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Analysis of desmin and vimentin phosphopeptides in cultured avian myogenic cells and their modulation by 8-bromoadenosine 3′,5′-cyclic monophosphate

(intermediate filament/skeletal muscle/tryptic peptide analysis)

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ABSTRACT **The intermediate filament proteins desmin and vimentin are two of the major ^{32}P phosphate acceptors in chicken myotubes differentiating in tissue culture. Analysis of the desmin and vimentin phosphopeptides by two-dimensional tryptic peptide mapping shows that both proteins are phosphorylated at multiple sites, giving rise to 5 phosphopeptides in desmin and as many as 11 in vimentin. Addition of the cAMP analogue 8-bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) to the culture medium of mature (8-day-old) myotubes results in a 2- to 3-fold increase in PO_4 incorporation into desmin and vimentin. Two-dimensional tryptic analysis of desmin and vimentin from 8-BrcAMP-treated myotubes shows increased $^{32}PO_4$ incorporation into a subset of the phosphopeptides observed in control cells. Comparison of phosphopeptides from the two proteins shows the presence of at least three comigrating peptides. All three comigrating peptides exhibit cAMP-dependent increases in $^{32}PO_4$ incorporation in vimentin, while only two of the three exhibit 8-BrcAMP-dependent responses in desmin. While these peptides are the only two that are sensitive to 8-BrcAMP in desmin, vimentin contains additional peptides that exhibit increased $^{32}PO_4$ incorporation in response to 8-BrcAMP. This result suggests the existence of both common and distinct phosphorylation sites between desmin and vimentin that may be differentially regulated by cAMP. Thus, desmin and vimentin, even though structurally related, may be capable of responding differently to physiological stimuli.**

Avian embryonic skeletal myotubes cultured *in vitro* express two intermediate filament (IF) proteins: desmin, the IF protein identified first in smooth muscle and subsequently as the major subunit of intermediate filaments in adult skeletal and cardiac muscle, and vimentin, the IF protein found in most mesenchymal cells and all cells in culture (for review, see ref. 1). Early in myogenesis, both filament proteins are found in an extensive network of cytoplasmic filaments (2, 3). Late in myogenesis, however, both proteins become associated with the Z lines of myofibril bundles, where they form a network of collars that interlink Z discs of adjacent myofibrils (3–5). The mechanisms that regulate this redistribution of intermediate filaments during myogenesis are not yet understood.

We have previously observed that both desmin and vimentin are phosphorylated in cultured skeletal muscle myotubes and that, in mature myotubes, such phosphorylation can be stimulated by the addition of β-adrenergic hormones or cAMP analogues to the culture medium (6). Sensitivity to cAMP-stimulated increases in IF protein phosphorylation is dependent on the state of differentiation of the myotubes. The onset of sensitivity appears to coincide chronologically with the redistribution of intermediate filaments to the Z line during myogenesis (6, 7). This result suggests that phosphorylation of intermediate filament proteins is intimately associated with filament distribution.

In this study, we have analyzed the sites of phosphorylation of vimentin from control and 8-BrcAMP-treated myotubes by two-dimensional tryptic peptide analysis and compared them with those previously identified in desmin. Our results show that vimentin, like desmin, is phosphorylated at multiple sites and that at least three (possibly four) of the vimentin phosphopeptides comigrate with phosphopeptides from desmin, suggesting sequence homology in the two proteins around these phosphorylation sites. Comparison of the phosphorylated peptides after exposure of mature myogenic cells to 8-BrcAMP shows that individual peptides differ in their response to 8-BrcAMP. This result shows that desmin- and vimentin-containing intermediate filaments, although structurally related, are capable of independent functional regulation.

MATERIALS AND METHODS

Cell Cultures. Cultures of embryonic myogenic cells were prepared from 10-day-old chicken embryos as described (3, 6).

$^{32}PO_4$ Incubation for Two-Dimensional Gel Electrophoresis. Duplicate cultures in 60-mm Petri plates were washed twice with 3 ml of phosphate-free minimal essential medium/1% horse serum and incubated in the same medium containing $^{32}PO_4$ at 50 μCi/ml (1 Ci = 3.7×10^{10} becquerels; New England Nuclear). 8-BrcAMP (Sigma) was added to one of the cultures to a final concentration of 1 mM for 45 min prior to the end of the 4-hr $^{32}PO_4$ incubation. Preliminary experiments indicated that this concentration of the cAMP analogue gave maximal stimulation in the phosphorylation of desmin and vimentin for the indicated period of time (6). Labeled cultures were washed once with cold phosphate-buffered saline (0.14 M NaCl/3 mM KCl/10 mM NaK phosphate, pH 7.2), scraped from the Petri dishes with a rubber policeman, and pelleted at top speed in an IEC tabletop centrifuge (approximately $1,000 \times g$). Cell pellets were then solubilized for 3–5 min at 100°C in 75 μl of sample buffer (0.2% $NaDodSO_4$/50 mM Tris·HCl, pH 6.8/1% 2-mercaptoethanol/15% glycerol). Samples were prepared immediately for electrophoresis (see below) or frozen at −20°C for later use. Two-dimensional electrophoresis was carried out as described (6).

Two-Dimensional Tryptic Mapping. Two-dimensional tryptic mapping of $^{32}PO_4$-labeled desmin and vimentin was carried out essentially as described (6). Briefly, 8-day-old myotubes (10^6 cells per 100-mm plate) were labeled as above for 4 hr with 100

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Abbreviations: 8-BrcAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; IF protein, intermediate filament protein.

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μCi of $^{32}PO_4$ per ml. 8-BrcAMP was added during the last hour of the labeling period. After two-dimensional isoelectric focusing/NaDodSO$_4$/polyacrylamide gel electrophoresis, staining and destaining, the phosphorylated variants of desmin and vimentin were excised and processed for tryptic digestion. Samples (approximately equal amounts of protein, representing 500–2,000 cpm) were spotted onto cellulose thin layer plates (Eastman 13255) and analyzed by two-dimensional electrophoresis/chromatography as described (6). The locations of phosphopeptides were determined by autoradiography.

RESULTS

Phosphorylation of Desmin and Vimentin in 8-Day-Old Myotubes is Stimulated by 8-BrcAMP. We have previously shown that the IF proteins desmin and vimentin are phosphorylated in avian embryonic myotubes cultured *in vitro* and that both proteins exist as a major nonphosphorylated variant and multiple more acidic phosphorylated variants (refs. 6, 7; see Fig. 1 *A* and *B*). In the pH range of the isoelectric focusing system used here (pH 4–7), the proteins most prominently labeled with $^{32}PO_4$ in 8-day-old myotubes were the muscle tropomyosins and the IF proteins, desmin and vimentin (Fig. 1 *A* and *B*). Comparison of the autoradiogram with the Coomassie blue-stained gel shows that, as expected (7), the most basic variant of both desmin and vimentin did not incorporate any $^{32}PO_4$. $^{32}PO_4$ was incorporated into at least two major desmin variants (termed α and α' in order of decreasing isoelectric point) and into one or more poorly resolved vimentin variants.

Treatment of 8-day-old myotubes with 1 mM 8-BrcAMP for the last 45 min of a 4-hr incubation with $^{32}PO_4$ resulted in an increase in incorporation of label into desmin and vimentin (Fig. 1*D*) with no apparent increase in labeling of tropomyosin (6). As previously shown, quantitation revealed a 3-fold increase in $^{32}PO_4$ incorporation into desmin and vimentin in myotubes treated with 8-BrcAMP (6). The 8-BrcAMP-induced increase in $^{32}PO_4$ incorporation into desmin and vimentin represents an actual increase in phosphorylation of these proteins; this increase is not due to protein phosphate turnover, is independent of protein synthesis, and is not caused by a 8-BrcAMP-induced change in the specific activity of the cellular ATP pool (6). This increase is not observed when cells are treated with 8-bromoadenosine monophosphate (6). Comparison of the autoradiogram of the 8-BrcAMP-treated cultures with its corresponding Coomassie blue-stained gel showed that the cAMP analogue causes no perceptible changes in the quantity of either desmin or vimentin. Furthermore, the analogue caused no perceptible changes in the ratios of desmin or vimentin isoelectric variants during the labeling period (4 hr) used, as revealed by Coomassie blue staining (see *Discussion*), suggesting that, although $^{32}PO_4$ incorporation is increased by a factor of 2 to 3, the actual increase in phosphate content is less. This disproportionate increase in $^{32}PO_4$ incorporation may result from the short labeling times used in these experiments, which do not allow equili-

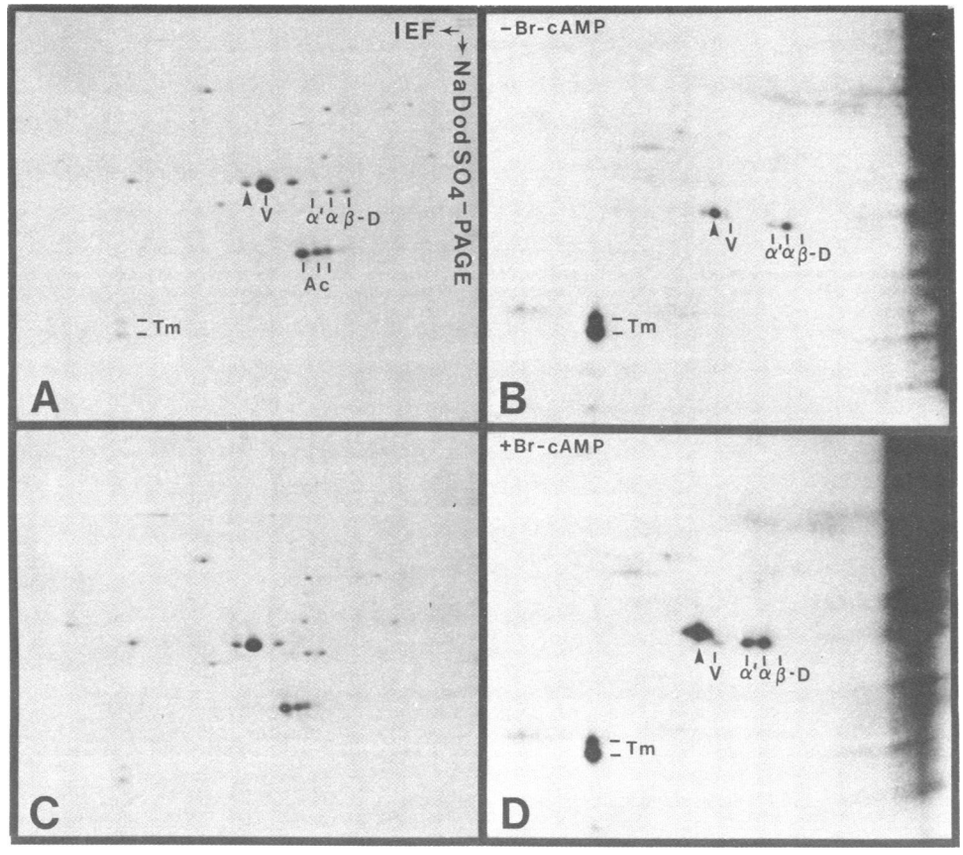

FIG. 1. Two-dimensional isoelectric focusing (IEF)/NaDodSO$_4$/polyacrylamide gel electrophoresis (PAGE) analysis of phosphorylation of intermediate filament proteins in control and 8-BrcAMP-treated 8-day-old myotubes. (*A* and *B*) Coomassie blue-stained gel and the corresponding autoradiogram of control cultures. Cells were labeled for 4 hr with $^{32}PO_4$. (*C* and *D*) Coomassie blue stained-gel and the corresponding autoradiogram of 8-BrcAMP-treated cultures. Cells were labeled as in *A* and *B* and treated with 1 mM 8-BrcAMP for the last 45 min of the $^{32}PO_4$ incubation. Note the increase in phosphate in both desmin (D) variants (α, α') and in the acidic vimentin (V) variants (arrowhead). Coomassie blue staining reveals no changes in the ratios of the desmin or vimentin isoelectric variants in control and 8-BrcAMP-treated cells. Autoradiograms were exposed for 18 hr. Isoelectric focusing is from right (basic end) to left (acidic end). pH range, 4–7. Ac, actin; Tm, tropomyosins.

bration between the protein and the ATP pool.

Tryptic Analysis of Desmin Phosphopeptides. A representative peptide map of $^{32}PO_4$-labeled desmin from 8-day-old myotubes is shown in Fig. 2*A*. Cultures were incubated with $^{32}PO_4$ for 4 hr and 8-BrcAMP-treated cells received 1 mM 8-BrcAMP during the last hour of the $^{32}PO_4$ incubation. We have previously shown (6) that the ^{32}P-labeled peptide maps of α- and α'-desmin are indistinguishable and therefore we have used total desmin (α and α') for the maps presented here. Total desmin obtained from $^{32}PO_4$-labeled control myotube cultures exhibited two or three major ^{32}P-labeled peptides (A, B, and C) and two or more minor ones (D and E; Fig. 2*A*). The ^{32}P-labeled peptides of desmin from 8-day-old myotubes treated with 8-BrcAMP were qualitatively similar to those of control desmin. The most noticeable difference was a large increase in $^{32}PO_4$ incorporation into peptides C and D (Fig. 2*B*), while peptides A, B, and E remained unchanged. We have previously shown that there is a 7- to 10-fold increase in incorporation of $^{32}PO_4$ into peptides C and D, which is sufficient to account for the 2- to 3-fold increase in $^{32}PO_4$ incorporation observed for total desmin (6).

Tryptic Analysis of Vimentin Phosphopeptides. At least 11 phosphopeptides were resolved in vimentin isolated from cultures labeled for 4 hr with $^{32}PO_4$ (Fig. 2*C*). Analysis of vimentin phosphopeptides from cultures incubated with 1 mM 8-BrcAMP during the last hour of $^{32}PO_4$ incubation showed that, of the 11 peptides, at least 8 exhibit a substantial increase in $^{32}PO_4$ incorporation (designated by arrowheads in Fig. 2*D*), in comparison with peptides from untreated cells. With the exception of peptide B, the majority of the 8-BrcAMP-sensitive phosphopeptides in vimentin were barely detectable in control myotube cultures indicating that, in control cells, they are minor sites or are labeled at a very low specific activity.

Comparative Analysis of Desmin and Vimentin Phosphopeptides. Coelectrophoresis of desmin and vimentin phosphopeptides from control (not shown) and 8-BrcAMP-treated cultures (Fig. 2*E*) revealed comigration of three vimentin phosphopeptides with phosphopeptides previously identified in desmin

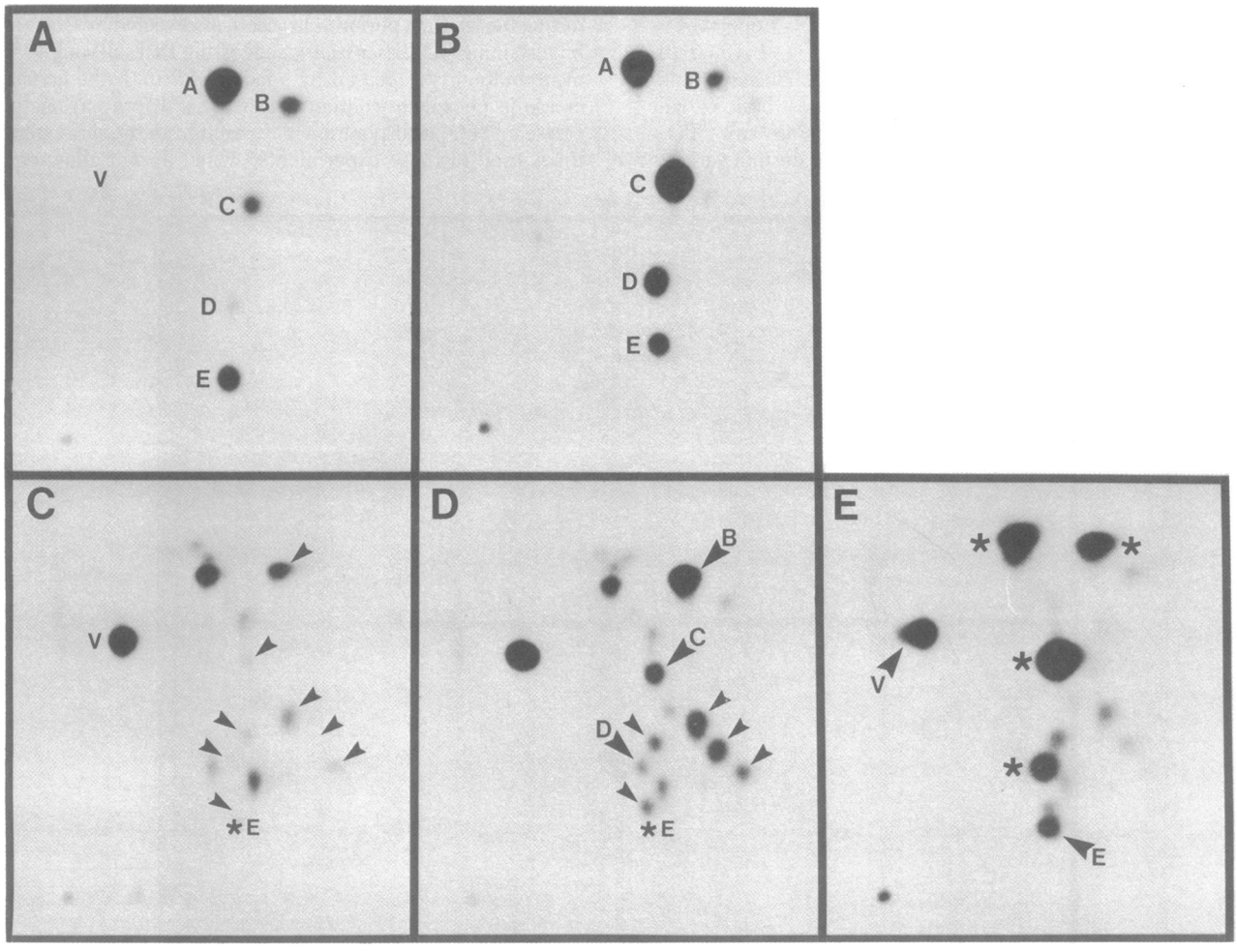

FIG. 2. Comparative peptide mapping of desmin and vimentin phosphopeptides in control and 8-BrcAMP-treated cultures. Desmin and vimentin phosphorylated *in vivo* (as described in Fig. 1) in the absence or presence of 8-BrcAMP were subjected to tryptic peptide analysis. High-voltage electrophoresis at pH 1.9 was from left to right; ascending chromatography was from bottom to top. (*A* and *B*) ^{32}P-Labeled peptides of desmin labeled in the absence (*A*) or presence (*B*) of 8-BrcAMP, showing a significant increase in $^{32}PO_4$ incorporation into peptides C and D in response to 8-BrcAMP. No changes are apparent with peptides A, B, and E. V, Location of a vimentin-specific peptide that is not observed in desmin. (*C* and *D*) ^{32}P-Labeled peptides from vimentin phosphorylated in the absence or presence of 8-BrcAMP. Arrowheads indicate those eight peptides showing reproducible increases in $^{32}PO_4$ incorporation in 8-BrcAMP-treated cells. ^{32}P-Labeled vimentin peptides that comigrate with desmin peptides are labeled accordingly (peptides B, C, and D). Stars in *C* and *D* denote the location of desmin peptide E, which is not observed in vimentin. (*E*) Comigration of peptides from desmin and vimentin (*B* and *D*) from 8-BrcAMP-treated myotubes. Stars indicate the location of comigrating peptides B, C, and D and the closely similar migration of desmin peptide A with a major vimentin peptide. The locations of the unique peptides V (vimentin) and E (desmin) are indicated.

(designated B, C, and D), and one phosphopeptide exhibited nearly identical migration (peptide A). Comparison of Fig. 2 *B*, *D*, and *E* shows that these four phosphopeptides (designated with stars in Fig. 2*E*) respond differently in the two proteins in 8-BrcAMP-treated myotubes. Peptide A showed no change in either desmin or vimentin, peptide B exhibited an increase in $^{32}PO_4$ incorporation in vimentin but not in desmin, while peptides C and D exhibited increases in both desmin and vimentin. In addition, both vimentin and desmin exhibited unique phosphopeptides, allowing us to rule out the possibility of cross-contamination between samples used for mapping. Peptide E is unique to desmin and did not respond to 8-BrcAMP (see *Discussion*). However, at least three of the peptides unique to vimentin exhibited a substantial increase in $^{32}PO_4$ incorporation in the presence of 8-BrcAMP (Fig. 2*D*) while at least one major peptide (designated as peptide V in Fig. 2*E*) did not.

DISCUSSION

We have previously shown that desmin and vimentin are two of the major phosphoproteins in 8-day (or older) cultures of chicken skeletal myotubes (6). Peptide mapping of desmin and vimentin metabolically labeled with $^{32}PO_4$ *in vivo* shows that both proteins are phosphorylated at multiple sites; in desmin, 5 phosphopeptides have been reproducibly identified while, in vimentin, a much more complex pattern with as many as 13 phosphopeptides is observed. This observation is consistent with the observed presence of multiple phosphorylated isoelectric variants in both desmin and vimentin (7).

Addition of 8-BrcAMP to myotube cultures during the $^{32}PO_4$ labeling period results in a rapid increase in $^{32}PO_4$ incorporation into desmin and vimentin while incorporation into other cellular proteins remains unchanged. This response has been concluded to indicate a cAMP-dependent increase in IF protein phosphorylation (6). This result is consistent with earlier findings that both desmin and vimentin serve as substrates for the cAMP-dependent protein kinases *in vitro* (8) and strengthens the conclusion that phosphorylation of intermediate filaments in cultured myotubes *in vivo* is in part due to the action of cAMP-dependent protein kinases. Phosphorylation of vimentin in several nonmuscle cell types has also been found to be modulated by cAMP (refs. 6, 9, 10; however, see ref. 11).

In this report, we have analyzed the response of individual phosphopeptides of desmin and vimentin to 8-BrcAMP. Tryptic peptide analysis of desmin and vimentin from myotubes labeled with $^{32}PO_4$ in the presence of 8-BrcAMP reveals an increase in radiolabel incorporated into specific subsets of the phosphopeptides identified in control cells. In desmin, two peptides (C and D) exhibit a 7- to 10-fold increase in $^{32}PO_4$ incorporation while three others (A, B, and E) remain at a basal level of incorporation. That only two peptides showed a response was somewhat surprising considering our previous observation that four out of five desmin peptides (E excluded) were identifiable in desmin phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase (6, 8). This result may indicate the site-specific control of cAMP-dependent phosphorylation in the two polypeptides.

In vimentin, at least eight peptides were found to exhibit substantially increased incorporation of $^{32}PO_4$ in response to 8-BrcAMP. Several of these peptides were not apparent, or were seen at only very low levels, in untreated control myotubes and showed increases estimated to be greater than 10-fold, while others (peptide B, for example) show less dramatic increases. At least three vimentin phosphopeptides exhibit little or no response to 8-BrcAMP.

It is important to note that the observed increases in $^{32}PO_4$ incorporation do not accurately reflect the actual increase in occupancy of a given phosphorylation site, since the short labeling period used for these experiments results in an ATP donor pool with a specific activity much higher than that of the phosphorylated protein prior to 8-BrcAMP addition. Thus, the increase in $^{32}PO_4$ incorporation is probably disproportionately larger than the increase in total phosphorylation of a given site. Nevertheless, these results reflect the response of each phosphorylation site in a qualitative manner.

Three of the 8-BrcAMP responsive peptides observed in vimentin (B, C, and D) were found to comigrate exactly with peptides identified and labeled correspondingly in desmin. In addition, peptide A of desmin may partially comigrate with a vimentin peptide. While peptides C and D respond to 8-BrcAMP in both desmin and vimentin, peptide B exhibits a 8-BrcAMP-mediated increase in phosphate incorporation in vimentin but not in desmin. The possibility of cross-contamination of the protein samples used for peptide mapping can be ruled out by the presence of prominent phosphopeptides unique to either desmin (peptide E) or vimentin (peptide V).

Considerable evidence has shown that desmin and vimentin are structurally related polypeptides (12, 13); direct sequence analysis of the carboxyl-terminal one-third of the protein molecules indicates a significant (approximately 60%) amount of amino acid sequence homology (14). Previous results (15), and those presented here, suggest that desmin and vimentin share at least three sites of sequence homology around these phosphorylation sites. We have shown that, in desmin, these sites contain phosphoserine as the phosphorylated amino acid (6, 8). Examination of the carboxyl-terminal protein sequences published for desmin and vimentin (14) reveals no serine-containing sequences that show similarity to previously established cAMP-dependent phosphorylation sites (16–18). This suggests that the majority of the phosphorylation sites may be clustered in the amino-terminal two-thirds of the polypeptides, indicating sequence homology in addition to that observed in the carboxyl terminus.

An important point raised by the heterogeneity of response of individual phosphopeptides to cAMP is the possibility of multiple modes of regulation of intermediate filaments by phosphorylation. This is evident in phosphopeptides that vary in their responses to exogenous stimuli such as cAMP. In addition, the presence of homologous phosphorylation sites on desmin and vimentin may indicate the presence of a coordinate regulation while unique phosphopeptides may allow for differential effects of stimuli on the two polypeptides. The functional significance of these potential regulatory sites is as yet unknown. Immunofluorescence studies of desmin and vimentin during myogenesis (3), and *in vitro* polymerization studies (19), have suggested that desmin and vimentin may polymerize into heteropolymer filaments. We have previously suggested that, by varying the subunit composition of intermediate filaments (i.e., the desmin/vimentin ratio), either globally or locally, the functional properties of the filaments themselves might be altered (3, 20). The sequential expression of IF subunits that occurs during muscle (3) and neural (21) differentiation lends support to this hypothesis. The differential response of desmin and vimentin phosphorylation to stimuli such as cAMP may be a manifestation of such functional differences between desmin and vimentin.

The function of intermediate filament protein phosphorylation during myogenesis remains to be established. It is interesting that the onset of sensitivity of phosphorylation to modulation by exogenous 8-BrcAMP coincides in time with the association of intermediate filaments with the myofibril Z line during myogenesis (3, 6). This suggests that phosphorylation of

desmin and vimentin may be one mechanism for the regulation of filament associations with the Z disc during myogenesis.

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