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Phosphorylation of subunit proteins of intermediate filaments from chicken muscle and nonmuscle cells

(desmin/tropomyosin/two-dimensional gel electrophoresis)

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ABSTRACT **The phosphorylation of the subunit proteins of intermediate (10-nm) filaments has been investigated in chicken muscle and nonmuscle cells by using a two-dimensional gel electrophoresis system. Desmin, the 50,000-dalton subunit protein of the intermediate filaments of muscle, had previously been shown to exist as two major isoelectric variants—α and β—in smooth, skeletal, and cardiac chicken muscle. Incubation of skeletal and smooth muscle tissue with $^{32}PO_4^{3-}$ reveals that the acidic variant, α-desmin, and three other desmin variants are phosphorylated *in vivo* and *in vitro*. Under the same conditions, minor components of α- and β-tropomyosin from skeletal muscle, but not smooth muscle, are also phosphorylated. Both the phosphorylated desmin variants and the nonphosphorylated β-desmin variant remain insoluble under conditions that solubilize actin and myosin filaments, but leave Z-discs and intermediate filaments insoluble. Primary cultures of embryonic chicken muscle labeled with $^{32}PO_4^{3-}$ possess, in addition to the desmin variants described above, a major nonphosphorylated and multiple phosphorylated variants of the 52,000-dalton, fibroblast-type intermediate filament protein (IFP). Filamentous cytoskeletons, prepared from primary myogenic cultures by Triton X-100 extraction, contain actin and all of the phosphorylated and nonphosphorylated variants of both desmin and the IFP. Similarly, these proteins are the major components of the caps of aggregated 10-nm filaments isolated from the same cell cultures previously exposed to Colcemid. These results demonstrate that a nonphosphorylated and several phosphorylated variants of desmin and IFP are present in assembled structures in muscle and nonmuscle cells.**

Muscle cells contain a cytoskeletal network of filaments, commonly referred to as 10-nm or intermediate filaments, that are intermediate in diameter to the actin and myosin filaments involved in contraction (1–3). Biochemical and immunological studies have demonstrated that the intermediate filaments from smooth muscle are comprised largely of a 50,000-dalton protein, desmin (4), which occurs as the same two isoelectric variants in chicken skeletal, smooth, and cardiac muscle (5). Immunofluorescent staining of muscle cells with a desmin-specific antibody has shown that desmin is present both in cytoplasmic filaments and in areas where actin filaments associate with the plasma membrane, such as the cardiac muscle intercalated discs and the peripheries of skeletal muscle Z-discs (4, 6–8). Thus, it was proposed that desmin-containing structures in muscle cells are involved in the mechanical integration of contractile units.

Cytoskeletal networks of 10-nm filaments also exist in many nonmuscle cells. Recent studies have demonstrated that, although the 10-nm filaments from various nonmuscle and muscle cells are morphologically similar, their subunit proteins may be cell-type specific. Neurofilaments, glial filaments, epidermal keratin filaments, the 10-nm filaments from fibroblasts, and muscle intermediate filaments are each composed of biochemically distinct subunits with little serological cross-reactivity (6, 9–13). Except for the keratin filaments that associate with desmosomes in epithelial and epidermal cells, the nonmuscle 10-nm filaments are not associated with well-defined cytoplasmic structures. The cytoskeletal networks formed by the intermediate filaments are stable, however, and remain insoluble after Triton X-100 extraction of the cells (14). The muscle intermediate filaments also remain insoluble after the removal of actin and myosin filaments with 1 M KI (15). Colcemid treatment of nonmuscle and muscle cells induces a rearrangement of the intermediate filaments into large perinuclear caps (6, 16–18). Biochemical characterization of the intermediate filaments present in the Triton cytoskeletons and Colcemid-induced caps of fibroblasts has identified the subunit as a protein with a molecular weight of 52,000–55,000 (14, 18).

Several contractile and structural proteins have been shown to be phosphorylated in muscle and nonmuscle cells, including the myosin light chains, α-tropomyosin, filamin, and spectrin (19–22). In the case of myosin, phosphorylation is required for the activation of myosin ATPase by actin (19), while phosphorylation of spectrin is required for its association with actin (22). In this paper, we show that desmin is present as a single nonphosphorylated and several phosphorylated variants in chicken smooth and skeletal muscle. All of these desmin variants are also present together with a nonphosphorylated and several phosphorylated variants of the fibroblast-type intermediate filament protein in primary cultures of embryonic thigh muscle. We have also observed that minor components of both the α and β forms of tropomyosin are phosphorylated in skeletal, but not in smooth, muscle cells.

MATERIALS AND METHODS

Protein Phosphorylation in Intact and Excised Muscle Tissues. Muscle proteins were labeled *in vivo* by injecting the right thigh muscle of a young chick (1 to 10 days old) with 0.5 mCi (1 Ci = 3.7×10^{10} becquerels) of neutralized [^{32}P]phosphoric acid (New England Nuclear, carrier-free). After 24 hr, the tissue was excised, minced, and homogenized in 3 vol of 8 M urea/2% (vol/vol) Nonidet P-40/0.5% 2-mercaptoethanol with a ground-glass homogenizer. The homogenized tissue was solubilized for 1 hr at room temperature. Insoluble material was removed by centrifugation at $500 \times g$ for 5 min, and the supernatant was applied directly to isoelectric focusing gels.

Small pieces of excised thigh or gizzard muscle were equilibrated for 30–60 min in phosphate-free minimal essential medium (GIBCO) and subsequently incubated in 0.5 ml of phosphate-free minimal essential medium to which $^{32}PO_4^{3-}$ was added to give a final concentration of 100–200 μCi/ml. After 4- to 5-hr incubation, the tissue pieces were removed, rinsed in phosphate-free minimal essential medium, and pro-

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IFP, intermediate filament protein.

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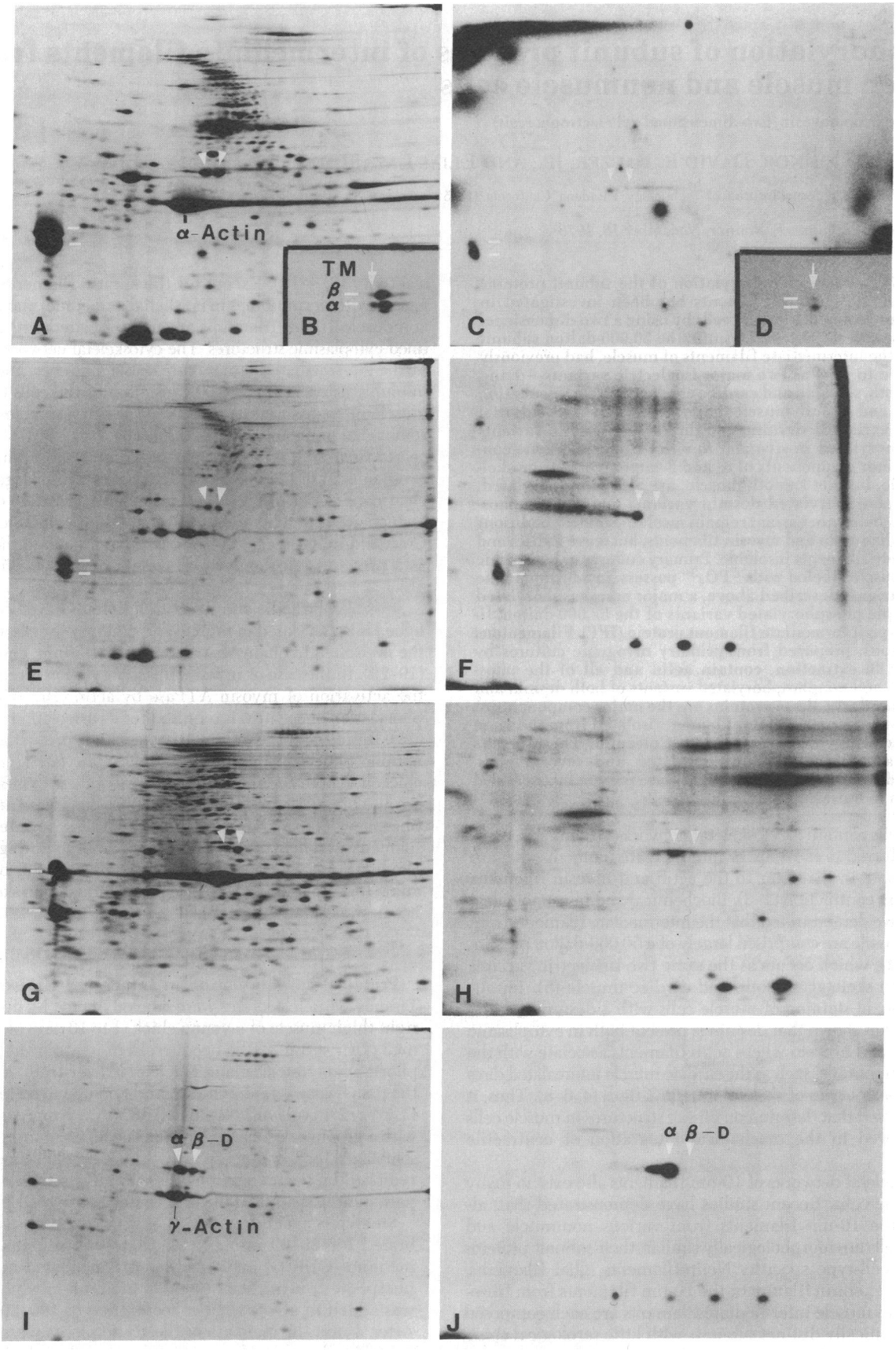

FIG. 1. Two-dimensional gel electrophoresis of the urea extracts of ^{32}P-labeled chicken muscle. Isoelectric focusing is in the horizontal dimension, with the acidic end of the gradient on the left. Electrophoresis in the presence of sodium dodecyl sulfate is in the vertical dimension, with the anode on the bottom. The Coomassie blue-stained gels are on the left and the corresponding autoradiograms are on the right. (*A* and *C*) Thigh muscle labeled *in vivo*. (*B* and *D*). Tropomyosin region from a gel with an expanded pH gradient from pH 4 to 6. Arrows point to the

cessed as above. In some cases, actomyosin was extracted from the minced gizzard or thigh tissue by homogenizing the tissue in half-strength phosphate-buffered saline (full-strength phosphate-buffered saline is 0.14 M NaCl/3 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4, pH 7.2) and extracting the residue at 4°C with 0.6 M KI/0.01 M $Na_4P_2O_7$/0.02 M $Na_2S_2O_3$/0.01 M NaH_2PO_4, pH 7.0, overnight. This final residue was prepared for gel electrophoresis as described above.

Protein Phosphorylation in Myogenic Cells Grown in Tissue Culture. Primary myogenic cell cultures were prepared as described by O'Neill and Stockdale (23), except that trypsin was used at a final concentration of 0.05% and preplating was done for 25 min. Cells were plated at an initial density of 0.6–0.8 $\times 10^6$ per 35-mm plate. They were labeled in 0.8 ml of phosphate-free minimal essential medium without serum or chicken embryo extract to which $^{32}PO_4^{3-}$ was added to give a final concentration of 60–100 μCi/ml. After 4–6 hr of incubation at 37°C, the cells were washed twice with phosphate-free MEM and each cell monolayer was solubilized for 30 min at room temperature with 100–200 μl of 8 M urea/2% Nonidet P-40/0.5%-mercaptoethanol. The extracts were then applied directly to isoelectric focusing gels.

Preparation of Triton Cytoskeletons and Colcemid-Induced Filament Caps. Cytoskeletons and caps were prepared essentially as described by Starger *et al.* (18). All operations were performed at 4°C. $^{32}PO_4^{3-}$-labeled myogenic cell cultures were washed twice with 1.3× phosphate buffered saline/10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). Cells were scraped off each plate with a rubber policeman into 1 ml of 1.3 × phosphate-buffered saline EGTA/0.1 mM phenylmethylsulfonyl fluoride. The cells were pelleted and subsequently digested for 10 min in a lysis buffer consisting of 0.6 M KCl, 1% Triton X-100 (Rohm and Haas, Philadelphia), 0.1 mM phenylmethylsulfonyl fluoride, and *p*-tosyl-L-arginine methyl ester·HCl, 1 mg/ml, to which pancreatic DNase I (Worthington; 5 mg/ml in 0.1 mM phenylmethylsulfonyl fluoride, 1 mg of tosylarginine methyl ester per ml) and $MgCl_2$ were added to final concentrations of 0.5 mg/ml and 10 mM, respectively. The cytoskeletons or caps were pelleted by centrifugation at 500 × *g* for 5 min, and the pellets were washed twice with 0.5 ml of 1.3× phosphate-buffered saline/10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride. The cytoskeletons or caps were solubilized for 30 min at room temperature in 8 M urea, 2% Nonidet P-40, 0.5% 2-mercaptoethanol, and the supernatants were applied directly to the isoelectric focusing gels.

Capping of intermediate filaments was induced by treating myogenic cultures for 24 hr with 5 μM Colcemid (Calbiochem). Cells were labeled with $^{32}PO_4^{3-}$ in the presence of Colcemid during the final 4–6 hr of Colcemid incubation as described above.

Two-Dimensional Electrophoresis. Two-dimensional electrophoresis was performed as described (13). The pH range of the isoelectric focusing dimension was from 4.5 to 7.0. The gradient was formulated with the following final concentrations of carrier Ampholines (LKB): 2% pH 5–7, 0.8% pH 4–6, 0.2% pH 3.5–10. The expanded acidic isoelectric focusing dimension of Fig. 1 *C* and *D* was formulated with the following final concentrations of carrier Ampholines: 2.8% pH 4–6, 0.2% pH 3.5–10. The gels were stained overnight with 0.25% Coomassie brilliant blue in 45% ethanol/10% acetic acid/45% water (vol/vol). After destaining in 12.5% ethanol/5% acetic acid/82.5% water, the gels were photographed and dried onto Whatman no. 1 paper for autoradiography. Autoradiography was carried out by using either Kodak NS-2T No-screen film at room temperature or Kodak X-omat XR5 film with a Du Pont Cronex intensifier screen at −70°C for various lengths of time. No-screen film was developed with Kodak D-19 developer and X-omat film with Kodak x-ray developer.

RESULTS

Skeletal muscle

Fig. 1 *A* and *C* show the stained gel and corresponding autoradiogram obtained from the solubilized thigh tissue of a 10-day-old chick injected with $^{32}PO_4^{3-}$. Several phosphorylated proteins can be identified on the basis of their comigration with known muscle proteins (5). The autoradiogram indicates that α-desmin, the acidic variant of desmin, is labeled *in vivo*, but that no phosphate is associated with β-desmin. In addition, both the α and β forms of tropomyosin are phosphorylated *in vivo*, with α-tropomyosin being more extensively labeled than the β variant. However, only minor components of the two tropomyosins are phosphorylated. Direct comparison of the autoradiogram with the stained and dried gel shows that only the acidic ends of the tropomyosin spots are labeled with phosphorus. This is more clearly shown in Fig. 1 *B* and *D*, which depicts a two-dimensional gel with an expanded pH gradient from pH 4 to 6. In this case, tropomyosin focuses at the midpoint of the isoelectric focusing gels, and all of the radioactive phosphorus is associated with the projections at the acidic ends of the stained tropomyosin spots. The tissue used for Fig. 1 *B* and *D* was labeled with $^{32}PO_4^{3-}$ *in vitro* rather than in *in vivo*. Desmin and tropomyosin are phosphorylated similarly under both conditions, but no $^{32}PO_4^{3-}$ is incorporated into actin in either case. Because the specific activity of the protein labeled *in vitro* is always higher than that of protein labeled *in vivo*, the remaining experiments were performed with tissue labeled with $^{32}PO_4^{3-}$ *in vitro*.

Previous studies have demonstrated that most of the skeletal muscle desmin is associated with myofibril Z-discs and remains insoluble under conditions that solubilize actomyosin (8). Likewise, Fig. 1 *E* and *F* indicates that phosphate remains associated with α-desmin after overnight extraction of myofibrils with 0.6 M KI. In addition, it is apparent that radioactive phosphate is associated with another polypeptide slightly more acidic than α-desmin, but of the same molecular weight. This satellite protein may represent an additional isoelectric variant of desmin (see below). The α- and β-tropomyosins that remain insoluble in the KI pellet also contain radioactive phosphate.

Smooth muscle

A slightly different pattern of phosphorylation is obtained from the excised gizzard tissue of a 4-day-old chick that has been incubated with $^{32}PO_4^{3-}$ *in vitro*. In this case, no radioactive phosphate is associated with the tropomyosins (Fig. 1 *G* and *H*) or the β-desmin variant, but α-desmin and its acidic satellite are clearly phosphorylated. As in the case of skeletal muscle, the phosphate remains associated with α-desmin after overnight extraction of the tissue with 0.6 M KI (Fig. 1 *I* and *J*).

phosphorylated variants of α- and β-tropomyosin (TM). (*E* and *F*) Insoluble proteins remaining after extraction of thigh muscle with 0.6 M KI. (*G* and *H*) Gizzard tissue labeled *in vitro*. The gel has been overloaded to demonstrate desmin phosphorylation, resulting in some streaking. (*I* and *J*) Insoluble proteins remaining after extraction of gizzard muscle with 0.6 M KI. White arrowheads indicate the position of α and β-desmin (D). White lines indicate the positions of α- and β-tropomyosin. Due to uneven shrinkage of the gels during drying, the spots on the autoradiograms are not entirely coincident with those in the photographs of the stained gels.

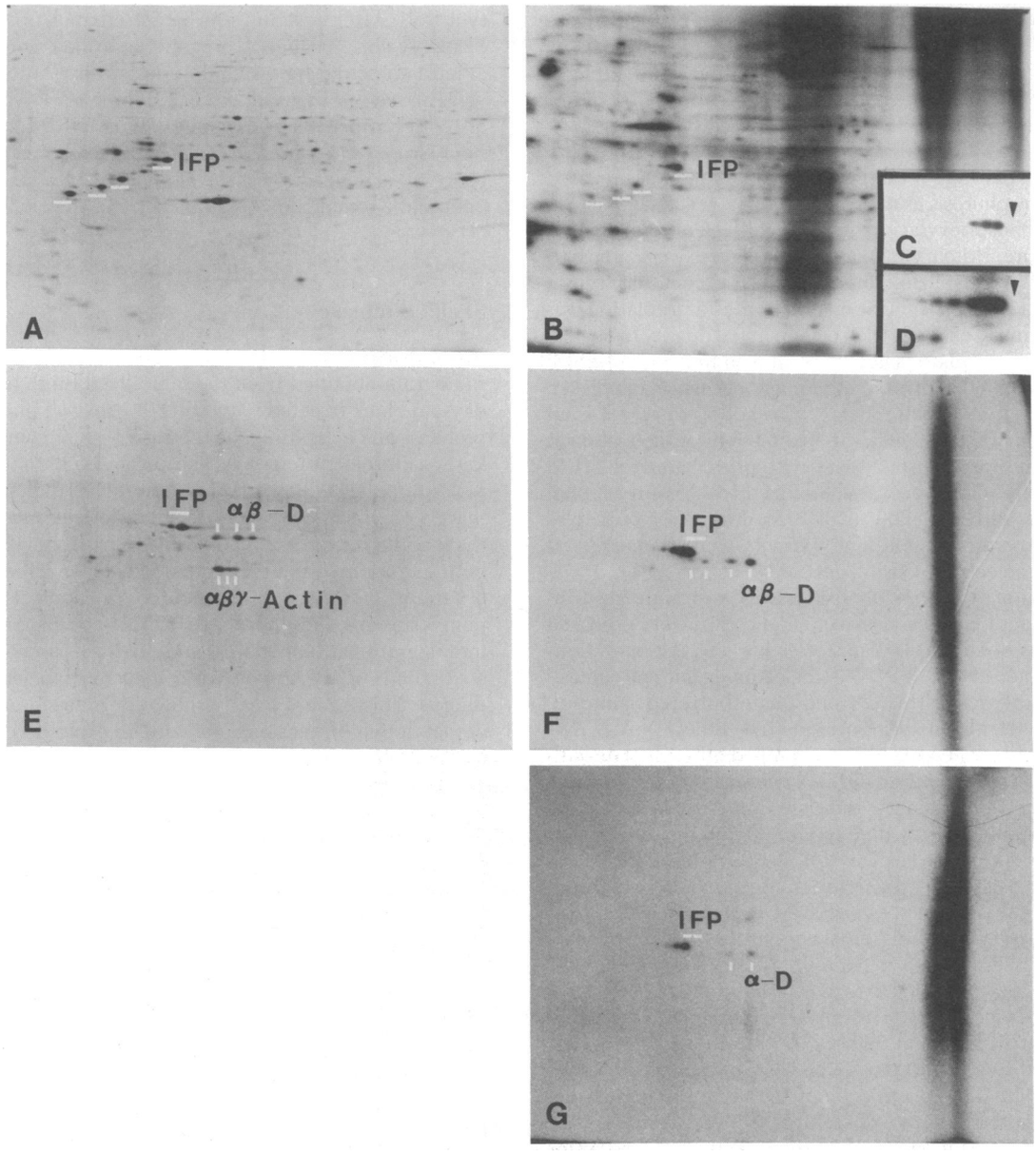

FIG. 2. Two-dimensional gel electrophoresis of urea extracts obtained from ^{32}P-labeled myogenic cultures. The Coomassie blue-stained gels are on the left, and the corresponding autoradiograms are on the right. (*A* and *B*) Total cellular protein. The positions of the nonphosphorylated variant and major phosphorylated variant of IFP and of the diagonal proteins are underlined. (*C* and *D*) Photographs depicting the IFP region of *B* have been underexposed or overexposed to demonstrate the presence of seven isoelectric variants. Black arrowheads indicate the position of the nonphosphorylated IFP variant. (*E* and *F*) Caps of intermediate filaments isolated from myogenic cultures. The positions of the desmin (D) and actin isoelectric variants are indicated by bars, and those of IFP are overlined. (*G*) Cytoskeletons prepared from myogenic cell cultures by extraction with 1% Triton X-100.

Myogenic cell cultures

Primary cell cultures derived from the thigh muscles of 11- and 12-day chicken embryos are initially composed of unfused myoblastic cells and less than 10% fibroblastic cells. By 4 to 5 days after plating, most of the myoblasts have fused to become multinucleated myotubes. Fig. 2 *A* and *B* shows the complex pattern of protein staining and phosphate incorporation obtained from a myogenic cell culture incubated with $^{32}PO_4^{3-}$ on the sixth day after plating. A prominent 52,000-dalton species is present that possesses the electrophoretic mobility on two-dimensional gels characteristic of the intermediate filament protein (IFP) previously described in fibroblasts (14). This protein is also prominent in Colcemid-treated cells (not shown) and is a major component of the isolated caps of aggregated filaments that form as a result of Colcemid treatment (Fig. 2 *E* and *F*). It is clear from Fig. 2 that several variants of the IFP coexist in cultured cells. The major IFP variant is not phosphorylated, but, depending on the exposure time, up to seven more acidic, phosphorylated IFP variants (Fig. 2 *C* and *D*) can be detected on the autoradiograms. Colcemid treatment does not alter the phosphorylation of the IFP variants.

Only a small number of protein species can be identified on the stained gels of the Colcemid-induced caps isolated from myogenic cell cultures (Fig. 2*E*). Actin, α- and β-desmin, and the IFP are the major components of the caps. In addition, the acidic variant of α-desmin described above, and a series of smaller, more acidic proteins that form a diagonal line with the fibroblast-type IFP on the gels (underlined spots in Fig. 2), can be resolved. The autoradiograms of Fig. 2 *B* and *F* reveal that the most densely stained diagonal proteins are not phosphorylated, but that each one has a phosphorylated satellite variant associated with it. Variable amounts of these diagonal

proteins always copurify with IFP. One-dimensional peptide maps of the diagonal proteins are highly homologous to the map of the 52,000-dalton IFP (D. Gard and E. Lazarides, unpublished data), suggesting that the diagonal proteins are derived from the 52,000-dalton forms either by intracellular proteolysis or by proteolysis during protein extraction or electrophoresis. The autoradiogram in Fig. 2*F* demonstrates that α-desmin, its acidic satellite, and two more acidic species of the same molecular weight are phosphorylated in the Colcemid-induced caps. Both the major desmin variants and the three more acidic species appear to be highly homologous by one-dimensional peptide mapping (D. Gard and E. Lazarides, unpublished data).

Results very similar to those of Fig. 2 *E* and *F* were obtained from the cytoskeletons remaining after Triton extraction of myogenic cell cultures, as shown in Fig. 2*G*. Exposure of a similar culture to cycloheximide at 40 μg/ml for 1 hr before and during the $^{32}PO_4^{3-}$ pulse does not affect the phosphorylation of desmin or the IFP (data not shown).

DISCUSSION

The subunit proteins of intermediate filaments in muscle and nonmuscle cells exist as both phosphorylated and nonphosphorylated forms. The two-dimensional gel system used resolves multiple variants of desmin and the fibroblast-type IFP in mature muscle and in mixed myogenic–fibroblastic cell cultures. Desmin, the subunit protein of muscle 10-nm filaments, had previously been shown to exist as the same two isoelectric variants in all types of chick muscle (5), but the distinctive presence of phosphate in α- but not β-desmin had not been reported. Although the presence of a phosphate group at neutral pH might be enough to account for the difference of 0.05 units in the isoelectric points of α- and β-desmin (13), further differences in amino acid sequence cannot be precluded. Additional phosphorylated proteins of the same molecular weight but lower isoelectric point than α- and β-desmin become apparent in partially purified preparations of desmin, including the KI-insoluble residue of mature smooth and skeletal muscle and the Triton cytoskeletons and Colcemid-induced filament caps of myogenic cell cultures. The copurification and homology in peptide maps of these more acidic species with α- and β-desmin suggest that they represent additional isoelectric variants of desmin.

The subunit protein of the fibroblast-type intermediate filaments similarly exists as nonphosphorylated and multiply phosphorylated variants, all of which are highly homologous as judged by peptide mapping. The difference in isoelectric point between some of the IFP species is less than that expected from altering a single charge unit on the protein (24). As in the case of the desmin variants, however, we cannot predict the number of distinct amino acid sequences. A single IFP amino acid sequence could possess multiple phosphorylation sites.

Extraction of skeletal muscle with 0.6 M KI leaves an insoluble scaffold of interconnected Z-discs composed predominantly of desmin and actin (8). Similarly, KI extraction of smooth muscle leaves an insoluble cytoskeletal network of desmin- and actin-containing intermediate filaments that remain attached to cytoplasmic dense body structures (2, 15, 25). The presence of phosphate associated with desmin in the KI residue of muscle cells demonstrates that phosphorylated desmin is integrated into these cytoplasmic structures.

All of the phosphorylated and nonphosphorylated desmin variants are also present in aggregates of intermediate filaments isolated from myogenic cell cultures as Triton cytoskeletons or as Colcemid-induced caps. Likewise, the nonphosphorylated and phosphorylated variants of the 52,000-dalton IFP are major constituents of these preparations. Because the cell cultures used here are a mixture of myoblasts, myotubes, and fibroblasts, we cannot determine whether desmin derives solely from myoblasts and myotubes or whether the IFP is solely of fibroblastic origin. Even though fibroblasts initially represented less than 10% of the cell population, they, unlike myoblasts, continue to replicate in culture. By the sixth day after plating, when these experiments were done, fibroblasts could represent a considerable fraction of the cell population. Despite this difficulty, phosphorylation of 10-nm filament subunits may be a general phenomenon, because one of the major neurofilament proteins is also phosphorylated (26).

Coincidently, in the course of these experiments we have observed the phosphorylation of minor acidic variants of both α- and β-tropomyosin in chicken skeletal muscle labeled with $^{32}PO_4^{3-}$ *in vivo*. The phosphorylation of α- but not β-tropomyosin has been reported previously in both rabbit and frog skeletal muscle (20). In contrast to skeletal muscle, the chicken smooth muscle tropomyosins are not phosphorylated. The presence of phosphate in skeletal muscle tropomyosin, but not in smooth muscle tropomyosin, may be of functional significance, because the controlling elements for actomyosin are different in the two cell types (19).

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