BIOCHEMICAL STUDIES ON THE 10 NM FILAMENTS OF AVIAN MUSCLE

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Bruce D. Hubbard

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This thesis is dedicated to a screwdriver, received on my first birthday, with which I explored the mysteries of doorknobs, managing in the process to remove every one from every door throughout our house. It is also dedicated to Uncle Dick's glow-in-the-dark owl clock, which courageously gave up its life for the cause of science one night not long afterwards. Most of all, it is dedicated to all of the people who have valued and encouraged my curiosity ever since, and who have been special friends to me: Ray and Marion Hubbard, Cha-Rie Tang, Don and Dorthea Kauffman, the Hubbard family, the Cohen family, Ben Wolf, Maury Raffenspurger, Eileen Clancy, Deanna and Les Goldstein, Blain and Candy Bocarde, Tony Loh, Jeff Lagarias, Cha-Mei Tang, Jonathan King, David Botstein, Larry Gold, Dick McIntosh, Mark Dubin, Noboru Sueoka, Ray Owen, Jean-Paul Revel, Dona Chikaraishi, Zach Cande, Dan and Patti Brower, Eva Griepp, Dave Balzer, Melinda Burbank, Dave and Cindy Helphrey, and Ilga Lielausis.

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Abstract

This thesis describes the purification and initial biochemical characterization of desmin, a major component of avian muscle 10 nm filaments.

1. Extraction of chicken gizzard with KI solubilizes most of the actomyosin and other fibrillar proteins, leaving behind a KI insoluble residue containing 10 nm filaments, desmin, and a small amount of insoluble actin. Antibodies prepared against electrophoretically purified desmin do not cross-react with other muscle proteins. In conjunction with indirect immunofluorescence, they demonstrate the presence of desmin in close association with the Z-lines and plasma membrane of skeletal muscle cells. Desmin also is associated with the Z-lines, intercalated discs, and intercellular adhesion sites of cardiac muscle cells.

2. Both desmin and the actin that remain in the KI extracted gizzard are insoluble in high salt, but are soluble at low pH or in agents that dissociate hydrophobic bonds. Desmin may be purified by repeated cycles of solubilization by 1 M acetic acid and subsequent precipitation by neutralization to pH 4. During this process, a constant ratio of actin to desmin is attained.

3. Gel filtration on Bio-Gel P300 in the presence of 1 M acetic acid reveals that actin is dissociated from the vast majority of desmin under these conditions. When the acetic acid is removed from actin-desmin solutions by dialysis against water, a gel forms that is composed of 12-14 nm diameter filaments. Both anti-actin and anti-desmin react uniformly with these filaments to a resolution limit of 250 nm.

4. Actin and desmin can be extracted from homogenized chicken gizzard in soluble form by 10 mM EGTA. Both proteins are retained on a DNase I affinity column and also coprecipitate in indirect immunoprecipitation with either anti-actin

or anti-desmin. Velocity sedimentation of the EGTA extract shows that the actin and desmin cosediment as a series of stable complexes that range in size from 6 to 60 S. The 50-60 S family has an actin to desmin stoichiometry of approximately 5 to 1.

5. Several proteases are investigated that selectively cleave desmin (mwt 50,000) to sequentially produce fragments of 43,500 mwt, 40,000 mwt, and a 35,000 mwt limit digestion product that lacks 82% of the tyrosine of the original desmin. The cleavage process results in the conversion of gelled desmin to a non-cohesive, powdery, precipitate. These proteases remove the desmin from isolated myofibril bundles and cause their lateral separation into individual myofibrils.

6. Based upon these observations, it is proposed that desmin is a major component of muscle 10 nm filaments and that it forms stable complexes with actin. It is further proposed that the cellular function of desmin is to mechanically interlink individual actomyosin contractile units, both to each other and to the plasma membrane.

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CHAPTER ONE

Introduction

The biochemistry of cellular motility is currently undergoing a renaissance. On one hand, there is explosive interest in the roles traditional muscle proteins such as actin, α-actinin, myosin, and tropomyosin, play in the motility of non-muscle cells. This is matched by an interest in the nature of the structural framework that appears to anchor these proteins to the plasma membrane and transmit the forces produced throughout the whole cell.

My thesis describes a series of studies on the structure and function of 10 nm filaments in avian muscle. The data obtained will be used to develop and support the hypothesis that 10 nm muscle filaments are part of a system responsible for the organized transmission of force throughout the muscle cell.

This introduction will review the literature on 10 nm filaments up to the point at which the research reported in this thesis begins. The concluding chapter will then finish the review, placing my research in its proper context with other contemporary work.

10 nm Filaments in General

Electron microscopy has identified a morphological class of cytoplasmic filaments whose average diameter of 10 nm (range 8-12 nm) lies between that of the actin thin filaments (5-6 nm) and the myosin thick filaments and microtubules (15-25 nm). Found in a wide variety of vertebrate and invertebrate cells, they have been called intermediate filaments (in muscle), neurofilaments (in central and peripheral neuronal axoplasm), glial filaments (in astrocytes), tonofilaments (in epithelia), 100 Å and 10 nm filaments (in a variety of mesodermal and endodermal cell types). Specific examples are given below. I shall refer to the entire morphological class of these filaments, except tonofilaments, by the generic

term 10 nm filaments. The epidermal tonofilaments are composed of keratin and are sufficiently different from the rest of the 10 nm filaments to be considered as a separate entity (Bennett et al., 1978; Franke et al., 1978a,b; Fuchs and Green, 1978; Brysk et al., 1977; Steinert et al., 1976). Morphologically identifiable 10 nm filaments are generally found in one of two characteristic cytoplasmic distributions. Typical of muscle fibers and neuronal axons are long bundles of separate 10 nm filaments (Ishikawa et al., 1968; Rash et al., 1970; Cooke and Chase, 1971; Behrendt, 1977; Wuerker, 1970). On the other hand, spreading cells in tissue culture and aortic epithelium in vivo exhibit a condensed state in which tightly aggregated bundles of 10 nm filaments form perinuclear rings and caps (Starger and Goldman, 1977; Blose and Chacko, 1976). This condensed state may be induced in the 10 nm filaments of a wide variety of cells by either of the microtubule depolymerizing drugs colchicine or vinblastine (De Brabander et al., 1975; Holtrop et al., 1974; Blose and Chacko, 1976; Wisniewski and Terry, 1967; Ishikawa et al., 1968; Croop and Holtzer, 1975). The effect occurs in the presence of 10^{-6}-10^{-8} M colchicine, takes from 24 to 48 hr to mature, and is reversible on the same time scale after removal of the colchicine. Certain agents, including aluminum salts and acrylamide, also cause the specific aggregation of neurofilaments (and dementia) when injected intradurally (Iqbal et al., 1975; Terry and Pena, 1965).

The concomitant reduction in microtubule number and increase in 10 nm filament bundles observed in colchicine-treated cells has been interpreted to indicate that 10 nm filaments arose as the breakdown products of microtubules (Ishikawa et al., 1968). Studies showing a lack of correlation between microtubule disappearance and 10 nm filament appearance, a lack of colchicine binding to neurofilaments, and dissimilarities between various 10 nm filament subunits and

tubulin have proven this to be false (Daniels, 1973; Shelanski et al., 1971; De Brabander et al., 1975; Lazarides and Hubbard, 1976; Starger and Goldman, 1977; Gilbert et al., 1975; Day, 1977; Cooke, 1976).

There is, at present, no satisfactory explanation for the colchicine effect. While many authors comment upon the dramatic increase in observable 10 nm filament bundles, and imply a proportional increase in 10 nm filaments, few have actually quantitated the effect. Daniels (1973) found that colchicine causes only a 2-fold increase in neurofilaments and that they return to nearly the original number after removal of the colchicine. It is similarly unclear if de novo protein synthesis is required for the colchicine effect. Two groups report the effect of inhibiting 95% of protein synthesis with 0.1 mM cycloheximide before adding colchicine. De Brabander et al. (1975) report that it blocks 10 nm filament aggregation in mouse cells while Croop and Holtzer (1975) report that it has no effect on aggregation in chick cells. Finally, aluminum phosphate causes perikarial aggregates of neurofilaments to form without affecting microtubules. As a result, it is not possible to relate the effect of colchicine to microtubule function in any simple way at present.

10 nm Filaments in Neural Cells

Several approaches have been employed in the isolation and purification of 10 nm filaments (Table 1), with most taking advantage of the relative insolubility of the filaments. Neurofilaments and glial filaments are insoluble at low ionic strength, but disaggregate when exposed to high salt concentrations ($\sim$1.0 M) and may be reversibly dissociated and reconstituted by raising and lowering the amount of salt in their environment (Day, 1977; Gilbert et al., 1975). They

can be isolated directly from single neurons and gliotic tissue, respectively, by lysing the cells in hypotonic media and collecting the released (insoluble) filaments by centrifugation. Neurofilaments can also be isolated from central white matter, but some method, usually density centrifugation (Shelanski et al., 1971), is required to separate the neurons from associated glial tissue. All three methods are associated with technical problems. Of critical importance is the presence, in nerve tissue, of calcium-dependent proteases that degrade neurofilament subunits to fragments with the same molecular weight as the glial filament subunit (Liem et al., 1978; Benitz et al., 1976; Gilbert et al., 1975). This, coupled with the facts that density centrifugation procedures generally do not effect a complete removal of glial filaments from neuronal axons, and that gliotic tissue is rarely completely axon free, has produced a mass of conflicting and contradictory data (Benitz et al., 1976; Liem et al., 1975; Shelanski et al., 1971; Yen et al., 1976; Goldman et al., 1978). The most reliable to date are probably those of Lasek and Hoffman (1976), Hoffman and Lasek (1975), Gilbert et al. (1975), and Liem et al. (1978), and are included in Table 1. The consensus of opinion now appears to be that vertebrate neurofilaments are composed of three subunits of approximately 200,000, 150,000, and 68,000 mwt.

10 nm Filaments in Fibroblastic Cells

A widely distributed class of 10 nm filaments has been isolated from a variety of fibroblastic cells. In contrast to the neural filaments, these are soluble in low salt buffers and insoluble in saline (Starger et al., 1978). They have been isolated from BHK-21 cells by lysing 10 nm filament cap containing cells in saline. The caps maintain their integrity under these circumstances and may be collected

by centrifugation (Starger and Goldman, 1977; Starger et al., 1978). A doublet of proteins at 54,000, 55,000 mwt, and a minor component of molecular weight ∿300,000 are found in the caps. It is believed that the 54-55,000 mwt proteins are the major subunits of the 10 nm filaments, while the mwt 300,000 component is fibronectin (Hynes, 1976). All three copolymerize from low salt solutions.

Extraction of cultured cells with the detergent Triton X-100 and high salt solubilizes most of the cytoplasm, leaving behind cytoskeletons composed of 6 nm and 10 nm filaments (Brown et al., 1976; Bennett et al., 1978; also Franke et al., 1978b). The subunit of these 10 nm filaments is described by these workers as ranging in molecular weight from 52,000 to 58,000 (Table 1), but appears to be the same protein in every case. The molecular weight discrepancies appear to arise from the different electrophoretic systems employed. Molecular weight comparisons between different workers are generally in disagreement by about 5000 for a 50-60,000 mwt protein. The cytoskeletons also contain actin and a large protein believed to be fibronectin (Brown et al., 1976). No physical association has been observed between the 10 nm and actin filaments of fibroblasts, however.

10 nm Filaments in Muscle Cells

Developing embryonic muscle contains numerous free filaments, originally believed to be actin, but now identified as a species of 10 nm filament (Alan and Pepe, 1965; Ishikawa et al., 1968, 1969; Rash et al., 1970; Cooke and Chase, 1971; Uehara et al., 1971). 10 nm filaments are present in all three types of adult muscle (cardiac, skeletal, and smooth) but are most abundant in adult smooth muscle,

which has led to its frequent adoption as a starting point for the purification and study of these filaments.

The 10 nm filaments in adult smooth muscle form an interconnected network that links the cytoplasmic dense bodies with membrane-bound dense plaques (Cooke and Chase, 1971; Cooke and Fay, 1972; Cooke, 1976; Ashton et al., 1975; Uehara et al., 1971). The dense bodies contain α-actinin and tropomyosin (reviewed in Schollmeyer et al., 1976). Morphological evidence also shows the insertion of thin filaments into dense bodies and plaques (Ashton et al., 1975; Rice and Brady, 1973), but this has been disputed (Cooke, 1976; Small, 1977).

Although common in embryonic striated muscle, 10 nm filaments become progressively less visible as the sarcomeres condense, and are rarely reported at all in adult muscle. 10 nm filaments have recently been found in adult cardiac muscle, however, following steroid-induced hypertrophy (Behrendt, 1977). The filaments are transversely oriented, encircling the myofibrils at their Z-bands and inserting into both the intercalated discs and sarcolemmal dense patches.

These morphological associations provide a structural starting point for investigating the biochemistry of any actin-10 nm filament or actin-membrane interactions, and muscle presents itself as an ideal system in which to study such interactions. First of all, the internal structure of muscle is both regular and stable. Second, the biochemistry and structure of muscle contractile filaments has been well characterized. Both of these will facilitate the investigation of new structural relationships. This is in marked contrast to the situation in non-muscle cells. Although they must possess analogous systems, their actomyosin structures are transitory, and their 10 nm filaments do not appear to be associated

with any well-defined cytoplasmic structures (Goldman et al., 1976; Lazarides, 1975; Hynes and Destree, 1978).

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Table 1

10 nm Filament Purification

Cell Type	Purification	Calcium[1]	Subunits		References
			Major	Minor	
			mwt x 10^3		
Chicken gizzard	Differential extraction	-	50,42	47,45,43	1,2
Chicken gizzard, hog stomach	Differential extraction	-	55		3,4
Chick embryo	Differential extraction	+	230,52,42	50,(45,43)[2]	5
Chick embryo	Differential extraction	0	58	43	
Chick gizzard	Differential extraction		55	43,(55-43)[2]	6
Chick brain	Hydroxyapatite chromatography		55	51,43	
Bovine axons	Flotation	0	103,74,54		7
Bovine glia	Flotation		54	47-40.5	
Human glia	DEAE and PC chromatography[3]	0	54,49,45,40		8
3T3	Differential extraction	0,-	57		9
Human neurons	Flotation	0	49		10
Human glia	Differential extraction		49	45	
3T3, fibroblasts	Autoimmune antibodies	n/a	56	30	11
Rat spinal cord	Slow transport process	n/a[4]	212,160,68		12
Bovine axons	Flotation	0	60	120	13
BHK-21	Isolated caps	0	55,54	230	14,15
Neurons, varied	Flotation	+	200,160,68		16
Rat peripheral neuron	Osmotic lysis	0	68		17

Footnotes to Table 1

1. +, calcium was added to the preparation; -, free calcium was reduced by the addition of a chelating agent; 0, calcium concentrations were not controlled; n/a, the procedure was such that the concentration of free calcium was irrelevant.
2. Numbers in parentheses represent proteins visible on electrophoretic gels but not discussed by the authors.
3. DEAE is diethylaminoethyl derivatized cellulose; PC is phospho cellulose.

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CHAPTER TWO

Immunological Characterization of the Subunit of the 100 Å Filaments from Muscle Cells

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Biochemistry

Immunological characterization of the subunit of the 100 Å filaments from muscle cells

(actin/antibodies/immunofluorescence/sodium dodecyl sulfate-gel electrophoresis/tonofilaments)

ELIAS LAZARIDES* AND BRUCE D. HUBBARD

Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colo. 80309

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ABSTRACT **We report the immunological characterization of the subunit of the intermediate sized (100 Å) filaments from muscle cells. The protein as isolated from smooth muscle (chicken gizzard) has an apparent molecular weight of 50,000. It is insoluble in buffers that solubilize myosin and the majority of actin, but becomes soluble in the presence of urea. Under a variety of experimental conditions, that include the presence of 8 M urea, this new protein comigrates with actin during purification studies. The two proteins can be separated from each other by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and antibodies have been elicited against the 50,000 dalton protein purified by using this technique. These antibodies crossreact with the partially purified protein in urea, but show no detectable cross reaction with actin or myosin. Indirect immunofluorescence reveals that in skeletal muscle this protein is found in close association with the Z lines of the sarcomeres and extends between the Z lines of adjacent myofibrils; it is also associated with filamentous structures that run along the length of a muscle fiber both in close association with the plasma membrane and between myofibrils. These filaments appear to connect myofibrils to each other or to the plasma membrane at the level of their Z lines. In heart muscle, the protein shows the same distribution as in skeletal muscle. In addition, it is found intimately associated with intercalated disks and areas of membrane interaction between laterally associated heart muscle cells. The immunofluorescent localization to the subunit of the 100 Å filaments suggests that in muscle cells this molecule may serve to link actin filaments at the level of the Z line (or intercalated disk) with the muscle plasma membrane. We believe that it functions in muscle primarily as a three dimensional matrix which interconnects individual myofibrils to one another and to the plasma membrane at the level of their Z lines. In this manner, this molecule may provide a framework that mechanically integrates all the contractile myofilaments during the contraction and relaxation of muscle. As a means of indicating its linking role in muscle, we have termed the protein desmin (from the Greek δεσμός = link, bond).**

Higher eukaryotic cells contain in their cytoplasm three major filamentous structures: microtubules, actin filaments, and a class of intermediate sized filaments known as tonofilaments or 100 Å filaments due to their characteristic diameter as seen in the electron microscope. Intermediate sized filaments are present in such diverse cell types as fibroblasts, epithelial cells, skeletal muscle, smooth muscle, nerve cells, and glial cells (1–10). However, little is known about the cellular function of these filaments, and even less is known about their biochemical composition. A presumptive proteinaceous subunit of these filaments has recently been isolated and characterized biochemically and immunologically from neurons and glial cells (7–10), but the relationship of the protein found in nervous tissues to that in other cell types is presently unknown.

One of the most characteristic morphological features of the intermediate sized filaments is their intimate association in a variety of cell types with desmosomes, those distinctive structures that provide for attachment between adjacent cells within a tissue (for a review see ref. 11). The cytoplasm of both developing and adult smooth muscle cells contains a great number of these filaments which comprise a class distinct from the actin and myosin filaments that are involved in cellular contraction (12, 13). These filaments are known to insert characteristically into desmosome-like structures associated with membrane which are somehow involved in maintaining a strong cell to cell interaction between smooth muscle cells (14). We used smooth muscle as a representative differentiated cell system for the isolation and characterization of the subunit of the 100 Å filaments. In this paper, we report its immunological characterization from this type of muscle, and use indirect immunofluorescence to probe its localization in smooth, skeletal, and cardiac muscle cells.

MATERIALS AND METHODS

Antibody Preparation. Analytical and preparative slab gel electrophoresis with 12.5% gels was performed according to the discontinuous Tris-glycine system of Laemmli (15). The 50,000 molecular weight protein (desmin), for immunization, was purified from the 8 M urea extract of smooth muscle (see legend to Fig. 1f), by introducing as a final purification step preparative sodium dodecyl sulfate ($NaDodSO_4$)/slab gel electrophoresis (refs. 16 and 17; see *Results*). After preparative electrophoresis of the 8 M urea extract (Fig. 1f), the gels were stained for 15 min in 0.25% Coomassie brilliant blue, made up in 50% methanol (vol/vol) and 7.5% acetic acid (vol/vol), and then destained for 3 hr in 20% methanol (vol/vol) 7.5% acetic acid (vol/vol). The bands corresponding to a molecular weight of 50,000 were removed with a razor blade, and neutralized in the $NaDodSO_4$ running buffer (per liter: 14.4 g of glycine, 3 g of Tris base, and 1 g of $NaDodSO_4$ at pH 8.3). Several such bands were pooled and the 50,000 dalton protein was eluted electrophoretically as previously described (16, 17). Some of the polyacrylamide bands were homogenized directly in phosphate-buffered saline at pH 7.2 with the use of a Dounce homogenizer. The eluted protein and the protein containing gel homogenate were separately emulsified by using Freund's complete adjuvant. Because the eluted protein still contained appreciable amounts of $NaDodSO_4$, it was difficult to emulsify it directly with the adjuvant. Complete emulsification was achieved by inducing the protein/$NaDodSO_4$ solution to form a precipitate with $AlCl_3$. This was done by making the eluted protein solution 1% in $AlCl_3$ (wt/vol), and slowly neutralizing the mixture to pH 7.4 with 1 M NaOH. The Al/$NaDodSO_4$/protein precipitate that formed could be now emulsified completely with the adjuvant. Two white rabbits (New Zealand females) were injected subcutaneously each with approximately 500 μg of the eluted antigen and approximately 200 μg of the

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol.

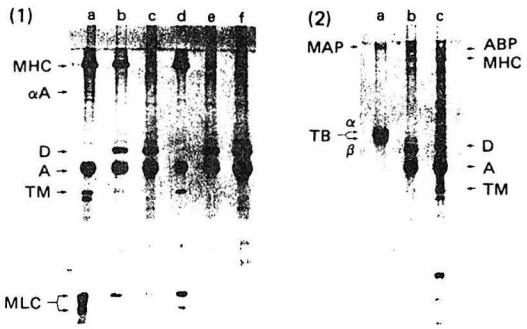
* To whom correspondence should be addressed at Division of Biology, California Institute of Technology, Pasadena, Calif. 91125.

homogenized gel at multiple separate sites on their backs. This procedure was repeated with the same quantities of antigen on the day 8, and the rabbits were bled by ear puncture on the day 15. Precipitating antibodies could be already detected by this time (Fig. 3B). The rabbits were reinjected subcutaneously on the day 21 with 500 μg of the eluted protein only, emulsified in Freund's incomplete adjuvant. The rabbits were bled by ear puncture a week later, and the last procedure of injection and bleeding was repeated once more. Serum was prepared by allowing the blood to clot, and the globulins were partially purified by precipitation with 48% ammonium sulfate at 4°. The precipitated globulins were dialyzed against 0.15 M NaCl, 0.02 M Tris·HCl at pH 7.8, 1 mM NaN_3, 5 mM ϵ-amino-n-caproic acid and stored at −20°, at an approximate concentration of 25 mg/ml. Both animals responded efficiently to the injected antigen. The results obtained with the globulin preparations from either of the two animals have been indistinguishable.

Indirect Immunofluorescence. For the preparation of skeletal myofibrils, skeletal muscle was allowed to stand for 48 hr at 4° in a muscle-relaxing solution containing 50% glycerol, 2.5 mM KCl, 0.014 M NaCl, 1 mM sodium phosphate buffer at pH 7.2, 0.2 mM $MgCl_2$, and 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid before blending. Heart muscle cells were incubated for 24 hr at 4° in the above relaxing solution in the presence of 25% glycerol (vol/vol). Exposure of heart muscle cells to low concentrations of Ca^{2+} ions causes the separation of individual heart muscle cells at their intercalated disks (18). The cardiac tissue was gently disrupted with a Dounce homogenizer, thus causing the dispersal of individual myocardial cells which retained one or both of their terminal intercalated disks as well as their lateral cellular connections. Frozen sections of skeletal muscle and intestine were obtained by using conventional techniques after embedding the material in OCT compound (Tissue-Tek II; Lab-Tek Products), and immersing them in liquid nitrogen. The sections, approximately 4 μm thick, were placed on slides, immersed into 95% ethanol at room temperature, and air dried before the application of the antibodies. Skeletal myofibrils and heart muscle cells were concentrated by centrifugation, and suspended directly in approximately 0.5 mg/ml of the globulin fraction containing antibody against desmin. An equivalent concentration of globulin from control serum showed no detectable reaction with any of the sarcomere structures. Indirect immunofluorescence was performed as previously described (19). The specimens were viewed under a Leitz microscope equipped with epifluorescent optics. Pictures were taken with TriX film using a 100X immersion lens, and developed in Diafine (Acufine, Inc.).

RESULTS

For the isolation and characterization of the putative subunit of the 100 Å filaments, we made use of the observation that in smooth muscle (chicken gizzard) this class of filaments is insoluble at salt concentrations that render the myosin and actin filaments soluble (14, 20). Fig. 1 shows that after prolonged extraction of actin and myosin with 0.6 M KCl, the insoluble residue (Fig. 1b) is enriched in a new protein with an apparent molecular weight of 50,000 (hereafter called desmin, see *Discussion*). The majority of the salt insoluble myosin and a substantial amount of the insoluble actin can now be extracted by moderate concentrations of potassium iodide (0.6 M KI; Fig. 1d), an agent that is known to depolymerize actin filaments and to dissociate actin from myosin (21). The KI insoluble material is highly enriched in desmin, but still contains a considerable amount of actin which does not seem to be soluble under conditions that solubilize the majority of muscle actin (Fig. 1c and e). Because such salt insoluble extracts do not contain any actin or myosin filaments, but are highly enriched in 100 Å filaments (20), we believe that the protein which is enriched in these salt insoluble extracts (desmin) is probably the major subunit of these filaments. The solubility properties of the actin that remains insoluble with desmin are unusual for conventional muscle actin. It has been tentatively identified as actin on the basis of its molecular weight (42,000), its reaction with antibodies raised against smooth muscle (gizzard) actin, and its isoelectric point. Actin and desmin can now be extracted in the presence of urea (Fig. 1f).



FIGS. 1 AND 2. *Fig. 1.* Partial purification of desmin from smooth muscle. Frozen chicken gizzards (500 g), were thawed and freed of the surrounding cartilagenous matrix. The muscle was thoroughly washed at 4° with 2 liters of distilled H_2O containing 1 mM dithiothreitol (DTT), 1 mM p-tosyl-L-arginine methyl ester, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 25 units/ml of Trasylol (Aprotinin, Sigma). The muscle was subsequently blended in the above solution in a Sorvall omnimixer for approximately 5 min, and the blended muscle was recovered at 8000 × g. The pellet was then successively extracted for 3 hr, 6 hr, 12 hr, 12 hr, and finally 24 hr in 500 ml of fresh buffer containing 0.6 M KCl, 20 mM Tris·HCl at pH 8.7, 1 mM DTT, 1 mM p-tosyl-L-arginine methyl ester, 0.5 mM phenylmethylsulfonyl fluoride, and 25 units/ml of Trasylol and the insoluble material was recovered at 8000 × g. (a) 0.6 M KCl final soluble proteins. (b) 0.6 M KCl final residue. This residue was freshly extracted three times for 12 hr each with 500 ml of 0.6 M KI, 20 mM sodium thiosulfate, 1 mM DTT, 20 mM Tris·HCl, at pH 8.7, and 1 mM ATP. Practically all of the residual actomyosin is solubilized within the first 2 hr. The insoluble material was recovered by centrifugation at 9000 × g. (c) Initial KI residue. (d) KI soluble material. (e) Final KI residue. This KI insoluble material (Fig. 1e) was extracted with 500 ml of a buffer containing 8 M urea (Ultra Pure, Schwarz/Mann), 2 mM DTT, 20 mM Tris·HCl at pH 8.7, for 12 hr, and the insoluble material was removed at 20,000 × g for 20 min and subsequently at 100,000 × g for 2 hr. (f) Final soluble proteins. *Fig. 2.* Comparative electrophoresis of porcine brain tubulin and desmin. Porcine brain tubulin was a generous gift of J. A. Snyder and J. R. McIntosh. (a) Approximately 8 μm of tubulin. (b) Ten micrograms of 8 M urea-soluble material of gizzard. (c) Approximately 10 μg of gizzard from a day-old chicken which was denatured and reduced directly in $NaDodSO_4$ sample buffer by placing in a boiling H_2O bath for 6 min. A, actin; D, desmin; MHC, myosin heavy chain; MLC, myosin light chains; ABP, actin binding protein (24, 25); α-A, α-actinin; TM, tropomyosin; TB, tubulin; MAP, microtubule associated proteins.

Assuming that the molecular weights of the α and β subunits of tubulin are 56,000 and 54,000, respectively (22), and that of actin is 42,000 (23), Fig. 2 indicates that the apparent molecular weight of desmin is 50,000.

Because desmin comigrates with the residual salt insoluble actin, we used preparative $NaDodSO_4$/gel electrophoresis as a final step in the purification of this protein prior to its use as

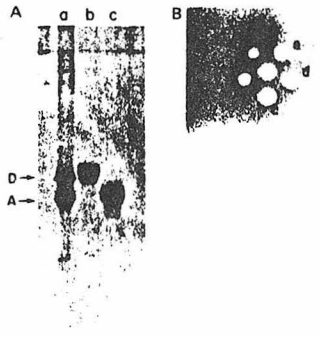


FIG. 3. (A) Analytical electrophoresis of desmin and actin after purification by preparative $NaDodSO_4$/polyacrylamide slab gel electrophoresis. (a) The 8 M urea extract (Fig. 1f) that was used for preparative purification of the two proteins. (b) Re-electrophoresed desmin and (c) re-electrophoresed actin. Preparations similar in purity to those depicted in this picture were used as antigens for the production of antibodies to desmin and actin (19). When only small amounts of desmin (approximately 1 μg) are re-electrophoresed, the protein separates into a closely spaced doublet that is poorly resolved under the reducing and nonalkylating conditions of electrophoresis employed here. (B) Double immunodiffusion of the partially purified desmin and the globulin preparation obtained against the electrophoretically purified antigen. The immunodiffusion plates were 1% agar equilibrated in 0.6 M KCl, 0.02 M Tris·HCl at pH 8.7. (a) Ten micrograms of the 8 M urea soluble extract (Fig. 1f). The buffer of this protein solution was 8 M urea, 0.02 M Tris·HCl at pH 8.7, 2 mM DTT. (b) Approximately a 10-fold dilution of the protein solution in (a). (c) Ten micrograms of smooth muscle actomyosin (Fig. 1a) in 0.6 M KCl, 0.2 M Tris·HCl at pH 8.7, 1 mM DTT, and the proteolytic inhibitors described in the legend of Fig. 1. (d) Eight microliters of the urea buffer of the samples in (a) and (b). (e) Five micrograms of skeletal muscle α-actinin in 0.6 M KCl, 0.02 M Tris·HCl at pH 8.7 (19). Immunodiffusion was for 72 hr at 4°. The agar plates were subsequently washed for 76 hr in 0.6 M KCl, 0.02 M Tris·HCl at pH 8.7, stained for 30 min with 0.25% Coomassie brilliant blue made up in 50% methanol and 7.5% acetic acid, and destained with 20% methanol and 7.5% acetic acid. For abbreviations, see legend to Figs. 1 and 2.

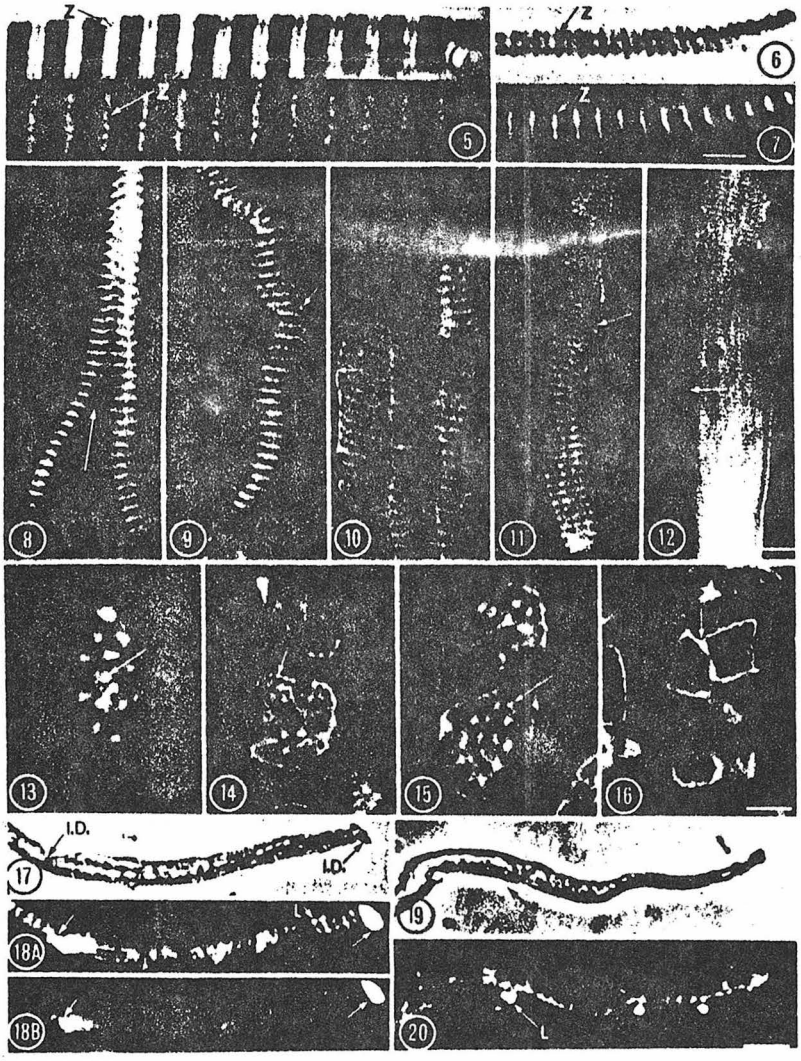
an antigen. Fig. 3A indicates that upon re-electrophoresis on an analytical $NaDodSO_4$/gel, desmin migrates with the same molecular weight as the original protein and it shows no detectable contamination by actin or any other protein of different molecular weight. In double immunodiffusion, the antiserum against desmin reveals only one detectable precipitin line with the protein partially purified in the presence of urea, but no detectable crossreaction with actin, myosin, α-actinin, or the 34,000 subunit of smooth muscle tropomyosin (Fig. 3B). These results, in conjunction with the immunofluorescent results reported below, indicate that the antiserum against desmin is specific for the 50,000 molecular weight desmin, and carries no detectable antibodies to any of the other known major contractile proteins of smooth muscle.

Indirect immunofluorescence shows desmin localized along the Z lines of isolated, relaxed, or contracted skeletal myofibrils (Figs. 4–7). Mechanical shearing of myofibrils results in the stretching of a slender desmin containing link between two adjacent Z lines (Fig. 8) that indicates this molecule extends between the Z lines of adjacent myofibrils. Myofibrils separated this way can sometimes be seen to contain a "knob"-like fluorescent structure at the ends of their Z lines (Fig. 9, arrow). These are presumably the sites at which desmin snaps back when the two adjacent myofibrils are finally sheared away from each other at their respective Z lines. The split appears to take place on one or the other side of each "knob"-like structure with the result that desmin snaps back only on one or the other Z line (compare Figs. 8 and 9). Within a muscle fiber, desmin is found associated with fluorescent filamentous structures that run along the long axis of the fiber both in close association with the plasma membrane and within the fiber between the Z lines. These structures appear to interconnect myofibrils to each other and to the plasma membrane at the level of their Z lines (Figs. 10 and 12, arrows). When a bundle of myofibrils is sheared away from the rest of the muscle fiber, the shearing takes place along the filaments found inside along the long axis of the fiber (Fig. 10 upper). The resulting myofibril bundles have the same fluorescent "knob"-like structures at the ends of their Z lines (Fig. 11), as they do in individual myofibrils. These "knob"-like structures appear to be the sites of attachment of the myofibril bundle's Z lines to the filaments that run along the length of the fiber either within the fiber or in close association with the fiber's plasma membrane. In a whole muscle fiber, desmin is localized in a diffuse form as well as at the Z lines and in close association with the plasma membrane (Fig. 12). The close association of desmin with filamentous structures that are in turn associated with the fiber's plasma membrane, is clearly demonstrated in cross sections of the fibers (Figs. 13–16). Desmin is also found in areas where two muscle fibers make contact (Fig. 16), as well as in a fine transverse filamentous matrix that appears to interconnect the filaments that run along the long axis of the fiber, within the fiber and parallel to the plasma membrane (Figs. 14–15).

In isolated heart muscle cells, desmin is found intimately associated with intercalated disks (Figs. 17–18), and with areas where adjacent heart muscle cells make contact (Figs. 19–20). In addition, desmin shows the same basic distribution as it does in skeletal myofibrils; it is found associated with cardiac myofibril Z lines and in filamentous structures that extend between the two intercalated disks of the myocardial cell and onto which a great number of Z lines appear to connect (Figs. 17–20). In frozen sections of intestine, the desmin antibody reacts with filamentous structures in the cytoplasm of the intestinal smooth muscle cells that run along the whole length of these cells (Fig. 21). The antibody does not seem to react with the intestinal brush border which can be shown to react intensely with antibodies to actin (unpublished observations). Furthermore, the desmin antibody also reacts with filamentous structures distinct from actin filaments in gizzard smooth muscle and in blood vessels. Desmin also shows a perinuclear localization in differentiating muscle cells growing in tissue culture and treated with colcemid, which is a characteristic distribution of the 100 Å filaments in cells exposed to this drug (1–3) (E. Lazarides, in preparation).

DISCUSSION

In smooth muscle, 100 Å filaments and actin filaments appear to terminate in electron dense material that is found associated all along the cell's plasma membrane, especially in areas where two smooth muscle cells share an intercellular junction (12, 14). This electron-dense material associated with membranes is morphologically indistinguishable from the electron-dense material of the fasciae adherentes which are the sites within a cardiac intercalated disk where the actin filaments of the terminal sarcomere terminate and insert into the plasma membrane. Quite frequently, the electron-dense material of the fasciae adherentes is continuous with intervening desmosomes and 100 Å filaments were observed in association with these two structures in both developing and adult cardiac muscle cells



FIGS. 4–20. *Figs. 4–7.* The localization of desmin in the Z lines of chicken skeletal myofibrils. Myofibrils were reacted with the desmin antibody in indirect immunofluorescence, and viewed with phase contrast (Figs. 4 and 6) and epifluorescent (Figs. 5 and 7) optics. The arrows termed Z refer to the localization of the Z line within a sarcomere. Figs. 4 and 5 are those of a relaxed myofibril (distance between two Z lines approximately 2.6 μm), while Figs. 6 and 7 are those of a contracted myofibril (distance between two Z lines approximately 1.6 μm; bar = 3.2 μm). Within a sarcomere, a I band seems to be the weakest part of the myofibril structure, and mechanical shearing of muscle breaks myofibrils through the I bands on one or the other side of the Z line (18). Such myofibrils have a Z line as their terminal structure. Note in Figs. 4–7 (right) that this is indeed the case in these myofibrils and that the terminal Z lines react with the desmin antibody. Occasional longitudinal fluorescent connections between Z lines are evident (sixth fluorescent Z line from the right in Fig. 7; see also below). *Figs. 8–12.* Sheared products of muscle revealing the localization of desmin in a muscle fiber. See *text* for figure explanation. Bar = 7 μm for Figs. 8–12. *Figs. 13–16.* The localization of desmin in frozen cross sections of skeletal muscle. For details see the *text*. Bar = 11 μm for Figs. 13–15. Fig. 16, bar = 8 μm. Pictures were taken using an oil immersion 63× lens. *Figs. 17–20.* The localization of desmin in isolated heart muscle cells. Figs. 17 (phase optics) and 18 (epifluorescent optics) show a heart muscle cell which has retained its intercalated disc (I.D.) on the right side and is still connected to another heart cell with its intercalated disc on the left side. Fig. 18B is a repeat of Fig. 18A, but which has been printed darker to reveal more clearly the presence of desmin at the I.D.s. Figs. 19 (phase optics) and 20 (epifluorescent optics) show the presence of desmin in areas where two heart cells make lateral (L, see also Fig. 18A) connections. Note the apparent attachment of these lateral fluorescent connections to fluorescent "knob" like material that is associated with the heart myofibril Z lines (bar = 7 μm in Figs. 17–20).

(26–28). Morphologically, the area of the fascia adherens was considered as the terminal Z line and it was assumed that these two structures are biochemically related (26–28). In developing skeletal or in heart muscle cells, Z lines are quite frequently seen to originate from intercellular desmosomes or from electron dense membrane continuations of such areas which resemble morphologically the electron-dense material of the fascia adherens (26, 27, 29). Similarly in adult cardiac or skeletal muscle,

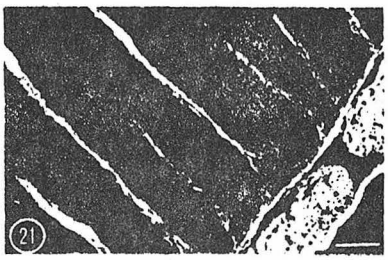


FIG. 21. The localization of desmin in intestinal smooth muscle. Similar results have been obtained with gizzard smooth muscle. Note that the epithelial cells also show a small amount of reaction which may indicate an antigenic crossreaction of the epithelial and muscle desmin (100 Å filaments; bar = 70 μm). Pictures were taken with a 25× lens.

Z lines are often seen in association with intercellular junctions that are located along the longitudinal surfaces of these cells (30). On the basis of this morphological evidence, the assumption was made that the electron-dense material found in association with the muscle Z lines and the muscle intercellular junctions are biochemically related and share at least one structural component (12, 26–28). The immunological evidence presented here indicates that one of the structural proteins they may share is the subunit of the 100 Å filaments.

By the immunofluorescent localization of this molecule, we believe that in muscle cells the 100 Å filaments and/or their protein subunit may mediate the attachment of actin filaments to specialized areas of the plasma membrane (Z lines to plasma membrane associated intercellular junctions terminal sarcomere actin filaments to fasciae adherentes in intercalated disks, and actin filaments to dense bodies in smooth muscle). Because muscle cells strongly interact at areas where 100 Å filaments insert to the plasma membrane, we envision that this class of filaments or their subunit functions as a three dimensional matrix which interconnects individual myofibrils to one another and to the plasma membrane at the level of their Z lines. Such an interconnecting matrix may provide tensile strength to the muscle and ensure that each and all the myofibrils, and each and all the muscle cells, are mechanically integrated during the contraction and relaxation of muscle. To indicate the linking function that this molecule might have in muscle cells and as a means of summarizing its fluorescent localization, we have termed the protein desmin (from the Greek δεσμός = link, bond).

The name desmin denotes that this protein, in addition to being the subunit of the 100 Å filaments, is also a component of muscle desmosome-like structures. It should be realized that we do not know yet whether the same protein is also a component of the more conventional types of desmosomes and 100 Å filaments that are seen in epithelial cells. Similarly, it should be emphasized that the conclusive identification of desmin as the subunit of the 100 Å filaments requires further purification of this molecule and the demonstration *in vitro* that it can assemble into filaments with an approximate diameter of 100 Å.

The role of the actin that copurifies with desmin in the structure of 100 Å filaments is presently unknown. Its comigration with desmin is intriguing, especially because it has solubility properties which are characteristically distinct from those of conventional muscle actin.

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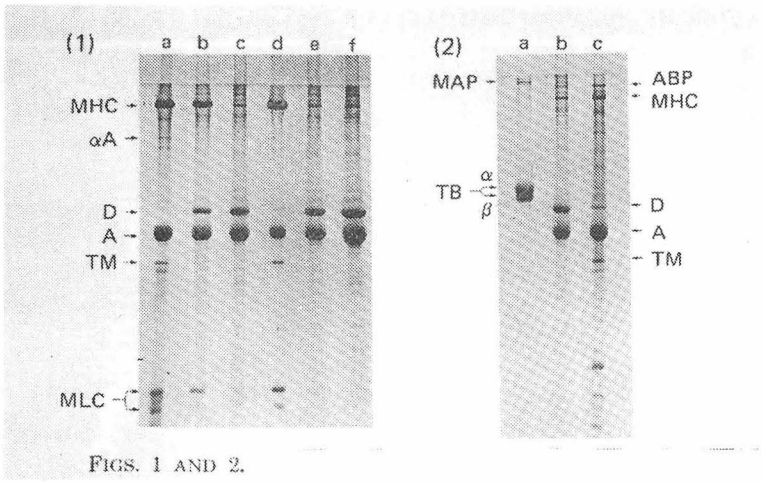


FIGS. 1 AND 2.

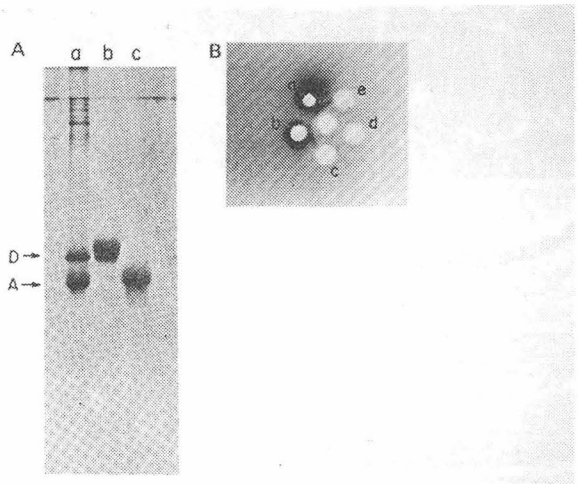
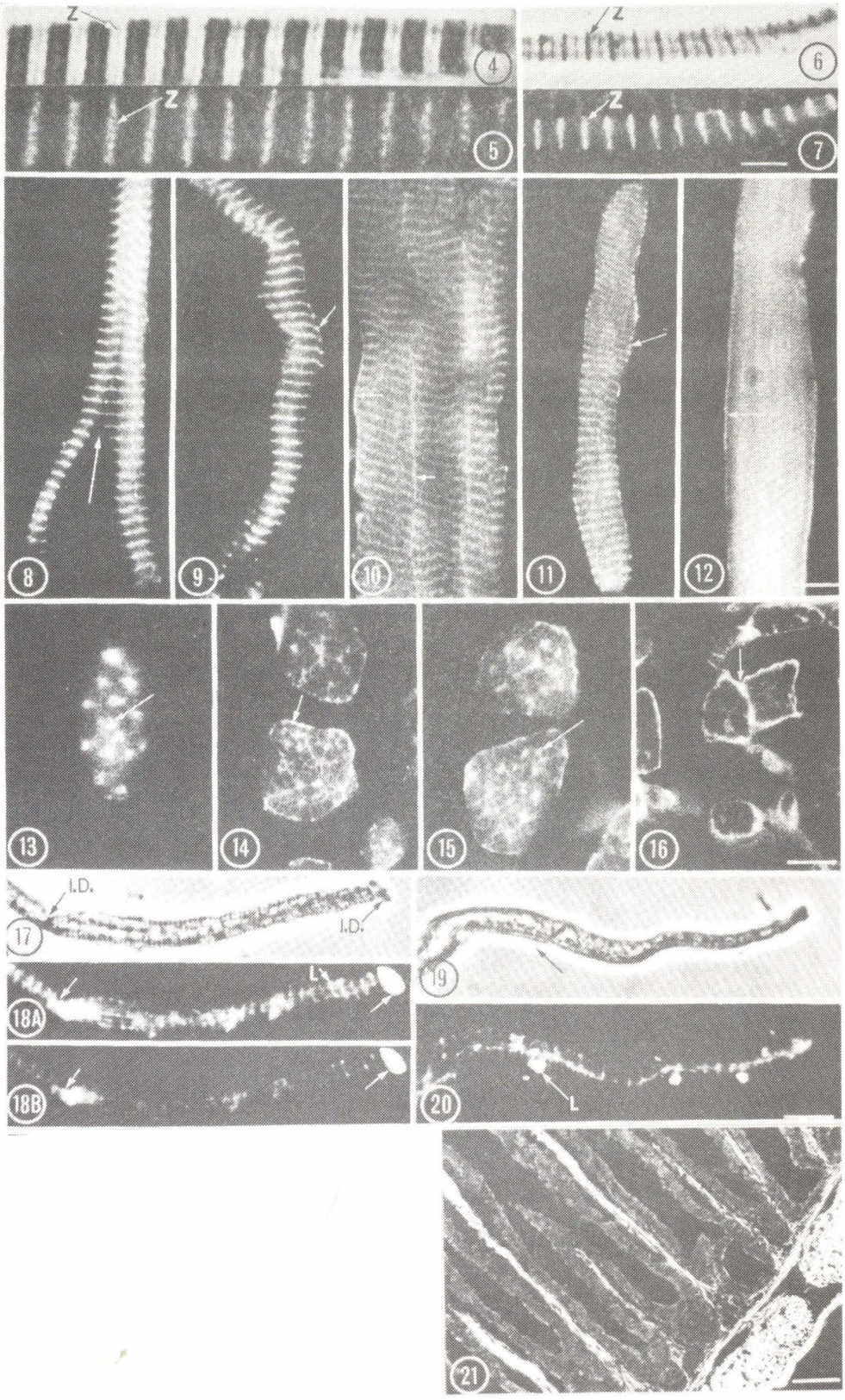


FIG. 3.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CHAPTER THREE

Copurification of Actin and Desmin from Chicken Smooth Muscle and Their Copolymerization *in vitro* to Intermediate Filaments

COPURIFICATION OF ACTIN AND DESMIN FROM CHICKEN SMOOTH MUSCLE AND THEIR COPOLYMERIZATION IN VITRO TO INTERMEDIATE FILAMENTS *

BRUCE D. HUBBARD and ELIAS LAZARIDES

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

ABSTRACT

Desmin is a 50,000-mol wt protein that is enriched along with 100-Å filaments in chicken gizzard that has been extracted with 1 M KI. Although 1 M KI removes most of the actin from gizzard, a small fraction of this protein remains persistently insoluble, along with desmin. The solubility properties of this actin are the same as for desmin: they are both insoluble in high salt concentrations, but are solubilized at low pH or by agents that dissociate hydrophobic bonds. Desmin may be purified by repeated cycles of solubilization by 1 M acetic acid and subsequent precipitation by neutralization to pH 4. During this process, a constant nonstoichiometric ratio of actin to desmin is attained.

Gel filtration on Ultrogel AcA34 in the presence of 0.5% Sarkosyl NL-97 reveals nonmonomeric fractions of actin and desmin that comigrate through the column. Gel filtration on Bio-Gel P300 in the presence of 1 M acetic acid reveals that the majority of desmin is monomeric under these conditions. A small fraction of desmin and all of the actin elute with the excluded volume. When the acetic acid is removed from actin-desmin solutions by dialysis, a gel forms that is composed of filaments with diameters of 120–140 Å. These filaments react uniformly with both anti-actin and anti-desmin antiserum. These results suggest that desmin is the major subunit of the muscle 100-Å filaments and that it may form nonstoichiometric complexes with actin.

KEY WORDS 100-Å filaments · immunofluorescence · α-actinin · Z disk

A group of filaments with diameters of ~100 Å has been described in several widely differing cell types. They are known as "neurofilaments" in neurons (31, 43), "glial filaments" in glial cells and astrocytes (43), "10-nm filaments" in cultured nonmuscle cells (3, 11, 13), and "intermediate filaments" in muscle (1, 5, 6, 39).

The putative subunits of 100-Å filaments have been isolated from nerve cells (9, 30), glial cells (2, 7, 28), cultured cells (13, 34), and smooth muscle cells (5, 22, 33). Immunological and biochemical comparisons have indicated that the subunits of neurofilaments, glial filaments, and intermediate filaments are chemically distinguishable (2, 7, 8, 21), and that they are apparently unrelated to the keratin which makes up epidermal tonofilaments (4, 35). 100-Å filaments thus form a heterogeneous group, although the extent of this heterogeneity has not yet been determined.

Biochemical characterization of the subunit of intermediate filaments has recently become possible with the demonstration that the extraction of smooth-muscle actomyosin at high ionic strength leaves an insoluble residue which is enriched in 100-Å filaments (6). There are two predominant

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proteins in this residue: actin and a 50–55,000-mol wt protein (22). This latter protein has been characterized as the major subunit of muscle intermediate filaments by several researchers (5, 22, 33). We have isolated it from chicken gizzard as a 50,000-mol wt protein which we call desmin (22).

In smooth muscle cells, one of the most characteristic morphological features of the intermediate-sized filaments is their insertion into cytoplasmic and membrane-bound dense bodies and their intimate associations with actin filaments at these sites (1, 5, 39). In skeletal muscle, immunofluorescence reveals that desmin is localized at the Z lines and where the Z lines come into apposition with the plasma membrane. Desmin is also found at the Z lines and intercalated disks of cardiac muscle (22). These are all sites where actin structures are linked either together or to membranes. From these distributions, we concluded that desmin forms a network in muscle cells which interlinks individual myofibrils, at their Z disks, into a single integrated mechanical unit and also functions in the linkage of this unit to the plasma membrane.

In this paper, we present evidence that actin and desmin copurify from extracts of chicken gizzard and copolymerize into 100-Å-like filaments. These results suggest that desmin and actin may form a stable complex and provide an insight to the molecular basis of how desmin may function to link actin filaments in muscle cells.

MATERIALS AND METHODS

Materials

From Eastman Organics (Rochester, N.Y.): acrylamide (No. 5221) for slab gels, acrylamide (No. X5521) for isoelectric focusing gels, and N,N'-methylene bisacrylamide (No. 8383) were used as supplied without further purification. Sarkosyl NL-97 (Sarkosyl) is a trademark of CIBA-GEIGY (Greensboro, N.C.); Nonidet NP-40 is a trademark of Shell Chemical Co. (New York). Sodium dodecyl sulfate (SDS) was obtained from Serva (Heidelberg, West Germany); Ultrogel and Ampholines were products of LKB Produkter (Bromma, Sweden); urea was the ultrapure grade from Schwarz/Mann (Orangeburg, N.Y.); Bio-Gel was a product of Bio-Rad (Richmond, Calif.). Fluoresceine-labeled IgG fraction of goat anti-rabbit IgG was obtained from Miles Laboratories (G5-173-1; Miles Labs Inc., Elkhart, Ind.). Chicken gizzard was obtained from Pel-Freeze (Rogers, Ark.). Plus-X film (Kodak) was developed in Diafine (Accufine Corp., Chicago, Ill.). Elvanol is a water-soluble polyvinyl alcohol (Du Pont 51-05, E. I. Du Pont de Nemours, Wilmington, Del.). All other chemicals were of reagent quality. Dialysis tubing was number 8 from Union Carbide (Chicago, Ill.). Ethylene glycol-bis-(β-aminoethyl ether)*N*,*N*'-tetra acetic acid is EGTA.

Analytical Methods

Protein concentration was determined relative to a bovine serum albumin (BSA) standard by an elevated temperature modification of the Lowry method (29). All buffer pH values were determined at 20°C. Electrophoretic recipes are all wt/vol.

Dialysis

Dialysis tubing was prepared by simmering it in 0.1 M NaOH and 10 mM EDTA for 8 h. The tubing was then neutralized with Tris-HCl, pH 6.8, extensively rinsed with water, and stored in water at 5°C.

One-Dimensional SDS Slab Gel Electrophoresis (SDS-PAGE)

One-dimensional electrophoretic analysis of proteins was performed on high-resolution SDS-polyacrylamide slab gels (SDS-PAGE) by a modification of the discontinuous Tris-glycine buffer system (18). The stacking gel contained: 5% acrylamide, 0.13% N,N'-methylene bisacrylamide, 0.125 M Tris-HCl, pH 6.8, and 0.1% SDS. The quantities of acrylamide and of bisacrylamide in the analytical (lower) gel were provided by a hyperbolic relationship: % acrylamide × % bisacrylamide = 1.3. Gels containing 12.5% acrylamide were used most often because of their high resolution in the molecular-weight range of actin and desmin. A 12.5% analytical gel contained: 12.5% acrylamide, 0.107% bisacrylamide, 0.386 M Tris-HCl, pH 8.7, and 0.1% SDS. Polymerization was catalyzed by the addition of 100 μl of 10% ammonium persulfate and 10 μl of N,N,N',N'-tetramethylethylenediamine/30 ml of gel solution. The same running buffer was used in both the upper and lower reservoirs: 0.025 M Tris base, 0.112 M glycine, and 0.1% SDS, final pH 8.5. Sample buffer (2 ×) contained 0.1 M dithiothreitol, 0.08 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and bromophenol blue. After electrophoresis, gels were stained overnight in 50% ethanol, 10% acetic acid, and 0.05% Coomassie Brilliant Blue R-250. Gels were destained by three changes of 10% ethanol, 5% acetic acid, and photographed over a light box with Polaroid PN-55 film (Polaroid Corp., Cambridge, Mass.), using an orange-colored filter to enhance contrast. In the figures, most of the well-stained, compact bands represent 2-4 μg of protein, while faint bands may represent <0.1 μg. In our experience, SDS-electrophoresis systems other than discontinuous Tris buffered slab PAGE do not adequately resolve or visualize significant minor components in desmin preparations.

The effluents of gel-filtration columns were analyzed

by SDS-PAGE. For fractions containing Sarkosyl NL-97, aliquots were mixed directly with 0.2 vol of 5 × SDS sample buffer and heated briefly before loading on gels. For fractions containing acetic acid, aliquots were dialyzed first against distilled water, lyophilized if necessary, and then mixed with SDS sample buffer.

Two-Dimensional Isoelectric-Focusing SDS-Gel-Electrophoresis (IEF/SDS-PAGE)

Two-dimensional electrophoresis was carried out according to the system of O'Farrell (24). The first dimension (isoelectric focusing) was prepared and pre-run as described (15), but with the following modifications: the gels (2.5 × 120 mm) contained 0.2% Ampholines, pH range 3.5–10; 0.8% Ampholines, pH range 4–5; and 2% Ampholines, pH range 5–7 (each supplied as 40% solution). Ampholines were not added to overlay solutions nor to lysis buffers. Samples (see below) were loaded, overlayed directly with 0.02 M NaOH, and run at 450 V for 16 h and then at 800 V for 1 h. Samples of native proteins were dissolved in 8 M urea at room temperature for 1 h. They were then made 1% in Nonidet NP-40 and 0.5% in 2-mercaptoethanol. The samples were subsequently isoelectric focused as described above, but without being heated.

Microscopy

Unless otherwise specified in the text, all desmin gels for microscopy were induced from cycle-2 acetic acid extracts (see below). Samples for electron microscopy were placed on carbon-coated 400-mesh copper grids. Because many of the samples were bulky, insoluble gels, it was found helpful to place a drop of water on the grid and then wipe the gel across it. These preparations were stained for 2 min with a 2% aqueous solution of uranyl acetate, excess stain was drawn off with a filter paper, and the grids were then air dried. The specimens were observed in a Philips EM 201 electron microscope operated at 80 kV, and photographed with a 35-mm camera. Final magnifications were determined from the calculated values for the 35-mm camera.

Samples of desmin gels for fluorescence microscopy were placed on glass coverslips and spread by gentle flattening against a slide. This causes some of the gel to adhere to the coverslip in a thin layer. The coverslips were then dehydrated for 10 min in 95% ethanol, rehydrated in calcium- and magnesium-free phosphate-buffered saline at pH 7.4 (PBS), and stained for indirect immunofluorescence as described (19). Antibodies against actin (19) and desmin (22) were the same as those described. They were prepared against proteins purified to apparent homogeneity from smooth muscle (chicken gizzard). The coverslips were mounted on slides with a drop of Elvanol as mounting medium. Photomicroscopy was performed on a Leitz microscope equipped with a fluorescence epi-illumination system and Leitz FITC filter module H. Samples were photographed through a × 100 oil immersion phase objective. Plus-X film was exposed at Din 28 and developed in Diafine. Magnification was determined by photographing the lines ruled on a hemacytometer.

Enrichment of Gizzard Preparations for Desmin (see Scheme I)

Desmin was extracted from chicken smooth muscle by modifications of previous methods (6, 22, 33). All procedures were performed at 5°C. The details are presented in Scheme I. During the extractions, gelatinous masses formed and were discarded. They were not analyzed. The final KI-insoluble residue (KI-residue) was washed with water to reduce the KI concentration to below 1 mM and stored as a thick slurry in the presence of 10 mM NaN_3. KI-residue stored as an actual pellet tended to solidify with time. Freezing (at −20°C) hastened this process.

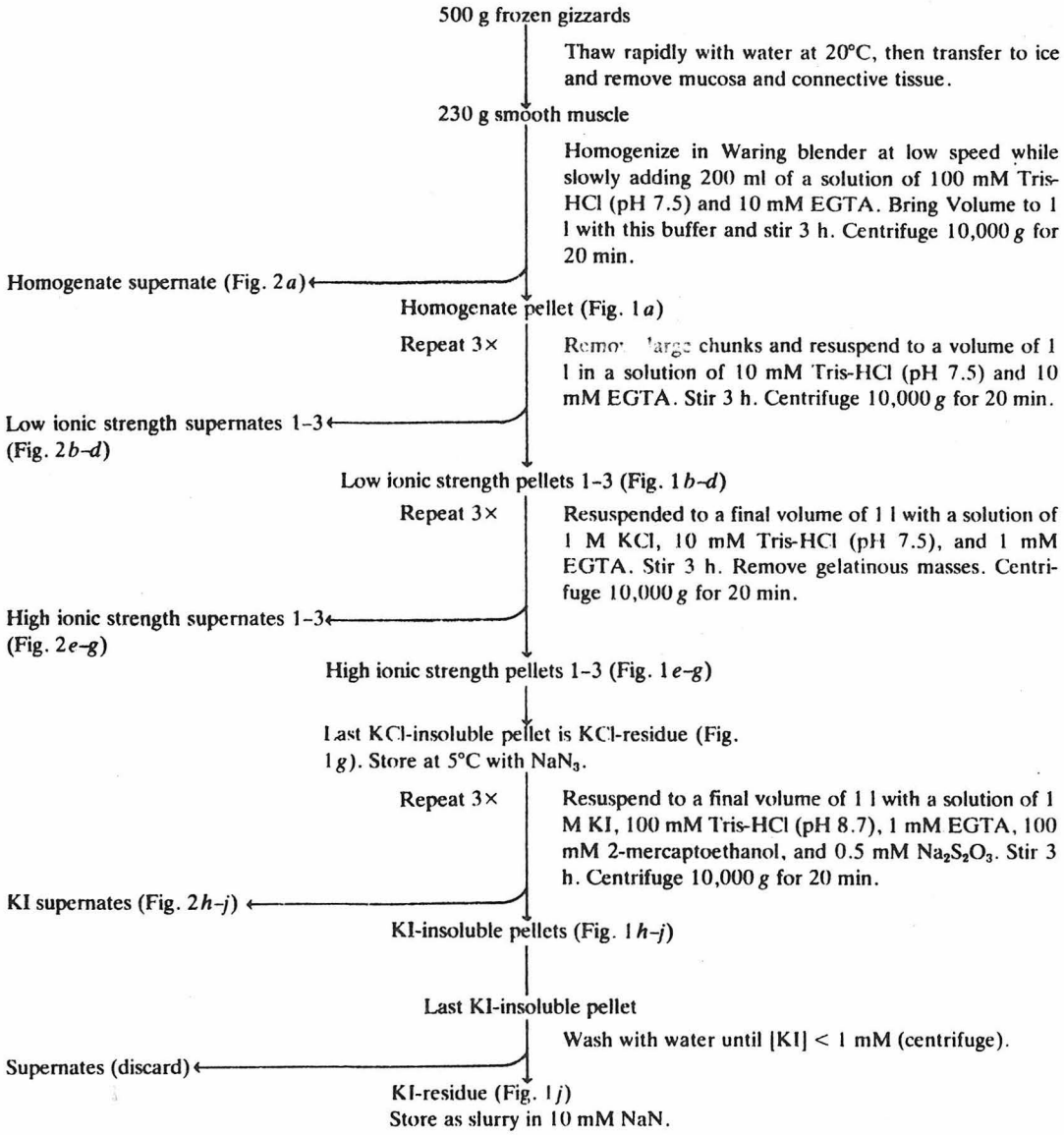
Water-washed KCl- or KI-residue pellets were made into acetone powders (KCl-AP, KI-AP) by suspending them in an equal volume of water to prevent clumping. This suspension was then mixed with 3–5 vol of cold acetone, stirred for 1 h, and spun out. This was repeated with more acetone until the final suspension contained <5% of the original water. The final acetone-insoluble residue was air dried overnight and stored at −20°C.

Purification of Desmin from KCl- and KI-residues (see Scheme II)

Desmin was extracted from KCl- or KI-residues with acetic acid at low temperatures, because this resulted in the least number of artifactual charge modifications observable by IEF/SDS-PAGE (see Results). The details of this procedure and the notations used for the various desmin extracts are given in Scheme II. Cycle 2 desmin was used in most experiments.

Desmin was extracted from acetone powders as described in Scheme II but with the following modification: the acetone powder was first extracted at room temperature with water and then washed on a Büchner funnel to remove soluble actin and tropomyosin. The washed material was then extracted with acetic acid as described above.

Purified desmin was occasionally stored by dissolving it in 1 mM HCl and keeping this solution frozen at −80°C. Desmin was stable for up to 2 wk under these conditions. If desmin was stored as a precipitate at pH 4 in acetic acid/acetate or at pH 7.5 in Tris-HCl, it tended to become acid insoluble with time. Freezing either KI-residues or desmin precipitates for several weeks also caused desmin to become acid insoluble. In the cyclic purification procedure of Scheme II, desmin was found to be soluble in acid only at low ionic strength. Desmin was not solubilized away from contaminating proteins if



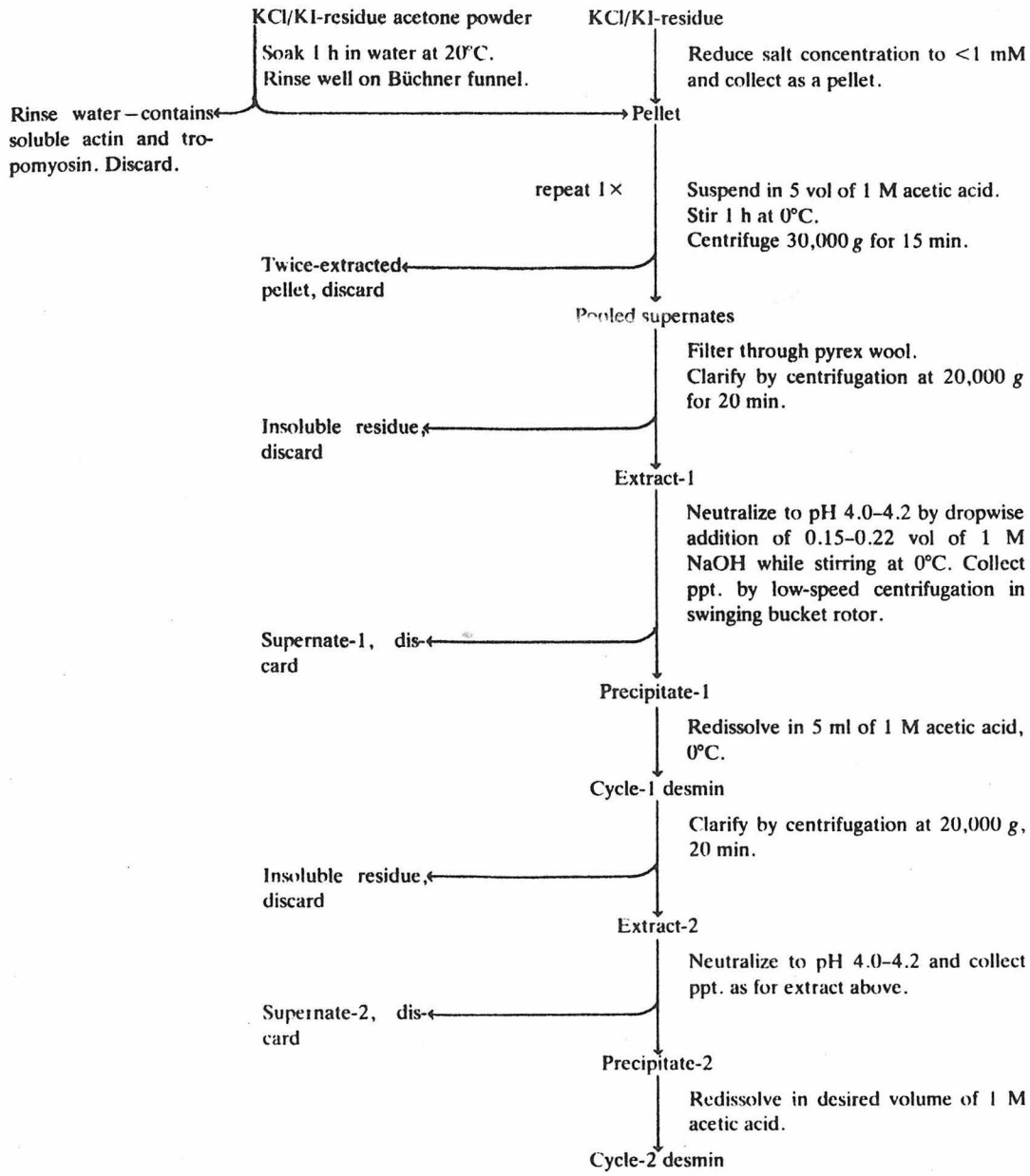
SCHEME 1
Preparation of KCl- and KI-Insoluble Residues of Gizzard

too much salt remained in the precipitate. Desmin precipitates are very sticky, especially towards glass, and the manipulation of them was minimized to reduce losses.

RESULTS

Copurification of Actin and Desmin

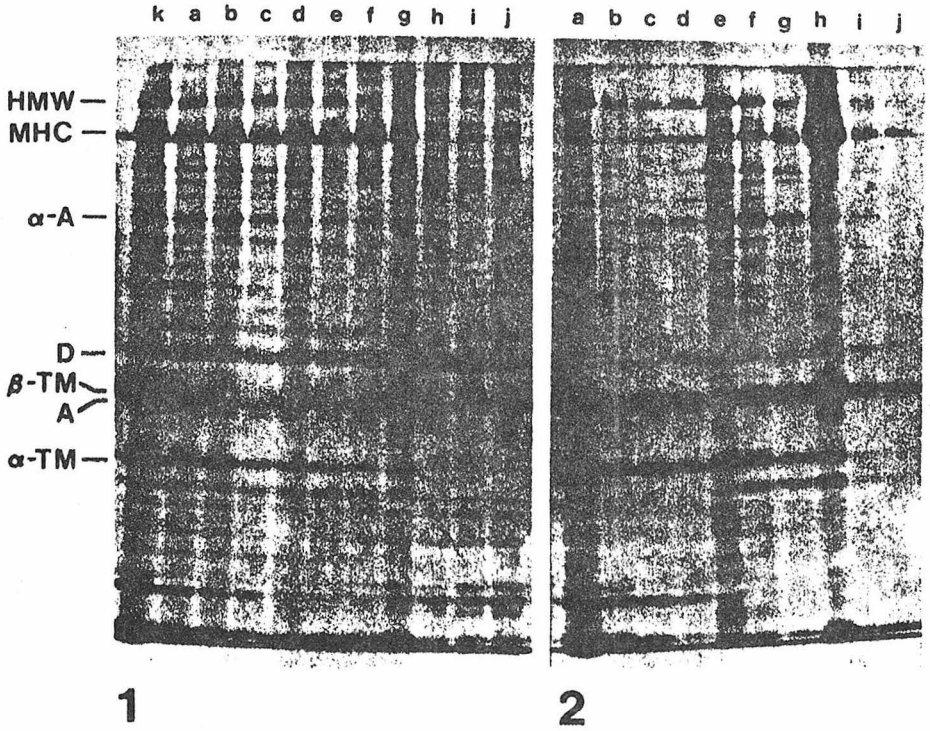
PREPARATION OF EXTRACTED CHICKEN-GIZZARD RESIDUES THAT ARE ENRICHED IN DESMIN (SCHEME I): Figs. 1 and 2 show the proteins that are extracted (Fig. 2) and that remain insoluble (Fig. 1) at each step of the extraction procedure described in Scheme 1. The presence of 100 mM Tris-HCl in the homogenization buffer releases some actomyosin (Fig. 2*a*). During the next three low ionic strength (LI) extractions (20 mM Tris-HCl), actin and tropomyosin, but not myosin, are released (Fig. 2*b–d*). As the LI extractions proceed, α-actinin, desmin,



SCHEME II

Cyclic Purification of Desmin Extracted with Acetic Acid

and a high molecular weight protein (HMW) begin to appear in the supernate, especially in LI supernate 3 (Fig. 2*d*). Two-dimensional gel electrophoresis of LI supernate 3 (Fig. 3) reveals the presence of the α and β components of desmin (15), the presence of β- and γ-actin (10, 15, 27, 36, 42), a pair of spots designated * (shown most clearly in Fig. 6), and three isoelectric variants of α-actinin. The HMW does not appear on our two-dimensional gels. We have adopted the conversion of labeling the actin isoelectric variants of smooth muscle as β and γ, with γ denoting the most basic



FIGURES 1 AND 2 The extraction of chicken gizzard proteins at low and high ionic strength, showing, respectively, the insoluble (Fig. 1) and soluble (Fig. 2) proteins at each step of the extraction. See Materials and Methods for details, and Results for discussion. The protein loads in the various wells have been roughly equalized to aid viewing and do not represent the concentrations actually present at that step. (1*k*) shows the initial homogenate in 100 mM Tris-HCl, pH 7.5. (1*a*) Pellet and (2*a*) supernate from the initial homogenate. Three successive extractions with 10 mM Tris-HCl, pH 7.5, produced insoluble pellets (1*b–d*) and soluble supernates (2*b–d*). Three subsequent successive extractions with 1 M KCl produced insoluble pellets (1*e–g*) and soluble supernates (2*e–g*). Finally, three subsequent successive extractions with 1 M KI produced insoluble pellets (1*h–j*) and soluble supernates (2*h–j*). The final KI-insoluble residue that is enriched in 100-Å filaments is (1*j*). Actin and β-tropomyosin form a doublet on less heavily loaded gels. Actin (*A*), α-actinin (*α-A*), desmin (*D*), high molecular weight protein (*HMW*), myosin heavy chain (*MHC*), tropomyosin (*TM*).

variant (10, 15).

High ionic strength (HI) extraction of the LI-insoluble residue with 1 M KCl (Fig. 2*e–g*) and then with 1 M KI (Fig. 2*h–j*), releases most of the actomyosin, α-actinin, HMW, and tropomyosin. The remaining KI-insoluble residue (Fig. 1*j*) contains mostly actin and desmin. We have not quantitated yields or fold purification during this purification because of the lack of a suitable quantitative assay for desmin. Comparison of Fig. 1*a* and *k* and examination of the solubilized proteins (Fig. 2), however, indicates that the purification is substantial. The total amount of recoverable desmin in gizzard is quite small, however, and 200 g of gizzard muscle will typically yield 100–200 mg of moderately pure desmin (such as the cycle-2 desmin shown in Figs. 5 and 6).

The actin and desmin that remain in the KI-insoluble residue are still associated with a considerable bulk of SDS-insoluble matrix, the composition of which is unidentified. Two-dimensional electrophoresis of the KI-residue reveals the presence of α- and β-desmin, of $*_1$ and $*_2$ (brackets), and of γ-actin (Fig. 4). A band is seen next to desmin on one-dimensional gels of some KI-residue preparations. It has never been unambiguously observed on the corresponding two-dimensional gels, however, and is most likely either the α- or β-component of desmin. We have not observed any marked tendency for desmin to be proteolyzed during our extraction procedures.

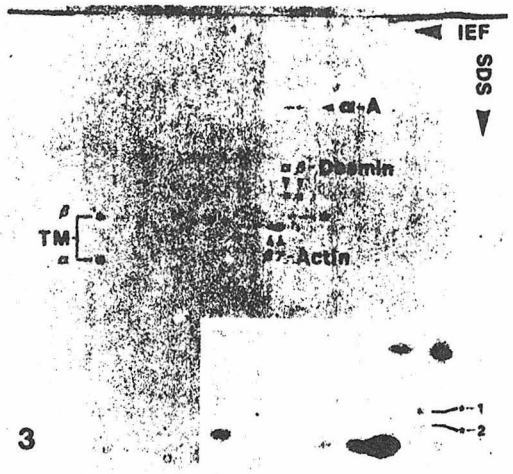


FIGURE 3 Two-dimensional gel of the low ionic strength supernate-3 (Fig. 2*d*). Indicated are α-actinin, α- and β-desmin, proteins $*_1$ and $*_2$, β- and γ-actin, and gizzard tropomyosin (α, β *TM*). α-Actinin focuses into three isoelectric variants. The acid side is on the left. The samples were loaded at the upper right (base side) for the first (isoelectric, *IEF*) dimension. The second (*SDS*-PAGE) dimension is top to bottom. The very high molecular weight proteins generally do not resolve on these gels.

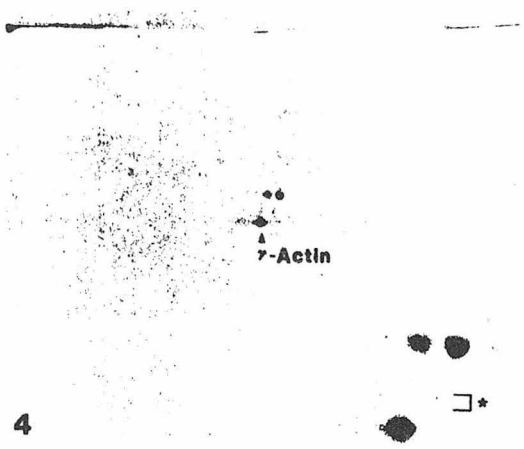


FIGURE 4 Two-dimensional gel of the KI-insoluble residue (Fig. 1*j*). α,β-desmin, proteins $*_1$ and $*_2$, and γ-actin are present.

The desmin from KI-extracted muscle has the same molecular weight and isoelectric variant composition as desmin from fresh muscle (15). Desmin, however, is slowly degraded at low pH.

SOLUBILIZATION OF DESMIN: Crude desmin may be solubilized from KCl- or KI-extracted gizzard residues by 1 M acetic acid (reference 33 and this paper), concentrated ethylene diamine, 0.5% Sarkosyl NL-97, 3 M sodium trichloroacetate, or 3 M urea. Of these, 1 M acetic acid at 0°C was chosen for routine extraction of desmin because it is easy to work with and to remove, is reasonably selective, and does not appear to denature or rapidly damage desmin. Although acetic acid at elevated temperatures is reported to give better yields of desmin (33), its use was avoided because it produced extensive charge heterogeneity in both actin and desmin, and also solubilized considerable quantities of collagen and myosin. The major difference between the KI-residue and the KI-residue acetone powder is that the latter yields much purer desmin.

CYCLIC PURIFICATION OF DESMIN SOLUBILIZED BY ACETIC ACID (SCHEME II): When the first acetic acid extract (0°C) is neutralized, a fine precipitate containing desmin forms at about pH 4.0. As the pH is increased to 4.2, the precipitate coalesces into small flocculent masses. These are extremely sticky and easily trap air bubbles. A desmin-containing precipitate also forms if a 1-M acetic acid extract is brought to 0.3–0.4 M in NaCl.

The first acid extract of a KI-residue (Fig. 5*a*) was purified by four cycles of precipitation and solubilization as described in Materials and Methods (see Scheme II). The supernate from each precipitate was dialyzed against water and lyophilized. Precipitates 1–4 are shown by one-dimensional SDS-PAGE in Fig. 5*b–e*, and concentrated supernates 1–4 in Fig. 5*f–i*. During this process of cyclic precipitation, a constant protein composition is attained, with >90% of the actin and desmin precipitating in each cycle. The small amount of actin and desmin which remains in the final supernate-4 are in the same relative proportion as the actin and desmin in precipitate-4. Desmin (mol wt 50,000) isolated in this manner is associated with four other proteins: myosin (mol wt 210,000), two intermediate-sized proteins (mol wt $*_1$: 45–47,000; mol wt $*_2$: 43–45,000), and actin (mol wt 42,000) (Fig. 6). Other proteins sometimes appear in the region between actin and desmin, but $*_1$ and $*_2$ predominate.

Precipitate-4 (Fig. 5*e*) was analyzed by two-dimensional electrophoresis. It contained both α- and β-desmin, $*_1$ and $*_2$, and actin. The actin has an isoelectric-focusing mobility corresponding to the γ-variant of this molecule (15). It can be

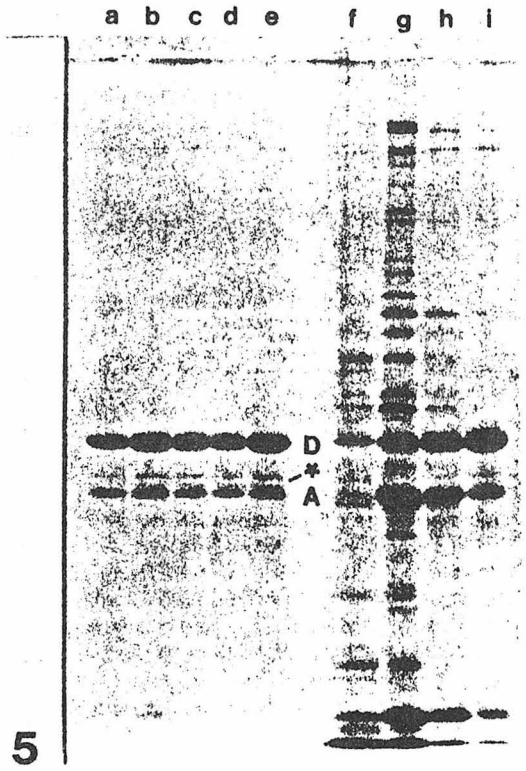


FIGURE 5 Proteins extracted from a KI-residue by 1 M acetic acid. The initial extract (extract-1) made with 1 M acetic acid at 0°C for 1 h. Extract-1 was cyclically precipitated with NaOH (at pH 4) and redissolved in acetic acid (see Scheme II) to yield precipitate-1 (5*b*), supernate-1 (5*f*), precipitate-2 (5*c*), supernate-2 (5*g*), precipitate-3 (5*d*), supernate-3 (5*h*), precipitate-4 (5*e*), and supernate-4 (5*i*). By cycle 4, a constant ratio of actin to desmin is attained both in the precipitate (5*e*) and in the supernate (5*i*).

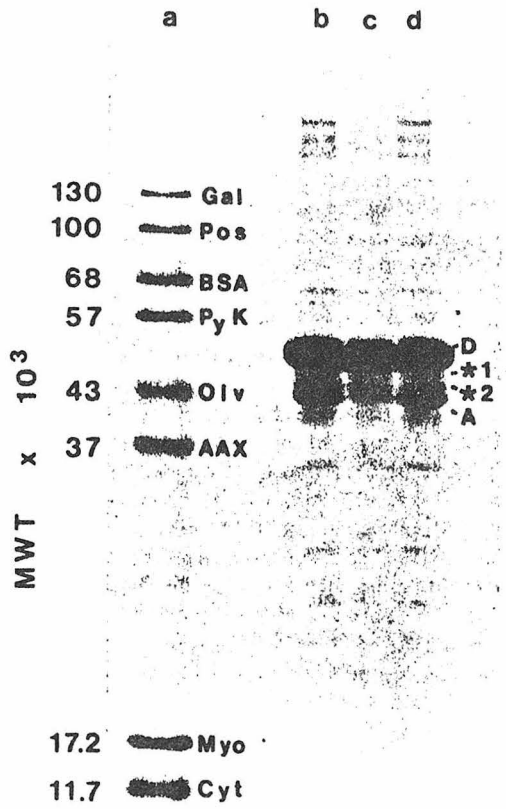


FIGURE 6 Cycle-2 desmin extracted by acetic acid from a KCl-residue acetone powder (KCl-AP; 6*b*), and from a KI-residue acetone power (KI-AP; 6*c*). Molecular weight (*MWT*) standards (6*a*) are β-galactosidase (*Gal*; 130,000), phosphorylase *a* (*Pos*; 100,000), bovine serum albumin (*BSA*; 68,000), pyruvate kinase (*PyK*; 57,000), ovalbumin (*Olv*; 43,000), D-amino acid oxidase (*AAX*; 37,000), myoglobin (*Myo;* 17,200), and cytochrome *c* (*Cyt*; 11,700). The SDS-PAGE is a 20% acrylamide gel. Calculated mol wt are: 50,000 for desmin; 47,000 for $*_1$; and 45,000 for $*_2$. Actin is given to have mol wt 42,000. Note that more actin is extractable from a KCl-acetone powder. When a KI-AP is washed with 1 M KCl before the preparation of a cycle-2 extract, the high-salt treatment reverses the effect of acetone powdering (6*d*).

further shown to be actin by reaction with anti-actin antibody (see below). The amount of myosin that is observed with desmin is variable and is virtually absent from desmin extracted from KI-residue acetone powders. Every nondenaturing purification scheme that we have investigated so far has failed to selectively solubilize desmin away from either the * proteins or from actin. It is of interest that both desmin and the proteins that copurify with it have P_I's of ~5.71 in 9 M urea/1% Nonidet NP-40.

Although there is a certain amount of variability in the relative proportions of α- and β-desmin in purified desmin, we have never observed the purification of one desmin variant without the other. At least some of this variability appears to be artifactual. α-Desmin is particularly susceptible to conversion to a more acidic variant. Urea, heating, and the use of pH 4–6 Ampholines will all promote this conversion. In the presence of the pH 4–6 Ampholines, the α-desmin spot splits into

two, with one remaining in the old position and one running ~0.05 P_I units more acidic (data not shown).

GEL CHROMATOGRAPHY OF ACTIN AND DESMIN: The consistent copurification of actin and desmin suggested that they might be associated together as a complex. Because purified desmin is insoluble under conditions of low or high ionic strength, we studied desmin that had been solubilized with the medium-strength anionic detergent Sarkosyl NL-97. This detergent does not appear to denature desmin, because desmin preparations that have been solubilized with Sarkosyl will form 100-Å filaments when the Sarkosyl is dialyzed away (see below). When Sarkosyl-solubilized desmin is chromatographed by gel filtration on a column of Ultrogel AcA 34 (range 20,000–340,000 mol wt), it is fractionated into two populations: one containing actin, desmin, and HMW that is excluded from the column; and one containing actin, desmin, $*_1$, and $*_2$ that is barely included in the column (Fig. 7). Two-dimensional electrophoresis (not shown) revealed the presence of α- and β-desmin and of γ-actin in both protein populations (fractions 17 and 37).

When desmin is chromatographed on Bio-Gel P300 in the presence of 1 M acetic acid and 0.05 M NaCl (Fig. 8), the vast majority of desmin elutes in what is probably a monomeric position and is not associated with actin under these conditions. A small fraction of the desmin elutes with the excluded volume. The actin present does not appear to be monomeric, and most of it also elutes with the excluded volume.

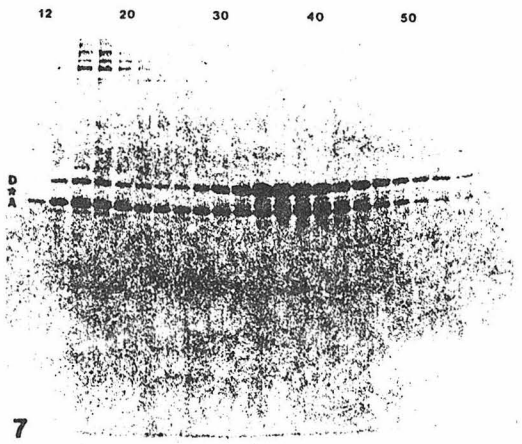


FIGURE 7 SDS-PAGE analysis of the fractions produced by the gel filtration of desmin and associated proteins on Ultrogel AcA 34 in the presence of 0.5% Sarkosyl NL-97. A KI-AP was washed with 1 M KCl and then with water. This resolubilizes some of the high molecular weight proteins. The washed pellet was extracted with 1 M acetic acid and this was cycled as described in Materials and Methods to produce a precipitate-2 (cycle-2 desmin). This was rinsed with 0.1 M Tris-HCl, pH 7.5, and then solubilized with 0.5% Sarkosyl NL-97, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, and 10 mM NaN_3. Ultrogel AcA 34 (range 20,000–350,000 mol wt) was equilibrated in this buffer and poured as a column of 2 × 40 cm. Blue dextran-2,000 eluted with a peak at fraction 17, and an included dye marker eluted with a peak at ~fraction 120. In the actual run, all of the protein eluted between fractions 12 and 55. Fractions were heated with 0.2 vol of 5 × SDS-sample buffer before analysis on SDS-PAGE. We have not determined whether the micell structure of Sarkosyl NL-97 had any effect on the elution profile.

Copolymerization of Actin and Desmin

FORMATION OF DESMIN GELS: When purified desmin is recovered from 1 M acetic acid (pH 2.4) by neutralization to pH 4.1, it generally precipitates as cohesive, cottonlike flakes. However, if the acetic acid is instead removed by dialysis against several changes of distilled water, three different and alternate phenomena are observed: (*a*) the spontaneous formation of a clear gel; (*b*) a clear solution; or (*c*) a cohesive precipitate. All three represent different states of desmin and of the proteins that copurify with it, as no differential participation of any of them is observed. A spontaneous gel is the most frequently observed state. The gels are extremely sticky, especially to themselves and to glass, but they will also coat the insides of plastic pipette tips. Desmin gels are strong enough to hold their shape when extruded from the dialysis bag. This includes any notches and wrinkles that resulted from their association with the nonuniform contours of the dialysis membrane. If they are left undisturbed, the clear gels contract slowly and become translucent over a period of several days. This spontaneous contraction can be speeded up, so that it is complete within 1 h, if the ionic strength or divalent cation concentration of the dialysis medium is raised. Fig. 9*a* shows an uncontracted gel in the dialysis bag in distilled water. Fig. 9*b* shows the same gel 1 h after the addition of 1 mM $MgCl_2$ to the dialysis medium.

The dialysis of cycle-purified desmin from acetic acid into water occasionally results in a metastable, nongelled solution (state 2). If left undis-

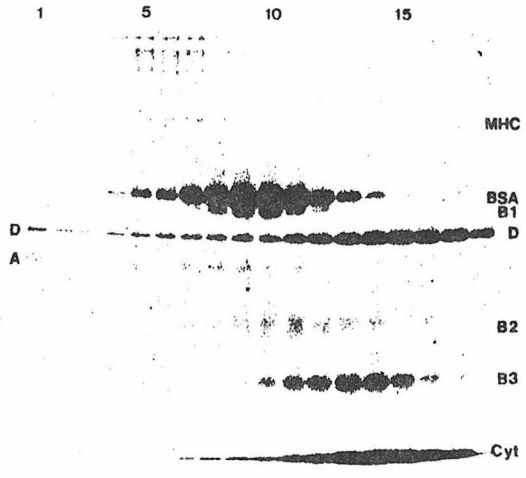


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FIGURE 8 SDS-PAGE analysis of the fractions produced by gel filtration of desmin and its associated proteins on Bio-Gel P300 in the presence of 1 M acetic acid and 0.05 M NaCl. Blue dextran-2,000 eluted in fractions 1 and 2. BSA and Cyt were mixed with the desmin before it was loaded on the column. The majority of desmin elutes as a monomer while a small fraction of desmin elutes with the excluded volume. All of the actin chromatographs in a manner similar to the partially excluded desmin. The elution profile for desmin is retarded relative to that expected for a 50,000 mol wt protein; one possible reason is that desmin may interact with Bio-Gel P300 under the above conditions. If this is so, caution should be exercised in assigning a monomeric molecular weight to this desmin fraction. The proteins *B1*, *B2*, and *B3* are most likely BSA degradation products and they are not present when desmin alone is run on the column. Other abbreviations are as in Figs. 1, 2, and 6.

turbed in the dialysis membrane, these solutions will remain in this state for days at 5°C. However, if even a small amount of an ionic substance (Table I) is dialyzed into the metastable solution, gelation is initiated and is complete within 1 h (state 1 above). Glass will also trigger gelation (Fig. 10). Syneresis of the gel occurs subsequently. While we have not measured the relative effectiveness of various ions in inducing gelation, we have not noticed any obvious requirements for any particular ions. All of the above gelation and syneresis phenomena are apparently passive in the sense that an external source of energy is not required.

Finally, the gelation phenomenon is not reversed by the removal of any of the substances listed in Table I (by extensive dialysis against water). The gels may be resolubilized in 1 M acetic acid, however, and the gelation procedure repeated.

Sarkosyl NL-97 solutions of desmin will also form spontaneous gels when the Sarkosyl is removed by extensive dialysis against water. These gels appear to be similar to the acetic acid gels, but were not investigated extensively. They are composed of fibrils with ~100-Å diameters (data not shown).

LIGHT MICROSCOPY OF DESMIN GELS: Phase microscopy of semicontracted desmin gels reveals a tangled network of branching fibers that are often embedded in an amorphous matrix (Fig. 11). These fibers are <1 μm wide and appear to be bundles of many smaller fibrils. No differences in morphology are seen when spontaneous gels or ion-initiated gels (10 mM KCl or 10 mM $MgCl_2$) are compared. The matrix and fibril bundles are intimately associated with each other (Fig. 11). We were unable to selectively solubilize either matrix or fibers at any of several concentrations of urea between 0.1 and 1.0 M. Desmin precipitated from acetic acid by rapid neutralization to pH 4.1 is usually amorphous, but occasionally exhibits fibril bundles similar to those observed from desmin gels produced by dialysis (data not shown).

In indirect immunofluorescence, both the fibrous and matrix components of the desmin gels are uniformly reactive with anti-desmin (Fig. 12) and anti-actin (Fig. 13) at the level of resolution of the light microscope. No periodicities or differential reactivity has been observed. Control preimmune antisera were unreactive.

ELECTRON MICROSCOPY OF DESMIN GELS: The insoluble gizzard residue which remains after extraction with 1.0 M KCl was investigated by negative staining to determine what fiber morphologies it contained before the extraction of desmin with acetic acid. High magnification pictures show tangled groups of well-preserved 100-Å filaments (Fig. 14*a–c*). The 100-Å-fiber configurations of Fig. 14*a–c* are similar to those seen in association with dense bodies (1, 5, 39). The measured diameters of the fibers in Fig. 14 range from 120 to 140 Å.

The most characteristic microscopic feature of desmin gels is that the long, tangled fibers seen at low magnification (Fig. 15*a*) actually represent a network of extensively intertwined fibrils that become visible at high magnifications (Fig. 15*b*, *c*). Neither microtubules nor F-actin, both of

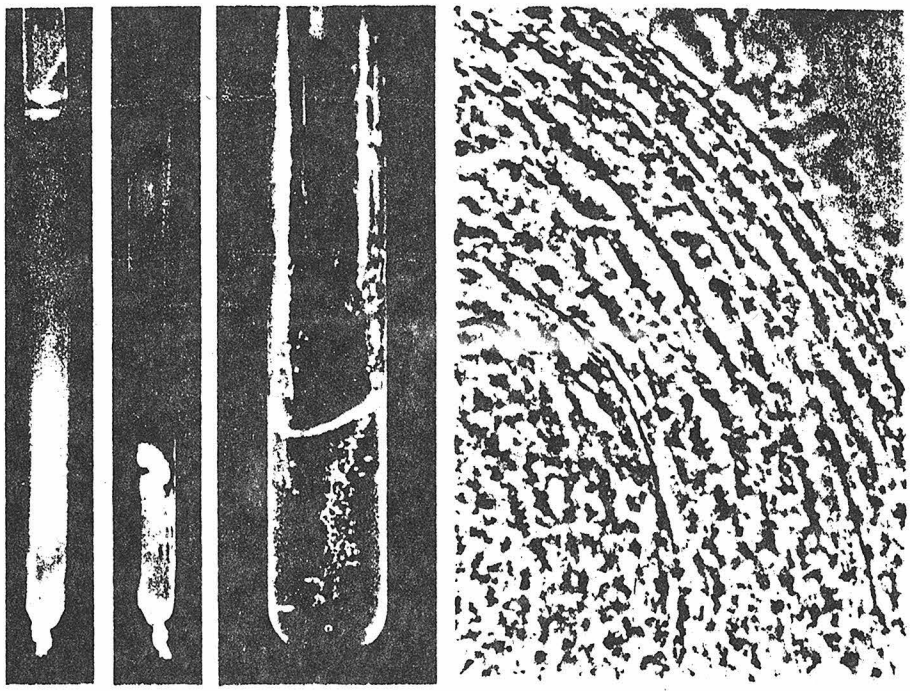


FIGURE 9 The formation of a spontaneous desmin gel. Acetic acid-solubilized cycle-2 desmin was dialyzed against several changes of water for 2 days at 4°C. The gel that resulted was photographed in the dialysis membrane (Fig. 9*a*). Dialysis was continued and 1 mM $MgCl_2$ was added to the dialysis medium to initiate contraction of the gel. The gel was rephotographed 1 h after the addition of $MgCl_2$ (Fig. 9*b*).

FIGURE 10 Gelation of metastable aqueous desmin upon contact with glass. A 1-M acetic acid extract of cycle-2 desmin was dialyzed against water to yield a metastable solution. The solution was removed from the dialysis membrane by inverting the open end of the membrane directly into a glass test tube. Gelation occurred within seconds, trapping air bubbles which appear suspended in the gel. The gel was photographed horizontally.

FIGURE 11 Light micrograph (phase optics) of a desmin gel similar to that depicted in Fig. 9*b*. Phase contrast dense fibers are seen embedded in an amorphous matrix. The fibers were probably oriented while the preparation was being flattened. Final magnification is × 1,000; 10 μm/cm.

which can form gel-like solutions, show this mode of twisting self-interaction. The fibril morphology of these gels is not uniform and a single gel may contain fibrils which exhibit regular profiles, twisted ribbon-like profiles, and intertwined profiles (Fig. 15*c*). The most consistent interpretation of these profiles is that they are actually flat ribbons of 120–140 Å width which have an inherent tendency to twist. The thin regions (60–80 Å; Fig. 15*c*, arrow) would correspond to nodes where the ribbon is parallel to the electron beam. We have not observed any profiles which can be interpreted as being strictly cylindrical along their entire lengths.

DISCUSSION

Solubility Properties of Desmin

Desmin is present in smooth muscle in two forms: a "soluble" one that is released during extraction at LI with EGTA, and an "insoluble" one that remains after extraction of the muscle cells at HI. In both cases, desmin is associated with actin.

The HI-insoluble desmin is solubilized either at low or high pH, or by agents that dissociate hydrophobic bonds. Two solubilizing agents were investigated in detail: acetic acid (1 M) and Sarkosyl NL-97 (0.5%). Acetic acid solubilizes des-

TABLE I

Conditions Causing the Gelation and Subsequent Contraction of Metastable Desmin Solutions

Salt	Concentrations tested
	mM
$MgCl_2$	0.1–100
ATP	0.1–10
Mg^{2+} + ATP	0.1, each
Mg^{2+} + K^+ + ATP	0.01, each
$CaCl_2$	0.1–50
EDTA pH 7.5	0.02–0.1
PIPES pH 6.9	100
Tris-HCl pH 6.9	10
KCl	10
NaCl	10
Glass	–

min along with a variety of other proteins from KCl- or KI-insoluble residues. Of these, only desmin, actin, and two proteins designated * precipitate quantitatively at pH 4.0 (Fig. 5). The pH-dependent precipitation of desmin is probably not a simple isoelectric phenomenon, however. First, desmin does not resolubilize above pH 4 until a very high pH is reached (e.g., with ethylene diamine). Second, the dialysis of desmin from acetic acid into water sometimes results in a metastable soluble state. Exposure of these solutions to an ionic environment causes the immediate coprecipitation of both actin and desmin. These properties are unlike any previously described for actin (26).

Desmin from avian muscle is resolved into two major isoelectric variants by two-dimensional electrophoresis (15, 21). Both variants are always present in every preparation and appear to behave identically in each of the purification schemes that we have employed (i.e., salt extractions, acetic acid cycling, and gel filtration in the presence of Sarkosyl). There is usually an excess of β-desmin over α-desmin in gizzard, however. One reason for this is the tendency of α-desmin to become modified and focus as two or more species. It is presently unknown whether α- and β-desmin are distinct gene products or if one arises by modification of the other.

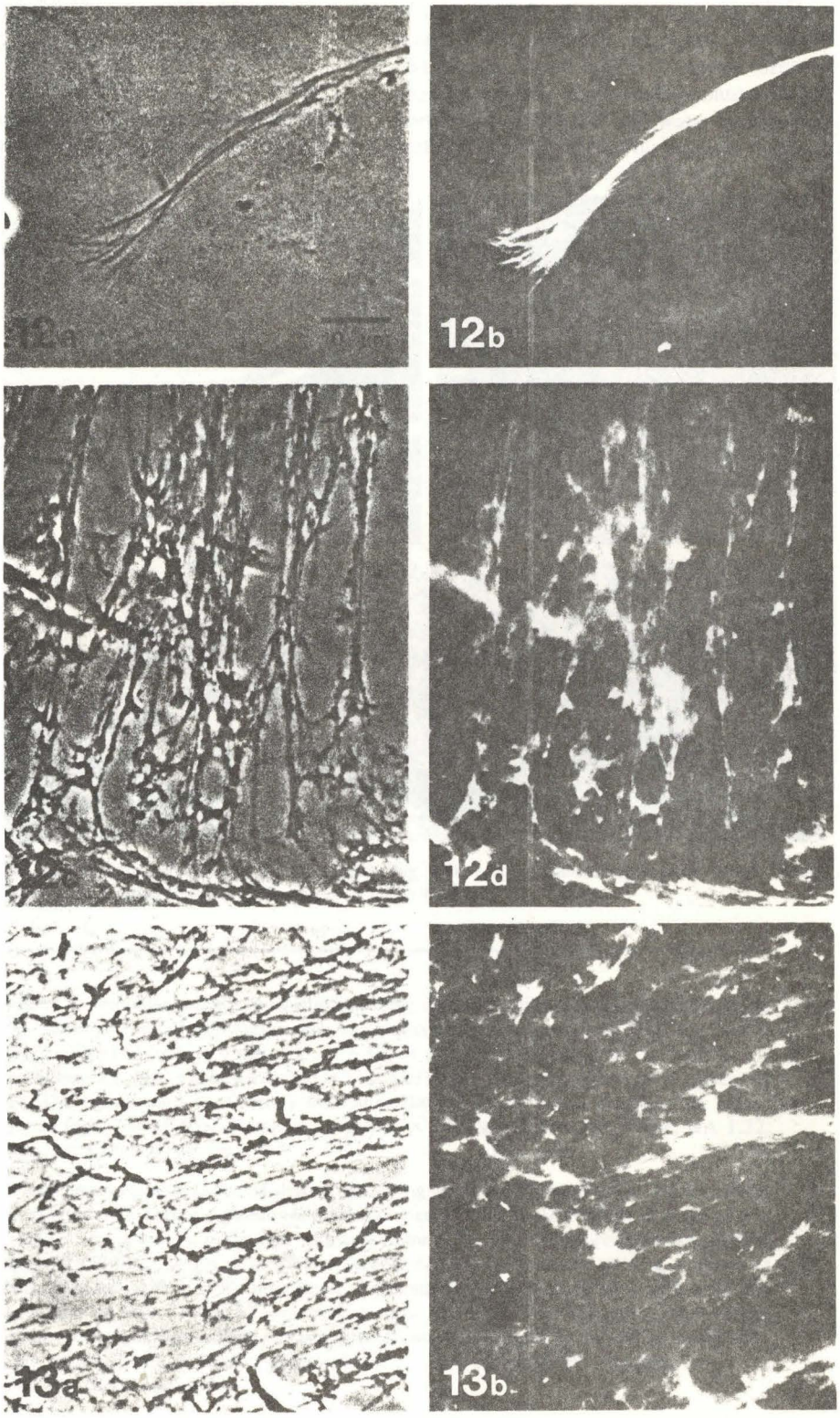
Copurification of Actin and Desmin

The most significant finding of this research is the suggestion that desmin and actin form nonstoichiometric complexes. The evidence for this is as follows: (*a*) A small fraction of gizzard actin has solubility properties that are different from the bulk of the actin but which are the same as for desmin. (*b*) Both actin and desmin copurify during repeated cycles of acetic acid solubilization and pH 4 precipitation. A constant ratio of actin to desmin is attained and this ratio is found in both the pH 4 precipitate and the supernate (Fig. 5*e* and *i*). (*c*) Gel filtration in the presence of 0.5% Sarkosyl NL-97 reveals an included fraction of actin and desmin that comigrate through the column. (*d*) Both actin and desmin appear to copolymerize from a metastable soluble state to form a single species of 100-Å-like filaments in which they are homogeneously distributed (see discussion below). The simplest interpretation of this is that actin and desmin are able to form stable, nonstoichiometric complexes with each other. We have hypothesized that desmin functions in muscle to bind separate actin-containing structures together into mechanically integrated units (22). The formation of an actin, desmin-containing polymer may provide a molecular basis for this hypothesis. It is important to note, however, that we have not excluded the possibilities of nonspecific interaction of actin and desmin or of separate populations of actin and desmin that simply have similar solubility properties.

Copurification of other Proteins with Desmin

At least two other proteins, termed $*_1$ and $*_2$, appear to be associated with desmin in a manner that is similar to that discussed for actin above. We wish to avoid giving these proteins names until we can determine whether they are specifically associated with desmin and whether or not they are cleavage fragments of desmin. This latter is a strong possibility. $*_2$ Comigrates with a known proteolytic fragment of desmin (in preparation), and both $*_1$ and $*_2$ are seen occasionally as isoelectric doublets on two-dimensional gels (data not shown). This further suggests that they may be derived from the cleavage of α- and β-desmin. It is intriguing to note that some preparations contain similar amounts of actin, $*_1$, and $*_2$ (Fig. 6*c*).

The remaining proteins that associate with desmin are HMW, myosin, α-actinin, and tropomyosin. These are all proteins that are known to bind to actin in the absence of desmin and are probably bound to the actin that copurifies with desmin. The HMW protein may be filamin, an actin-binding protein from smooth muscle (40, 41). How the cell chemically specifies the interactions of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actin with these proteins remains a matter for speculation.

Copolymerization of Actin and Desmin to 100-Å-like Filaments

Desmin appears to exist as an insoluble hydrophobic polymer under physiological conditions. These polymers may be solubilized by conditions of low or high pH (at low ionic strength) or by agents which dissociate hydrophobic bonds (5, 22, 33). Acetic acid-solubilized desmin forms either spontaneous gels or metastable solutions when the acetic acid is replaced with water by dialysis. The metastable solutions rapidly convert to gels if the

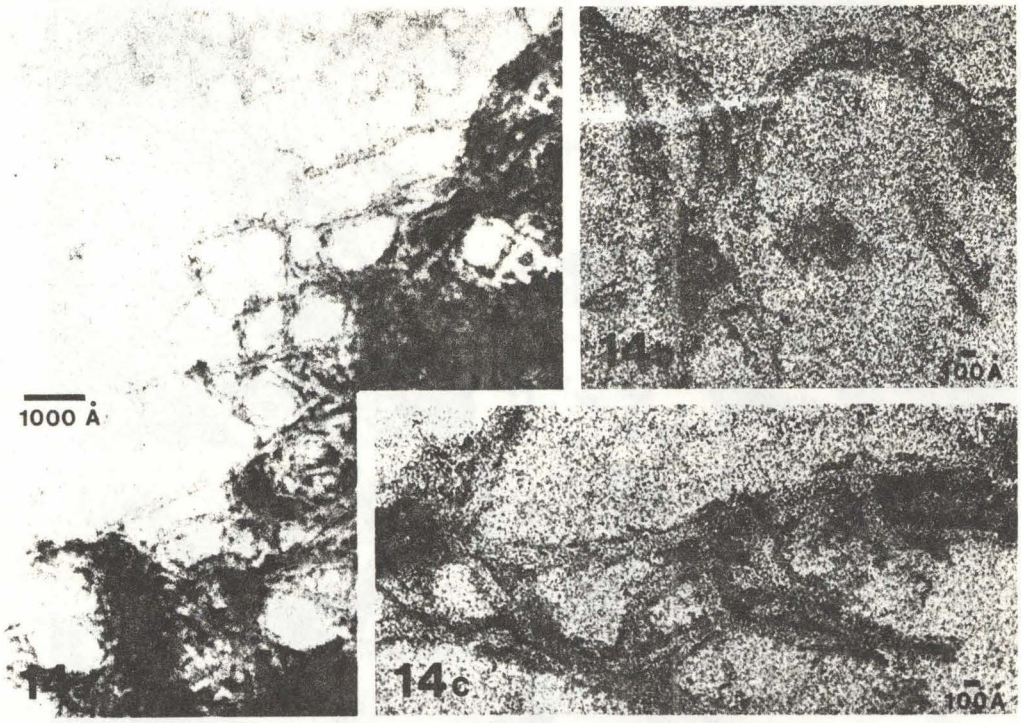

FIGURE 14 100-Å-diameter fibers in a KCl-residue. Fig. 14*a–c* shows tangled groups of 100-Å fibers with a well-preserved substructure and diameters of 120–140 Å.

FIGURE 12 Indirect immunofluorescence of desmin gels using desmin specific antibodies. Desmin fibers (described in Fig. 13) were reacted with anti-desmin and observed with phase contrast (Fig. 12*a* and *c*) and epifluorescence (Fig. 12*b* and *d*) optics. Fig. 12*b* shows a fine fluorescent network, between the fluorescent-filament bundles, that is nearly invisible in 12*a*. The larger fibers thus appear to be aggregates of thinner fibers. The fluorescence is uniformly distributed throughout the whole length of the fibers. Preimmune antisera (not shown) were completely negative. × 100 oil-immersion objective, NA 1.32; final magnification is × 1,000; 10 μm/cm.

FIGURE 13 Indirect immunofluorescence of desmin gels using actin specific antibodies. Desmin fibers from a gel were reacted with anti-smooth-muscle-actin and viewed with phase contrast (Fig. 13*a*) and epifluorescence (Fig. 13*b*) optics. As in the case with anti-desmin (Fig. 12), the fluorescence is uniformly distributed throughout the whole length of the fibers. No periodicities or differential staining of matrix or fibers is seen with either anti-actin or with anti-desmin. Preimmune antisera (not shown) were uniformly negative. The gels used for Figs. 12 and 13 were cycle-1 acetic acid extracts of KI-residue. The extracts were dialyzed against water and the resulting metastable solutions were induced to gel with 10 mM $MgCl_2$. These extracts are relatively rich in desmin-associated actin. × 100 oil-immersion objective, NA 1.32; final magnification × 1,000; 10 μm/cm.

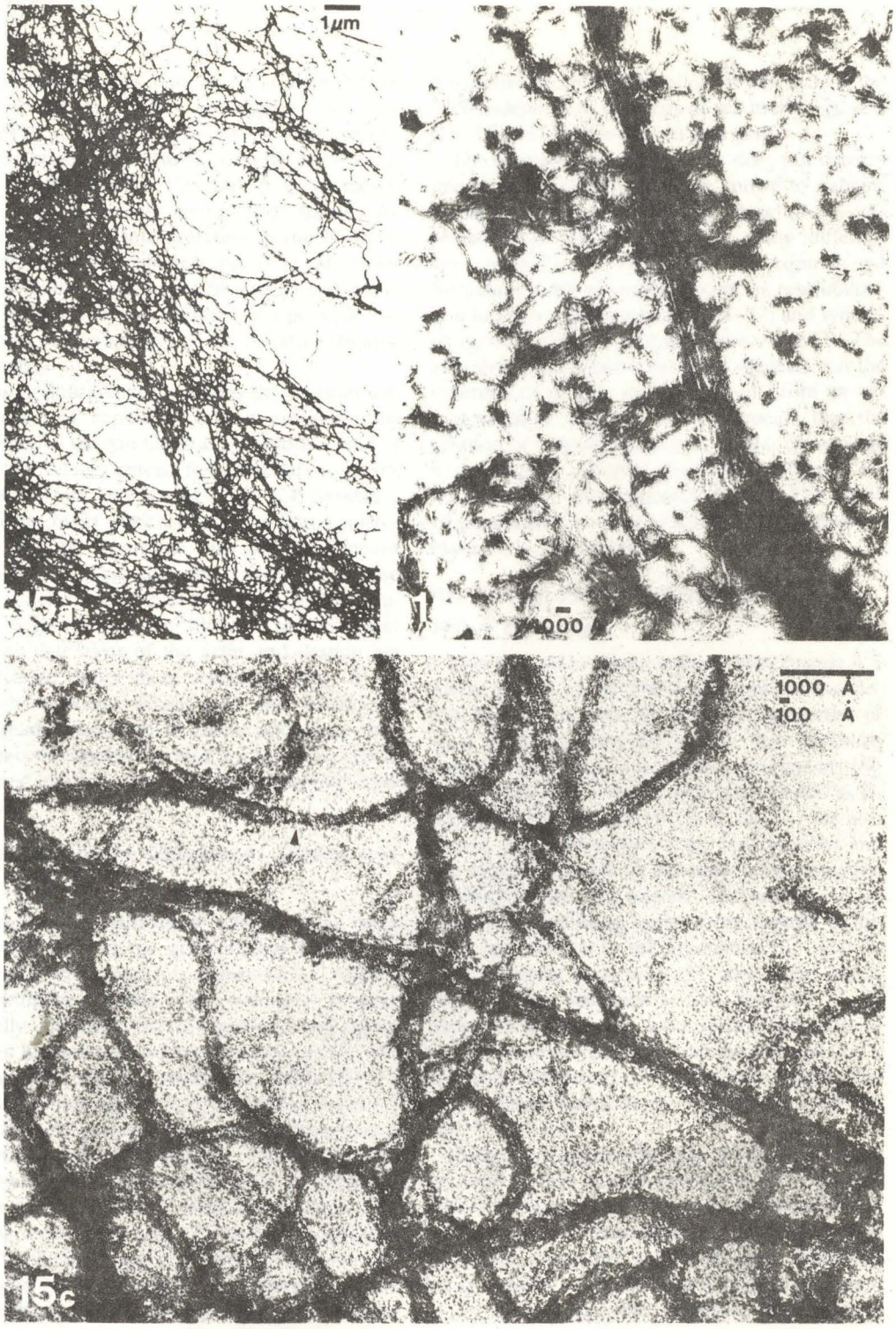

FIGURE 15 Typical gels produced by the dialysis of cycle-2 acetic acid extracts of KI-residue acetone powders against water. Many long, straight fibers can be seen in Fig. 15*a* and *b*. Most of these are composed of twisted and intertwining fibrils of 130 Å diameter (15*b*). Ribbonlike characteristics are evident in Fig. 15*c*. Occasional 11–15-Å profiles, which may be protofilaments, are visible in Fig. 15*c*. The substructure in Fig. 15*c* resembles that of the 100 Å filaments shown in Fig. 14*b* and *c*. The arrow indicates a region of apparent twisting. Final magnifications: (15*a*) × 5,200, 2 μm/cm; (15*b*) × 21,000, 4,750 Å/cm; (15*c*) × 140,000, 700 Å/cm.

concentration of ions rises above roughly micromolar values or if they are exposed to ionic surfaces (e.g., glass). These gels are characteristically composed of a network of highly intertwined fibrils which measure 120–140 Å in diameter. Most of the negatively stained fibril images are consistent with an interpretation of the fibrils as flat ribbons. These ribbons appear to intertwine to build up the macroscopic fibers that are visible in the light microscope, although it is possible that the fibers are an artifact of the negative staining procedure.

Immunofluorescence indicates that desmin and the actin that copurifies with it are uniformly distributed in desmin fibers at a resolution limit of 2,500 Å (Raleigh criteria for self-luminous points at λ of 530 nm and NA of 1.32; 32). Similarly, there is no overt evidence at the electron microscope level of separation into distinct filament morphologies. The gels also contain the same ratios of actin and desmin that the ungelled solution did. In addition, gel filtration in acetic acid indicates that most of the actin and desmin are unassociated under these conditions (Fig. 8). If the high molecular weight actin and desmin are not already associated in acetic acid, then they must become associated once it is removed. Thus, actin and desmin appear to copolymerize from solution.

Comparison of the Gelation of Desmin and Actin

A variety of cytoplasmic extracts have been discovered to undergo gelation and subsequent syneresis in vitro, and it is of interest to compare these with the gelation and syneresis of desmin. Typically, an extract that is capable of undergoing gelation is produced by homogenizing cells at 0°C in a buffered solution containing ATP, EGTA, and sucrose or glycerol. Upon being warmed, these extracts gel and then undergo syneresis if the gelled state is maintained. The gel-forming components of *Acanthamoeba* (23, 25), *Dictyostelium* (38), pulmonary macrophages (12, 37), and sea urchin eggs (16, 17) have been fractionated. While these are not identical systems, gel formation generally appears to depend upon the polymerization of G-actin to F-actin and on the subsequent cross-linking of this F-actin by one or more accessory proteins. In most cases, syneresis is magnesium-ATP dependent and is based upon the interaction of the cross-linked F-actin with myosinlike proteins. Exceptions to this include the ATP-independent syneresis of sea urchin egg gels (16, 17) and F-actin-filamin gels (41).

The formation of desmin gels does not depend upon any conditions which are known to stabilize F-actin: gel formation can be triggered by subphysiological concentrations of many different ions; neither ATP nor calcium is required; gel formation is not reversible except by resolubilization in acetic acid or Sarkosyl NL-97; and syneresis occurs in the absence of myosin or ATP. The adhesiveness of desmin for itself suggests that the syneresis of desmin gels may result from an autoaggregation process. It thus appears that the gelation of extracts based predominantly on actin is significantly different from the gelation of extracts that are predominantly desmin. The physiological significance of gelation and syneresis remains to be determined.

Are Desmin Filaments Related to 100-Å Filaments?

Two lines of evidence indicate that desmin is a major subunit of the 100-Å filaments of muscle. The first is based on the solubility properties of these filaments. Smooth muscle that has been extracted at high ionic strength is enriched in 100-Å filaments and contains actin and desmin as its major protein constituents (5, 6, 22, 33. See also above). Urea solubilizes these two proteins and also removes the 100-Å filaments from extraction enriched muscle (5). Second, the subsequent removal of solubilizing agents from desmin by dialysis has resulted in the production of ~100-Å-sized filaments from urea (5), acetic acid (reference 33 and this paper), and Sarkosyl NL-97 (data not shown). We have shown that most of the actin can be removed from desmin and that it will still polymerize to intermediate-sized filaments. These filaments are very similar to in vivo 100-Å filaments.

There are apparently two major differences between in vitro desmin fibrils and in vivo muscle 100-Å filaments. First, if desmin fibrils are in fact ribbonlike, then they differ from in vivo 100-Å filaments, which are shown to have cylindrical cross sections in the vast majority of preparations (1, 6, 39). It is possible, however, that the negative staining and drying procedures induced artifactual flattening and twisting in otherwise cylindrical desmin fibrils. Second, the adhesiveness of in vitro desmin fibrils and precipitates is unex-

pected because the 100-Å filaments of smooth muscle are not aggregated under normal circumstances. This phenomenon may result from either our in vitro assembly conditions or from an interaction of desmin with one of the proteins that copurifies with it. However, the formation of 100-Å-filament aggregates in colcemid-treated cultured cells has been reported for a variety of cell types, including striated muscle (14), cardiac muscle (20), and smooth muscle (unpublished observations). Although the mechanism of this aggregation remains unknown, it may reflect the unmasking of adhesive properties in vivo which are similar to those shown by the desmin fibrils in vitro.

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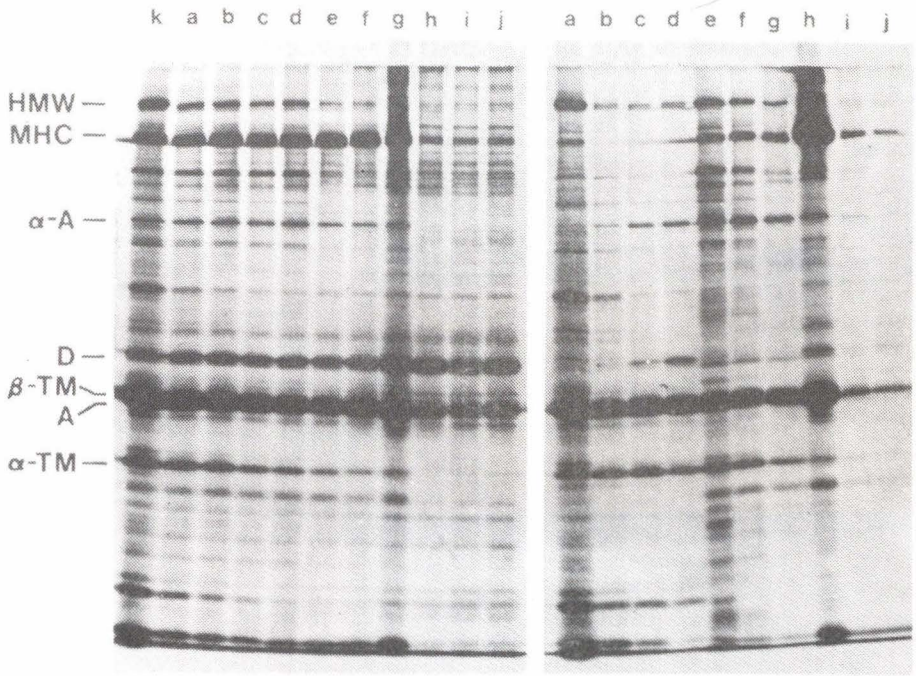
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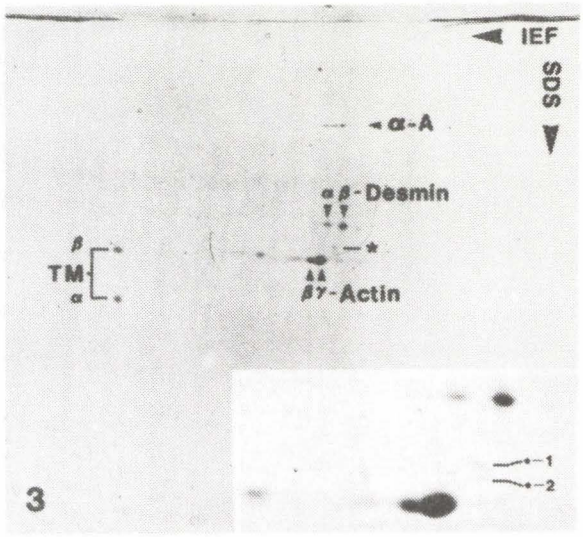
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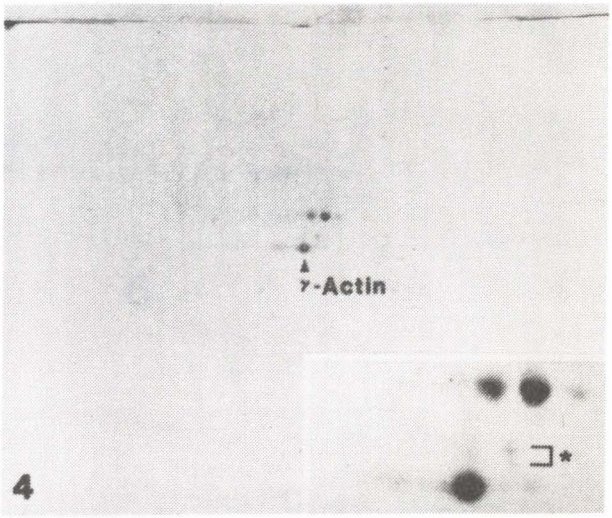
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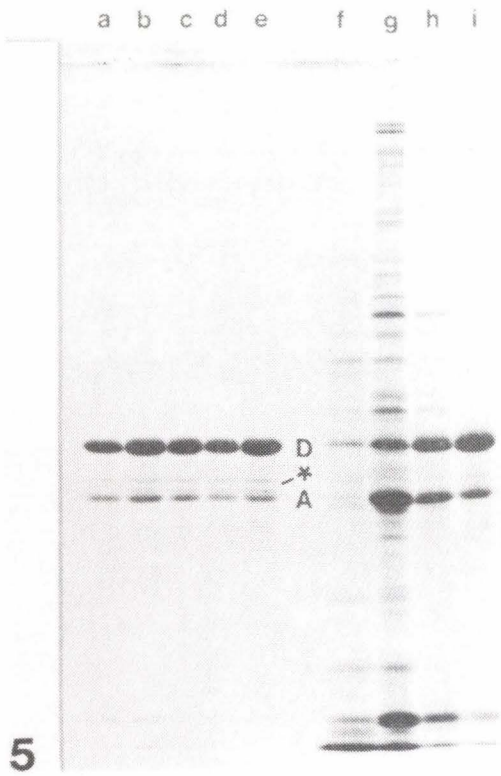
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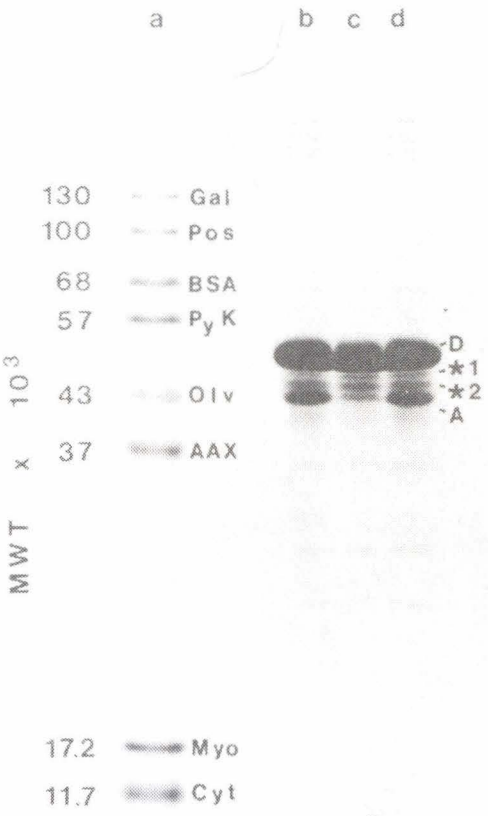
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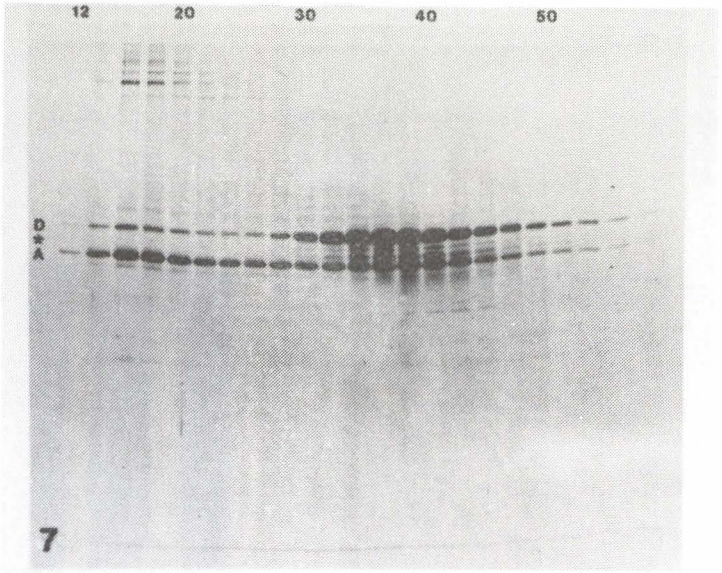
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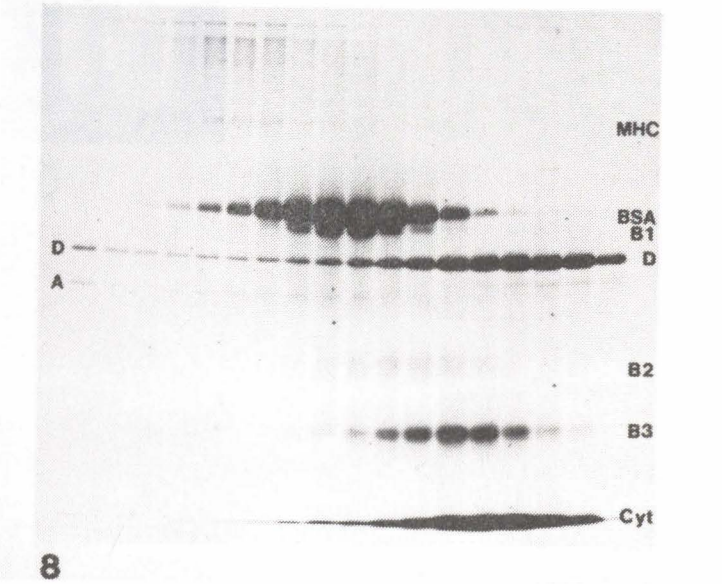
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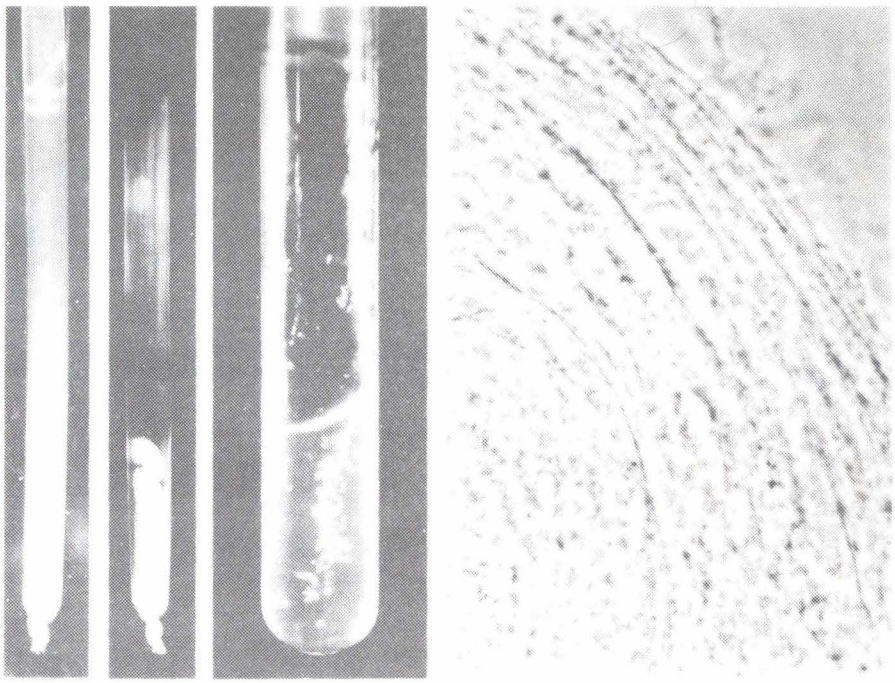

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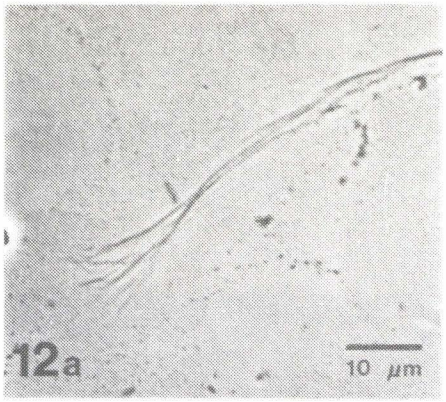
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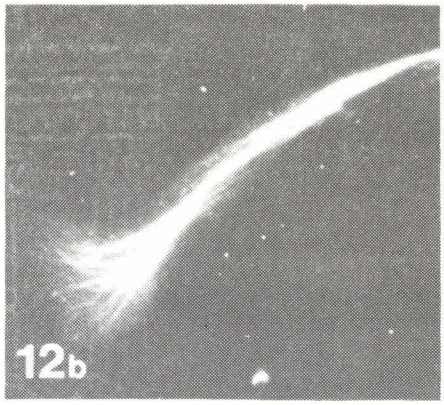
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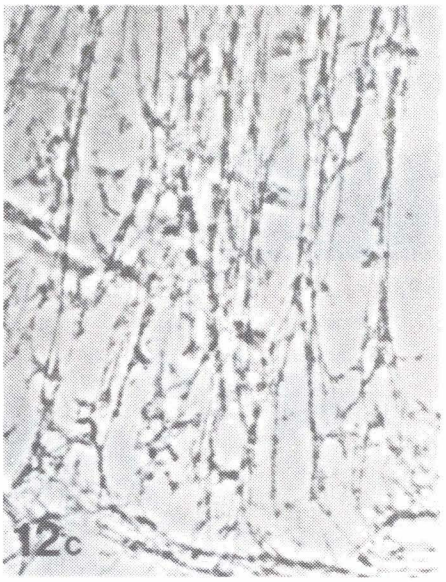
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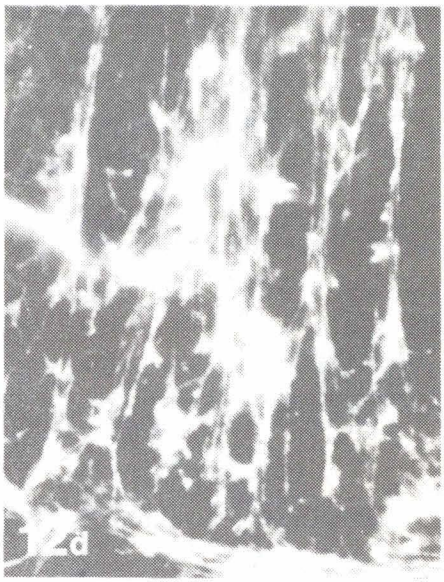
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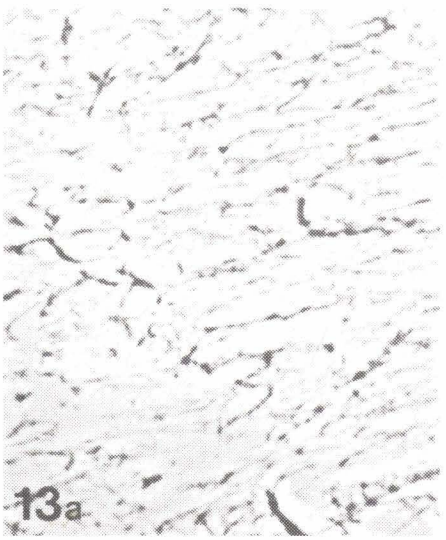
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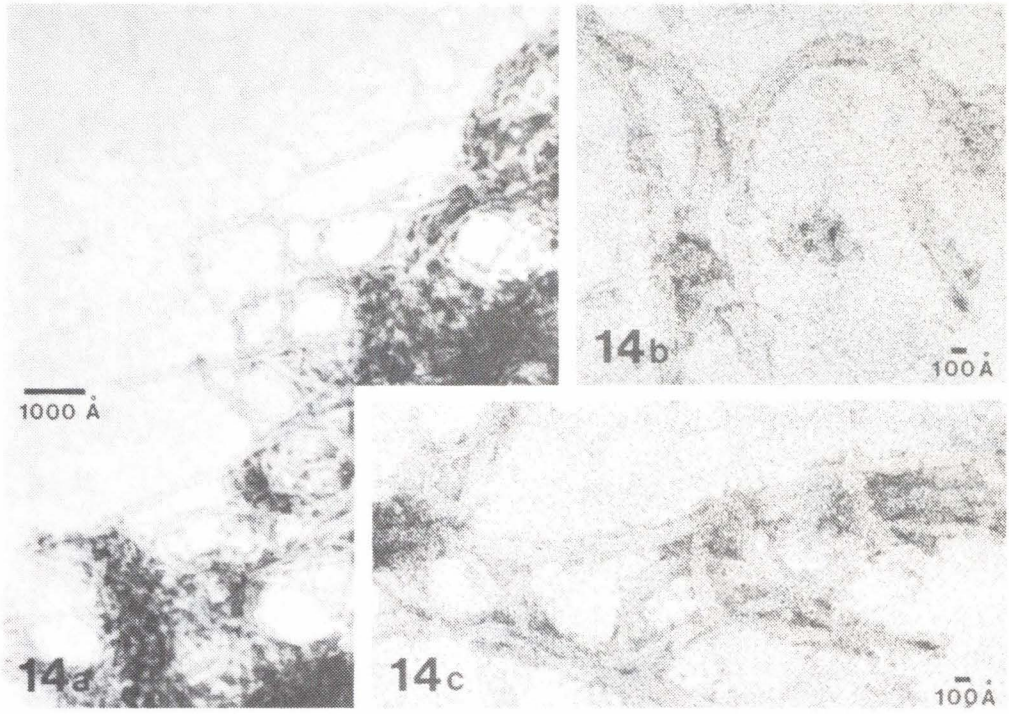
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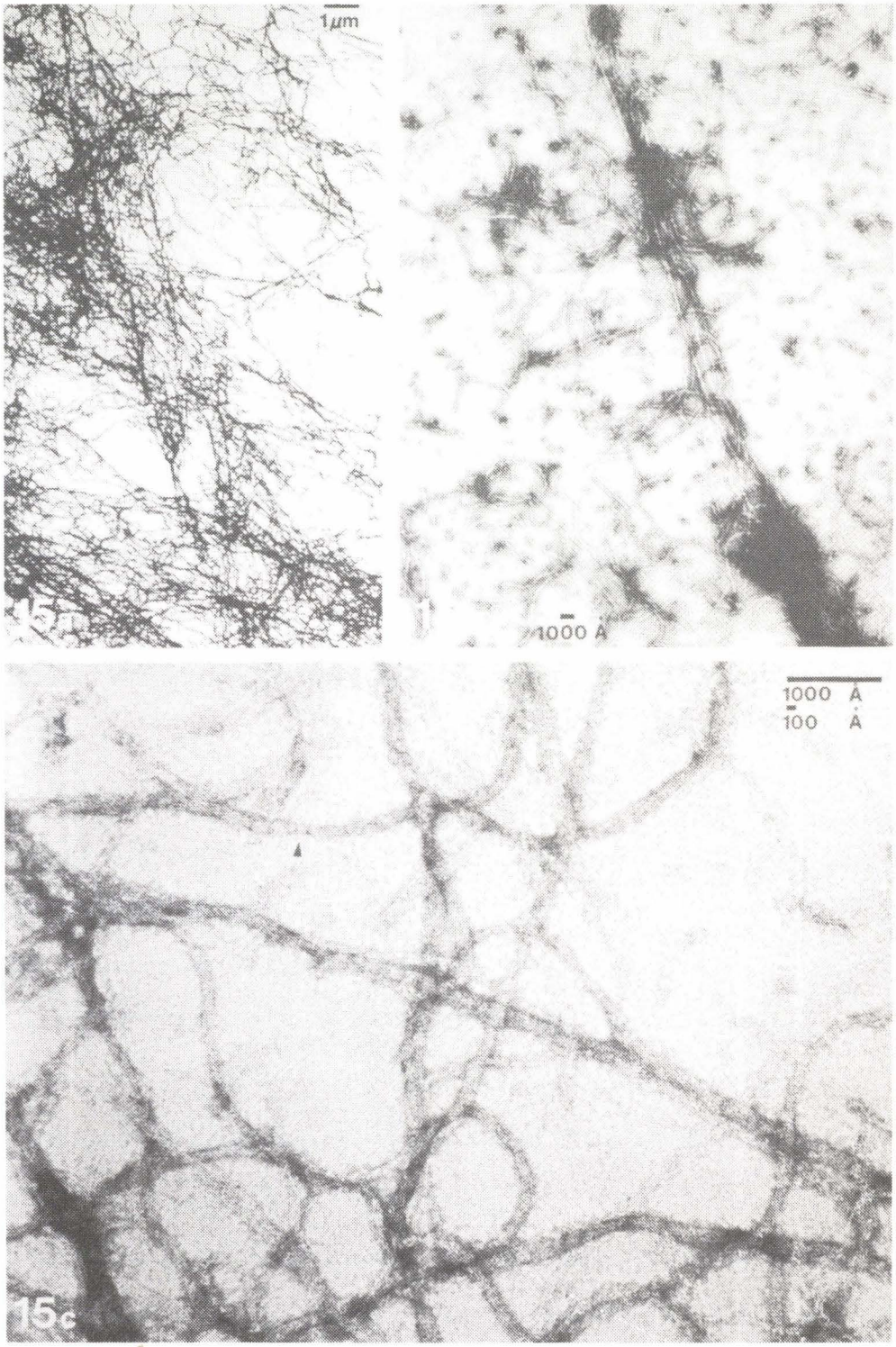
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CHAPTER FOUR

An Actin-Desmin Complex from Chicken Smooth Muscle

Abstract

Extraction of chicken gizzard muscle with 10 mM EGTA results in the solubilization of a small number of proteins including actin, desmin, tropomyosin, α-actinin, and filamin. The retention of both actin and desmin on a DNase I affinity column, and their coprecipitation in indirect immunoprecipitation with either anti-actin or anti-desmin specific antisera indicates that these two proteins may be solubilized in the form of a complex. Velocity sedimentation of the EGTA extract indicates that actin and desmin form a series of complexes ranging in size from 6 to 60 S. On isolation and resedimentation of the 50-60 S actin-desmin species, it resediments to its original position on the gradient. The 50-60 S actin-desmin component has an approximate stoichiometry of 5 actin molecules per desmin molecule, and contains no free actin, as shown by anti-desmin affinity chromatography.

Introduction

Several lines of evidence suggest that desmin, the major subunit of avian smooth muscle 100 Å filaments (Cooke, 1976; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979), associates with actin in vivo. Morphological studies have shown that 100 Å filaments coterminate with actin filaments in the dense bodies of smooth muscle and in the intercalated discs of cardiac muscle (Uehara et al., 1971; Ashton et al., 1975; Cooke, 1976). Immunofluorescent staining with specific antibodies has also indicated that both actin and desmin are present at the peripheries of striated myofibril Z-discs (Lazarides and Hubbard, 1976; Granger and Lazarides, 1978). During the extraction and purification of desmin from smooth muscle, a small proportion of the total cellular

actin consistently copurifies with the desmin (Hubbard and Lazarides, 1979). This mixture of actin and desmin will polymerize in vitro to form 120 Å diameter filaments. Immunofluorescence and electron microscopy indicate that both actin and desmin are present in these filaments (Hubbard and Lazarides, 1979).

The form of desmin described above was insoluble, except at pH extremes or in the presence of denaturing agents, making the unequivocal demonstration of an actin-desmin complex difficult. As we previously reported, however, a portion of the total smooth muscle actin and desmin becomes soluble during extraction at low ionic strength in the absence of calcium (Hubbard and Lazarides, 1979). In this paper we present evidence that this soluble desmin is extracted from smooth muscle as a family of actin-desmin complexes.

Materials and Methods

Materials

Biogel A-15m and A-150m are products of BioRad (Richmond, CA), and Sephadex G-200 is a product of Pharmacia (Piscataway, NJ). DNase I was RNase free grade DPFF from Worthington Biochemicals (Freehold, NJ). Triton X-100 is a trademark of Rohm and Haas (Philadelphia, PA). All other materials were as described previously (Hubbard and Lazarides, 1979).

Analytical Methods

Protein concentrations were determined as described previously (Hubbard and Lazarides, 1979).

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Laemmli, 1970; Hubbard and Lazarides, 1979). Two-dimensional electrophoresis (IEF/SDS-PAGE) was performed using the previously described modification of the O'Farrell method (O'Farrell, 1975; Hubbard and Lazarides, 1979).

Extraction of Gizzard at Low Ionic Strength

Chicken gizzards were homogenized and subsequently extracted with 10 mM Tris-HCl, pH 7.5, 10 mM EGTA (TE buffer; EGTA is ethylene glycol-bis-[β-aminoethyl ether] N,N,N',N'-tetraacetic acid) to produce low ionic strength supernatants as previously described (Hubbard and Lazarides, 1979). The third such low ionic strength supernatant was clarified by centrifugation for 30 min at 30,000 x g and used at a concentration of 2 mg protein per ml (TE extract). This supernatant was stored at 4°C in the presence of 10 mM NaN_3; it precipitated if subjected to repeated cycles of freezing and thawing.

Gel Filtration, Affinity Chromatography, and Velocity Sedimentation

The details are given in the appropriate figure legends.

Immunoprecipitation

The preparation of antibodies against electrophoretically pure actin (Lazarides, 1976) and desmin (Lazarides and Hubbard, 1976) has been described. These antibodies exhibit no cross-reactivity with any other smooth muscle proteins or with each other, as judged by both immunodiffusion and by adsorption analysis

(Lazarides, 1976; Lazarides and Hubbard, 1976). Antisera were purified by ammonium sulfate precipitation and reconstituted in phosphate buffered saline (PBS). Control sera were completely unreactive on myofibrils by immunofluorescence (1:10 dilution).

For immunoprecipitation, antibodies were diluted 1:10 with 100 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2% Nonidet NP-40, 1 mM 1,10-phenanthroline (TEN buffer). Formalin-fixed, heat-killed, protein A bearing Staphylococcus aureus (SpA, 7) was used as a 10% suspension in TEN buffer. The 1,10-phenanthroline was included to inhibit a SpA associated protease that degrades desmin. 100 µl of diluted antibody and 50 µl of TE extract (100 µgm) were mixed and allowed to react for 1 hr at room temperature. 100 µl of 10% SpA was then added and allowed to react an additional 15 min. The SpA were collected by centrifugation and washed 3 times in TEN; 3 times in PBS, 10 mM EGTA, 2% NP-40; and once in water. The bound proteins were dissociated from the final SpA pellet in 50 µl of 8 M urea, 0.1 M β-mercaptoethanol, 2% NP-40 for 15 min at room temperature. The bacteria were removed by centrifugation for 5 min at 7000 x g and the supernatant was loaded onto IEF/SDS-PAGE gels. Since desmin and the heavy chain of IgG have similar molecular weights but different isoelectric points, the two molecules are not resolved by the one-dimensional SDS-PAGE employed here, but are well resolved by two-dimensional IEF/SDS-PAGE.

Results

Extraction and Gel Filtration of EGTA Soluble Desmin and Actin

The repeated extraction of homogenized chicken gizzard with TE buffer (20 gm gizzard muscle per 100 ml TE buffer) slowly solubilizes a small number

of proteins, including β- and γ-actin; α-actinin; α- and β-desmin; α- and β-tropomyosin; a high molecular weight protein, presumed to be filamin (Wang et al., 1975; Wang and Singer, 1977); and uncharacterized proteins of 118K, 129K, and 137K molecular weight (Fig. 1). Of these, filamin is extracted least efficiently, and its presence in the TE extract is variable. At least 50% of the total cellular desmin can be solubilized during the three successive extractions employed in the Methods. As the release of these proteins from gizzard is dependent on the presence of EGTA, we investigated the effect of readdition of divalent cations to the TE extract. Actin, desmin, 129K protein, and filamin are induced to precipitate upon the addition of 10 mM Ca^{2+} to the TE extract (Fig. 2). However, if the extract is first dialyzed against water to remove the EGTA (which does not induce precipitation), then the addition of 0.1-0.2 mM Ca^{2+} is sufficient to induce precipitation. This precipitation is inhibited at higher ionic strengths, e.g. 0.1 M NaCl. Mg^{2+} ions have the same effect as Ca^{2+} ions, while manganese or zinc ions cause the quantitative precipitation of every protein in the TE extract (data not shown). The proteins in the TE extract do not precipitate under conditions known to polymerize G-actin to filaments in vitro (i.e., dialysis first against water to remove the EGTA, then against 50 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM ATP, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM β-mercaptoethanol). Electron microscopy of this latter preparation after negative staining reveals much aggregated material, but no clearly identifiable filaments (data not shown).

We attempted to isolate actin-desmin complexes from the TE extract by several methods, including gel filtration, affinity chromatography, immunoprecipitation, and velocity sedimentation. Gel filtration of the TE extract on Biogel A-150m shows (Fig. 3) that desmin migrates independently of α-actinin,

tropomyosin, and 137K protein, and that the chromatographic profile of actin overlaps with that of each of the other proteins.

DNase I Affinity Chromatography

The elution profile of the TE extract on a Biogel A-15m DNase affinity column is shown in Figure 4e-g. Approximately 50% of the total protein applied to the column does not bind and washes through with the loading buffer (Fig. 4e). A 0.75 M guanidine-HCl wash, which releases nonspecifically bound protein (Lazarides and Lindberg, 1974), fails to elute any further protein (Fig. 4f). The 3.0 M guanidine-HCl wash, which releases specifically bound actin from DNase I (Lazarides and Lindberg, 1974), elutes actin, desmin, 129K, and 137K proteins together (Fig. 4g). Tropomyosin and α-actinin do not bind to the column, but small amounts of filamin are present in overloaded gels of the 3.0 M guanidine-HCl wash.

Since calcium is required for DNase I activity (Hitchcock et al., 1976), a control column of underivatized Biogel A-15m was run to rule out the possibility that these results were due to calcium induced precipitation of actin and desmin in the column. None of the TE extract proteins are retained by the control column in the presence of calcium (Fig. 4b-d).

Indirect Immunoprecipitation with Actin and Desmin Specific Antisera

The existence of an actin-desmin complex is further demonstrated by indirect immune precipitation with anti-actin or anti-desmin antibodies and protein A bearing Staphylococcus aureus. Actin and desmin are the major components of the indirect immune precipitates produced with either anti-desmin (Fig. 5a)

or anti-actin (Fig. 5b). Tropomyosin and α-actinin are not precipitated by the antibodies, but 129K protein, 137K protein, and filamin appear as minor components of both anti-actin and anti-desmin precipitates on one-dimensional SDS-PAGE gels (data not shown). Immunoprecipitates of the TE extract with either preimmune sera or SpA alone were completely negative, and the SpA bacteria did not contribute significant amounts of extraneous protein under our conditions (Fig. 5c).

Velocity Sedimentation

More direct evidence on the size, stability, and stoichiometry of the actin-desmin complex was obtained by velocity sedimentation. Figure 6 shows the distribution obtained by sedimenting the TE soluble proteins through a 10-30% sucrose gradient. Both actin and desmin sediment as broad, overlapping bands that range in size from about 6 S to 60 S. α-Actinin and tropomyosin sediment as sharp bands that are independent of each other, desmin, filamin, and most of the actin. Filamin, 137K protein, 129K protein, and a minor protein at 95K all sediment as broad bands between about 6 S and 40 S.

The stability of the actin-desmin sedimentation distribution produced by the experiment of Figure 6 was investigated by dividing 5 similar gradients into four partitions (P1 through P4, Fig. 6), and then resedimenting each pooled partition individually. P4, P2, and P1 are shown in Figures 7b-9b, respectively (P3 has been omitted); the corresponding resedimented distributions are shown in Figures 7a-9a, respectively. Each partition retains its original actin-desmin distribution during recentrifugation, although some spreading occurs. This spreading may indicate that the components of the complex are in a slow dynamic equilibrium or it may be due to the effects of hydrostatic pressure on the complex during centrifugation.

The 50-60 S actin-desmin component, identified and isolated by centrifugation, was examined for the presence of free actin by chromatography through a Biogel A-15m anti-desmin affinity column. An aliquot of the P1 partition was repeatedly passed through the column until all of the desmin was adsorbed (Fig. 10). The final flowthrough, shown in Figure 10a, contains no residual actin or desmin. Approximately 50% of the recoverable actin-desmin can be eluted by TE buffer in the presence of 0.1 M NaCl (Fig. 10b). This may represent the partial dissociation either of actin-desmin complexes in 0.1 M NaCl, or of low affinity desmin-antibody complexes. The remainder of the actin-desmin can be eluted with 8 M urea (Fig. 10c). Since no IgG heavy chain is released during this process, the relative amount of desmin present in each fraction could be determined from one-dimensional SDS-PAGE.

The stoichiometry of the 50-60 S actin-desmin complex has been obtained from densitometric tracings of Coomassie brilliant blue stained gels of the P1 partition (Fig. 11). The actin:desmin density ratio ranges from 3.5:1 to 4.5:1 at 580 nm. These same ratios are present in both the 0.1 M NaCl (Fig. 10b) and the 8 M urea (Fig. 10c) eluates from the anti-desmin affinity column. The actin to desmin (density) ratio in the unfractionated TE extract is 10:1, suggesting that about 40% of the actin present may be involved in the formation of complexes with desmin. The composition of actin-desmin complexes smaller than 50 S has not yet been investigated.

Discussion

The Existence and Properties of an Actin-Desmin Complex

In the present paper we have investigated the properties of the actin

and desmin that are slowly released from chicken gizzard smooth muscle during extraction with Tris-HCl buffered EGTA. The results presented here show that actin and desmin form a series of stable complexes ranging in size from 6 S to 60 S which can be isolated without the use of potentially denaturing solubilizing agents. The existence of these complexes is supported by 1) the specific coretention of actin and desmin on a DNase I affinity column, 2) the coprecipitation of actin and desmin by either anti-actin or anti-desmin antibodies, and 3) the isolation of stable size classes of actin-desmin complexes by velocity sedimentation. The majority of these complexes sediment in the 30-40 S range. The stoichiometry of the largest class of actin-desmin complexes (partition P1, 50-60 S) is approximately 5 actin molecules per desmin molecule. About 40% of the total TE soluble actin is involved in the formation of actin-desmin complexes.

We have previously reported that both desmin and a portion of the total cellular actin remain insoluble in residues of chicken gizzard that had been enriched in 100 Å filaments by extraction with 1 M KI (Lazarides and Hubbard, 1976; Hubbard and Lazarides, 1979). It was found that this actin and desmin had similar solubility properties and that they always copurified with each other throughout a variety of different extraction procedures. They also comigrated during gel filtration in the presence of the detergent Sarkosyl NL-97. Preparations that were highly enriched in desmin would reassociate from acetic acid to form filaments with diameters of 120-140 Å. The residual actin that was still present appeared to bind tenaciously to these filaments. From these data, we concluded that desmin is the major component of avian muscle 100 Å filaments and that actin and desmin form a complex (Lazarides and Hubbard, 1976; Hubbard and Lazarides, 1979).

The previously described actin-desmin complex was only soluble in agents such as acetic acid, detergents, and urea, and it either precipitated or formed insoluble filaments when these agents were replaced with water. These solubility properties differ from those of the EGTA solubilized complexes described in these Results. These latter complexes remain soluble if the EGTA is removed by dialysis, so long as the divalent cation concentration remains low. These differences may be due, in part, to the differing procedures used to isolate the complexes. From our results, at least half, and perhaps all, of the desmin in freshly homogenized gizzard may be extracted by TE buffer. In contrast, once the extracted gizzard muscle has been dehydrated with acetone, TE buffer can no longer solubilize desmin from it (unpublished observations). Similarly, actin-desmin containing filaments that have been prepared from acetic acid extracts are not soluble in 20 mM EDTA (Hubbard and Lazarides, 1979). Thus, the exposure of desmin to acetic acid, Sarkosyl NL-97, urea, or dehydration may alter its solubility properties (Hubbard and Lazarides, 1979).

Other major proteins present in the TE extract are actin, α-actinin, tropomyosin, 129K protein, and 137K protein. The TE solubilized β- and γ-actin retains both its antigenicity and DNase I binding activity in spite of prolonged exposure to low calcium, low ATP conditions (up to 5 months). This is in contrast to α-actin, which permanently loses its DNase I binding activity after even brief exposure to low calcium conditions (Hitchcock et al., 1976). It is not known if this marked stability of TE soluble actin in low Ca^{2+} is a characteristic property of smooth muscle actin alone, or if it is due to a stabilizing effect from one or more accessory proteins found in the EGTA extracts. The DNase column data indicate that such a stabilizing effect might be due to desmin, 129K protein, or

137K protein. Neither α-actinin nor tropomyosin were observed to associate with actin under our conditions. This is consistent with data indicating that the binding of skeletal muscle tropomyosin to α-actin requires Mg^{2+} and/or KCl, but is dissociated by high salt (Eaton et al., 1975). Not all of the actin (or actin associated desmin) bound to the DNase column. This appears to be a reproducible, but presently unexplained result.

The Origin of the Actin-Desmin Complex from Smooth Muscle

We have previously proposed that desmin serves to mechanically integrate the individual contractile units of striated muscle (Lazarides and Hubbard, 1976; Granger and Lazarides, 1978; Hubbard and Lazarides, 1979). This might occur through an actin-desmin complex similar to that described in these Results. Considering the stability of this complex in 0.75 M guanidine-HCl (Fig. 4f) it seems likely that the same associations may exist in vivo. It has been shown that the removal of calcium promotes the efficient extraction of both actin and desmin (these Results) and of dense bodies (Schollmeyer et al., 1976). In addition, thin filaments (actin) and 100 Å filaments (desmin) are seen to associate with and insert into dense bodies (Uehara et al., 1971; Ashton et al., 1975; Cooke, 1976). Finally, α-actinin and tropomyosin are associated with dense bodies (Schollmeyer et al., 1976) and are extracted along with the actin and desmin above. These data suggest that the low calcium soluble actin-desmin complexes may be a constituent of the smooth muscle dense bodies. From these results, we suggest that actin-desmin complexes exist in smooth muscle as components of a calcium stabilized structure(s), presumably dense bodies. Removal of the calcium by chelation

at low ionic strength would allow the slow dissociation of these structures to occur with the concomitant appearance of soluble actin-desmin complexes of various sizes.

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Figure 1 SDS-PAGE analysis of the proteins extracted from chicken gizzard by low calcium, low ionic strength (TE) buffer. 20 μl of TE extract at 2 mg/ml of protein was loaded onto the gel. Present are actin (A), α-actinin (α-A), desmin (D), filamin (F), α- and β-smooth muscle tropomyosin (α-TM, β-TM), and unidentified proteins of molecular weight 118,000 (118), molecular weight 129,000 (129), and molecular weight 137,000 (137). These molecular weights were determined by relative comigration, on SDS-PAGE, against standards of myosin heavy chain (molecular weight 200,000), β-galactosidase (molecular weight 130,000), phosphorylase A (molecular weight 100,000), bovine serum albumin (molecular weight 68,000), pyruvate kinase (molecular weight 57,000), and actin (molecular weight 42,000).

Figure 2 Precipitation of TE soluble proteins by 10 mM $CaCl_2$. The TE extract was dialyzed against 10 mM NaN_3, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, and the resulting precipitate (2a) was separated from the remaining soluble proteins (2b) by centrifugation at 7000 x g for 5 min and analyzed by SDS-PAGE.

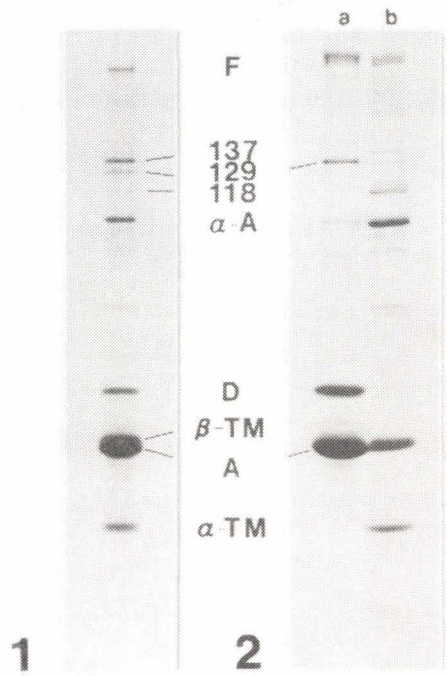
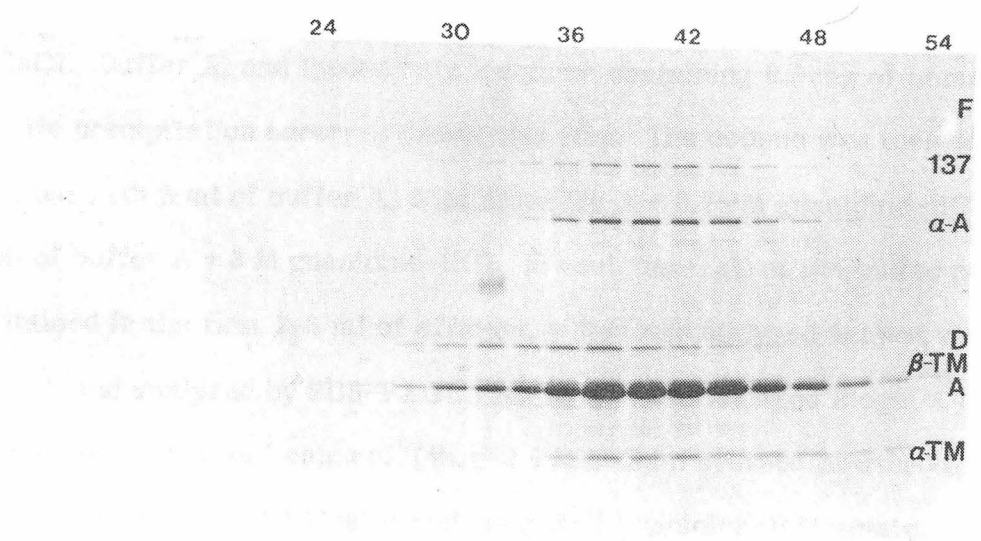

a
b
F
137
129
118
α-A
D
β-TM
A
α-TM
1
2

Figure 3 SDS-PAGE analysis of the fractions produced by gel filtration of the TE soluble proteins on Biogel A-150m. 0.5 ml (1 mg) of TE extract was loaded on a 1.2 x 50 cm column of Biogel A-150 (molecular weight limits of 5×10^6-1.5×10^8) that was equilibrated in 10 mM Tris-HCl, pH 7.5, 1 mM EGTA. The material was eluted in the same buffer at a flow rate of 12 ml/hr. 1 ml fractions were collected, lyophilized, and fractions 8, 10,..., 54 were redissolved in SDS sample buffer and analyzed by SDS-PAGE. A void volume marker of T4 phage began eluting in fraction 18 while an included dye marker eluted in fractions 47 to 55. Little filamin was present in this TE extract. Abbreviations are as in Figure 1.



3

Figure 4 DNase I affinity chromatography of the TE soluble proteins. DNase I was coupled to Biogel A-15m agarose at the rate of 1 mg DNase I per 1 ml of damp-packed resin as described (Lazarides and Lindberg, 1974). The DNase I derivatized resin was treated with 1 mM phenylmethylsulfonyl fluoride for 1 week before use. 250 µl of TE extract (0.5 mg) was diluted 1:1 with 0.5 M sodium acetate, 1 mM $CaCl_2$ (buffer A) and loaded onto a column containing 0.5 mg of immobilized DNase. No precipitation occurred during this step. The column was then sequentially eluted with 6 ml of buffer A, 6 ml of buffer A + 0.75 M guanidine-HCl, and 6 ml of buffer A + 3 M guanidine-HCl. In each case, all of the eluted protein was contained in the first 2-3 ml of effluent, which was dialyzed against water, lyophilized, and analyzed by SDS-PAGE. (4b-d) an underivatized Biogel A-15m column and (4e-g) DNase I column. (4b,e) 0.5 M sodium acetate flowthrough; (4c,f) 0.75 M guanidine-HCl eluate; and (4d,g) 3 M guanidine-HCl eluate. (4a) shows the initial sample. Abbreviations are as in Figure 1.

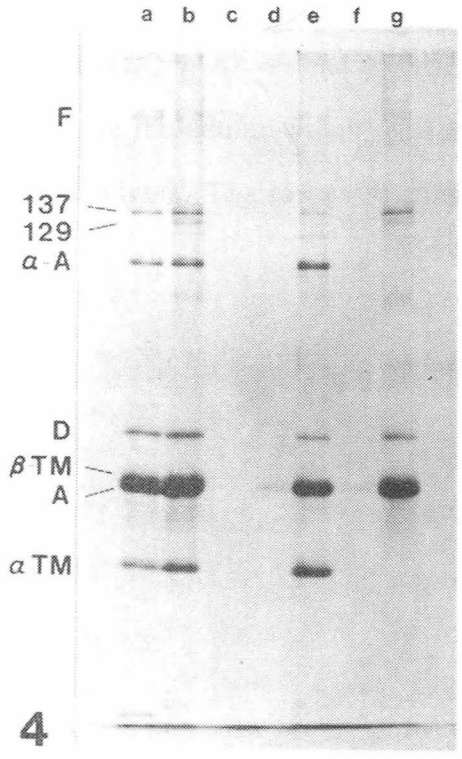
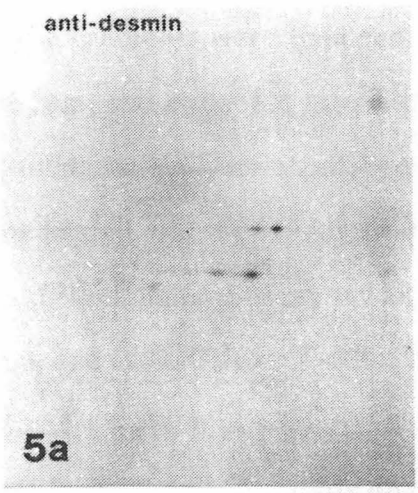
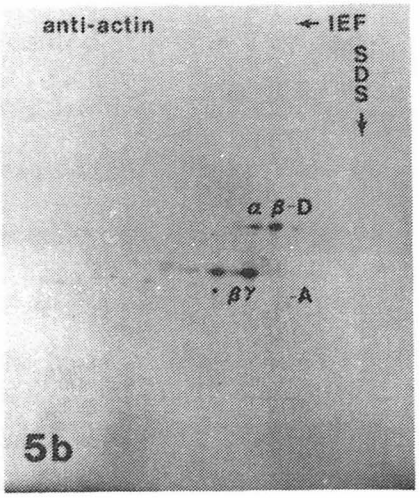

a b c d e f g
F
137
129
α A
D
β TM
A
α TM
4

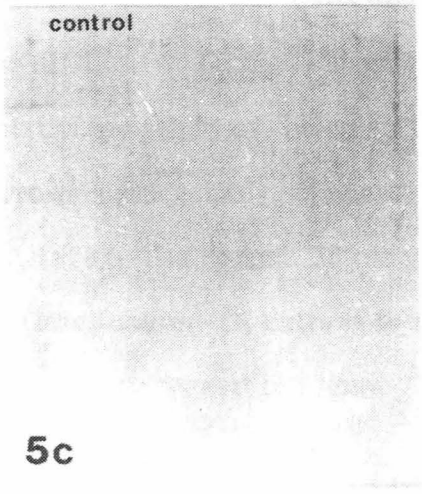
Figure 5 IEF/SDS-PAGE analysis of the TE soluble proteins precipitated by anti-desmin (5a), anti-actin (5b), and control anti-serum (5c). Indirect immunoprecipitations were preformed using SpA and analyzed as described in the Methods. The basic side of the first (IEF) dimension is in the upper right and focusing occurred from right to left. The second dimension of SDS-PAGE proceeded from top to bottom. Labeled in (5b) are: β- and γ-actin (β-, γ-A), α- and β-desmin (α-, β-D), and a protein marked ○ which has the same molecular weight and antigenicity as actin, but which is otherwise uncharacterized. The same proteins are present in (5a).



5a

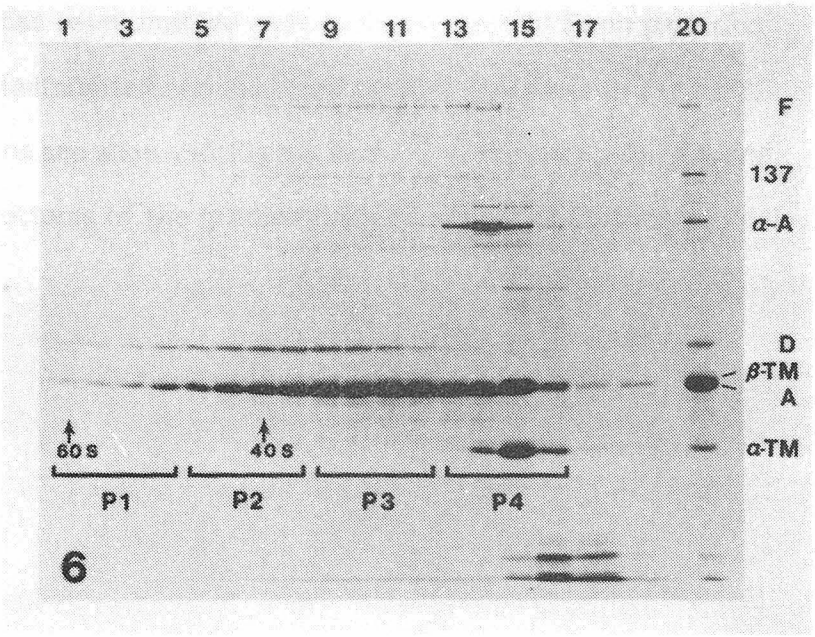


5b



5c

Figure 6 Sedimentation profile of the TE soluble proteins on 10-30% sucrose density gradients. Gradients of 10 to 30% (w/w; 0.303 to 0.988 M) sucrose in TE buffer were prepared and 400 µl of TE extract (0.8 mg protein) was layered on top of each gradient. The gradients were then sedimented at 5°C in a SW50.1 rotor for 9 hr at 40,000 rpm, and collected into 5-drop fractions (0.3 ml each). The fractions of one gradient were lyophilized, redissolved in 100 µl of SDS sample buffer, and 50 µl of this loaded onto SDS-PAGE. Ribosomes were prepared by homogenizing 10-day-old chick embryo thigh muscles in 0.12 M KCl, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, and .05% Triton X-100. The homogenate was clarified at 15,000 x g for 15 min at 4°C. 400 µl aliquots of the supernatant were layered on top of 10-30% RNase free sucrose gradients containing 0.12 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5. The ribosome peaks were located by their OD at 260 nm. The relative positions of the small and large subunits are indicated by the 40 S and 60 S arrows, respectively. The sedimentation constant of α-actinin is 6.25 S (Robson et al., 1970). The bottom of the gradient is in well 1, the top is in well 18, and the unfractionated TE extract is shown in well 20. The designations P1-P4 are explained in the legend to Figure 7; all other abbreviations are as in Figure 1.


1
3
5
7
9
11
13
15
17
20
F
137
α-A
D
β-TM
A
α-TM
60 s
40 s
P1
P2
P3
P4
6

Figures 7, 8, 9 Resedimentation of actin-desmin complexes. The fractions corresponding to P1-P4 in Figure 6 were pooled from 5 identical gradients. Each pooled partition, P1-P4, was dialyzed against TE buffer to remove the sucrose and its volume then reduced from 5 ml to 0.5 ml by immersing the dialysis bag in dry Sephadex G-200. The concentrated P4 is shown in Figure 7b, P2 in Figure 8b, and P1 in Figure 9b; P3 has been omitted as it is similar to P2. Each partition was then individually resedimented and analyzed by SDS-PAGE as in Figure 6. The resulting distributions are shown in Figure 7a for P4, Figure 8a for P2, and Figure 9a for P1. The bottoms of the gradients are in well 1. All other abbreviations are as in Figure 1.

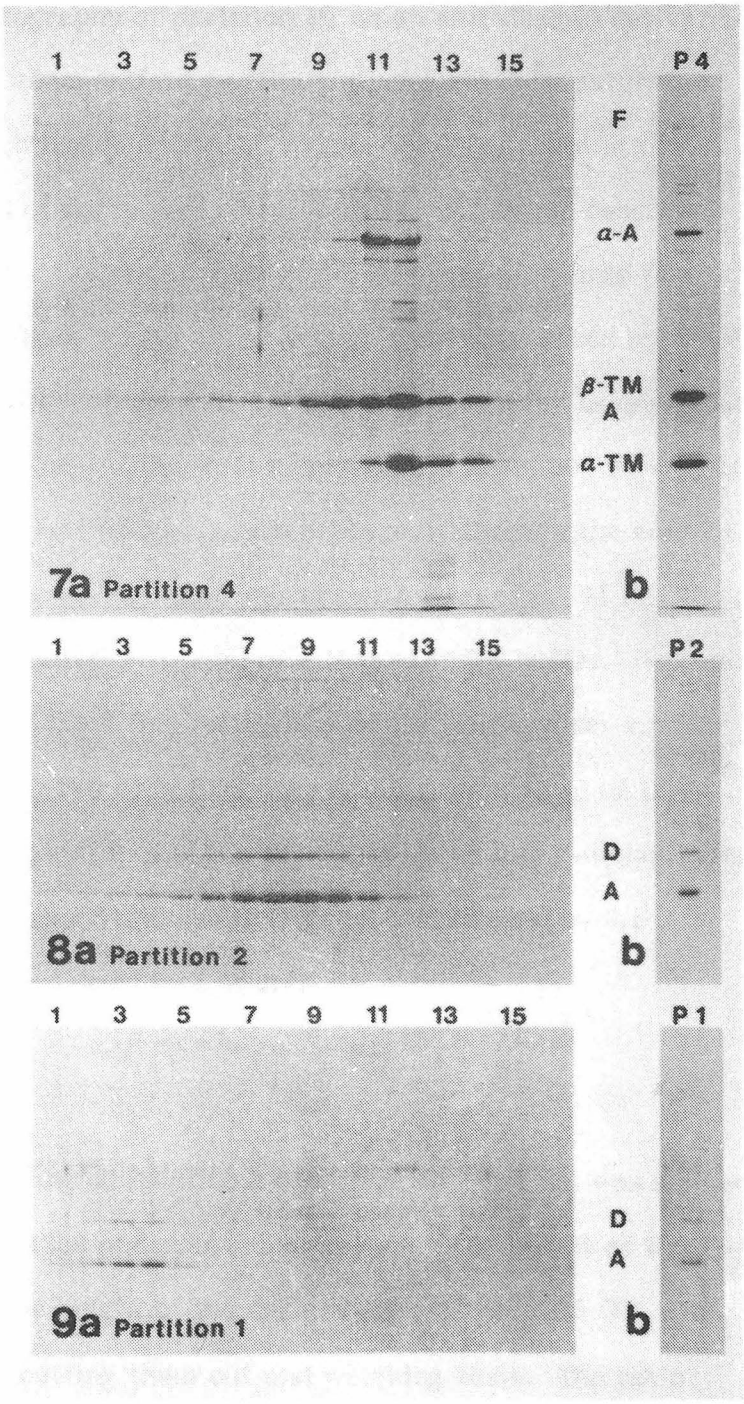
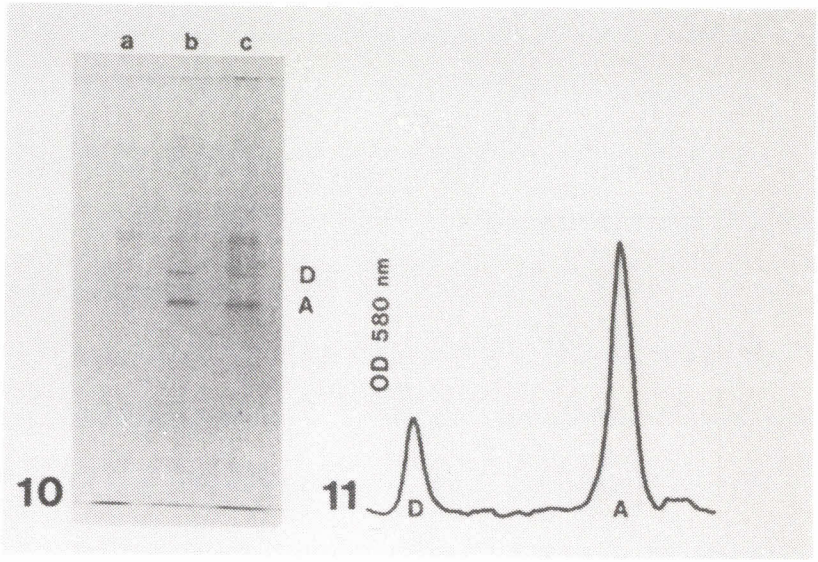

1 3 5 7 9 11 13 15
P 4
F
α-A
β-TM
A
α-TM
7a Partition 4
b
1 3 5 7 9 11 13 15
P 2
D
A
8a Partition 2
b
1 3 5 7 9 11 13 15
P 1
D
A
9a Partition 1
b

Figure 10 Affinity chromatography of partition P1 on an anti-desmin derivatized Biogel A-15m column. Ammonium sulfate purified anti-desmin (50% saturation cut) was coupled to cyanogen bromide activated Biogel A-15m agarose in the ratio of 2 mg protein per 1 ml of damp-packed resin using the method described (Lazarides and Lindberg, 1974). Columns containing 2 ml of coupled resin in a 5 ml syringe were prepared. These were washed with 8 M urea, followed by 0.1 M NaCl, and finally by TE buffer before use. P1 partition was prepared as described in Figures 6 and 7, but was not dialyzed or concentrated, and contained 27% sucrose and 30 μgm protein/ml. 1.0 ml of this was passed repeatedly through the column for a total of 10 times. The column was then washed with 6 ml of 0.1 M NaCl in TE buffer and subsequently eluted with 6 ml of 8 M urea in TE buffer. No protein bled from one step to the next. Each final effluent was dialyzed against 1 mM NaCl, lyophilized, redissolved in 150 μl of SDS sample buffer and 50 μl of this loaded on SDS-PAGE. (10a) shows the protein remaining in the input aliquot after the tenth pass through the column. (10b) shows the protein released by 0.1 M NaCl. (10c) shows the protein released by 8 M urea.

Figure 11 Densitometer tracing of fraction 4 of Figure 9a. The gel was stained with Coomassie brilliant blue R250 and scanned at 580 nm. The height of the actin (A) peak is 0.1 OD and the height of the desmin peak (D) is 0.035 OD. The peak areas were measured by cutting them out and weighing them. The ratio A/D here is 3.50. This ratio ranges from 3.5 to 4.5 on various partition 1 preparations. If an average stain density ratio of 4.0 is assumed, this gives a molar ratio of 5 actin molecules to 1 desmin molecule (mwt:actin = 42,000; desmin = 50,000).


a
b
c
D
A
10
OD 580 nm
11
D
A

CHAPTER FIVE

Studies on the Proteolytic Digestion of Desmin

Abstract

Clostridial collagenase was found to cleave desmin, sequentially producing fragments of 43,500 mwt, 40,000 mwt, and a limit digestion product of 35,000 mwt. The 35,000 mwt product lacks 60% of the methionine and 82% of the tyrosine of the original desmin (molecular weight 50,000). The cleavage process results in the conversion of gelled desmin to a non-cohesive, powdery, precipitate. β- and γ-actin from smooth muscle are also degraded, but α-actin, α-actinin, and myosin are all comparatively resistant. Proteolysis requires the presence of Ca^{2+}, can be inhibited by EGTA, and 1,10-phenanthroline, but was unaffected by either PMSF or N-ethylmaleimide.

Fractionation of the collagenase by gel filtration on Ultrogel AcA-34 produced four fractions of differing protein composition that exhibit desmin cleaving activity (DCA). These did not coincide with the major peak of collagenase activity and three of the four desmin cleaving activities had no collagenase activity at all. Two other proteases that produced similar cleavages of desmin were identified by comparing two-dimensional gels of the fragments. One of the proteases is associated with Staphylococcus aureus, while the other is the calcium activated factor of muscle (Dayton et al., 1976a,b).

The effect of the collagenase associated DCA on the structure of polymeric desmin was investigated by gel filtration and bifunctional protein cross-linking reagents. Possible physiological roles of the DCA are discussed.

Introduction

Living skeletal muscle cells display a characteristically striated appearance, resulting from the regular transverse alignment of their component myofibrils.

This alignment is maintained in the small bundles of myofibrils that are produced by mechanical disruption of the original cells, and does not depend upon either actin-myosin interaction or the plasma membrane (see, for example, Granger and Lazarides, 1978). During the course of other experiments, it was noticed that clostridial collagenase caused the separation of these bundles of aligned myofibrils into single myofibrils. This was a surprising observation inasmuch as collagen is not generally believed to be an intracellular component of striated muscle. More surprising was the subsequent observation that collagenase also removed the desmin, observable by indirect immunofluorescence, from these myofibrils. This chapter describes the fractionation of clostridial collagenase into its active components, and the effect of these components on the structure of desmin.

Materials and Methods

Materials

Desmin was extracted from acetone powders of KI extracted chicken gizzard and purified through two cycles of precipitation and resolubilization as described (Hubbard and Lazarides, 1979). The cycle-2 desmin was stored at -80°C in 1 mM HCl; its shelf life was about three weeks under these conditions. α-Actin was prepared from chicken breast muscle as described (Spudich and Watt, 1971).

Reagents

Digestion buffer contains 200 mM NaCl, 10 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.5, and 2 mM PMSF, pH 7.5. Cross-linking buffer contains 0.5 M NaCl, 10 mM $NaCl_2$, and 0.5 M triethanolamine-HCl, pH 8.0. EGTA/P solution contains 10 mM EGTA and 20 mM O-phenanthroline in 50% aqueous ethanol. Dithiobis (succinimidyl

propionate), DTSP, was prepared as a stock solution of 20 mg/ml in anhydrous acetone and stored dessicated in the dark. It was diluted 1:10 with water just before use to produce a working solution of 2 mg/ml in 10% acetone. Non-reducing SDS sample buffer for SDS-PAGE of cross-linked proteins was the same as reducing sample buffer, except that the reducing agent was omitted (Hubbard and Lazarides, 1979).

Collagenase (CLS-3), soluble calf skin collagen (CLCS), and TPCK-treated trypsin (TRTPCK) were purchased from Worthington Biochemical Corporation (Freehold, NJ). Dithiobis (succinimidyl propionate) was from Pierce Chemical Co. (Rockford, IL). PMSF is phenylmethylsulfonyl fluoride; TLCK is tosyllysine chloromethylketone; TPCK is tosylphenylalanine chloromethylketone; O-phenanthroline is 1,10-phenanthroline; EGTA is ethylene glycol bis(β-aminoethyl ester) N,N,N',N'- tetracetate.

Methods

Electrophoresis, protein concentration, and molecular weight determinations were performed as previously described (Hubbard and Lazarides, 1979).

Amino Acid Analysis

Desmin for amino acid analysis was solubilized directly from KI insoluble residues of chicken gizzard with hot 1% SDS, 10 mM 2-mercaptoethanol and clarified by centrifugation at 40,000 x g for 20 min at 20°C. Desmin was fractionated from the SDS extract by gel filtration on a 110 x 2 cm column of Ultrogel AcA-34 equilibrated with 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM NaN_3. The column was eluted at a rate of 12-15 ml/hr and 1 ml fractions were collected.

The fractions were assayed by SDS-PAGE and those containing the purest desmin were pooled and dialyzed against water, causing the desmin to precipitate. The purified desmin was collected by centrifugation and dissolved in 6 N redistilled HCl in thick walled glass tubes. The tubes were purged with nitrogen, sealed under a pressure of 1.5×10^{-2} Torr, and heated for 2, 4, 6 or 8 hr at 145°C (Roach and Gehrke, 1970). The hydrolyzed desmin was lyophilized and subjected to automated amino acid analysis on a Durrum D-500 amino acid analyzer (Durrum Instrument Co., Palo Alto, CA). Hydroxyproline was determined by the method of Bergman and Loxley (1963).

Results

Proteolysis

Cycle-2 desmin (containing about 15% actin) was initially proteolyzed with unfractionated Worthington CLS-3 collagenase, a preparation described as being especially low in extraneous proteolytic activities (Worthington Enzyme Manual). Desmin was first precipitated from acid solution as a cohesive mass by rapid neutralization to pH 7.5, then treated with 1% collagenase (w/w) in the presence of 5 mM Ca^{2+}. Typical results are shown in Figure 1. The desmin (molecular weight 50,000) is sequentially cleaved to yield major fragments D1 of molecular weight 43,500, D2 of molecular weight 40,000, and limit digestion fragment D3 of molecular weight 35,000. During this process, the cohesive desmin precipitate is converted into a fine and non-cohesive powder. The desmin-associated actin (β- and γ-isoelectric variants) is slowly degraded into unidentified products. This occurs at about the same rate as the cleavage of desmin so that the complete conversion of desmin to D3 corresponds with the disappearance of identifiable

actin from SDS-PAGE. These cleavages appear to be fairly specific as both bovine serum albumin and α-actin are completely resistant to digestion. Both α-actinin and myosin are also found to be comparatively resistant to degradation in muscle extracts containing these proteins (data not shown). Neither trypsin nor chymotrypsin cleaves desmin in the same limited fashion that collagenase cleaves it in.

Analysis of the CLS-3 collagenase by SDS-PAGE revealed the presence of several protein bands. These were fractionated by gel filtration on Ultrogel AcA-34, the effluent fractions divided into four major groups, designated F1 through F4 (Fig. 2), and assayed for activity against desmin (Fig. 3). Fraction F1 has no activity, while fractions F2 and F4 have the previously described limited proteolytic activity against desmin. This requires Ca^{2+} and is inhibitable by either EGTA or O-phenanthroline. PMSF, N-ethylmaleimide, TAME, TPCK, TLCK, arginine, and soybean trypsin inhibitor have no effect on it (data not shown). Fraction F3, containing at least two activities, causes extensive degradation of desmin and cannot be completely inhibited by either EGTA or O-phenanthroline. Again, neither PMSF nor N-ethylmaleimide has any effect on fraction F3 activity. These fractions were also assayed for collagenase activity against soluble calf skin collagen (Fig. 4). The strongest collagenase activity is found in fraction F1 (Fig. 4, column 1), while a weaker activity occurs in fraction F3 (Fig. 4, column 4). Both of these activities are Ca^{2+}-dependent and can be inhibited by EGTA or O-phenanthroline (data not shown). From this comparison of fractions F1, F2, and F4, it appears that the anti-desmin and anti-collagen activities in commercial collagenase are unrelated.

The limited proteolysis of desmin by fraction F2 was investigated by IEF/SDS-PAGE as shown in Figure 5. Both α- and β-desmin appear to be cleaved in a fashion that maintains a relative P_I differential between them. While there is very little change in P_I values after cleavage 1 (desmin to D1) or cleavage 3 (D2 to D3), a major change occurs after cleavage 2 (D1 to D2) that results in a shift in the average P_I from 5.7 for D1 to 5.2 for D2.

Two other proteases that produce limited digestions of desmin that are very similar to those produced by the above described collagenase fractions have been discovered. One is a protease that is associated with the Cowan I strain of Staphylococcus aureus that is used as a source of protein A (Kessler, 1975). It is active in the presence of EGTA, but is inhibited by O-phenanthroline. The other is the calcium activated protease of muscle (Dayton et al., 1976a,b). Both of these produce the same pattern of desmin fragments on IEF/SDS-PAGE as was shown above in Figure 5 (data not shown).

The effect of fraction F4 cleavage on the polymeric state of desmin has been investigated with gel filtration and bifunctional cross-linking agents. Both desmin and its cleavage products are essentially insoluble in aqueous buffers at pH 7-8. Consequently, the gel filtration and cross-linking was done in the presence of Sarkosyl NL-97. Previous work has shown that Sarkosyl solubilizes cycle-2 desmin by dissociating it into two major actin-desmin-containing components, one of which migrates through Ultrogel AcA-34 with an apparent molecular weight of 200,000, while the other is excluded from such a column (Hubbard and Lazarides, 1979). Sarkosyl does not appear to cause the type of extensive denaturation that SDS does, as 100 Å-like filaments can still be formed by desmin that has been treated with Sarkosyl, but not with SDS.

Cycle-2 desmin was digested with F4 to yield a mixture containing roughly equal proportions of D1, D2, D3 and uncleaved desmin. These were solubilized in 0.5% Sarkosyl NL-97 and analyzed by gel filtration on Ultrogel AcA-34 as shown in Figure 6. The dissociation of desmin into two major components is similar to that previously reported. Of interest is the presence of fragments D1-D3 in the high molecular weight fraction, and also the apparently independent migration of desmin, D1, D2, and D3 in the lower molecular weight component. This lower molecular weight component migrates in the 150,000-200,000 mwt region of the column. It contains considerably less actin than the higher molecular weight component.

Three bifunctional cross-linking agents were investigated, and dithiobis (succinimidyl propionate) was found to be considerably more satisfactory under our conditions than either dimethylsuberimidate or methyl-4-mercaptobutyrimidate. In the absence of Sarkosyl, dithiobis (succinimidyl proprionate), DTSP, cross-links both desmin and its cleavage fragments into high molecular weight products that are too large to enter SDS-PAGE stacking gels (data not shown). The results of cross-linking desmin in the presence of Sarkosyl are shown in Figure 7. Figure 7a shows selected fractions from the column of Figure 6, representing both the high and low molecular weight components of solubilized desmin. Figure 7b shows these fractions after being partially cross-linked with DTSP under non-reducing conditions. Fractions 13 to 17 produce cross-linked products in the molecular weight range of 100,000-200,000 while fractions 7 to 11 produce only very large products that fail to enter the gel. DTSP cross-linking of pure D3 in the presence of Sarkosyl produces products in the molecular weight range of 90,000-110,000

(data not shown). Under these conditions, DTSP did not produce any cross-linking of desmin in the presence of SDS instead of Sarkosyl (data not shown).

The amino acid compositions for desmin and fragment D3 have been obtained as described in the Methods, and are shown in Table 1. Desmin is comparatively rich in ASX and GLX residues, but it is not known how many of these are in the amide form. A peak running between phenylalanine and histidine comigrated with D-galactosamine, indicating that desmin may be a glycoprotein. During the cleavages that result in the production of D3, unusually high percentages of both methionine and tyrosine are lost from the molecule. No hydroxyproline was detected in desmin (Bergman and Loxley, 1963).

Discussion

The Specificity of Desmin Cleaving Enzymes

In this paper I report the discovery of a new proteolytic activity from Clostridium histolyticum and characterize its action on desmin, the subunit of 100 Å filaments in avian muscle. The desmin cleaving activity (DCA) was initially discovered as a contaminant in clostridial collagenase preparations, from which it was fractionated into three partially overlapping fractions, F2, F3, and F4, by gel filgration. F4 contains components of molecular weight $\leq$17,200, F3 contains proteins of molecular weights 31,000 and 38,000, and F2 contains a protein of 42,000 mwt. While the low molecular weight components of F4 certainly contain DCA, incomplete separation of the F4 components from F3 and F2 during gel filtration makes it impossible to assess the relative contribution to DCA of the other proteins present. The proteolytic and inhibition properties of F3 differ

from those of F2 and F4, however, indicating that it probably contains at least one additional activity that is not present in either F2 or F4. It also exhibits a weak collagenase activity.

The F4 DCA requires Ca^{2+} for activity and can be inhibited by EGTA. It can also be inhibited by O-phenanthroline, even in the presence of Ca^{2+}, indicating the additional presence of a bound transition element. O-phenanthroline forms high stability complexes with transition elements, of which iron, cobalt, nickle, copper, and zinc are of most interest from a biochemical point of view. In particular, zinc is an essential component of many proteases, including carboxypeptidases, aminopeptidases, collagenases, and neutral proteases (Boyer, 1971). In the case of carboxypeptidase A, there is one atom of zinc situated at the active site where it serves to polarize the carbonyl group of the peptide bond that is under attack (Boyer, 1971).

DCA is resistant to inhibition by either PMSF or N-ethylmaleimide, indicating the absences of both serine and cysteine from the active site. Finally, DCA is active in the pH range of 7 to 8. While we have not determined the specificity of cleavage of DCA, it is not appreciably inhibited by any of arginine, tosylphenylalanine chloromethylketone, or tosyllysine chloromethylketone.

These properties serve to classify DCA in the category of neutral bacterial proteases (Boyer, 1971). As a group, the neutral proteases show a preference for bulky hydrophobic amino acids, especially leucine, phenylalanine, and tyrosine, on the amino side of the bond being cleaved. While most of the neutral proteases do not appear to be highly selective, the specificity studies performed with them have generally involved synthetic peptides and denatured proteins as substrates. Native proteins would not be expected to contain sequences of hydrophobic amino

acids on their surfaces and this consideration could lend greater specificity to the neutral proteases under our conditions.

In the following section, similarities between the specificities of clostridial DCA and a calcium-dependent protease of brain and muscle will be discussed. If this may be anticipated, Guroff (1964) has determined that the brain protease selectively cleaves the oxidized β-chain of insulin at the tyr-leu bond in the sequence NH_2-glu-ala-leu-tyr·leu-val-cys-gly. The protease was inactive, however, on a wide range of di- and tripeptides and on a collection of ten enzymes involved in intermediary metabolism (Drummond and Duncan, 1968). The tyr-leu bond is one of the most widely recognized by neutral proteases, but they are also active on the tyr-leu dipeptide, however, and on related bonds such as his-leu, ala-leu, leu-val, phe-phe, and phe-tyr (Boyer, 1971). Consequently, the adjoining amino acids must be of considerable importance in determining the specificity of DCA. The IgA protease produced by certain strains of streptococci and neisseria is one example of a very specific neutral protease (Plaut, 1978). It is specific for the pro-thr bond in the sequence thr-pro-pro·thr-pro-ser-pro-ser that occurs only in the hinge region of human IgA-1.

During the course of this investigation we encountered two other proteases with DCA activity. One is produced by Cowan I Staphylococcus aureus and is most similar to the clostridial F3 DCA. The other is a calcium-activated protease that selectively degrades the Z-disc densities of striated muscle (Dayton et al., 1976b). This protease is one of a growing number of highly specific calcium-dependent proteases discovered in mammalian tissues in recent years. Included in this group are a protein kinase activating enzyme from brain (Inoue et al., 1977), an estrogen receptor cleaving enzyme (Puca et al., 1977), a protease that is specific

for certain proteins found in platelets (Phillips and Kakabova, 1977) and a calcium-dependent phosphorylase b kinase activating factor bound in both brain and muscle (Drummond and Duncan, 1968; Meyer et al., 1964; Guroff, 1964). Comparative work by Drummond and Duncan (1968) and by Dayton et al. (1976a) has shown that the phosphorylase b kinase activating factors (KAF) of brain and muscle are essentially identical to each other and to the calcium-activated protease of muscle (CAF).

The in vivo functions of KAF and CAF have not yet been elucidated. There are several problems. First is that these proteases require the presence of roughly millimolar concentrations of calcium before showing appreciable activity in vitro. Second is that the kinase activation produced by KAF is irreversible, which does not fit into the accepted scheme of cAMP-dependent phosphorylase b kinase activation/inactivation (Drummond and Duncan, 1968; Rubin and Rosen, 1975). Based upon its ability to degrade Z-bands, Dayton et al. (1976a,b) suggest that CAF is involved in the catabolism of muscle proteins, especially in damaged or dysfunctional muscle.

I would like to suggest an alternate approach to the functions of KAF and CAF, as follows. When an enzyme is assayed in vitro, one is looking for conditions that produce extensive activation and substrate turnover during a time course that is usually measured in minutes. This level of activation may be entirely inappropriate in terms of the in vivo function. For example, 10^{-5} M calcium may produce a low level activation of KAF that operates satisfactorily over a time span of days, instead of minutes. There may also be as yet undiscovered activating factors that amplify the effects of low calcium concentrations. In terms of a specific model, it is possible that CAF is a muscle restructuring enzyme and not simply a catabolic agent. For example, it has been observed that desmin surrounds

each myofibril Z-disc in striated muscle like a collar (Granger and Lazarides, 1978). During the hypertrophy of muscle, the myofibrils split longitudinally and new desmin is deposited between them (Goldspink, 1971; Granger and Lazarides, 1978; Shear and Goldspink, 1971). It is possible that the old collar of desmin would have to be loosened during this process, which CAF could accomplish in its capacity as a desmin cleaving activity. The increased calcium concentration transients associated with exercise might produce just enough active CAF to fulfill this function, but not enough to degrade the entire Z-band as occurs in the in vitro assay.

CAF, in its capacity as KAF, could also produce a long-lived but low-level activation of phosphorylase b kinase. This would result in increased glycogen catabolism and allow a higher level of metabolic activity in resting muscle, which would also aid in the process of hypertrophy. Such an effect has been observed in heavily exercised muscle. During single episodes of muscle activation, there is a rapid conversion of phosphorylase b to phosphorylase a, followed by a return to the previous resting level of phosphorylase a after activation (Danforth and Helmreich, 1964; Danforth et al., 1962). In muscle that has been subjected to a regimen of training, however, there is a 20-100% increase in the resting phosphorylase a levels over the untrained levels (Taylor et al., 1972; Lamb et al., 1969; Exner et al., 1973; Staudte et al., 1973). The actual mechanism underlying this increase in the basal level of phosphorylase a is presently unknown.

The Cleavage of Desmin by DCA

DCA sequentially cleaves desmin to produce major fragments D1 of molecular weight 43,500, D2 of molecular weight 40,000, and D3 of molecular

35,000. During this process, the P_I shifts from 5.7 for desmin to 5.2 for D3 and approximately 82% of the tyrosine is lost. This is accompanied by a loss of cohesiveness of the precipitated desmin, although D3 is not appreciably soluble in aqueous buffers at pH 7.5. The desmin-associated β- and γ-actins are degraded during the cleavage of desmin and while it is not known if these two processes are connected, the net result is to effect the release of D3 from the associated actin.

DCA also causes the release of desmin from skeletal myofibril bundles and the concomitant separation of these bundles into single myofibrils (data not shown). This provides additional evidence that the function of desmin involves mechanically interlinking the individual myofibrils.

The gel filtration and cross-linking data permit some conclusions to be drawn on the effect of DCA on desmin-desmin interactions. The ability of DTSP to cross-link the desmin family that migrates with an apparent molecular weight of 200,000 during filtration, into a complex that migrates with the same molecular weight during SDS-PAGE, indicates that this family actually represents a polymeric structure and is not simply the result of anomalous migration in the presence of a detergent. The cross-linked molecular weight of 200,000 could accommodate a tetrameric structure of desmin. The possible contribution to this from actin will be ignored since relatively little actin is present in this family. Similar considerations apply to the >350,000 mwt family, although why this family is relatively resistant to both Sarkosyl dissociation and to DCA is not clear.

The DCA cleaved products of the molecular weight 200,000 family appear to migrate independently on gel filtration but cross-link to complexes in the 100,000 to 200,000 mwt range. One explanation for this apparent anomaly would require

that only desmin could associate with desmin, D1 with D1, D2 with D2, and D3 with D3, which seems unlikely. A more appealing explanation is that this apparent independent migration is actually a superposition of all of the 35 possible tetramers of desmin and its fragments: D.D.D.D, D.D.D.D1, D.D.D1.D1, ..., D2.D3,D3.D3, D3.D3.D3.D3. A theoretical calculation of the sizes and frequencies of each of these species, in a digest containing equimolar amounts of D, D1, D2 and D3, matches the distribution observed in Figure 6 reasonably well. The calculation also matches the distribution of bands in fraction 17 of Figure 7b fairly closely with one exception. The shape of the theoretical distribution is a skewed bell-shaped curve that does not contain appreciable amounts of material in its upper and lower quadrants. The cross-linked complexes do contain dense bands in these regions. This may be due either to a more complex process than the simple one described here, or to non-uniform cross-linking.

In summary, a proteolytic activity has been discovered that produces specific cleavages of desmin. These activities are widespread in nature and may range from aiding in pathogen invasiveness to the structural modification of muscle that occurs during periods of use and disuse.

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Table 1[1]

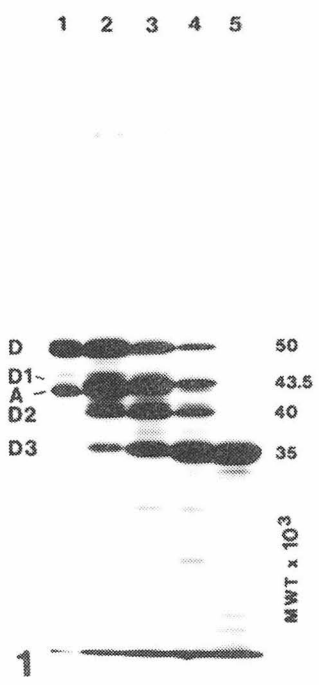
Amino Acid	Mole Percent		Corresponding Amino Acid Residues[5]		Loss of Amino Acid Residues from Desmin to D3 (%)
	Desmin	D3	Desmin	D3	
ASX[2]	9.3	9.7	42	31	26
SLX[2]	16.8	19.5	76	62	18
HIS	1.7	1.7	8	5	38
LYS	5.2	5.7	24	17	29
ARG	7.6	8.9	35	28	20
Avg.	40.5	45.6	185	143	26
Thr	6.0	5.3	27	17	37
Ser	6.1	5.2	28	17	39
Cys[3]	0.3	0	1	0	100
Tyr[4]	2.5	0.6	11	2	82
Avg.	14.9	11.1	67	36	53
Pro	2.4	2.1	11	7	36
Gly	6.6	6.5	30	21	30
Ala	9.3	9.1	42	29	31
Val	6.2	6.1	28	19	32
Met	2.3	1.1	10	4	60
Ile	4.7	5.1	21	16	24
Leu	9.5	10.0	43	32	26
Phe	3.5	3.1	16	10	38
Avg.	44.5	43.3	202	138	35
Avg.					30
Total	100	100	454	317	

Footnotes to Table 1

1. These data are the averaged values of timed hydrolyses at 145°C for 2, 4, 6, or 8 hr. The values of threonine, serine, and tyrosine are represented by least squares estimates of the amounts present at 0 hr.
2. Asparagine and glutamine were not determined separately.
3. Cysteine was not reliably discriminated by the computer program that analyzed the peak area data. It was always visible, however, as a shoulder on the value peak.
4. The values for tyrosine were variable, and this estimate is probably low.
5. This is for an average amino acid molecular weight of 110.

Figure 1 Timed digestion of cycle-2 desmin by unfractionated Worthington CLS-3 collagenase. 100 μgm of cycle-2 desmin in 100 μl of 1 mM HCl was precipitated by the addition of 100 μl of pH 7.5 digestion buffer. 1 μgm of CLS-3 collagenase in 10 λ of digestion buffer was added at time 0, and the reaction was allowed to proceed at 20°C. 20 μl aliquots were removed at the indicated times and stopped with 2 μl of EDTA/P solution. The entire aliquot was then mixed with hot 2X SDS sample buffer and analyzed by SDS-PAGE as described in the Methods. (1) cycle-2 desmin standard; (2) 3 min; (3) 9 min; (4) 30 min; (5) 90 min. Identical results are obtained by using increasing concentrations of collagenase for a fixed time interval. Desmin (D) has molecular weight 50,000; actin (A) has molecular weight 42,000; fragment D1 has molecular weight 43,500; fragment D2 has molecular weight 40,000; and fragment D3 has molecular weight 35,000. Molecular weights were determined as in Hubbard and Lazarides (1979).

Figure 2 Fractionation of CLS-3 collagenase by gel filtration on Ultrogel AcA-34. A 1.5 x 40 cm column of Ultrogel AcA-34 was equilibrated in a buffer containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.5 and 5 mM $CaCl_2$. 20 mg of Worthington CLS-3 collagenase was dissolved in this buffer + 1 mM PMSF and eluted at a rate of 8 ml/hr into 1 ml fractiontions. Protein (OD 280) eluted in fractions 30 to 70 while an included dye marker began eluting with fraction 100. Selected fractions were analyzed on SDS-PAGE, as indicated. The fractions were stored at -20°C and were stable to repeated freezing and thawing. Individual fractions within each group F1, F2, F3, or F4 were used interchangeably for experiments described in the text. CL is a standard of unfractionated collagenase.


1 2 3 4 5
D
D1
A
D2
D3
50
43.5
40
35
MWT x 10³
1

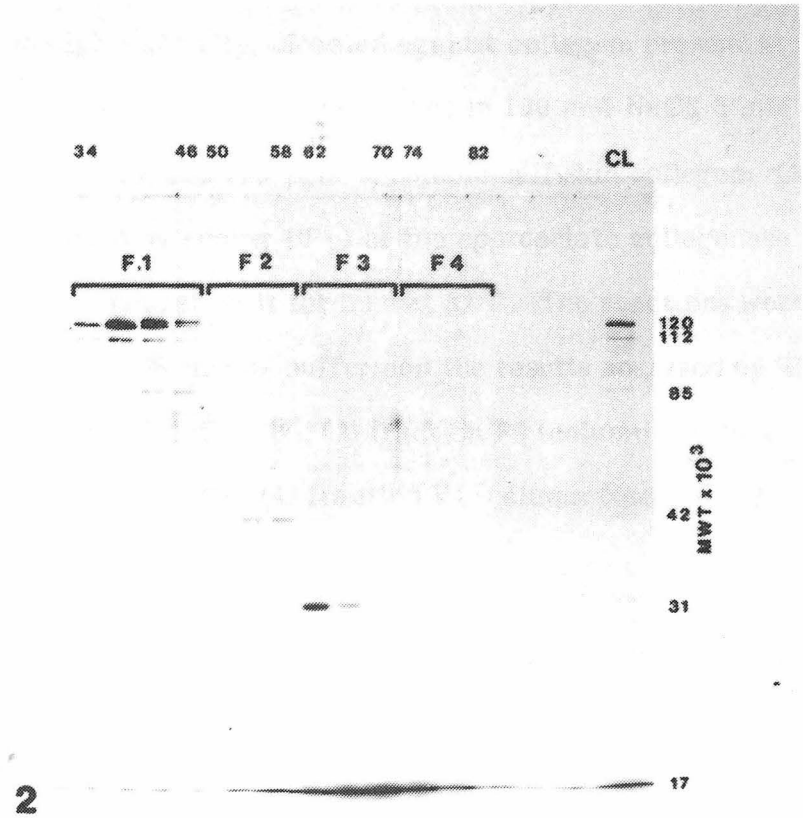
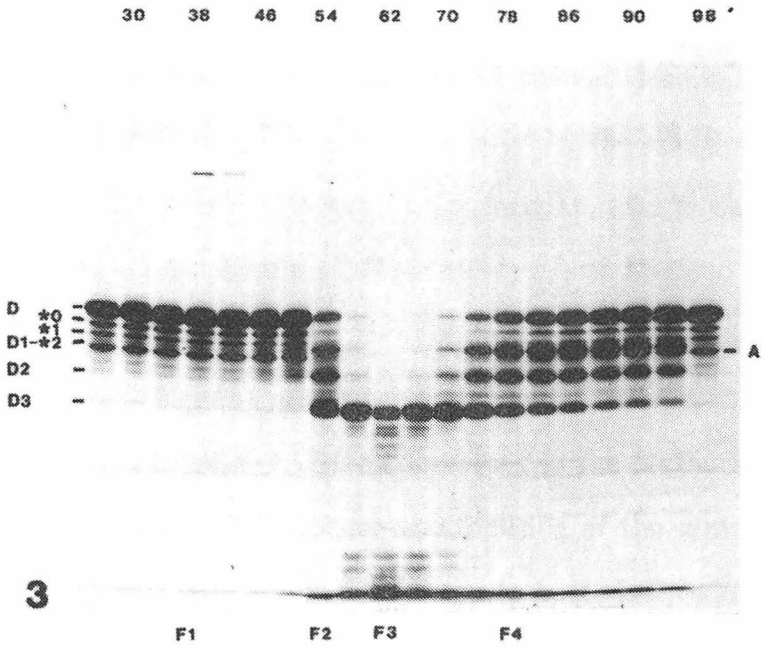
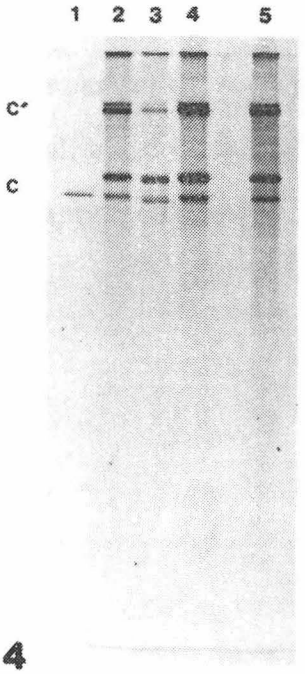

34
46 50
58 62
70 74
82
CL
F.1
F 2
F 3
F 4
120
112
85
42
31
17
MWT x 10³
2

Figure 3 Assay of proteolytic activity, directed against desmin, present in fractionated CLS-3 collagenase. Aliquots of 10 µl of 1 mM HCl were precipitated with 10 µl of pH 7.5 digestion buffer. Each aliquot was then mixed with 2 µl of a selected collagenase fraction from the column in Figure 2 and incubated for 60 min at 20°C. The reactions were stopped with 10 λ aliquots of hot 2X SDS sample buffer and the results analyzed by SDS-PAGE. The collagenase fractions used are indicated at the top of the figure. The group that each fraction belongs to is indicated by F1-F4 at the bottom of the figure. Proteins designated * have been previously described (Hubbard and Lazarides, 1979). D1 comigrates with *2.

Figure 4 Assay of proteolytic activity, directed against collagen, present in fractionated CLS-3 collagenase. Each assay contained in 100 mM NaCl, 5 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.5, and 700 µgm of soluble calf skin collagen. Collagenase activity was identified by adding 10 µl of the appropriate collagenase fraction to an assay and then incubating it for 5 hr at 37°C. The reactions were stopped by the addition of hot SDS sample buffer and the results analyzed by SDS-PAGE. (1) fraction F1 (column fraction 42), (2) fraction F2 (column fraction 54), (3) fraction F3 (column fraction 62), (4) fraction F4 (column fraction 82), (5) collagen standard bands C, C'.



3

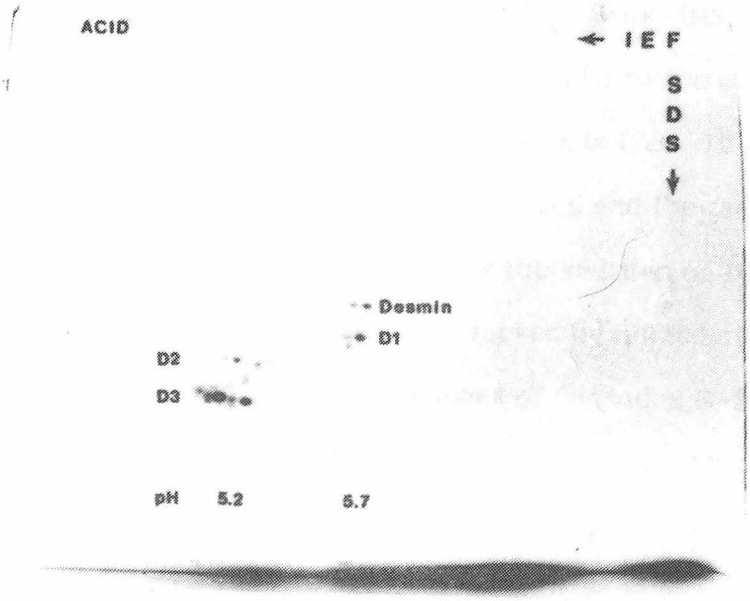


4

Figure 5 Two-dimensional electrophoretic analysis of cleaved desmin. 125 μgm of cycle-2 desmin in 125 μl of 1 mM HCl was precipitated by the addition of 125 μl of pH 7.5 digestion buffer. 25 μl of F2 (as column fraction 58, Fig. 3) was added and the reaction was incubated for 90 min at 20°C before it was stopped by the addition of 25 μl of EGTA/P solution. Then, 25 μl of 2-mercaptoethanol, 100 μl of 10% Nonidet NP-40, and 0.45 gm of urea were added and dissolved. 50 μl of this solution was analyzed on IEF/SDS-PAGE as described in the Methods. The basic side of the gel is in the upper right corner and focusing of the sample occurred from right to left, while SDS-PAGE occurred from top to bottom.

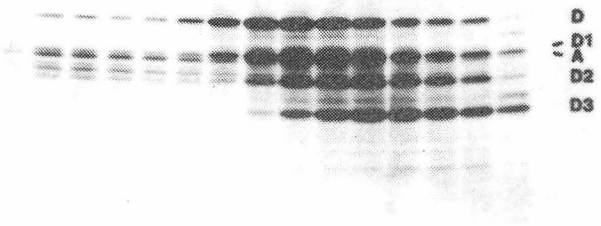
For the determination of isoelectric points, first dimensional gels were stained by a modification of Reisner et al. (1975). The focusing gels were soaked overnight in 15% perchloric acid and then stained in 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid. If the gels turned dark, the stain was changed every few hours until the background became orange, revealing green protein bands. This method appears to be at least as sensitive as normal staining with Coomassie R-250 in acetic acid/methanol, but does not suffer from high backgrounds due to the ampholines. Microgram amounts of protein are easily visualized with it.

Figure 6 Analysis of F4 digested cycle-2 desmin on Ultrogel AcA-34 in the presence of 0.5% Sarkosyl NL-97. A 2 x 43 cm column of Ultrogel AcA-34 was equilibrated in 100 mM NaCl, 0.5% Sarkosyl NL-97, 10 mM Tris-HCl, pH 7.5, 10 mM NaN_3^-, and 10 mM 2-mercaptoethanol. 2 mg of cycle-2 desmin in 0.5 ml HCl, pH 3.0, was precipitated with 0.1 volume of pH 8.0 cross-linking buffer and digested with 70 µl of EDTA/P solution and 0.5% Sarkosyl was added. The digested desmin was loaded on the column in 0.8 ml and eluted into 0.5 ml fractions at the rate of 6 ml/hr. 10 ml were discarded before fraction counting began; blue dextran 2000 began eluting with fraction 5, while an included dye marker eluted from fraction 45. 30 µl aliquots of the indicated fractions were heated with 8 λ of 5X SDS sample buffer and analyzed by SDS-PAGE.



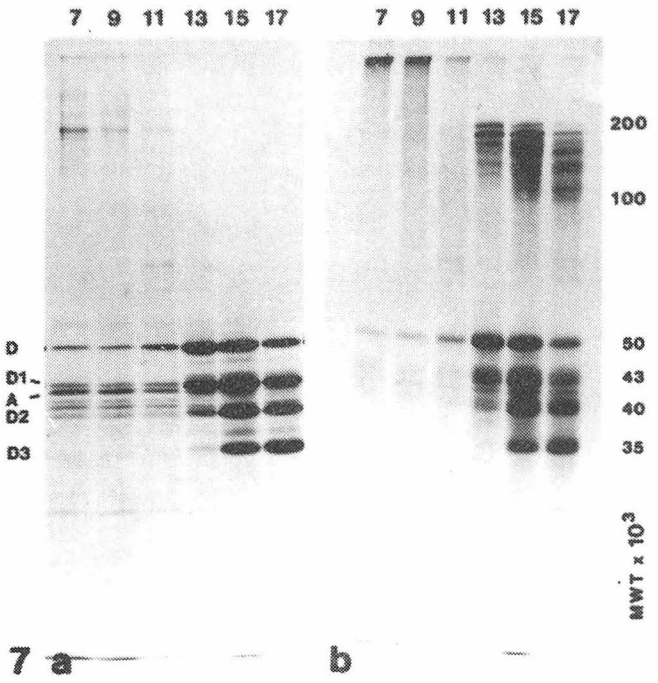
5

6 7 8 9 10 11 12 13 14 15 16 17 18 19 28 45



6

Figure 7 Cross-linking of desmin cleavage fragments with dithiobis (succinimidyl propionate). 100 µl aliquots of selected fractions from the gel filtration experiment of Figure 6 were mixed with 11 µl of pH 8.0 cross-linking buffer. 10 µl of freshly prepared DTSP at 2 mg/ml in 10% acetone was added and the reactions were incubated for 5 min at 20°C before being stopped by the addition of 14 µl of 1 M lysine-HCl. The excess DTSP and lysine were removed by immediately centrifuging the reaction mixtures through 1.0 ml columns of Sephadex G-25 that were equilibrated in non-reducing SDS sample buffer (Neal and Florini, 1973). The resulting samples were analyzed by SDS-PAGE without being reduced. (a) Fractions 7, 9, 11, 13, 15 and 17 from the filtration experiment of Figure 6. (b) The cross-linked fractions corresponding to those shown in (a). The cross-linked products in fractions 13-17 range in molecular weight from 100,000 to 200,000. More extensive treatment with DTSP would have cross-linked all of the residual desmin and fragments into products in this size range. Prior treatment of the proteins with SDS prevents their being cross-linked, even when 10-fold higher concentrations of DTSP are used (data not shown).


7 9 11 13 15 17
7 9 11 13 15 17
200
100
50
43
40
35
MWT x 10³
D
D1
A
D2
D3
7 a
b

CHAPTER SIX

Conclusions

The Biochemical Properties of Desmin

The extraction of smooth muscle with 0.6 M KCl solubilizes most of the actomyosin and leaves an insoluble residue that is enriched in 10 nm filaments (Cooke and Chase, 1971; Cooke, 1976; Small and Sobieszek, 1977). This residue contains predominantly actin, myosin, and a novel 50-55,000 mwt protein (Cooke, 1976; Hubbard and Lazarides, 1979; Lazarides and Hubbard, 1976). The variability in the molecular weight of the 50-55,000 mwt protein reflects actual differences in the systems used to measure its size. It migrates slower than tubulin in Cooke (1976) but faster than tubulin in Lazarides and Hubbard (1976). A comparison of amino acid analyses, physical properties, and chemical properties indicates that it is the same protein in every case, however, and I will refer to it as desmin, molecular weight of 50,000 (Cooke, 1976; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977; Hubbard and Lazarides, 1979; Chapter Five of this Thesis). Desmin occurs as two isoelectric variants, α and β, which are always found together in avian muscle cells (Izant and Lazarides, 1977). Recent evidence indicates that α-desmin is phosphorylated *in vivo* (O'Connor *et al.*, 1979).

Hubbard and Lazarides (1979) studied the properties of the insoluble actin and desmin remaining in KI extracted chicken gizzard residues. It was found that this actin and desmin had similar solubility properties and always copurified with each other throughout a variety of extraction procedures. They also comigrated during gel filtration in the presence of the detergent Sarkosyl NL-97. Preparations that were highly enriched in desmin would reassociate from acetic acid to form filaments with diameters of 12-14 nm (also Small and Sobieszek, 1977). Immunofluorescence indicated that the residual actin was still tenaciously bound to these filaments. From these results, it was concluded that a portion

of the cellular desmin was stably associated with actin. This conclusion is complicated by the lack of simple actin-desmin stoichiometries in the system and by the insolubility of the actin and desmin under physiological conditions. Consequently, a second study concentrated on the actin and desmin that were extracted from freshly homogenized chicken gizzard into low salt, low calcium buffers (Chapter Four of this Thesis). Velocity centrifugation indicates that this actin and desmin form a series of complexes that range in size from about 6 S to 60 S. Further evidence for the existence of these complexes includes (1) the specific co-retention of actin and desmin on a DNase I actin affinity column, (2) the co-precipitation of actin and desmin by either anti-actin or anti-desmin antibodies, and (3) the isolation of stable size classes of actin-desmin complexes by partitioning the velocity centrifugation gradients. The 50-60 S complex isolated in this way had an actin:desmin stoichiometry of approximately 5:1.

Finally, a variety of evidence indicates that desmin is the major subunit of (avian) muscle 10 nm filaments. Urea extracts both desmin and 10 nm filaments from KCl insoluble smooth muscle residues (Cooke, 1976). Desmin will polymerize in vitro to form 10 nm-like filaments (Cooke, 1976; Hubbard and Lazarides, 1979; Small and Sobieszek, 1977). Anti-desmin antibody reacts with colcemid cappable 10 nm filaments in cultured cardiac muscle cells, and the filaments thus visualized are different from those shown by either anti-actin or anti-tubulin (Lazarides, 1978a,b).

Distinguishable Classes of 10 nm Filaments

The various 10 nm filaments all have a variety of properties in common. These include varying degrees of insolubility, their appearance, colcemid cappability,

and similar amino acid compositions (see Small and Sobieszek, 1977; Starger et al., 1978; Brysk et al., 1977, for tables showing comparative amino acid analyses). These similarities underline the question of how closely related the different 10 nm filament families are. The main evidence on this question comes from serology.

While desmin occurs in avian cardiac skeletal, and smooth muscle cells, anti-desmin does not cross-react with avian non-muscle cells nor with muscle from mammalian sources (Lazarides and Balzer, 1978). Chicken muscle cells do not label with either anti-neurofilament or anti-fibroblast 10 nm filament antisera, and desmin, tubulin, and neurofilaments have differing peptide maps (Franke et al., 1978b; Bennett et al., 1978; Davison et al., 1977). Thus, desmin is serologically unrelated to both non-muscle 10 nm filaments and non-avian muscle 10 nm filaments.

Serological analysis has indicated that neurofilaments and glial filaments are immunologically distinguishable from both each other and the other 10 nm filaments (Bennett et al., 1978; Liem et al., 1978; Lazarides and Balzer, 1978). This agrees with the results of partial peptide mapping (Davison et al., 1977) and has led the above workers to suggest that the neurofilaments and glial filaments form two distinct classes of 10 nm filaments. These results are somewhat complicated by earlier reports of serological identity between neurofilament and glial filament subunits (Yen et al., 1976). The work of Liem et al. (1978), however, makes it appear likely that such cross-reactions were due to cross-contamination of filament types during purification.

Antibodies prepared against the fibroblast 10 nm filament subunit react with a wide variety of different cells by immunofluorescence. Included are myogenic, retinal pigment, spinal ganglion, gizzard, and chondrogenic cells (Bennett

et al., 1978), PtK2, 3T3, melanocyte, neuroblastoma, endothelial, and glial cells (Franke et al., 1978b), and neuroblastoma (Starger et al., 1978). In contrast, anti-neurofilament is specific for neurons, anti-glial filament is specific to glia, anti-keratin is specific to epithelia (PtK2 cells [Franke et al., 1978a,b]), and anti-muscle 10 nm filaments are specific to muscle. This leads to the conclusion that neurofilaments, glial filaments, fibroblast 10 nm filaments, muscle 10 nm filaments, and tonofilaments are all distinguishably different classes of 10 nm filaments (Lazarides and Balzer, 1978; Davison et al., 1977; Bennett et al., 1978). The fibroblastic 10 nm filaments may coexist with other 10 nm filament classes in many cell types, however.

The Cytological Localization of Desmin in Striated Muscle

Although 10 nm filaments are rare in adult skeletal muscle, the presence of desmin has been demonstrated by both electrophoresis and immunofluorescence (Lazarides and Hubbard, 1976; Izant and Lazarides, 1977; Granger and Lazarides, 1978). Before describing the immunofluorescence results, I would like to present a nomenclature for describing the structure of the Z-line (Lazarides and Hubbard, 1978; Granger and Lazarides, 1978). The sarcomeres of a single myofibril are connected longitudinally by individual Z-discs. The Z-disc is therefore confined to a single myofibril and is the smallest unit that I will be considering. In a bundle of myofibrils, the Z-discs of each fibril are in lateral register and define a Z-plane. When a myofibril bundle is viewed laterally (side view), the Z-discs in each Z-plane superimpose to form the Z-line observed in whole muscle fibers by light microscopy.

Lazarides and Hubbard (1976) originally discovered that desmin is localized in the Z-lines of skeletal muscle. More recently, Granger and Lazarides (1978)

have developed a technique for performing immunofluorescent localization on the surface of a single Z-plane. They find that desmin is restricted to the boundary region that separates the individual Z-discs from each other in the Z-plane, and that desmin is not a component of the Z-disc itself. This distribution is visible in the transverse muscle fiber sections (Lazarides and Hubbard, 1976), is also very similar to that described above for the 10 nm filaments in cardiac cells. Finally, the distribution of desmin overlaps that of the T-tubule membrane system in rat and chicken (Lazarides and Granger, 1978; Behrendt, 1977; and my own observations). The T-system is an elaborate invagination of the plasma membrane that is electrically active and provides a pathway for coupling the excitation of the plasma membrane with the interior of the muscle cell (Franzini-Armstrong and Porter, 1964; Peachey, 1965, 1968; Huxley, 1971).

The Function of Desmin in Muscle

For many years, the 10 nm filaments have been assigned the vague function of "playing a structural role." This was based as much upon their lack of involvement in any obvious cellular activity as it was upon known attributes. The data reviewed above all demonstrate a particular relationship between muscle 10 nm filaments, actin, and the plasma membrane. Based upon this, it has been proposed that the function of desmin and muscle 10 nm filaments is to mechanically interlink individual actomyosin contractile units, both to each other and to the plasma membrane. Such a system would provide for the uniform transmission of force from the interior to the exterior of the muscle cell (Lazarides and Hubbard, 1976). An additional consequence of a system that interlinked Z-discs might be the regular alignment of Z-discs into Z-bands that characterizes striated muscle. Additional

evidence for this is provided by the separation of myofibrils that occurs after desmin has been removed by a desmin cleaving activity (Chapter Five of this Thesis).

Not enough is presently known about the structure of contractile units in smooth muscle to permit specific statements about the function of its 10 nm filament network (Shoenberg and Needham, 1976). There are no structures in smooth muscle that are obviously equivalent to the striated muscle Z-line. Whether or not the 10 nm filament-dense body network performs an analogous function has been the subject of controversy for some years (Prosser et al., 1960; Bois, 1973; Ashton et al., 1973; Small and Squire, 1972; Cooke, 1976; Small, 1977; reviewed by Schollmeyer et al., 1976). Most of the evidence against this equivalence is indirect or negative. Positive evidence for their equivalence includes (1) the association of desmin with Z-discs and dense bodies; (2) the insertion of actin filaments into Z-discs and dense bodies; and (3) the presence of α-actinin and tropomyosin in Z-bands and dense bodies (Lazarides and Burridge, 1975; Schollmeyer et al., 1974, 1976). The Z-discs and dense bodies thus appear to be similar, both in composition and in function as interlinking structures. The internal structure of the two appears to be vastly different, however. The Z-disc has a mesh-like structure, while the dense body is amorphous (Franzini-Armstrong and Porter, 1974; Hay, 1963; Kelly, 1967; Knappeis and Carlsen, 1962; Ashton et al., 1975; Cooke, 1976; Small, 1977). Consequently, the equivalence of the dense body and the Z-disc remains unresolved.

Our observations that desmin is complexed with β- and γ-actins in smooth muscle (Chapter Four of this Thesis) raises the question as to whether or not the Z-bands of striated muscle contain unusual actin variants. This is important in

light of our observation that α-actin is resistant to DCA. Neither β- or γ-actin has yet been observed on two-dimensional gels of adult skeletal muscle (Garrels and Gibson, 1976). Measurements of the ratio of Z-band area to I-band area indicates, however, that less than 5% of the total actin is in the Z-band. It is unlikely that β- or γ-actin would be detectable at the 5% level due to the tendency of α-actin to smear in the first dimension (Izant and Lazarides, 1977).

The Role of the Actin-Desmin Complex

It is not known how these actin-desmin complexes fit into the physiology of 10 nm filaments. Several studies have failed to find heavy meromyosin binding to various types of 10 nm filaments (Ishikawa et al., 1969; Burton and Kirkland, 1972; Chang and Goldman, 1973; Goldman et al., 1976). It is possible, of course, that if a population of 10 nm filaments did bind heavy meromyosin, then they would be misclassified as actin filaments. It is also possible that desmin masks the presence of actin in the complex, although this was not the case with either the in vitro actin-desmin filaments or the soluble actin-desmin complexes. Anti-actin and anti-desmin antibodies reacted with both (Hubbard and Lazarides, 1979; Chapter Three of this Thesis). Anti-actin and anti-desmin also reveal different populations of filaments in cultured cells (Lazarides, 1975, 1978a,b). A possible exception to this spatial separation of actin and desmin is found in Granger and Lazarides (1978). They found actin distributed over the entire Z-plane, including the interdisc boundary region where desmin is localized. It is hard to determine, however, if this actin was present in the boundary region in vivo, or if it was deposited there during an actomyosin extraction step during the production of the Z-planes.

Possibly, the actin-desmin complex represents the "joint" between an actomyosin unit and a desmin interlink. This would require some mechanism for localizing the actin-desmin interaction. No data are presently available on either the existence or the nature of such a mechanism, but there are several possibilities. First, the actin-desmin interaction may require a third protein. Candidates for such a protein are the * proteins that copurify with actin and desmin in the 40-50,000 mwt range (Hubbard and Lazarides, 1979; Chapter Five of this Thesis). Evidence against this is the absence of these proteins from the 50-60 S soluble actin-desmin complex, and also evidence that the * proteins are desmin cleavage products (Chapter Five of this Thesis). Second, an analogous situation is encountered in bacteriophage morphogenesis where one structure serves as the nucleating site for the self-assembly of a second (Casjens and King, 1975). Thus, some actin configuration may initiate the growth of a pure desmin filament from that site. Unsolved by this hypothesis is how the growing end of the desmin filament becomes anchored on another actin structure. Third, specific variants of the molecules involved may be responsible for the actin-desmin interaction. For example, there are α-, β-, and γ-isoelectric variants of actin and α- and β-isoelectric variants of desmin in muscle (Izant and Lazarides, 1977; Garrels and Gibson, 1976). Although α-actin is restricted to skeletal muscle while β- and γ-actins occur in smooth and non-muscle cells, it has not yet been possible to demonstrate any differences in function or spatial localization for any of the desmin variants. Finally, there may be different types of desmin polymer in muscle cells. Evidence for this comes from the observation that the number of visible 10 nm filaments decreases during myogenesis. This suggests that the 10 nm filaments in striated muscle may be a storage form of desmin. These would be progressively disassembled as the desmin

was required for the formation of inter-Z-disc linkages. This model would require that desmin not bind to actin while in its storage form, but places no limit on the extent of actin-desmin interaction elsewhere. It should be noted, however, that desmin does not simply bind to actin wherever it encounters it within the cell. Other proteins, such as tropomyosin or actin isoelectric variants, may play a role in mediating this restriction.

The Rheological Properties of Cytoplasmic Gels

By comparing the physical properties of desmin based polymers with those of various actin based polymers, it is possible to infer several principles that may be of general importance. The biochemistry of these two systems is compared elsewhere (Hubbard and Lazarides, 1979; Pollard and Weihing, 1974; Pollard, 1976a; Taylor, 1977) and will be considered here only as it relates to their rheological properties. Pure F-actin prepared from skeletal muscle by the Spudich and Watt procedure (1971) forms viscous solutions that exhibit non-newtonian flow. Non-newtonian flow is that in which the apparent viscosity is dependent upon the rate of shear (Tanford, 1966) and is common property of solutions of asymmetric or interactive molecules. F-actin solutions exhibit complex, non-Newtonian, behavior under shear, but they may be described as being thixotropic in the simplest analysis (Maruyama et al., 1974; Brotschi et al., 1978). A thixotropic solution is one in which the viscosity decreases as the steady state shear rate increases. A simple and satisfactory explanation for this behavior can be provided for the ideal case of stiff, asymmetric molecules. These are normally randomly oriented due to thermal agitation, but tend to become aligned parallel to the direction of flow in a velocity gradient (Tanford, 1966; Meares, 1965).

Light scattering measurements on F-actin are consistent with this picture of freely diffusing rods (Carlson and Fraser, 1974). Studies with a rotational viscometer, however, show that the viscosity of F-actin is inversely proportional to the velocity gradient over a wide range of values (Maruyama et al., 1974). This is in contrast to the behavior of molecules such as flagella, collagen, and tropomyosin aggregates, which exhibit constant viscosities in low velocity gradients (Maruyama et al., 1974). This has been interpreted as indicating that stable but weak interactions exist between the F-actin molecules that result in their being cross-linked into large networks. This may be due to the presence of small amounts of cross-linking contaminants in the actin preparation.

A striking property of many cytoplasmic extracts is their ability to undergo gelation and subsequent syneresis in vitro (Kane, 1975, 1976; Bryan and Kane, 1978; Kane et al., 1977; Hartwig and Stossel, 1975; Stossel and Hartwig, 1976; Maruta and Korn, 1977; Taylor, 1977; Pollard, 1976b; Weihing, 1976). These gels are based upon the interaction of F-actin with one or more accessory proteins that appear to cross-link the actin filaments into bundles and networks. The cross-linking proteins vary from system to system and have been tabulated by Taylor (1977). These gels are frequently described as being physically strong. They do not flow and break into chunks if sheared (Weihing, 1976; Stossel and Hartwig, 1976; Pollard, 1976a). The gels formed in vitro by 12-14 nm desmin filaments behave similarly (Hubbard and Lazarides, 1979).

Several groups have investigated the effects of some presumed cross-linking proteins on F-actin. Both α-actinin and heavy meromyosin increase the viscosity and modulus of elasticity of F-actin solutions (Abe and Maruyama, 1973, 1974; Holmes et al., 1971; Ebashi and Maruyama, 1965; Maruyama and Ebashi,

1965). These results are interpreted as indicating the formation of limited networks, although the solutions remain thixotropic. Additional evidence for cross-linking comes from (univalent) myosin subfragment 1, which produces only 10% of the increase in visco-elasticity that (divalent) heavy meromyosin does (Abe and Maruyama, 1974). In the case of α-actinin, its binding to F-actin saturates at 12-16 mole percent, or about 1 α-actinin per turn of the actin helix (Holmes et al., 1971; Abe and Maruyama, 1973). The F-actin precipitates under these conditions and electron microscopy reveals loosely bound bundles of actin fibers (Kawamura et al., 1970).

Myosin, filamin and actin-binding protein (ABP) all produce gels when mixed with F-actin (Wang and Singer, 1977; Stossel and Hartwig, 1976). The physical characteristics of these gels has been investigated by Brotschi et al. (1978). They find that skeletal muscle actin alone is thixotropic and recovers 50% of its initial rigidity by 15 min after shearing. In contrast, while myosin, filamin, and ABP form gels that are 4-5 times as strong as F-actin alone, these are permanently destroyed by shearing. They do not spontaneously reform after being disrupted. Brotschi et al. (1978) also find that these gel forming, actin cross-linking proteins are extremely active. The minimum effective molar ratios for gel formation is 1:100 for myosin, 1:190 for filamin, and 1:740 for ABP (actin:cross-linker ratio). This is in agreement with measurements made on the gel forming components of amoeba and sea urchin egg cytoplasm (Maruta and Korn, 1976; Bryan and Kane, 1978).

Desmin, Cytoplasmic Gels, and the Cytoskeleton

Amoeba cytoplasm is not only contractile, but it also exhibits localized

and reversible changes in its viscosity (reviewed by Allen, 1961). More recently, Thompson and Wolpert (1963) found that extracts of amoeba cytoplasm can be made to gel and contract in vitro by warming them in the presence of ATP. The discovery that in vitro gelation is due to actin has stimulated considerable speculation about the possible role of actin in the control of cytoplasmic structure in vivo. This possibility has been reviewed at length by Pollard (1976a) and is of interest here because desmin forms a physically similar, although biochemically distinct, gelation and syneresis system (Hubbard and Lazarides, 1979).

In the previous section, it was shown that gel forming polymers could be divided into two classes: those that are thixotropic and those that are not. Thixotropic gels would be suitable for the maintenance of temporary structures, perhaps ones that are intended to be dynamically remodeled. Non-thixotropic gels, in contrast, are capable of withstanding considerable force and would be suitable for constructing permanent structures or for transmitting forces from one site to another. Desmin gels are of this type and it is consequently evident that they are eminently suited for the proposed role of transmitting force laterally through muscle cells.

Why does the cell employ desmin to serve this function, instead of some other actin cross-linking molecule such as α-actinin of filamin? The answer is, at present, unknown. I would like to suggest, however, a reason that is based upon the principle of hierarchical design (Simon, 1962). Early in myogenesis, actin is forming actomyosin and the sarcomeres are assembling. This requires lateral bonding between myofilaments. In order to ensure the formation of compact structures, this bonding should be restricted to operating only over short distances. Thus, molecules like α-actinin would be employed in forming the Z-discs. After

the myofibrils have formed, the requirements for lateral bonding are somewhat different, because the spacing between myofibrils is greater and exhibits less uniformity than that between myofilaments. A molecule that can bond over wide gaps and intervening membranes is now required. An actin-binding polymer fills this requirement very nicely, which is just the type of structure that desmin can form.

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