Intermediate Filament Reconstitution in Vitro

THE ROLE OF PHOSPHORYLATION ON THE ASSEMBLY-DISASSEMBLY OF DESMIN*

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Desmin, the myogenic intermediate filament protein, is a phosphoprotein containing phosphoserine, in vivo. The role of phosphorylation on assembly-disassembly and organization of the desmin filament has remained obscure. We report here on a stable and purified system which enables a biochemical examination of desmin filament assembly and disassembly. Using this in vitro system, we carried out stoichiometrical phosphorylations by purified protein kinases. The extent of polymerization-depolymerization was estimated using procedures related to centrifugation and electron microscopy. The evidence we obtained suggests that disassembly of the desmin filament and inhibition of the NaCl-dependent polymerization of the soluble desmin can reversibly occur with either cAMP-dependent or Ca²⁺-activated, phospholipid-dependent desmin phosphorylation.

Protein phosphorylation is an established mechanism by which intracellular events in mammalian tissues are controlled by external physiological stimuli (1, 2). Cyclic nucleotides, Ca²⁺-calmodulin, and diacylglycerol are regulators, which are neither tissue- nor species-specific, and affect a large number of cellular functions. Biological functions of these regulators seem to be manifest through protein phosphorylations by cyclic nucleotides, Ca²⁺-calmodulin-dependent protein kinases, and by protein kinase C (3, 4).

The cytoskeletal proteins are apparently potential targets for these protein kinases because drastic alterations in their architecture occur in response to stimulation by extracellular signals. Some contractile and cytoskeletal proteins can serve as substrates for these protein kinases, both in vitro and in vivo. Both cAMP- and Ca²⁺-dependent protein kinases may be regulators of microtubule function as they phosphorylate microtubule-associated proteins (5–11). The Ca²⁺-dependent phosphorylation of the 20,000-Da light chain of myosin is considered to play a central role in regulating the actin-myosin interaction (12–18).

In addition to microtubules and microfilaments, cells contain intermediate filaments, another class of cytoplasmic filaments. While an in vitro system of assembly-disassembly has been established in the case of microtubules and microfilaments (19–23), the rapid disassembly of intermediate filaments at neutral pH was not shown in vitro. We set up a stable condition of ionic strength and MgCl₂ concentrations that facilitated biochemical examinations of the assembly-disassembly of purified intermediate filament proteins, at neutral pH. Using this in vitro system, we found that phosphorylation of vimentin filaments by cAMP-dependent protein kinase results in a depolymerization (24). In the present experiment, we obtained further evidence on the role of phosphorylation of intermediate filament proteins as follows. 1) Phosphorylation of desmin and vimentin not only induced depolymerization of the filament, it also inhibited the NaCl-dependent polymerization. The extent of polymerization-depolymerization was quantitatively estimated, using ultracentrifugation procedures. Polymerization-depolymerization was detected only by electron microscopy in the previous experiment (24). 2) The phosphorylated desmin resumed NaCl-dependent polymerization immediately after dephosphorylation by protein phosphatases. 3) Effects of CaCl₂ and protein kinase C on the assembly-disassembly of desmin and vimentin were clearly shown. In the previous study, phosphorylation of vimentin filament by protein kinase C occurred to a lesser extent and had little effect on filament formation because of the presence of high concentrations of calcium ions. Thus, we have established a common irreversible and purified in vitro reconstitution system that is applicable to either vimentin or desmin. We present evidence that both protein kinase C and cAMP-dependent protein kinase phosphorylate desmin and vimentin, and functional alteration ensues.

EXPERIMENTAL PROCEDURES

Preparation of Protein Kinases—The catalytic subunit of cAMP-dependent protein kinase was prepared from bovine heart by the method of Beavo et al. (25). Protein kinase C was prepared from rabbit brain by the method of Inagaki et al. (26). Myosin light chain kinase was prepared from chicken gizzard (27), and calmodulin was from bovine brain (28). Calmodulin-dependent glycogen synthase kinase was prepared from rabbit liver (29). Casein kinase I from rabbit liver nuclei was prepared by the method of Meggio et al. (30). Casein kinase II from rabbit skeletal muscle was prepared according to Huang et al. (31).

Phosphorylation of Intermediate Filament Proteins—Desmin (3.8 μM) or vimentin (3.6 μM) was phosphorylated by incubation with 5 μg/ml protein kinase C, 0.1 mM [γ-²P]ATP, 0.3 mM MgCl₂, 0.1 mM CaCl₂, 30 mM NaCl, 0.5 μg/ml phosphatidylserine, 2.5 μg/ml diacylglycerol, 25 mM Tris-HCl, pH 7.0 at 25 °C, or by incubation with 5 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-²P]ATP, 0.3 mM MgCl₂, 30 mM NaCl, 25 mM Tris-HCl, pH 7.0 at 25 °C. In case of the glycogen synthase kinase and myosin light chain kinase assays, the reaction mixture consisted of 0.1 mM [γ-²P]ATP, 25 mM Tris-HCl, pH 7.0, 0.5–3 mM MgCl₂, 0.1 mM CaCl₂, 30 mM NaCl, 10 μg/ml calmodulin, 3.8 μM desmin, and 5 μg/ml of each enzyme. For the casein kinase I and casein kinase II assays, the reaction mixture consisted of 0.1 mM [γ-²P]ATP, 25 mM MgCl₂, 30 mM NaCl, 25 mM Tris-HCl, pH 7.0 at 25 °C. In case of the glycogen synthase kinase and myosin light chain kinase assays, the reaction mixture consisted of 0.1 mM [γ-²P]ATP, 25 mM Tris-HCl, pH 7.0, 0.5–3 mM MgCl₂, 0.1 mM CaCl₂, 30 mM NaCl, 10 μg/ml calmodulin, 3.8 μM desmin, and 5 μg/ml of each enzyme. For the casein kinase I and casein kinase II assays, the reaction mixture consisted of 0.1 mM [γ-²P]ATP, 25 mM MgCl₂, 30 mM NaCl, 25 mM Tris-HCl, pH 7.0 at 25 °C.

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Tris-HCl, pH 7.0, 0.5-3 mM MgCl2, 30 mM NaCl, 3.5 μM desmin, and 5 μg/ml of each enzyme. Purification of Intermediate Filament Proteins—Purified desmin was obtained by extraction of the intermediate filament preparation (32) from chicken gizzard (100 g). Desmin was solubilized by overnight extraction of the intermediate filament preparations with 20 mM Tris-HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, and 8 μg urea (Buffer B). The homogenate was centrifuged for 60 min at 100,000 × g. The supernatant (67 mg of protein) was applied to a DEAE-cellulose column (inner diameter, 1.5 × 20 cm) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, and 7 μg urea (Buffer C), and the dialyzed fraction was applied to a CM-cellulose column (inner diameter, 1.5 × 20 cm) previously equilibrated with Buffer C. The column was washed with 50 μl of the same buffer. Desmin was eluted from the column by application of a 300-ml linear concentration gradient of NaCl (0.0-0.25 M) in Buffer B at a flow rate of 15 ml/h. Desmin was eluted as a sharp peak between 70 and 100 mM NaCl. The DEAE fraction (31 mg of protein) was dialyzed against 20 mM formate buffer, pH 4.0, containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, and 7 μg urea (Buffer C), and the dialyzed fraction was applied to a CM-cellulose column (inner diameter, 1.5 × 20 cm) previously equilibrated with Buffer C. The column was washed with 50 μl of the same buffer. Desmin was eluted from the column by application of a 300-ml linear concentration gradient of NaCl (0.0-0.25 M) in Buffer B at a flow rate of 15 ml/h. Purified desmin (20 mg) was eluted as a sharp peak between 70 and 120 mM NaCl. Removal of the urea by dialysis for 24-36 h yielded a protein that was mostly tetramer in 10 mM Tris-HCl, pH 8.8 (32-35). The soluble desmin (proportion of 0.5-0.6 mg/ml) then assembled into a 12-nm diameter filament during dialysis at 4 °C against 50 mM NaCl, 10 μg/ml leupeptin, 2 mM EDTA, and 10 mM imidazole-HCl, pH 7.0. When 150 mM NaCl was used instead of 50 mM NaCl, the resulting suspension of desmin filament was highly viscous and gel-like in consistency (33), and a small portion could not be obtained from this preparation.

Crude vimentin preparations from Furch's murine mastocytoma cells were prepared essentially as described by Zackroff and Goldman (36). Mastocytoma cells were lysed in a phosphate-buffered saline (−) solution (10 ml/1 g of cells) containing 0.6 M KCl, 2 mM PMSF, 100 mM 2-mercaptoethanol, 2 mM EDTA, 1% Triton X-100, 10 mg/ml MgCl2, and 10 μg/ml leupeptin. DNase I (from Sigma) was added to a final concentration of 0.5 mg/ml, and this solution was washed twice in phosphate-buffered saline (−) containing 1 mM PMSF, 100 mM 2-mercaptoethanol, and 2 mM EDTA. Vimentin was quantitatively solubilized by an overnight extraction of the crude intermediate filament preparations with Buffer A at 4 °C. The homogenate was centrifuged for 50 min at 100,000 × g, and the solubilized vimentin sample was applied to a DEAE-cellulose column followed by application to a CM-cellulose column. Three to 6 mg of vimentin was prepared from 10 g of mastocytoma cells. The soluble vimentin (protein concentration of 0.3 to 0.8 mg/ml) then assembled into the 7-12-nm diameter filament during dialysis for 30 min. When soluble desmin preparations were incubated at 25 °C for 120 min with various concentrations of NaCl and then fractionated, desmin was present exclusively in the s10 fraction at a NaCl concentration between 10 and 50 mM. Increasing the NaCl concentration (>100 mM) yielded an increase in the p10 fraction (Fig. 1a). Following the addition of 150 mM NaCl, a half-elevation of the p10 fraction was measurable at 5 min. At 20 min, the p10 fraction reached a plateau (Fig. 1b). As shown in Fig. 1e, the NaCl-induced p10 fractions were morphologically similar to the native intermediate filaments. When desmin filament preparations were incubated at 25 °C for 120 min with various concentrations of NaCl and then fractionated, desmin was present exclusively in the p10 fraction at a NaCl concentration between 10 and 150 mM (Fig. 1c). Each desmin sample was also examined using electron microscopy (Fig. 1, d-f). Similar results were obtained with the soluble vimentin and the vimentin filament (data not shown). To separate the soluble vimentin from the vimentin filament, the preparations were subjected to higher speed centrifugation (20,000 × g).

Identification of the Protein Kinase Responsible for the Intermediate Filament Protein Phosphorylation in Vitro—As a next step toward defining the regulatory mechanism, we analyzed the phosphorylation of desmin, using six different protein kinases. The smooth muscle myosin light chain kinase, liver calmodulin-dependent glycogen synthase kinase, casein kinase I, and casein kinase II did not significantly phosphorylate desmin (data not shown). In contrast, desmin served as an excellent substrate for both protein kinase C and CAMP-dependent protein kinase. These results indicate that the protein kinases responsible for desmin phosphorylation are the same as those for the phosphorylation of vimentin (24). The time courses of phosphorylation of the soluble desmin and desmin filament by protein kinase C or by the catalytic subunit of CAMP-dependent protein kinase are shown in Fig. 2. The properties of soluble desmin remained fairly constant between 10 and 50 mM NaCl, and the properties of desmin filament remained constant between 10 and 150 mM NaCl when the samples were incubated at 25 °C (see Fig. 1). The high MgCl2 concentration (>3 mM) favored formation of the desmin filament from the soluble desmin, as was the case with vimentin (data not shown) (24). Therefore, all experiments related to desmin phosphorylations were performed in the reaction mixture containing 25 mM Tris-HCl, pH 7.0, 0.1 mM ATP, 30 mM NaCl, and 0.3 mM MgCl2. Protein kinase C incorporated 3 mol of phosphate into 1 mol of the desmin molecule when soluble desmin was used as the substrate. When the desmin filament was used as the substrate for protein kinase C, 2 mol of phosphate was incorporated per mol of desmin molecule at the end of 6 h. The catalytic subunit of CAMP-dependent protein kinase incorporated 3 mol of phosphate into 1 mol of desmin filament when soluble desmin was used as the substrate. When the desmin filament was used as the substrate for protein kinase C, 2 mol of phosphate was incorporated per mol of desmin molecule at the end of 6 h.

**RESULTS**

Determination of the Assembly or Disassembly of the Reconstituted Intermediate Filaments—As a first step toward defining regulatory mechanisms, we prepared a purified and stable condition that facilitates biochemical examinations of the intermediate filament proteins. To separate the soluble desmin (tetramer) from the desmin filament, these preparations were subjected to high speed centrifugation (10,000 × g) for 30 min. We obtained a supernatant (s10) that contained predominantly soluble desmin, and a pellet (p10) of desmin filament. The assembly and disassembly of intermediate filament formation of 10-12 nm filaments from the high-purity intermediate filament proteins are influenced by ion strength (33-35). With this technique, we examined the effect of NaCl concentrations on the states of soluble desmin and desmin filaments at a physiological pH (25 mM Tris-HCl, pH 7.0). When soluble desmin preparations were incubated at 25 °C for 120 min under various ionic strengths and then fractionated, desmin was present exclusively in the s10 fraction at a NaCl concentration between 10 and 50 mM. Increasing the NaCl concentration (>100 mM) yielded an increase in the p10 fraction (Fig. 1a). Following the addition of 150 mM NaCl, a half-elevation of the p10 fraction was measurable at 5 min. At 20 min, the p10 fraction reached a plateau (Fig. 1b). As shown in Fig. 1e, the NaCl-induced p10 fractions were morphologically similar to the native intermediate filaments. When desmin filament preparations were incubated at 25 °C for 120 min with various concentrations of NaCl and then fractionated, desmin was present exclusively in the p10 fraction at a NaCl concentration between 10 and 150 mM (Fig. 1c). Each desmin sample was also examined using electron microscopy (Fig. 1, d-f). Similar results were obtained with the soluble vimentin and the vimentin filament (data not shown). To separate the soluble vimentin from the vimentin filament, the preparations were subjected to higher speed centrifugation (20,000 × g).

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Phosphorylation-dependent Disassembly of the Desmin Filament

FIG. 1. Fractionation of soluble desmin and desmin filament (a–c) and electron microscopy of the negatively stained desmin samples (d–f). a, effect of NaCl on soluble desmin; b, time course of NaCl-dependent filament formation of soluble desmin; c, effect of NaCl on the desmin filament. The soluble desmin (3.8 μM) or desmin filament (3.8 μM) preparation was incubated in 25 mM Tris-HCl, pH 7.0, and 0.3 mM MgCl₂ with various concentrations of NaCl for 2 h at 25 °C, and the samples were subjected to high speed centrifugation (10,000 × g). Electrophoresis was carried out on 9% polyacrylamide slab gels in the presence of 0.1% SDS at 15 mA, using the discontinuous buffer system of Laemmli (49). d–f, electron microscopy of the negatively stained desmin samples. Soluble desmin was incubated in 25 mM Tris-HCl, pH 7.0, and 0.3 mM MgCl₂ with 10 mM NaCl (d) or 150 mM NaCl (e) for 120 min. The desmin filament sample is shown in f. Scale bars = 200 nm, magnification × 44,175.

FIG. 2. Time course of phosphorylation of soluble desmin (○) and desmin filament (●) by protein kinase C (A) and by the catalytic subunit of cAMP-dependent protein kinase (B). Desmin (3.8 μM) was phosphorylated by incubation with 5 μg/ml mol of phosphate into 1 mol of desmin molecule when soluble desmin was used as the substrate. When desmin filament was used as the substrate for the catalytic subunit of cAMP-dependent protein kinase, 2 mol of phosphate was incorporated per mol of desmin molecule at the end of 6 h. With high concentrations of protein kinase C or the catalytic subunit of cAMP-dependent protein kinase, approximately 3 mol of phosphate per mol of desmin molecule in the desmin filament was incorporated.

The Role of Phosphorylation on the Assembly-Disassembly of Desmin Filaments

protein kinase C, 0.1 mM [γ-32P]ATP, 0.3 mM MgCl₂, 0.1 mM CaCl₂, 30 mM NaCl, 50 μg/ml phosphatidylycerine, 2.5 μg/ml diacylglycerol, 25 mM Tris-HCl, pH 7.0 at 25 °C, or by incubation with 5 μg/ml of the catalytic subunit of cAMP-dependent protein kinase, 0.1 mM [γ-32P]ATP, 0.3 mM MgCl₂, 30 mM NaCl, 25 mM Tris-HCl, pH 7.0 at 25 °C.
Phosphorylation-dependent Disassembly of the Desmin Filament

The soluble desmin was preincubated with protein kinase C or the catalytic subunit of cAMP-dependent protein kinase for 1.5 h, and the NaCl-dependent filament formation of soluble desmin was assayed by centrifugation. Phosphorylation of the soluble desmin either by protein kinase C or by the catalytic subunit of cAMP-dependent protein kinase resulted in a nearly complete inhibition of the NaCl-dependent filament formation of soluble desmin (Fig. 3A). When the desmin filament was preincubated with protein kinase C or the catalytic subunit of cAMP-dependent protein kinase for 1.5 h, over 50% desmin was present in the s10 fraction (Fig. 3B). The effect of these phosphorylations on the states of desmin filament was also determined by electron microscopy of the negatively stained desmin filament samples described above. The phosphorylation of desmin filament by protein kinase C or the catalytic subunit of cAMP-dependent protein kinase induced a dramatic transition toward the depolymerized state. Typical images of the phosphorylated samples are shown in Fig. 3C.

Neither protein kinase C (the catalytic subunit of cAMP-dependent protein kinase) nor MgATP alone produced disassembly of the desmin filament.

To elucidate whether the disassembly of desmin filament and the inhibition of polymerization after treatment with protein kinases were due to the ensuing phosphorylation, two control experiments were done: 1) we examined whether the two directional effects of the protein kinases would be inhibited by each protein kinase inhibitor, and 2) whether or not the desmin would be able to assemble into filaments when dephosphorylated by phosphoprotein phosphatase. As shown in Fig. 4, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine (H-7), a potent inhibitor of protein kinase C in vitro (18, 26, 38), decreased the incorporation of the radioactive phosphate into the soluble desmin and correspondingly enhanced the NaCl-dependent filament formation from soluble desmin, in the presence of protein kinase C. Similar results were obtained with N-(2-aminoethyl)-5-isouquinolinesulfonamide (H-8) and the catalytic subunit of cAMP-dependent protein kinase (Fig. 4).

**Fig. 3.** Separation of soluble desmin from desmin filament (A and B) and electron microscopy of the negatively stained desmin samples phosphorylated by protein kinase C (C-b) or the catalytic subunit of cAMP-dependent protein kinase (C-c). A, effect of phosphorylation on the NaCl-dependent filament formation of soluble desmin. B, effect of phosphorylation on the state of desmin filament. Desmin was phosphorylated as described under “Experimental Procedures.” The samples taken at 90 min were incubated with 30 or 150 mM NaCl for a further 30 min. Fractionation of the desmin samples was performed as described in the legend to Fig. 1. C, unphosphorylated desmin filament (C-a), desmin filament phosphorylated by protein kinase C (C-b), and desmin filament phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (C-c). Electron microscopy of the negatively stained desmin samples was performed as described under “Experimental Procedures.” The samples taken at 90 min were negatively stained. In the control experiment, the same assay was carried out, but without ATP. Scale bars = 200 nm, magnification × 38,475.
4. In the second control experiment, desmin was previously phosphorylated by either the catalytic subunit of cAMP-dependent protein kinase or protein kinase C for 60 min and then dephosphorylated by the catalytically active α-subunit of rat liver phosphatase IB (39, 40) in the presence of each protein kinase inhibitor. The resulting dephosphorylated desmin was assembled into the filaments (Fig. 5). These observations strongly suggest that the inhibitory effects we observed after treatment with protein kinases are indeed due to the ensuing phosphorylation and not to protease or to other contaminations of the protein kinase preparations.

Characterization of the Sites of Desmin Phosphorylation—
After the desmin preparations had been phosphorylated by either protein kinase C or by the catalytic subunit of cAMP-dependent protein kinase, the samples were separated on SDS-polyacrylamide gels, eluted, and digested with chymotrypsin. The digested fragments were analyzed by two-dimensional peptide mapping, using electrophoresis in the first dimension and thin-layer chromatography in the second (Fig. 6). When the desmin phosphorylated by protein kinase C was digested, three major phosphopeptides were obtained follow-

![Two-dimensional peptide mapping](image)

**Fig. 4. Effect of protein kinase inhibitors, H-7 and H-8, on desmin phosphorylation and polymerization in the presence of protein kinase.** A, effect of H-7 on the phosphorylation of desmin by protein kinase C. B, effect of H-8 on the phosphorylation of desmin by the catalytic subunit of cAMP-dependent protein kinase. Desmin was phosphorylated as described under “Experimental Procedures” in the presence or absence of drugs. C, NaCl-dependent filament formation of unphosphorylated soluble desmin (control), NaCl-dependent filament formation of soluble desmin phosphorylated by protein kinase C (a), NaCl-dependent filament formation of soluble desmin phosphorylated by protein kinase C (b), NaCl-dependent filament formation of soluble desmin phosphorylated by protein kinase C (c), NaCl-dependent filament formation of soluble desmin phosphorylated by protein kinase C (d), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (e), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (f), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (g), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (h), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (i), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (j), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (k), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (l), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (m), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (n), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (o), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (p), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (q), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (r), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (s), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (t), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (u), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (v), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (w), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (x), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (y), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (z), NaCl-dependent filament formation of soluble desmin phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Serine was the major phosphoamino acid resulting from the phosphorylation of desmin, either by protein kinase C or by the catalytic subunit of cAMP-dependent protein kinase (Fig. 7).

![Phosphorylation-dependent Disassembly of the Desmin Filament](image)
Phosphorylation-dependent Disassembly of the Desmin Filament

**Calcium Ion and Intermediate Filaments**—Intermediate filaments are a heterogeneous class of protein fibrils which can be subdivided into five groups, based on their subunit composition (41–44). The similarities among intermediate filament subunit proteins are exemplified in the case of vimentin and desmin. The proteins have similar molecular weights and isoelectric points and may co-exist in the same cell. In addition, they apparently can form heteropolymers (45). Our results indicate that the protein kinases responsible for the desmin phosphorylation are the same as those for the phosphorylation of vimentin (24). In our previous studies on vimentin filaments, phosphorylation by protein kinase C occurred to a lesser extent (0.5 mol of phosphate/mol of vimentin), and this phosphorylation had little effect on filament formation (24). On the other hand, our present studies indicate that protein kinase C phosphorylates the desmin filament (3 mol of phosphate/mol of desmin) and induces disassembly of this filament. This difference seems to relate to the difference of each condition of phosphorylation by protein kinase C and not to differences in each protein molecule. In the previous studies on vimentin filament (24), protein kinase C phosphorylations were performed under conditions of high CaCl₂ concentration (0.8 mM), without diacylglycerol, instead of the low CaCl₂ concentration (0.1 mM) with 2.5 μg/ml diacylglycerol used in the present work. As shown in Fig. 8, the increase of CaCl₂ concentration is reflected by alteration in the rate and extent of both vimentin and desmin phosphorylations by protein kinase C. The high CaCl₂ concentration (>0.5 mM) seems to promote the formation of vimentin or desmin filament from each soluble protein and to inhibit
disassembly of these intermediate filaments. As well as desmin filament phosphorylation, protein kinase C activated by diacylglycerol and a low concentration of CaCl₂ phosphorylated the vimentin filament at a significantly higher rate and extent (>8 mol of phosphate/mol of vimentin). These extensive phosphorylations induced disassembly of the vimentin filament and inhibition of the NaCl-dependent filament formation of soluble vimentin (Fig. 9). The effects of ionic strength and MgCl₂ and CaCl₂ concentrations on desmin and vimentin were similar.

DISCUSSION

Although intermediate filaments appear to play a significant role in the maintenance of the organization of cytoplasmic space (42), the cytoplasmic regulatory mechanisms that govern the assembly-disassembly of these structures are not well characterized. Until recently, most information on the regulatory mechanisms of filament assembly-disassembly has come from the other two classes of cytoskeletal filaments, microfilaments and microtubules, which, together with bat-

![Graph and images](image-url)
Phosphorylation-dependent Disassembly of the Desmin Filament

A, effect of phosphorylation by protein kinase C on the NaCl-dependent filament formation of soluble vimentin. B, effect of phosphorylation by protein kinase C on the state of vimentin filament. Each phosphorylated vimentin sample taken at 90 min was incubated with 30 or 150 mM NaCl for a further 30 min, and the samples were subjected to high speed centrifugation (20,000 × g). C, unphosphorylated vimentin filament (C-a), vimentin filament phosphorylated by protein kinase C in the presence of 0.8 mM CaCl₂ (C-b) or of 0.1 mM CaCl₂ and 2.5 µg/ml diacylglycerol (C-c). Electron microscopy of the negatively stained vimentin samples was performed as described under “Experimental Procedures.” In the control experiment, the same assay was carried out, but without ATP. Scale bars = 200 nm, magnification × 39,900.

FIG. 9. Separation of soluble vimentin from the vimentin filament (A, B) and electron microscopy of the negatively stained vimentin samples (C). A, effect of phosphorylation by protein kinase C on the NaCl-dependent filament formation of soluble vimentin. B, effect of phosphorylation by protein kinase C on the state of vimentin filament. Each phosphorylated vimentin sample taken at 90 min was incubated with 30 or 150 mM NaCl for a further 30 min, and the samples were subjected to high speed centrifugation (20,000 × g). C, unphosphorylated vimentin filament (C-a), vimentin filament phosphorylated by protein kinase C in the presence of 0.8 mM CaCl₂ (C-b) or of 0.1 mM CaCl₂ and 2.5 µg/ml diacylglycerol (C-c). Electron microscopy of the negatively stained vimentin samples was performed as described under “Experimental Procedures.” In the control experiment, the same assay was carried out, but without ATP. Scale bars = 200 nm, magnification × 39,900.

teries of associated proteins, have been extensively characterized (19–23). In previous studies (24) and this study, we have demonstrated the role of phosphorylation of desmin and vimentin in vitro as follows: desmin and vimentin phosphorylated by either cAMP-dependent protein kinase or protein kinase C do not polymerize, and the polymerized filaments tend to depolymerize after phosphorylation. Moreover, dephosphorylation by phosphoprotein phosphatase induced the reassembly of soluble desmin into filaments. Thus, our results suggest the possibility that reversible enzymatic phosphorylation-dephosphorylation of desmin and vimentin may be involved in modulating the state of polymerization of desmin and vimentin and their reversible disassembly in various cell functions.

Another hypothesis for the regulatory mechanism that governs the assembly-disassembly of these structures has been reported (34, 35). Nelson and Traub (34) described a Ca²⁺-activated neutral proteinase which appears to have a high substrate specificity for vimentin and desmin. The role of the vimentin- and desmin-specific Ca²⁺-activated proteinase appears to involve the limited proteolysis of the 9-kilodalton amino-terminal fragment, which is essential for the assembly of protofilaments into intermediate filaments (34). A question arises that which of the proteolytic mechanisms or the phosphorylation mechanisms predominantly regulate the assembly-disassembly of the intermediate filaments in vivo? The proteolytic mechanism could be related to the observation that vimentin and desmin are very susceptible to proteolysis in vivo (42). Our findings could be related to earlier observations that the organization of intermediate filament changes in cells with phosphorylation events (46–48). At the present time, it is not clear if one or both of these mechanisms are preferentially involved during the dynamic rearrangement of the intermediate filaments in vivo. Whether these two mechanisms are involved or whether other modifications are effective during the dynamic rearrangement of the cytoplasm in a variety of different cells are interesting areas for further experimentation.

Further investigations of these subjects using the reversible in vitro reconstitution systems will lead to a greater understanding of the structure and function of intermediate filaments.
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