper [11]) and the almost identical migration rates on SDS gels of the respective $M_r \sim 220,000$ components (Fig. 1) led us to suspect initially that M1 antibody might, in fact, recognize fibronectin and some of its proteolytic breakdown products (for a review on fibronectin, see reference 24). That this is not the case was first evidenced by the different proteolytic cleavage patterns, suggesting different primary structures for the two molecules (Fig. 2). The experiments described here convinced us that the polypeptides precipitated by M1 antibody

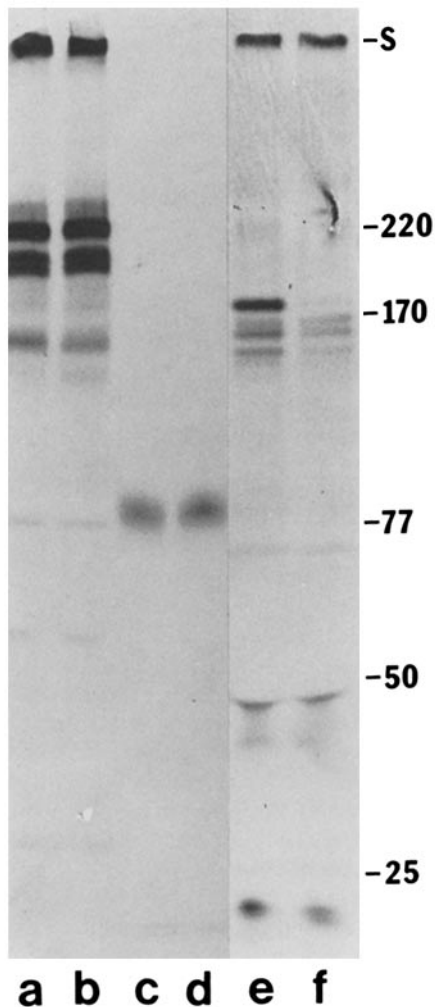


FIGURE 3 Polypeptides precipitated by M1 antibody contain a pepsin-resistant domain, but are not digested by bacterial collagenase. 100- μ l aliquots of conditioned medium from [35 S]methionine-labeled chick muscle cultures were either incubated directly with M1 antibody (a and b) or anti-chick collagen antiserum (e and f) or first digested with pepsin (see Materials and Methods) before incubation with M1 antibody (c and d). Second antibody was added and resulting immunoprecipitates were washed and treated with bacterial collagenase (b, d, and f) or with buffer alone (a, c, and e) as described in Materials and Methods. Immunoprecipitates were then run on an SDS PAGE gel which was fluorographed. Neither the native polypeptides (b) nor a M_r \sim 80,000 pepsin-resistant fragment (d) precipitated by M1 antibody were attacked by the collagenase.

clearly differ from fibronectin in many respects (Figs. 4 and 5).

Fibronectin and presumptive myotendinous antigen were isolated from fibroblast conditioned medium as described in Materials and Methods. Both preparations contained a major, somewhat heterogeneous component of M_r \sim 220,000 (Fig. 4, a and b). Samples of the two preparations were run in pairs in adjacent SDS PAGE lanes and blotted to nitrocellulose sheets that were incubated with either M1 or B3 (antifibronectin) antibody followed by iodinated second antibody. As seen in Fig. 4d, M1 antibody bound to the M_r \sim 220,000 region of the myotendinous antigen preparation, but did not bind to any material in the fibronectin preparation (Fig. 4c); the reverse was true for B3 antibody (Fig. 4, e and f), thereby

confirming the identity of the M_r \sim 220,000 component binding to gelatin-Sepharose as chick fibronectin.

Several important conclusions can be drawn from this experiment. First, fibronectin and myotendinous antigen are antigenically distinct molecules. Second, the antigenic site recognized by M1 antibody is localized on the major M_r \sim 220,000 polypeptide found in M1 immunoprecipitates. (On immunoblots of other preparations, polypeptides of lower M_r were stained as well; not shown.) Third, whereas fibronectin is selectively retained by gelatin-Sepharose, myotendinous antigen is not, pointing to a functional difference between these two extracellular matrix components. This fact also demonstrates that fibronectin and myotendinous antigen are

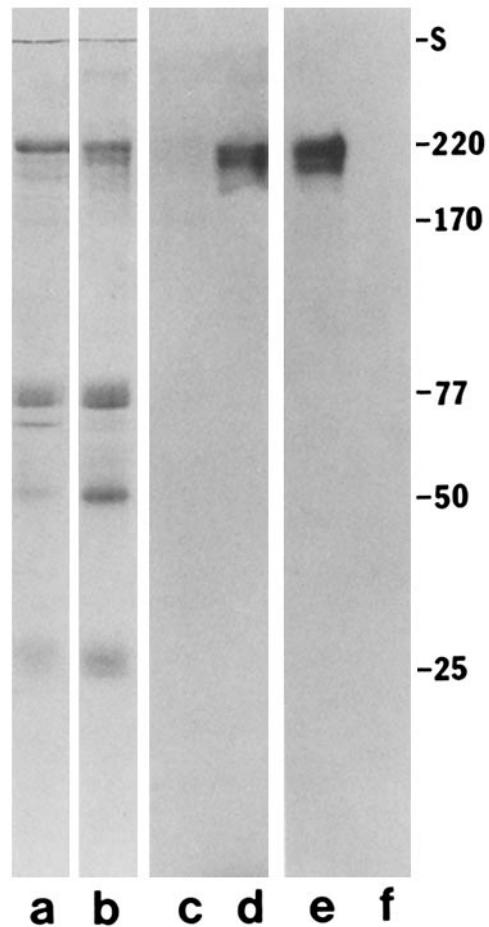


FIGURE 4 Immunoblotting of M1 antigen and fibronectin isolated from conditioned cell culture medium. Chick fibroblasts were grown in medium containing fibronectin-free serum (see Materials and Methods). Secreted chick fibronectin and myotendinous antigen were isolated from the medium by affinity chromatography on gelatin and M1 antibody, respectively, coupled to Sepharose (see Materials and Methods). Eluate samples from the gelatin-Sepharose column (a, c, and e) and the M1 antibody-Sepharose column (b, d, and f) were run on an SDS PAGE gel. Polypeptide bands were either stained with Coomassie Blue (a and b) or transferred to nitrocellulose sheets which were probed with M1 antibody (c and d) and antifibronectin antibody B3 (e and f), respectively, followed by iodinated second antibody. Prominent polypeptide band(s) around M_r \sim 220,000 were present in both preparations (a and b). However, M1 antibody only reacted with corresponding polypeptide(s) in the eluate of the M1 antibody-Sepharose (d), but not the gelatin-Sepharose (c); B3 antibody gave the opposite result (e and f). The bands of lower M_r in (a and b) are contaminating horse serum proteins.

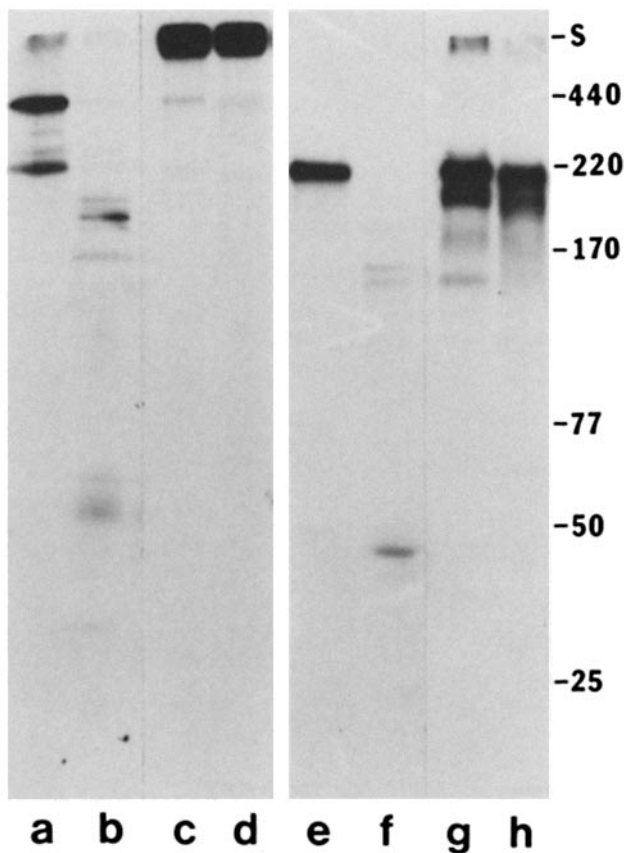


FIGURE 5 Digestion of immunoprecipitated myotendinous antigen and fibronectin with α -chymotrypsin. 200- μ l samples of [35 S]methionine labeled fibroblast conditioned medium were incubated with antifibronectin antibody B3 (a and b and e and f) or M1 antibody (c and d and g and h), respectively, followed by second antibody. Immunoprecipitates were incubated with buffer alone (a, c, e and g) or with α -chymotrypsin (b, d, f, and h) as described in Materials and Methods. Reactions were stopped by adding SDS sample buffer without (a-d) or with (e-h) β -mercaptoethanol, respectively, and samples were run on an SDS PAGE gel which was fluorographed. Whereas immunoprecipitated fibronectin was digested under these conditions, the large, disulfide-linked myotendinous antigen complexes (c and d) as well as their reduced subunits (g and h) were barely affected.

not complexed with each other in fibroblast conditioned medium, but can easily be separated. It might be added here that we could not detect any material reacting with M1 antibody in chicken serum or plasma that contains fibronectin (not shown). Fourth, comparable amounts of myotendinous antigen and cellular fibronectin, respectively, could be isolated from fibroblast conditioned medium. (The yield of fibronectin was calculated to be 0.6–0.7 mg/liter.) This means that myotendinous antigen is a major component secreted by cultured chick fibroblasts, a finding which is confirmed by the metabolic labeling experiments (see, e.g., Fig. 1).

Myotendinous Antigen Is Structurally Different from Fibronectin

Further experiments were done to compare the structure of myotendinous antigen and fibronectin released by chick cells into the medium. In SDS PAGE under nonreducing conditions, immunoprecipitated fibronectin runs mainly as a disulfide-linked dimer of $M_r \sim 450,000$, while a certain fraction

is reduced to monomeric size (Fig. 5a). In contrast, myotendinous antigen appears to be a huge disulfide-linked complex that barely enters a 5–15% (Fig. 5c) or a 3.5–15% (not shown) polyacrylamide gradient gel in the absence of reducing agents. Obviously, polypeptides of all the apparent size classes precipitated by M1 antibody ($M_r \sim 220,000$, $\sim 200,000$, and $\sim 150,000$) are disulfide-linked to each other in antigen complexes, since none of these bands is visible on nonreducing gels.

Digestion of native, immunoprecipitated fibronectin with chymotrypsin (Fig. 5, b and f) produces large fragments which are monomeric, i.e., no longer disulfide-linked, because C-terminal intrachain S-S bridges are removed (see, e.g., reference 13). In contrast, the native myotendinous antigen complex was not affected by an identical treatment with chymotrypsin: its subunits obviously remained disulfide-linked, since the complex still ran on top of the separating gel under nonreducing conditions (Fig. 5d). Analysis of a parallel sample reduced with β -mercaptoethanol revealed that chymotrypsin converted the major polypeptides of the antigen (Fig. 5g) into bands of only slightly faster migration rates (Fig. 5h). (Perhaps surprisingly, we did not observe a conversion of the $M_r \sim 220,000$ band into the $M_r \sim 200,000/190,000$ doublet.) This experiment demonstrated that myotendinous antigen differs from fibronectin in terms of quaternary structure and protease cleavage sites within the native molecule.

To find out whether the myotendinous antigen complexes found in conditioned medium are homogeneous or heterogeneous with respect to subunit composition, we did velocity sedimentation experiments. Samples of [35 S]methionine-la-

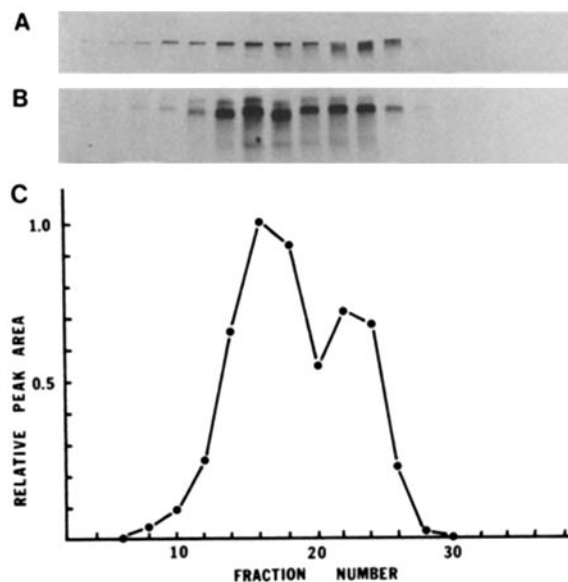


FIGURE 6 Velocity sedimentation analysis of myotendinous antigen complexes in conditioned medium. [35 S]Methionine-labeled chick fibroblast conditioned medium was run on 5–20% sucrose gradients in BBS (pH 8.4) containing 1% Triton X-100. Fractions were collected and incubated with M1 antibody followed by second antibody. Immunoprecipitations derived from consecutive gradient fractions were analyzed by SDS PAGE without prior reduction (A) or after reduction with β -mercaptoethanol (B). Densitometric analysis of the $M_r \sim 220,000$ myotendinous antigen band on the gel shown in B is depicted in C. Myotendinous antigen complexes migrated as two peaks around 9S and 12S, which revealed similar subunit heterogeneity (B) but differed in migration rate on nonreducing SDS PAGE gels (A).

beled, fibroblast-conditioned medium were run on 5–20% sucrose gradients in BBS containing 1% Triton X-100. Gradient fractions were analyzed by immunoprecipitation with M1 antibody, followed by SDS PAGE and fluorography of the gels. The antigen was found to sediment in two peaks of about 9S and 12S (Fig. 6C). Native, dimeric serum fibronectin was found to co-migrate with the myotendinous antigen peak of 12S, a value expected for fibronectin under these conditions (see reference 1). When analyzed on SDS PAGE under reducing conditions, the 9S and 12S peak were found to contain differently sized myotendinous antigen polypeptides in about the same ratio, i.e., antigen complexes found in the two peaks were *not* composed of either only smaller or only larger polypeptides, respectively (Fig. 6B). However, electrophoretic analysis of nonreduced samples revealed that M1 antigen complexes found in the 12S peak stuck on top of the 5–15% acrylamide running gel, whereas the material of the 9S peak entered the running gel to a certain extent (Fig. 6A). It is thus likely that the two peaks with different sedimentation rates represent two classes of myotendinous antigen oligomers containing different numbers of polypeptides, i.e., that the antigen released by cells does not possess a constant subunit stoichiometry. Since myotendinous antigen complexes migrate more slowly than laminin ($M_r \sim 10^6$) in SDS PAGE without reduction (not shown), the “minimum size” myotendinous antigen found in conditioned medium is at least a pentamer.

Heterogeneity of Myotendinous Antigen: Differences between Muscle and Fibroblast Cultures

As mentioned above, two kinds of myotendinous antigen heterogeneity were observed: first, a pattern of related subunits having different migration rates, and second, differences in this pattern in material isolated from muscle as compared to fibroblast cultures. Experiments were done to find out when, and possibly how, this heterogeneity is generated.

In myotendinous antigen isolated from muscle culture medium, the $M_r \sim 220,000$, 200,000, and 190,000 bands were always labeled in a ratio of about 2:1:1 when measured by densitometric scanning of autoradiographed gels (see Figs. 1 and 7). In contrast, the $M_r \sim 220,000$ component was always the major polypeptide in antigen derived from fibroblasts, containing at least four times as much radioactivity as any of the minor bands (Figs. 6 and 7). To see whether these different polypeptide patterns were generated by proteolytic cleavage after the antigen was released from the cells, we labeled primary muscle and fibroblast cultures for different time intervals (from 1 to 6 h) with [35 S]methionine. As seen in Fig. 7, myotendinous antigen was detected in the medium after a lag of more than an hour; the rate of accumulation was determined to be linear between 2 and 6 h and about seven times higher for the fibroblast as compared to the muscle cultures. Interestingly, the antigen polypeptide pattern was always typical for the respective culture type and did not change with increasing labeling time. Moreover, labeled conditioned medium could be stored for up to 2 wk at 4°C without any further change in the typical gel pattern of material precipitated by M1 antibody (not shown). The heterogeneity and culture type specific pattern of myotendinous antigen is thus created at or before its release from the cells and is not likely to be caused by proteolysis (or some other

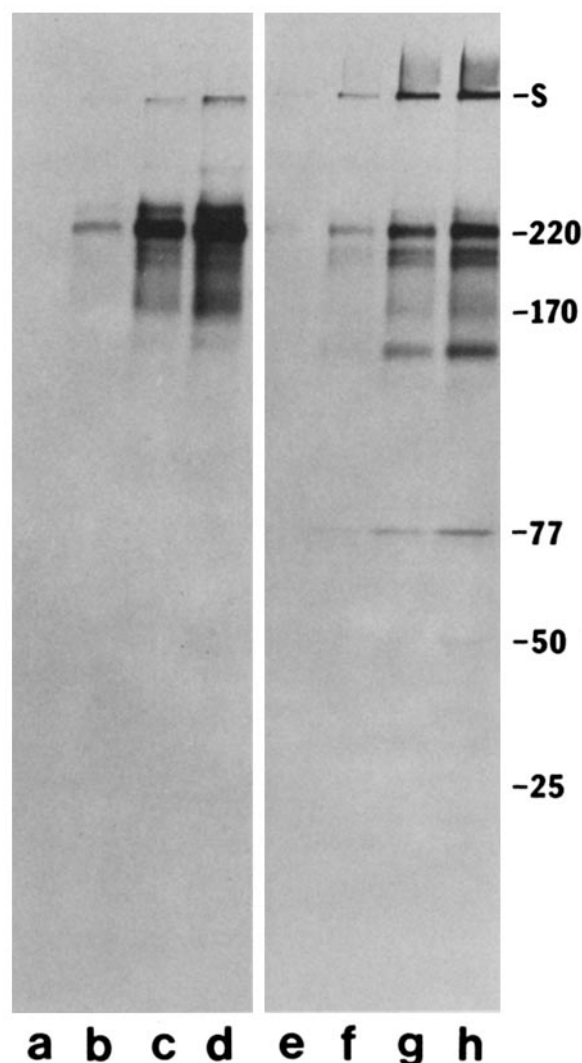


FIGURE 7 Differences in the polypeptide pattern of myotendinous antigen isolated from fibroblast and muscle culture conditioned medium, respectively. Primary chick fibroblast (a–d) and muscle (e–h) were labeled for 1 (a and e), 2 (b and f), 4 (c and g), and 6 (d and h) h with [35 S]methionine. Samples of 100 μ l (fibroblast medium) or 200 μ l (muscle culture medium) were immunoprecipitated with M1 antibody. Precipitates were analyzed on an SDS PAGE gel which was fluorographed for 1 d (a–d; fibroblasts) or 3 d (e–h; muscle cultures) to achieve similar intensity of labeled bands. The pattern of polypeptides precipitated by M1 antibody differs between the two culture types, but does not vary with increasing labeling time.

processing) in the culture medium. We have evidence that the polypeptide heterogeneity of the antigen arises intracellularly or at the cell surface: immunoprecipitates from deoxycholate extracts of cultures pulse-labeled for 1 h revealed the same cell type specific subunit heterogeneity as antigen isolated from conditioned medium (not shown).

As mentioned above, we have been unable to extract myotendinous antigen from the extracellular matrix of cell cultures or tissues in a form compatible with antibody binding. We therefore do not yet know its actual rate of biosynthesis and whether the same subunit heterogeneity is found in antigen incorporated into extracellular structures.

We compared the accumulation of newly synthesized myotendinous antigen in the medium of primary skin fibroblast and skeletal muscle cultures. In this experiment, the muscle

cultures were enriched for myotubes as described in Materials and Methods; the percentage of nuclei in multinucleated myotubes averaged 84%. After labeling cultures for 6 h with 100 $\mu\text{Ci/ml}$ [^{35}S]methionine, the antigen was immunoprecipitated from aliquots of the conditioned media. 160 ± 80 cpm ($n = 6$) per 10^4 nuclear equivalents were found to be incorporated into the antigen in muscle culture medium, as opposed to $1,060 \pm 170$ cpm ($n = 4$) per 10^4 cells in fibroblast culture medium. The actual difference in myotendinous antigen release between fibroblasts and myogenic cells might be even greater than the 6.7-fold observed. 7.5% contaminating fibroblasts (as was estimated by counting flat, multipolar cells) present in the muscle cultures used could account for about half of the observed antigen secretion; in this case, fibroblasts would produce about twelve times as much myotendinous antigen as myogenic cells.

Sulfate Labeling of Myotendinous Antigen and Its Association with Sulfated Glycosaminoglycans in Muscle Cultures

M1 antibody immunoprecipitates of muscle culture medium always contained, together with the M_r ~150,000–220,000 antigen polypeptides, material migrating within the stacking gel even under reducing conditions (Fig. 1). This very high molecular weight substance was heavily labeled by [^3H]glucosamine (Fig. 1*i*), but only weakly by radioactive amino acids (Fig. 1, *a*, *d*, and *g*). We suspected that it might consist of sulfated proteoglycans (for a review, see reference 20) coprecipitated with myotendinous antigen. Therefore, muscle and fibroblast cell cultures were labeled with [^{35}S]sulfate and aliquots of the collected conditioned media incubated with the antibodies M1, B3 (antifibronectin), and P3x63 (nonspecific antibody), respectively. Both conditioned media and immunoprecipitates were analyzed SDS PAGE as shown in Fig. 8. Most of the sulfate-labeled material in fibroblast conditioned medium was found on top of the 5–15% acrylamide running gel (Fig. 8*g*). In the case of muscle conditioned medium, however, most radioactivity migrated within the 3% acrylamide stacking gel (Fig. 8*h*). Most interestingly, this sulfate-labeled, very high molecular weight material in muscle conditioned medium seemed to be co-precipitated specifically by M1 antibody (Fig. 8*d*), since it was not found in immunoprecipitates of B3 or P3x63 antibody (Fig. 8, *e* and *f*). From fibroblast conditioned medium, which contained much less of this material to start with, accordingly less (and only slightly more than in the controls) was co-precipitated by M1 antibody (Fig. 8*a*). In SDS PAGE in the absence of reducing agents, M1 antigen polypeptide complexes were still separated from the high molecular weight sulfate-labeled material by entering the running gel to some extent (Figs. 5 and 6). Therefore, M1 antigen polypeptides are disulfide-linked to each other rather than to the high molecular weight material.

When M1 antibody immunoprecipitates from conditioned medium of radiolabeled muscle cultures were incubated with chondroitinase ABC, all the sulfate-labeled material migrating in the stacking gel was digested (Fig. 9*b*), whereas the pattern of methionine-labeled antigen polypeptides was not affected (Fig. 9*d*). This experiment provides evidence that the very high molecular weight material indeed contains sulfated glycosaminoglycans which, together with its size, are indicative of a chondroitin sulfate proteoglycan (20).

M1 antibody has an isoelectric point of lower than 8 and is

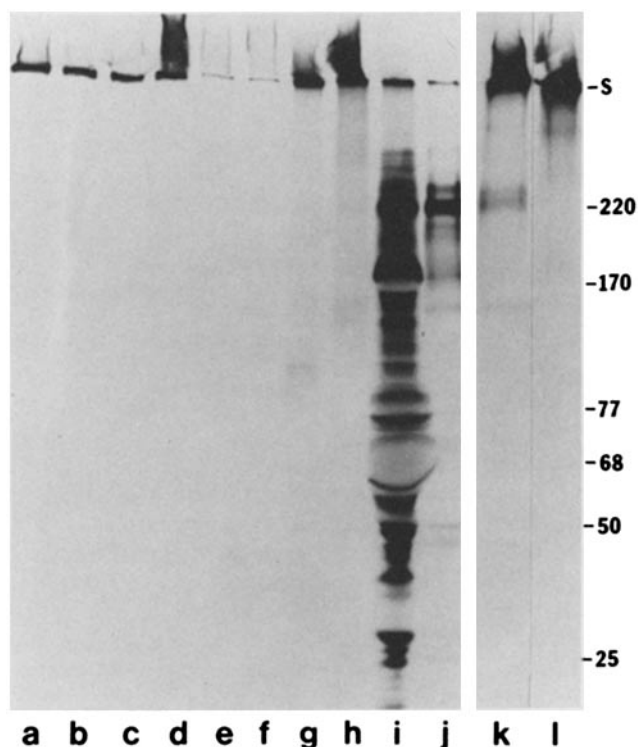


FIGURE 8 High molecular weight sulfated material is co-precipitated with M1 antigen polypeptides. Chick fibroblast (*a–c* and *g*) and muscle (*d–f* and *h*) cultures were labeled with [^{35}S]sulfate for 6 h. 100- μl aliquots of the conditioned media were incubated with M1 antibody (*a* and *d*), B3 antifibronectin antibody (*b* and *e*) and P3 \times 63 nonspecific antibody (*c* and *f*), respectively, followed by second antibody. Precipitates were run on an SDS gel which was fluorographed. 20- μl samples of the whole [^{35}S]sulfate-labeled fibroblast (*g*) and muscle (*h*) conditioned media were run on the same gel. 20 μl [^{35}S]methionine labeled fibroblast medium (*i*) and an M1 antibody immunoprecipitation of 50 μl of the same medium (*j*) are shown for comparison. Note the sulfate-labeled material in muscle conditioned medium that migrates in the stacking gel (*h*) and that is precipitated by M1 antibody (*d*) but not other antibodies (*e* and *f*). Lane *k* is identical to lane *a*, except that the gel was exposed three times longer, thereby revealing two sulfate labeled polypeptide bands that co-migrate with the M_r ~220,000 and ~250,000 components of M1 antigen (*j*). Lane *l* depicts a parallel, but nonreduced, sample of the one shown in lane *k*: the two sulfate-labeled bands are not visible and probably migrate on top of the gel.

thus unlikely to precipitate glycosaminoglycans nonspecifically by electrostatic interaction. The immunoblotting experiments suggest (but cannot rule out completely) that this material is not immunologically related to the polypeptides recognized by M1 antibody. It is, however, likely that M1 antibody precipitates the sulfate-labeled material indirectly if the latter is bound to myotendinous antigen polypeptides in conditioned medium.

Longer exposure times of the gel shown in Fig. 8 revealed two fuzzy, [^{35}S]sulfate-labeled polypeptide bands in M1 antibody immunoprecipitates in addition to the material on top of the running gel (Fig. 8*k*). These two bands co-migrated with the methionine-labeled M1 antigen bands of M_r ~220,000 and M_r ~240,000 (Fig. 8*j*) and were not found in the absence of reducing agents, where all the sulfate label migrated on top of the gel (Fig. 8*l*). Since this behavior is in accordance with the observed migration of myotendinous

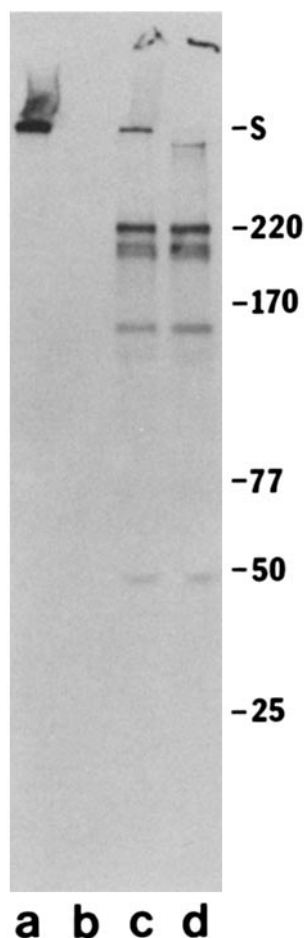


FIGURE 9 The sulfate labeled, high molecular weight material coprecipitated with M1 antigen is chondroitinase sensitive. Muscle cultures were labeled with [^{35}S]sulfate (a and b) or [^{35}S]methionine (c and d). 100- μl aliquots of the conditioned media were incubated with M1 antibody followed by second antibody. The immunoprecipitates were either treated with buffer alone (a and c) or chondroitinase ABC (b and d) as described in Materials and Methods. Chondroitinase digested the sulfate labeled material migrating in the stacking gel of M1 antibody immunoprecipitates (b) without attacking the methionine-labeled M1 antigen polypeptides (d).

antigen on SDS PAGE gels in the presence and absence of β -mercaptoethanol, it seems likely that the M_r ~220,000 and M_r ~240,000 components of the antigen (but not the components of lower M_r) are sulfated polypeptides.

DISCUSSION

Myotendinous antigen, which is recognized by monoclonal antibody M1, exhibits an interesting temporal and spatial pattern during chick limb morphogenesis (11). In this paper, we investigated some aspects of the antigen's structure and tried to establish its relationship, if any, to other known extracellular matrix components. We have shown that myotendinous antigen differs from fibronectin (24) both structurally and functionally. Likewise, comparison of myotendinous antigen with laminin (39), which could be immunoprecipitated by monoclonal antibody 31-2 (Bayne, E. K., M. J. Anderson, and D. M. Fambrough, manuscript in preparation), revealed differences in the migration rates of both the non-reduced and reduced polypeptides in SDS PAGE (not shown). Moreover, judged by our localization studies (11),

myotendinous antigen apparently is not a typical basal lamina component like laminin (39) or type IV collagen (5). Type IV (3) and type V (16) procollagens have electrophoretic mobilities similar to myotendinous antigen polypeptides in SDS-PAGE under reducing conditions. However, both these collagens have at least two distinct types of subunit chains (3, 5, 16), whereas myotendinous antigen polypeptides seem to be very similar. Also, myotendinous antigen is not susceptible to digestion by bacterial collagenase under conditions where the major collagens including type IV (17, 26) and type V (16) are. Other known extracellular matrix components like chondronectin (21), thrombospondin (33), entactin (7), sulfated glycoproteins (22), and minor collagenous polypeptides (14, 34, 37) are known to differ from myotendinous antigen both in tissue distribution and subunit size.

One possibility needs further consideration, namely that myotendinous antigen is a "microfibrillar protein" (35). Non-collagenous extracellular matrix fibrils of 2–12-nm diameter, called "microfibrils," have been described in vertebrate embryos (29), around elastic fibers (35), and in myotendinous junctions (41). Microfibrils in chick embryos have been implicated with neural crest cell migration and were shown to contain fibronectin by immunoperoxidase labeling techniques (29). Perhaps another type of microfibrils surrounds elastic fibers and might be involved in their morphogenesis (35). A "microfibrillar protein" of bovine elastic fibers has been isolated by Ross and Bornstein (35); its heavily disulfide-linked subunits exhibited an M_r of ~220,000 on SDS gel, which happens to be the approximate value for both fibronectin monomers and the major myotendinous antigen polypeptide. The same authors isolated a similar protein from extracellular matrix fibrils of smooth muscle cell cultures, and concluded that the latter "microfibrillar protein" might indeed be fibronectin (31). However, the amino acid compositions of the smooth muscle and the elastic fiber protein differed (31, 35). An antiserum against elastic tissue "microfibrillar protein" has been generated (25); it is not clear whether this antiserum crossreacts with fibronectin (38). Part of the apparently confusing results could easily be explained if the major M_r ~220,000 band found upon SDS gel electrophoresis of extracellular matrices (9), surface-iodinated cells (23), and conditioned media (30) was in fact a mixture of several polypeptides. Of these, fibronectin (24) certainly is the most abundant. Even so, the major polypeptide of myotendinous antigen might contribute 20% or more to the M_r ~220,000 band found in fibroblast conditioned medium (see, e.g., Fig. 1).

In adult chick feet, M1 antibody labeled tendons intensely, whereas staining of elastic fibers was not obvious (not shown). It is therefore likely that myotendinous antigen differs from the elastic fiber "microfibrillar protein." Judged from its biochemical characteristics and its tissue distribution, myotendinous antigen might well be a component of microfibrils in myotendinous junctions and tendons; we are further investigating this possibility.

Several aspects of the structure of myotendinous antigen, as we know it so far, are still puzzling. Compared to their very slow migration rates in SDS PAGE without prior reduction, M1 antigen complexes, which consist of several disulfide-linked subunits, exhibit quite small sedimentation values. This suggests a high frictional coefficient and hence an extended configuration of the antigen (1). Surprisingly, however, the antigen complexes are quite resistant to proteolysis. This is also the reason why we do not know yet how the polypeptide

heterogeneity of myotendinous antigen is generated. We know that the heterogeneous pattern arises early during the antigen's biosynthesis and/or assembly and seems to be fibroblast or muscle culture specific. More work is needed to find out whether myotendinous antigen is processed differently by different cell types or whether different genes encoding the antigen are expressed in different cell types.

Myotendinous antigen does not bind to gelatin (i.e., denatured collagen) and, at least in conditioned medium, seems not to be complexed with procollagens or fibronectin. However, high molecular weight sulfated glycosaminoglycans (20) are co-precipitated with the antigen, at a higher extent from muscle than from fibroblast conditioned medium. This is interesting because myotubes (but not fibroblasts) are known to synthesize a proteoglycan with very large chondroitin sulfate side chains (8). The possibility that myotendinous antigen specifically interacts with this muscle-specific proteoglycan needs further investigation. If this turns out to be the case, it would provide a mechanism by which the antigen binds to muscle fiber surfaces in myotendinous junctions, and support our hypothesis that myotendinous antigen represents a functional link between muscles and tendons.

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