

# Modulation of Vimentin Containing Intermediate Filament Distribution and Phosphorylation in Living Fibroblasts by the cAMP-dependent Protein Kinase

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**Abstract.** Microinjection of the purified catalytic subunit of the cAMP-dependent protein kinase (A-kinase) into living rat embryo fibroblasts leads to dramatic changes in vimentin intermediate filament (IF) organization, involving the collapse of the filaments into tight bundles. In some cell types, this rearrangement of the IF proceeds further, leading to an apparent loss of filament integrity, resulting in a punctate staining pattern throughout the cytoplasm. Both these types of IF rearrangement are fully reversible, and similar to structural changes previously described for IF during mitosis. As shown by electron microscopy, in rat embryo fibroblasts these changes in IF structure do not involve the loss of the 10-nm filament structure but instead correspond to the bundling together of 25 or more individual filaments. Metabolic pulse labeling of injected cells reveals that accompanying these

changes in IF organization is a dramatic increase in vimentin phosphorylation which appears maximal when the IF are fully rearranged. However, this increase in IF phosphorylation is not accompanied by any significant increase in soluble vimentin. Analysis of the sites of phosphorylation on vimentin from injected cells by either V8 protease cleavage, or two-dimensional tryptic peptide mapping, revealed increased de novo phosphorylation of two vimentin phosphopeptides after microinjection of A-kinase. These data strongly suggest that the site-specific phosphorylation of vimentin by A-kinase is responsible for the dynamic changes in IF organization observed after injection of the kinase into living cells, and may be involved in similar rearrangement of the IF previously described during mitosis or after heat shock.

THE intermediate filaments (IF), which comprise one of the three major cytoskeletal networks spreading throughout the cytoplasm of eucaryotic cells, are characterized by their 10-nm diameter and marked insolubility over a wide range of pH and ionic strength (for review see references 33, 52). To date, five classes of IF have been described on the basis of the tissue-specific expression of their constituent proteins and include the vimentin containing filaments in fibroblasts, neurofilaments in brain tissue, cytokeratins in epithelial cells, desmin in muscle tissue, and glial fibrillary acid proteins in glial cells (46, 47). Although intermediate filament organization is altered dramatically during mitosis (1, 3, 8, 16, 17, 27, 51, 58), heat-shock (48, 55), or after microtubule disassembly (4, 25), the mechanisms

through which IF assembly and distribution are modulated is still poorly understood (22). Since a number of IF proteins are predominant phosphoproteins (33), changes in IF phosphorylation have been suggested as a potential mechanism for modulating their assembly and distribution (7, 11, 19-22, 24, 37, 38, 42, 45). Indeed, treating cells with defined drugs that elevate intracellular cAMP results in both an increase in the level of vimentin phosphorylation (11, 19, 20, 37) and changes in IF distribution in vivo (11, 12). Other reports have shown that it is the amino-terminal regions of vimentin or desmin that contain the bulk of the phosphorylatable sites (14) which with respect to chicken desmin contains at least three specific sites that can serve as substrates for the cAMP-dependent protein kinase (A-kinase) in vitro (23). Nevertheless, both soluble and filamentous forms of vimentin that do not differ with respect to their overall phosphate content have been isolated (43), therefore the suggestion that changes in subunit phosphorylation alone may modulate the assembly properties of the IF in vivo remains questionable. More recently, Inagaki et al. (29, 30) demonstrated that both vimentin and desmin have specific sites for phosphorylation in vitro, by two major cellular protein kinases, A-kinase and the

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1. *Abbreviations used in this paper:* A-kinase, cAMP-dependent protein kinase; C-kinase, calcium phospholipid-dependent protein kinase; IB, injection buffer; IF, intermediate filaments; REF-52, rat embryo fibroblasts.

calcium/phospholipid-dependent protein kinase (C-kinase). Furthermore, these investigators (and others [28]) showed that the soluble monomeric form of vimentin was highly phosphorylated by C-kinase (but not A-kinase) and that this could influence IF assembly properties *in vitro*. However, these findings were obtained *in vitro*, and since the bulk of cellular intermediate filament protein is in the filamentous form and therefore not a likely substrate for C-kinase under these conditions; reference 43) the mechanism(s) that account for IF reorganization *in vivo* still await to be elucidated.

We have previously demonstrated that after the elevation of A-kinase levels in living cells through the microinjection of the free catalytic subunit into the cytoplasm, there occur transient but pronounced changes in both cell shape and the integrity of the actin microfilament cytoskeleton (31). To assess the possible involvement of A-kinase-dependent phosphorylation in the modulation of intermediate filaments, we have examined the consequences of microinjecting A-kinase into living rat embryo fibroblasts on IF structure and phosphorylation. Such microinjection led to reversible changes in vimentin organization, consisting of the clustering together of the filaments into bundles of 25 or more individual filaments, or in some cell types, a further collapse into punctate structures spread throughout the cytoplasm which are similar to the granular aggregates previously described for keratin or vimentin in some type of mitotic cells (16, 17, 27, 32, 51). By analyzing the changes in protein phosphorylation in injected living cells by two-dimensional electrophoresis and both one- and two-dimensional phosphopeptide mapping, we have found a temporal correlation between the changes in IF organization and the phosphorylation status of two vimentin phosphopeptides *in vivo*. Interestingly, this increased phosphorylation of vimentin was not accompanied by any increase in soluble vimentin in A-kinase-injected cells. These results indicate that A-kinase can play a key role in modulating major changes in the higher order structure of the IF without affecting their solubility. Moreover, such modulation of filament distribution appears dependent upon the phosphorylation status of vimentin and in particular two sites within the vimentin. The potential implications of our findings are discussed further in the context of the changes in both vimentin phosphorylation and IF organization which have been described *in vivo* immediately before cell division (1, 3, 5, 8, 14–18, 27, 32, 42, 51, 56–58) or during heat shock (48, 55).

## Materials and Methods

### Cell Culture

Rat embryo fibroblast cells (REF-52) were cultured in a humidified atmosphere containing 5% CO<sub>2</sub>, 95% air as described previously (31). Gerbil fibroma fibroblasts (CCL-146) were cultured essentially as described for REF-52 with the exception that DME was supplemented with 5% FCS. Cells were plated 2–3 d before use for microinjection studies.

### Microinjection and Immunofluorescence

REF-52 or CCL-146 growing on glass coverslips were microinjected essentially as described previously (31), using glass capillary needles. The free catalytic subunit of the A-kinase, purified from bovine heart was a generous gift from Dr. David Glass, (School of Medicine, Atlanta, GA). For microinjection, the purified A-kinase catalytic subunit was diluted into injection buffer (IB) (100 mM potassium glutamate, 40 mM potassium citrate, 1 mM

MgCl<sub>2</sub>, pH 7.2) to a final concentration of 1.0 mg/ml for REF-52 or 0.5 mg/ml for CCL-146, which we estimate elevates the endogenous levels of A-kinase two- to fourfold (assuming that the level of A-kinase in cells is ~0.2–2.0 μM, and we are injecting a 25-μM solution of the catalytic subunit which is diluted 20-fold by injection). At various times after injection, the cells were fixed for 5 min in 3.7% (wt/vol) Formalin in PBS followed by a 30-s extraction in –20°C acetone. The distribution of the vimentin intermediate filaments was analyzed by indirect immunofluorescence using monoclonal antibodies directed against vimentin (V9; Boehringer Mannheim Diagnostic, Inc., Houston, TX) and subsequently visualized by a 30-min incubation with affinity-purified fluorescein-conjugated goat anti-mouse antibody (Organon Teknika-Cappel, Malvern, PA) diluted 1:100. The cells were analyzed on a Zeiss photomicroscope III using a Planapo 63X (1.3 NA) lens. Fluorescent images were recorded onto Tri-X pan (Eastman Kodak Co. Rochester, NY) and processed as described earlier (31).

### Electron Microscopy

Cells were grown on 35-mm petri dishes and after 2–3 d suitable areas marked with an ink circle on the bottom of the dish. After microinjection of either IB or A-kinase solutions the cells were incubated for 45 (CCL-146) or 60 (REF-52) min. After incubation, the cells were fixed for 20 min with 2% (wt/vol) glutaraldehyde in PBS followed by further fixation in 75 mM cacodylate, pH 7.4, supplemented with 4.5% (wt/vol) sucrose. Cells were washed with PBS and postfixed with 1% (wt/vol) OsO<sub>4</sub> in 75 mM cacodylate, pH 7.4, for 10 min. After a water wash, the cells were “en-bloc” stained with saturated aqueous uranyl acetate for 5 min. After staining and washing in water, the cells were embedded in Epon 812 before sectioning. Sections containing injected cells were mounted on gold grids and poststained with lead citrate before analysis on a Phillips 200X electron microscope at 100 kV.

### Metabolic Pulse Labeling of Injected Cells

Microinjection studies involving metabolic pulse labeling used small (1 mm<sup>2</sup>) glass coverslips onto which cells were plated and allowed to grow for 2–3 d in serum-supplemented DME (31). For studies involving changes in protein phosphorylation, immediately after injection (~10 min/coverslip), the cells were transferred to humidified chambers before labeling in 5 μl phosphate free DME (supplemented with dialyzed serum to 10%), containing 250–500 μCi [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> (carrier free; Amersham Corp., Arlington Heights, IL) as described previously (31). For the analysis of the changes in protein solubility cells were either injected and labeled with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> as described above or alternatively selected coverslips were labeled for 24 h with 250 μCi of [<sup>35</sup>S]methionine in 1.0 ml before and for 1 h after injection. After labeling, the cells were briefly washed in PBS before being either extracted in Triton high salt as described below or lysed in 20 μl of SDS sample buffer (62.5 mM Tris-Cl [pH 6.8], 5 mM DTT, 0.5% [wt/vol] SDS, 7.5% [wt/vol] glycerol, and 2% [wt/vol] NP-40) containing ~5 × 10<sup>6</sup> unlabeled cells as carrier protein. After boiling, samples were lyophilized and resuspended in 10 μl of 1.0 mg/ml DNase and 1.0 mg/ml RNase in 50 mM Tris-Cl (pH 7.4), 10 mM MgCl<sub>2</sub>, 20 mM sucrose, and incubated for 5 min at 37°C. Samples were then re-lyophilized and diluted with either 30 μl SDS sample buffer before analysis by one-dimensional PAGE as described previously (2) or 100 μl of IEF sample buffer (9.5 M urea, 2% [wt/vol] NP-40, 5% [wt/vol] DTT, 2% [wt/vol] 6–8 Ampholines) such that the final concentration of SDS was <0.1% (wt/vol). The latter samples were analyzed by two-dimensional electrophoresis as described below.

### Two-Dimensional Electrophoresis

Samples were analyzed by two-dimensional electrophoresis using the method of O'Farrell (39). First dimension electrofocusing gels contained 9.5 M urea, 2% (wt/vol) ampholines, 2% (wt/vol) NP-40, 5% (wt/vol) DTT. The ratio of ampholine was 60% (wt/vol) ampholine, pH 3–10, 40% (wt/vol) ampholine, pH 5–7. Second dimension gels contained either 12.5% (wt/vol) acrylamide, 0.1% (wt/vol) bis-acrylamide (2). Immediately after electrophoresis, both one- and two-dimensional gels were stained for the molecular weight markers and after drying, the proteins were visualized by autoradiography at –70°C on XAR-5 film (Eastman Kodak Co.), using Cronex Lightening Plus intensifying screens as described before (31).

### Differential Extraction of Soluble and Filamentous Cellular Proteins Using High Salt and Triton X-100

Confluent 60-mm dishes of REF-52 cells were washed once in PBS before being scraped and collected by centrifugation at 13,000 g for 2.0 min. After

aspirating the PBS supernatant, coverslips containing microinjected cells labeled with either [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> or [<sup>32</sup>S]methionine were added to the cell pellet and extracted in 50  $\mu$ l of 600 mM KCl, 1.0% (vol/vol) Triton X-100, 1.0 mM MgCl<sub>2</sub>, 50 mM MES (pH 7.2) for 10 min at 4°C. The insoluble cellular proteins were collected by centrifugation at 13,000 g for 5 min after which the soluble cellular protein in the supernatant was removed. After three cycles of extraction, the insoluble cellular protein pellet and the pooled soluble cell fractions were denatured by the addition of 100  $\mu$ l of boiling 2 $\times$  sample buffer. Fractions were lyophilized and resuspended in 150  $\mu$ l 2 $\times$  sample buffer and 100  $\mu$ l water before analysis by electrophoresis. The inclusion of potassium iodide or other phosphatase inhibitors did not affect either the distribution of the proteins or phosphate label.

### **Reorganization of Vimentin Containing Intermediate Filaments in In Situ Lysed Cell Models**

Rat embryo cells growing on glass coverslips were lysed with 300 mM KCl, 0.1% Triton X-100, 50 mM MES (pH 7.2), 1 mM MgCl<sub>2</sub> for 1–3 min at 37°C, to differentially extract cellular proteins. After extraction, lysed cells were incubated in 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub>, in the presence or absence of the purified 25 ng/ml catalytic subunit or 1.0 mM ATP. After a 3-min incubation at 37°C, cells were fixed and stained for vimentin as described above. Alternatively, the cells were incubated with 1.0 mM ATP and 25 ng/ml catalytic subunit while in the presence of PKI, the specific peptide inhibitor of A-kinase (a generous gift from Dr. David Glass, School of Medicine, Atlanta, GA; reference 9) before fixation and staining as described above.

### **Peptide Mapping**

**V8 Phosphopeptide Mapping.** Vimentin phosphorylated *in vivo* was obtained from REF-52 cells injected with either microinjection buffer alone or A-kinase, labeled for 30–60 min postinjection and separated by two-dimensional electrophoresis as described above. Phosphovimentin was excised from the dried gels and subjected to peptide mapping using the technique of Cleveland et al. (10). The excised bands were equilibrated for 2 h in 125 mM Tris–Cl (pH 6.8), 0.1% SDS, 7.5% glycerol, 2.5 mM DTT, before being loaded into the enlarged wells of a 17.5% (wt/vol) acrylamide gel with a 5-cm 3.0% (wt/vol) stacking gel. Gel slices were overlaid with 0.1  $\mu$ g of V8 protease in 20% glycerol and run to the stacking/resolving gel interface at which point the power was turned off for 30 min. After digestion the proteins were separated by electrophoresis at 250 V until fully resolved. Gels were dried and exposed as described above.

### **Tryptic Peptide Mapping**

Phosphovimentin was derived from living cells injected with either IB or A-kinase as described for Cleveland mapping above. For labeling *in vitro*, vimentin (together with actomyosin) was Triton high salt-extracted from confluent 60-mm dishes of REF-52 cells through three cycles in 600 mM KCl, 1.0% (vol/vol) Triton X-100, 1.0 mM MgSO<sub>4</sub>, 50 mM Tris-acetate (pH 7.2) at 37°C as described above for the analysis of changes in protein solubility. After extraction, the enriched vimentin pellet was incubated in 25 nM A-kinase 10 mM Pipes (pH 6.9), 1.0 mM EGTA, 0.1 mM DTT in the presence of 10  $\mu$ Ci [<sup>32</sup>P] ATP (3,000 Ci/mol; Amersham, International, Amersham UK) for 5 min at 30°C. After labeling the reaction was stopped by the addition of boiling 2 $\times$  SDS sample buffer and the labeled phosphoproteins separated by electrophoresis as described above. After electrophoresis and exposure, the vimentin-containing band was excised and the radioactive vimentin eluted from the gel slice during digestion with trypsin at 0.2 mg/ml in 200 mM ammonium bicarbonate for 36 h at 37°C (20). Labeled tryptic phosphopeptides were separated by two-dimensional electrophoresis and chromatography on thin layer silica plates (HPTLC; Whatman International Ltd., Maidstone, UK). First-dimension electrophoresis was performed using acetic acid/formic acid/water (15:5:80) for 40 min at 900 V. For chromatography, the plates were developed in butanol/pyridine/acetic acid/water (32:25:5:20). After chromatography, the plates were dried and exposed on XAR film (Eastman Kodak Co.) as described above.

## **Results**

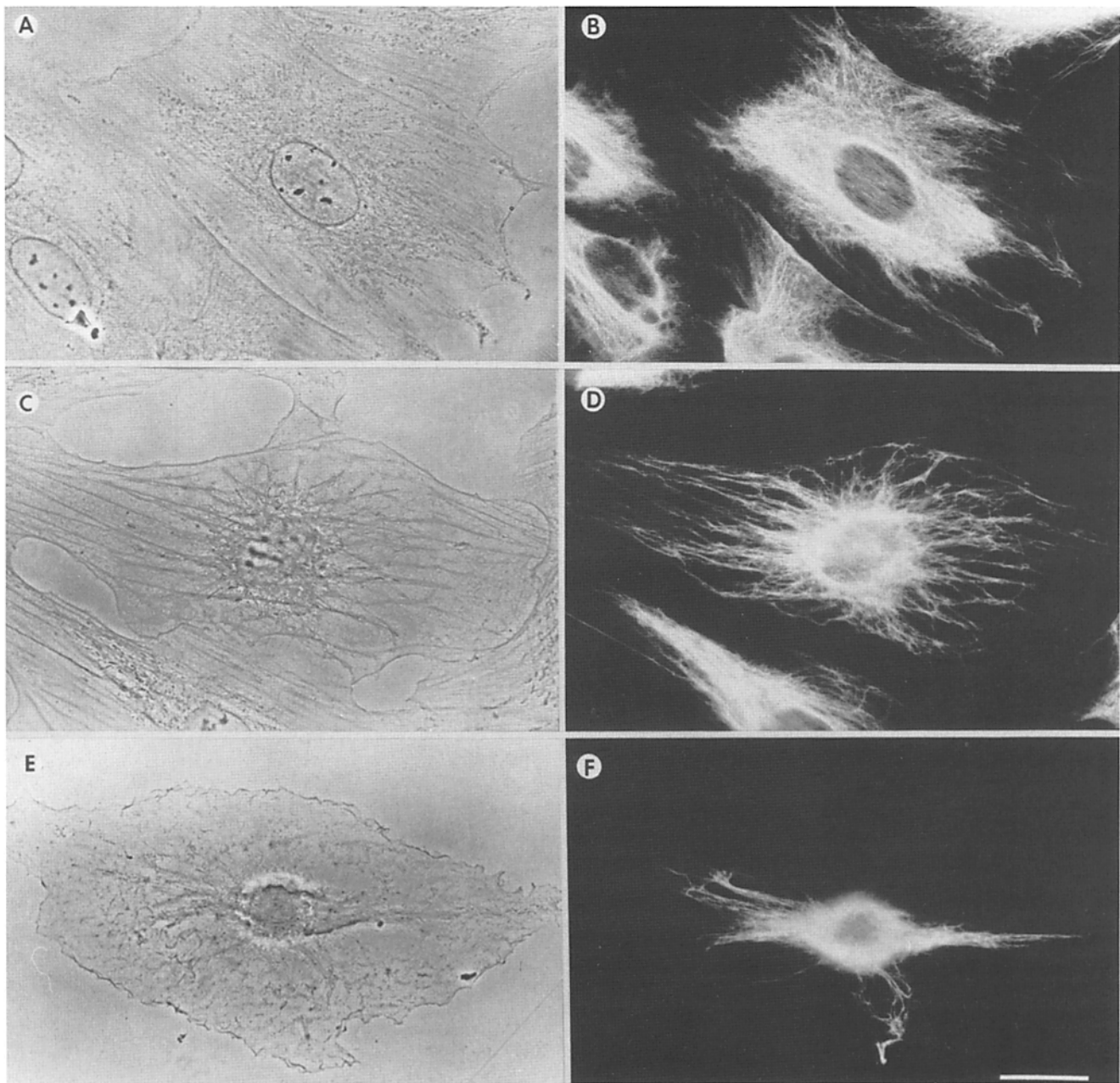
### **Intermediate Filament Reorganization In Vivo**

Previous reports have described changes in cell shape and the organization of the intermediate filaments after activation of

the endogenous A-kinase via defined drugs (11, 12). We were concerned that in addition to elevating intracellular cAMP levels, these drugs might have other pleiotropic effects. Therefore, to ascertain more directly whether A-kinase may be involved in these changes in cell morphology and intermediate filament integrity, we examined the consequences of elevating intracellular A-kinase levels directly by microinjection of the purified catalytic subunit into the cytoplasm of living cells.

We have previously shown that microinjecting REF-52 cells with the catalytic subunit of A-kinase induces a dramatic change in cell morphology together with the disassembly of the actin microfilament cytoskeleton (31). We have used a similar approach to investigate the fate of the intermediate filament network in REF-52 cells. Initially, using indirect immunofluorescence, we have examined the changes in IF organization after injection of A-kinase (1 mg/ml; Fig. 1). Within 30 min of elevating A-kinase levels, the normally fine fibrillar and well-spread distribution of the intermediate filaments became markedly reorganized as the filaments appear to bundle together and collapse (Fig. 1, C and D). In contrast, uninjected cells or cells injected with control solutions (BSA or microinjection buffer alone) exhibited no similar rearrangements of the IF (Fig. 1, A and B). By 60 min postinjection, the IF had become markedly reorganized into a few thick bundles which appeared to run both along the main axis of the cell or in a position closely juxtaposed to the nucleus (Fig. 1, E and F). By this time, >90% of cells injected with A-kinase showed a similar redistribution of the IF (data not shown) which also correspond to maximal changes in cell morphology and microfilament distribution as previously detailed (31) (note in Fig. 1 E, the birefringent cap around the nucleus reflecting the morphological changes induced by A-kinase injection). When using a number of other monoclonal and polyclonal antibodies specific to vimentin, similar redistribution of the IF were observed, thereby indicating that these alterations did not reflect the loss or masking of the epitope for the anti-vimentin V9 antibody (data not shown). These changes in IF organization induced by A-kinase were fully reversible: by 180 min after the injection the filaments had begun to redistribute back into the peripheral cytoplasm. However, up to 20–24 h were required after microinjection before the distribution of the IF became indistinguishable from that of uninjected cells (data not shown).

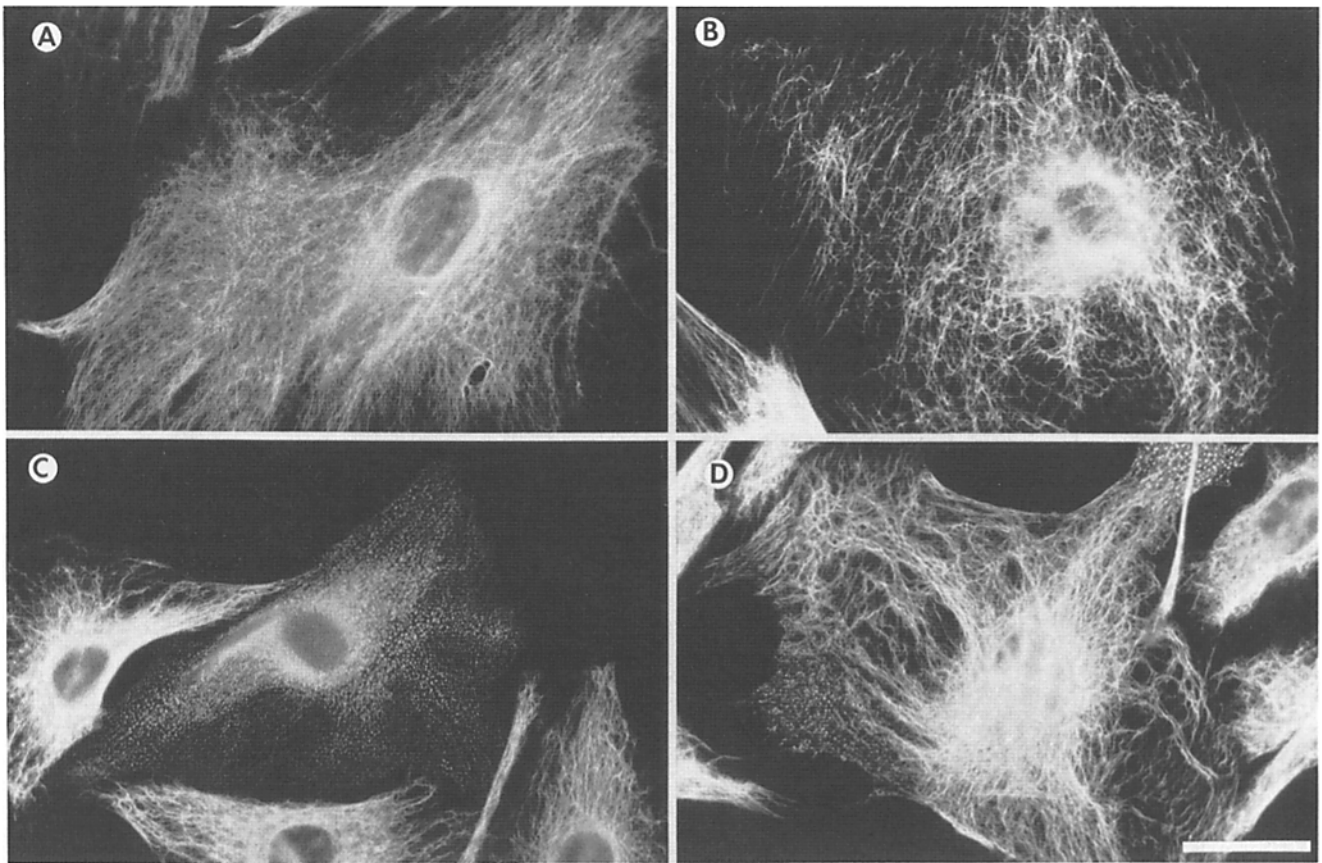
To determine if these changes in IF distribution after injection of A-kinase were common to cell types other than rat embryo fibroblasts, we examined the effect of microinjecting the catalytic subunit in Gerbil fibroma cells (CCL-146). CCL-146, like REF-52 cells, express vimentin as their single intermediate filament protein type dispersed throughout the cytoplasm in a fine fibrillar display (Fig. 2 A). Injection of A-kinase again induced an extensive reorganization of IF in the CCL-146 cells (Fig. 2, B–D). By 30 min after injection, IF had changed markedly, bundling together in a similar manner to that observed in REF-52 cells, whereas the injection of control solutions had no such effect (compare in Fig. 2, A [control] with B [kinase injected]). Incubation of the cells for longer periods after injection revealed the continued reorganization of the intermediate filaments such that by 60 min filamentous IF staining was no longer discernible. Instead, the cytoplasm contained a complex punctated vimentin staining pattern (Fig. 2 C). These punctate structures appeared similar to the distribution of vimentin or cytokera-



**Figure 1.** Changes in the intermediate filament integrity after microinjection of A-kinase. Subconfluent rat embryo fibroblast cells (REF-52) were cultured on glass coverslips and microinjected as described in Materials and Methods with either IB (100 mM potassium glutamate, 40 mM potassium citrate, 1 mM MgCl<sub>2</sub>, pH 7.2) or a solution of the purified catalytic subunit of A-kinase in IB at 1.0 mg/ml. At various times thereafter the cells were fixed and stained for the distribution of vimentin with monoclonal antibody V9. Shown are phase contrast (A, C, and E) and the corresponding fluorescence micrographs (B, D, and F). (A and B) A control cell injected with microinjection buffer alone and further incubated for 60 min. (C and D) A cell injected with purified A-kinase and further incubated for 30 min. (E and F) A single cell injected with purified A-kinase and further incubated for 60 min. Bar, 10  $\mu$ m.

tin filament networks, described in some cell types during mitosis (16, 17, 27, 32, 51) or after injection of anti-cytokeratin antibodies (49, 50). To confirm that these patterns of staining reflected a true loss of filamentous IF staining rather than a simple loss or masking of the epitope recognized by the monoclonal antibody, we examined injected cells using a number of different mono- and polyclonal antibodies directed against vimentin. Again in all cases we did not detect any filamentous intermediate filaments in CCL-146 cells

60 min after injection of A-kinase (data not shown). After longer incubations in the presence of the kinase we observed that, similar to REF-52 cells, these changes in IF organization were fully reversible. By 90 min after injection the majority of the injected cells had begun to regain some filamentous IF staining usually involving the spreading of the filaments towards the periphery. However, such cells still contained both heavily bundled vimentin filaments (as opposed to the thin fibers seen in control cells) and a proportion

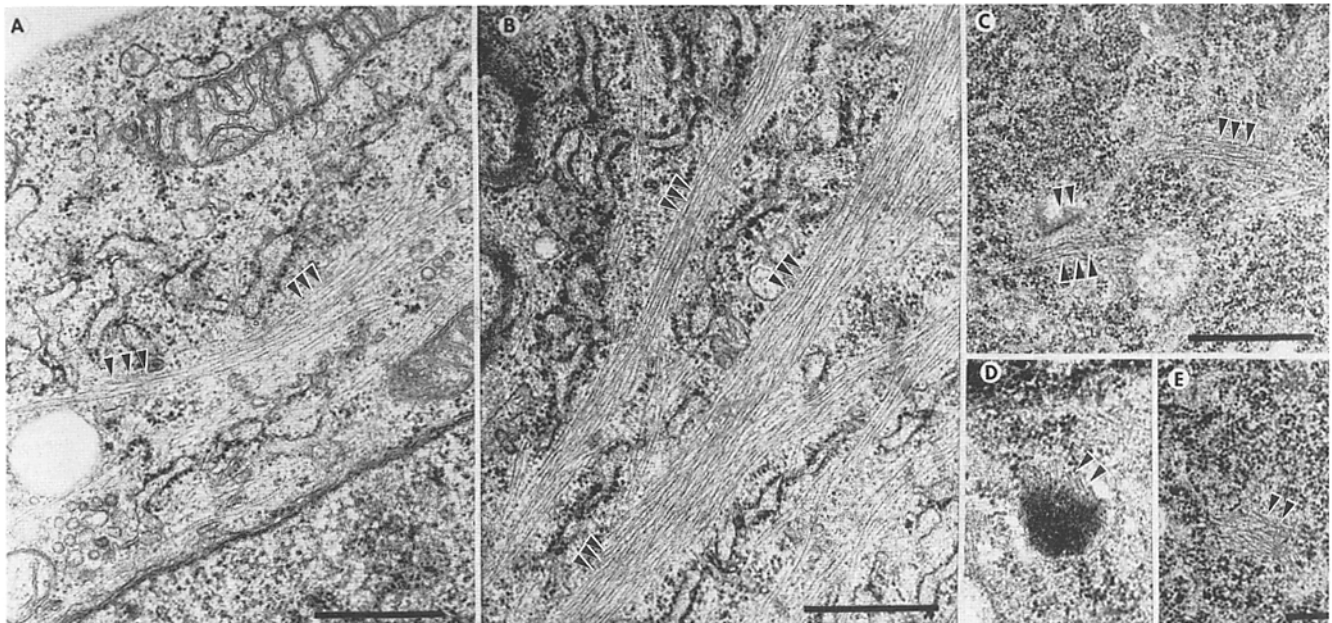


**Figure 2.** Microinjection of A-kinase induces a further reorganization and complete collapse of filamentous intermediate filaments in Gerbil fibroma cells. Gerbil fibroma fibroblasts (CCL-146) growing on glass coverslips were injected with either microinjection buffer or 0.5 mg/ml of the purified catalytic subunit in injection buffer and the distribution of the vimentin containing intermediate filaments analyzed by indirect immunofluorescence at varying times afterwards as described above for the REF-52 cells (see legend to Fig. 1). Shown only are the fluorescence micrographs of the intermediate filament distributions in microinjected cells at various times after injection. (A) Cells microinjected with buffer and fixed and stained 60 min afterward. (B) A cell injected with A-kinase and fixed 30 min after. (C) A cell injected with A-kinase and fixed 60 min after. (D) A cell injected with A-kinase and fixed 90 min after. Bar, 10  $\mu$ m.

of the vimentin organized into punctate structures within the peripheral regions of the injected cell (Fig. 2 D). As in REF-52 cells, by 24 h after injection, the IF had fully reformed into structures indistinguishable from uninjected cells (data not shown). Other cell lines examined for the effect of A-kinase microinjection on IF integrity, revealed patterns of vimentin redistribution similar either to that found in REF-52 cells (e.g., collapsed filaments in the case of NIH-3T3 or NRK [rat kidney] cells), or to the further reorganization observed in CCL-146 cells (e.g., punctate patterns in the case of CHO [hamster ovary] or 1493 human fibroblast] cells) (data not shown). We have not discerned any species or tissue-specific correlation with respect to the extent of IF reorganization after A-kinase injection. However, both types of IF reorganization induced after A-kinase injection, closely mimic the redistribution and structural changes in intermediate filaments observed previously in mitotic cells (1, 3, 16, 17, 27, 32, 58).

We next analyzed the IF distribution in A-kinase injected cells by serial section electron microscopy to determine if the changes observed truly reflected the bundling together of the filaments in a change of their higher order structure as opposed, for example, to other explanations such as the disas-

sembly of the filamentous polymer. Uninjected REF-52 cells exhibited mostly individual fibrillar IF, although a few bundles close to the nucleus could also be identified (Fig. 3 A, arrowheads). In marked contrast, in cells injected with A-kinase, the bulk of the IF were heavily bundled into groups of between 20 and 30 filaments (Fig. 3 B, arrowheads) both in the region closely juxtaposed to the nucleus (shown in Fig. 3 B) or along the axis of the cells, and this distribution accounted for virtually all the vimentin filaments in these cells. Such heavy groups of filaments were never observed in uninjected cells or cells injected with buffer. With respect to the other type of IF reorganization observed in CCL-146 cells, during the early times after injection, serial section electron microscopy revealed similar thick bundles as observed in REF-52 cells. However, these bundles were often found associated with groups of short fibers (Fig. 3 C, arrowheads). Analyzing IF distribution in CCL-146 after longer periods after kinase injection revealed the cytoplasm to contain little or no evidence of intermediate filaments either in bundles or single fibers in the cytoplasm. Rather these cells contained densely staining spheroidal structures (Fig. 3 D) and individual groups of short filaments similar to those arrowheaded in Fig. 3 C, but not associated with IF bundles (Fig. 3 E).



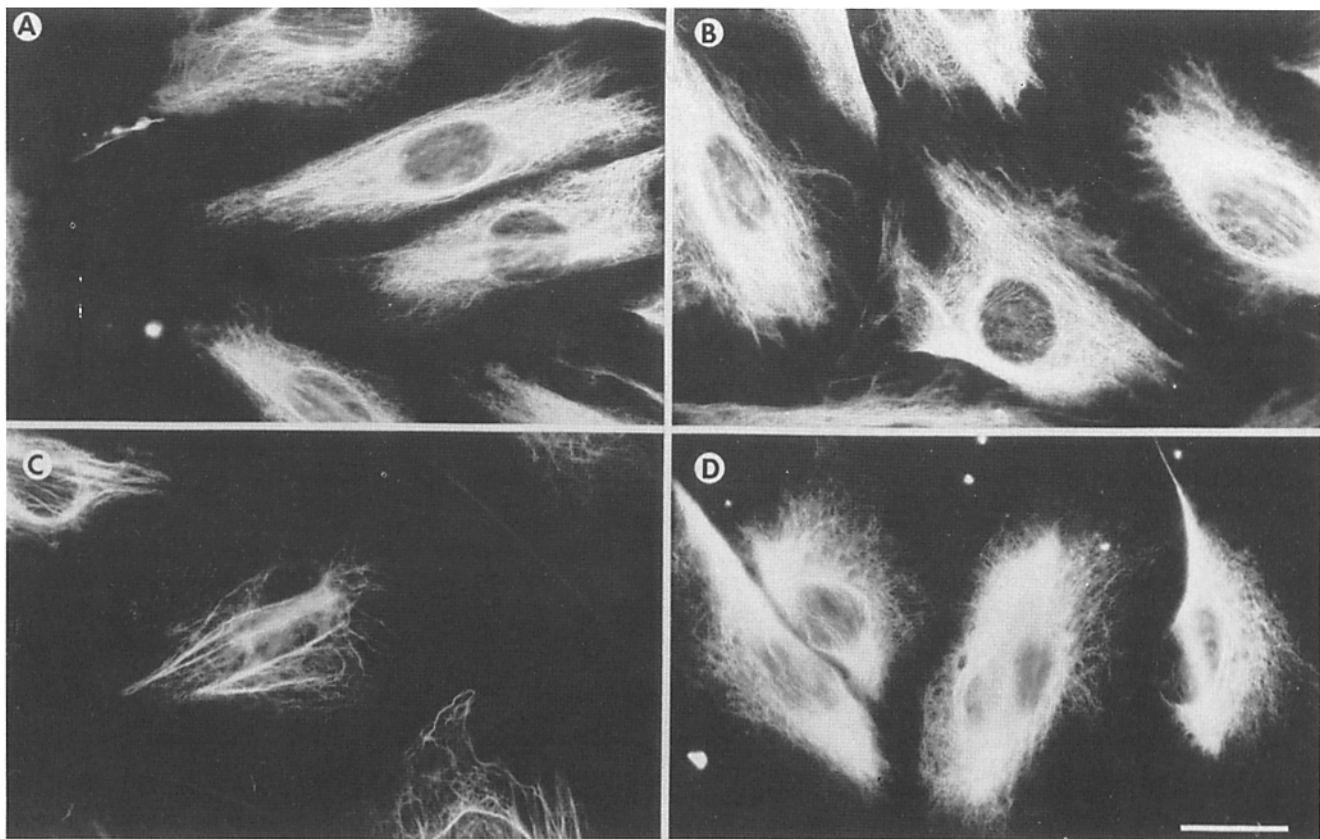
**Figure 3.** Analysis of the effects of A-kinase injection on intermediate filament integrity, as analyzed by thin section electron microscopy. In an experiment similar to that described in Fig. 1, REF-52 cells were injected with either injection buffer or purified A-kinase solution and 60 min after injection the cells were fixed in 1% glutaraldehyde in PBS. Alternatively, subconfluent CCL-146 cells growing in 35-mm dishes were injected with A-kinase essentially as described for Fig. 2. Injected cells were incubated for a further 45 or 60 min before fixation in 1% glutaraldehyde in PBS for 30 min. After processing for electron microscopy as described in Materials and Methods the distribution of the intermediate filaments was analyzed in thin sections. Shown are comparative areas of the cells in sections close to vertical midpoint of the cell in regions adjacent to the nucleus. Enlargements (*D* and *E*) show typical intermediate filament structures. (*A*) The distribution of intermediate filaments in a REF-52 cell injected with microinjection buffer alone. (*B*) The distribution of intermediate filaments in a REF-52 cell injected with the catalytic subunit of A-kinase. (*C*) The distribution of intermediate filaments in a CCL-146 cell 45 min after injection of the catalytic subunit. (*D*) An enlargement of an area of CCL-146 cells injected with A-kinase and analyzed 60 min afterward showing one of the characteristic types of vimentin containing structure observed. (*E*) A similar enlargement to that described in *D*, but showing another type of intermediate filament containing structure. Bars: (*A*–*C*) 100 nm; (*D* and *E*) 20 nm.

Both such types of structure have been previously described in electron microscopic analysis of the mitotic bodies formed by IF in a number of different cell types (16, 17). However, neither in the reorganization seen in REF-52 cells or in the further redistribution found in CCL-146 after kinase injection have we observed any evidence of filament disassembly, using electron microscopy. Interestingly, we have also found that microinjection of A-kinase into the epithelial rat kangaroo cell line PtK2, induces the bundling of the vimentin containing IF while causing the cyokeratin filaments to punctate. We are currently further investigating the molecular basis for these differences in filament behavior in different cells types (Fernandez, A., and N. J. C. Lamb, manuscript in preparation).

#### **Filament Reorganization In Situ**

To further characterize the potential role of A-kinase in mediating the reorganization of the intermediate filaments networks, we investigated if A-kinase could directly induce changes in intermediate filaments in cells briefly extracted with detergents. Previously, *in situ* cell models have been used to study the assembly of mitotic microtubules (41). Our approach involved briefly extracting cells with Triton X-100 to release the bulk of soluble cellular proteins, followed by direct addition of the purified kinase to the lysed cell skeleton. After a brief incubation, the cells are then fixed and the

distribution of vimentin analyzed by indirect immunofluorescence. Fig. 4 shows the immunofluorescence staining pattern of intermediate filaments in extracted cells incubated in the presence or absence of ATP and A-kinase. The brief cellular extraction with 0.1% Triton X-100 was carried out at 37°C since lower temperatures have been shown to lead to microtubule disassembly and subsequent reorganization of the IF (25). Clearly, the extraction procedure alone does not affect the organization of the IF (Fig. 4 *A*), nor does incubating the extracted cells for 3 min in the presence of ATP alone (Fig. 4 *B*). The same result can be obtained by incubating the cells in the presence of A-kinase without ATP (data not shown). Likewise, when we incubated cells with A-kinase in the presence of other nucleotides (cAMP, cGMP, AMP, ADP, GTP, GDP, CTP, or CDP) no changes in the organization of the filament networks took place (data not shown). However, when extracted cells were treated with A-kinase in the presence of ATP, dramatic changes in the IF filaments, similar to those seen after A-kinase injection, were observed (Fig. 4 *C*). In these cells, the intermediate filaments tightly bundled around the nucleus and the bulk of peripheral filamentous staining disappeared. To ascertain whether such changes in the distribution of IF were due to the added kinase, we performed the same experiment, but included also in the incubation a specific inhibitory peptide: PKI, which inhibits A-kinase activity (9). When cells were treated with



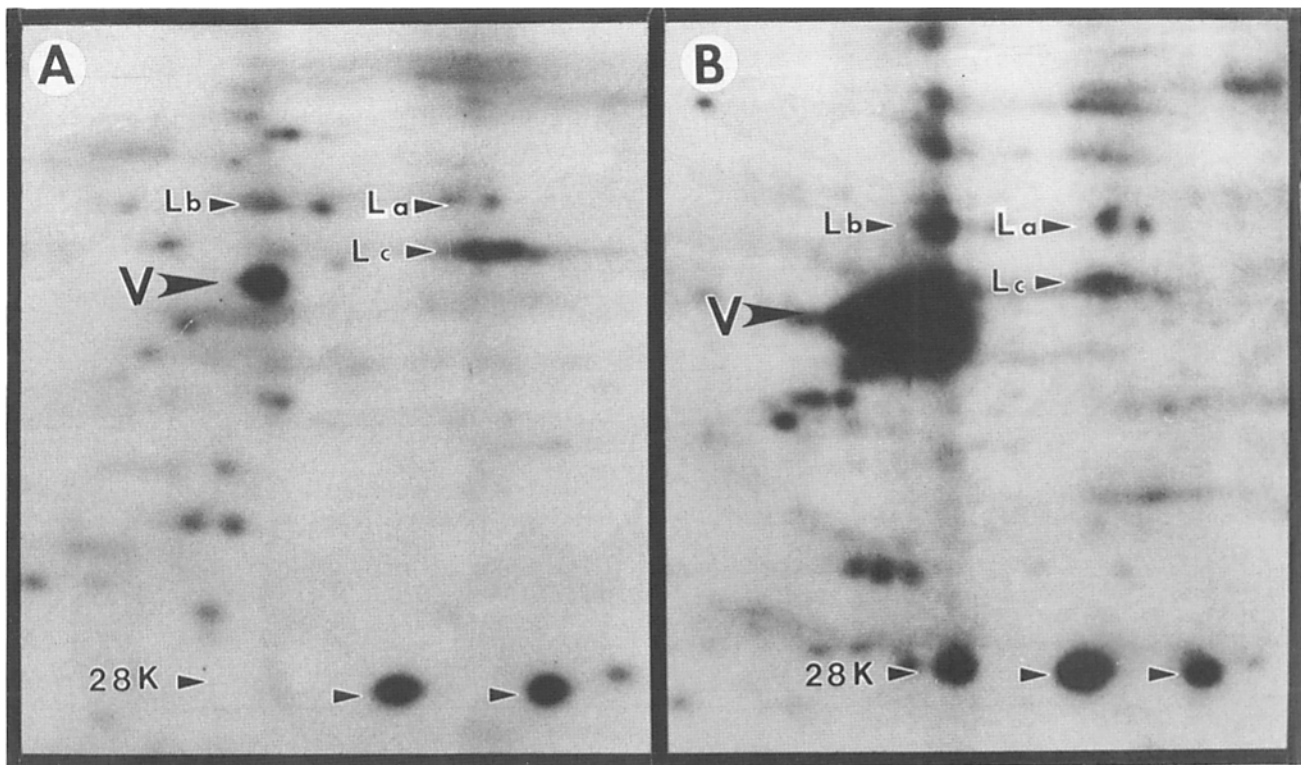
**Figure 4.** Reorganization of vimentin containing intermediate filaments in in situ lysed cell models. Rat embryo cells growing on glass coverslips were lysed with 300 mM KCl, 0.2% Triton X-100, 50 mM Tris-Cl (pH 7.2), 1 mM MgCl<sub>2</sub> for 1 min at 37°C, to differentially extract the soluble pool of cellular proteins as described in Materials and Methods. After extraction, lysed cells were incubated in 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub>, in the presence or absence of 25 ng/ml purified catalytic subunit and 1.0 mM ATP. After a 3-min incubation at 37°C, the cells were washed, fixed, and stained for vimentin as described above. Alternatively, the cells were incubated with 1.0 mM ATP and catalytic subunit which had been preincubated with PKI, the specific peptide inhibitor of A-kinase before fixation and staining as described above. Shown are typical fields of the distribution of vimentin in (A) extracted cells incubated in 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub> alone; (B) extracted cells incubated with 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub> containing 1.0 mM ATP; (C) extracted cells incubated with 25 ng/ml of the catalytic subunit in 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub>, supplemented with 1.0 mM ATP; and (D) extracted cells incubated with 25 ng/ml catalytic subunit in 100 mM Pipes (pH 7.2), 1 mM MgCl<sub>2</sub>, containing 1.0 mM ATP and 50 nM of PKI. Bar, 10 μm.

A-kinase and PKI, in the presence of ATP, no redistribution of the IF was observed (Fig. 4 D), whereas incubating the kinase with a nonspecific peptide still resulted in a complete reorganization of the filaments (data not shown). These data strongly indicate that A-kinase is itself sufficient to directly induce the rapid and dramatic changes in IF distribution.

#### **Changes in Vimentin Phosphorylation and Solubility**

Since the effects of A-kinase are presumably mediated via phosphorylation events, we next examined changes in the phosphorylation status of cellular proteins after microinjection of A-kinase. For these studies, cells were plated on small 1-mm<sup>2</sup> glass coverslips and all the cells on each coverslip injected either with A-kinase or with the microinjection buffer alone. At various times thereafter the cells were metabolically labeled with a very high specific activity with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> as described previously (31), before being solubilized and analyzed by two-dimensional gel electrophoresis. The phosphoprotein pattern of cells (~200 cells total) injected with buffer (i.e., control) and pulse labeled for the

period 30–60 min after injection are shown in Fig. 5 A. Among a generally low level of protein phosphorylation, a number of distinct phosphoproteins can be discerned and have been identified via immunoblotting to be vimentin (V), nuclear lamins A, B, and C (L), and the 28-kD heat shock proteins (28K) (only that region of the gels containing vimentin and the surrounding proteins are shown). After injection of the kinase (Fig. 5 B), an overall increase in the level of protein phosphorylation, especially prominent on vimentin was observed. This effect reached a maximum 30–60 min after injection, when an 8–12-fold increase in the levels of vimentin phosphorylation can be observed (Fig. 5 B). The level of vimentin phosphorylation already increased markedly during the period 0–30 min after injection, although to a lesser extent than that found during the period 30–60 min after injection (data not shown). The increased phosphorylation vimentin closely coincides with the maximal reorganization of the intermediate filaments observed by immunofluorescence. By 60–90 min postinjection, a gradual decrease in overall protein phosphorylation began to occur. However,



**Figure 5.** Analysis of phosphoprotein changes after microinjection of the catalytic subunit of A-kinase using two-dimensional electrophoresis. REF-52 cells, microinjected with either microinjection buffer alone or a solution of the catalytic subunit, were metabolically pulse labeled for 30–60 min after injection with  $[^{32}\text{P}]\text{H}_3\text{PO}_4$  before the cells were solubilized and the labeled phosphoproteins analyzed by two-dimensional electrophoresis as described in Materials and Methods. Shown are a portion of the autoradiograms containing vimentin and the surrounding proteins only. Indicated in the figure are vimentin (*V*), *La*, *Lb*, and *Lc*, the three nuclear lamins and three major isoforms of the 28-kD heat-shock proteins (*28K* and *arrows*) (55). (*A*) Phosphoproteins from 200 cells injected with microinjection buffer and labeled for the 30–60-min period. (*B*) Phosphoproteins from 100 cells injected with A-kinase (1.0 mg/ml) and labeled for the 30–60 min period.

both vimentin and the nuclear lamin proteins continued to show an increased phosphorylation when compared to the cells injected with control solutions (data not shown). The level of vimentin phosphorylation, like the distribution of the filaments, returned to normal 20–24 h after injection. In addition to vimentin, two of the nuclear lamins (*A* and *B*) showed an increased level of phosphorylation that coincided with the maximal increase in vimentin phosphorylation after kinase injection. The observation that the nuclear lamins are also phosphorylated may be related to the apparent close sequence homology between the amino-terminal sections of the nuclear lamins and vimentin (35, 40). However, this does not clearly explain why the third lamin (lamin C, which is highly homologous to lamin A) does not show any increased phosphorylation after kinase injection. We are currently investigating a number of possible explanations for example a different tertiary conformation of lamin C and/or its reduced accessibility within the nuclear envelope.

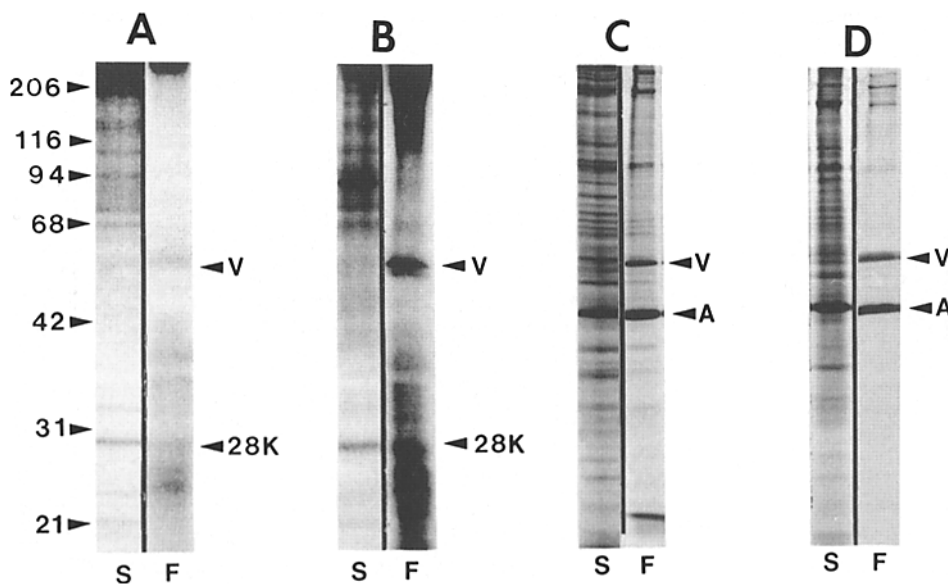
Since previous reports have suggested a correlation between vimentin and desmin phosphorylation and IF disassembly (29, 30), we next analyzed the changes in vimentin solubility after injection of cells with A-kinase, by extracting cells after incubation with  $[^{32}\text{P}]\text{H}_3\text{PO}_4$ . Fig. 6, *A* and *B* show the soluble and filamentous phosphoprotein fractions from cells injected with either buffer or A-kinase, respectively. Under conditions that selectively extract the insoluble cyto-

skeletal proteins (21), we have observed (Fig. 6 *B*) that the vimentin phosphorylated by A-kinase in vivo remains associated with the Triton high salt-insoluble pellet (along with actomyosin microtubule-associated proteins and the nuclear envelope proteins). This situation is similar to that seen in control cells (Fig. 6 *A*), and allow us to conclude that no phosphorylated vimentin is solubilized after A-kinase injection. To confirm that there were no quantitative changes in vimentin solubility, the cellular protein pool was prelabeled for 24 h with  $[^{32}\text{S}]\text{methionine}$  before injection of the kinase and extraction. Under such conditions, the bulk of the vimentin is associated with the insoluble protein pool in cells injected with control solutions (Fig. 6 *C*) as well as in cells injected with A-kinase (Fig. 6 *D*), strongly suggesting that the effects of A-kinase on IF distribution do not reflect the solubilization of the vimentin. A similar result was obtained with CCL-146 (data not shown), in which microinjection of A-kinase led to a more extended reorganization of the IF (see Fig. 2), and strongly implies that the phosphorylation of vimentin by A-kinase affects only the higher order structure of the filaments and does not induce any significant IF disassembly.

#### Vimentin Phosphopeptides

Since a number of different kinases have been shown to phos-



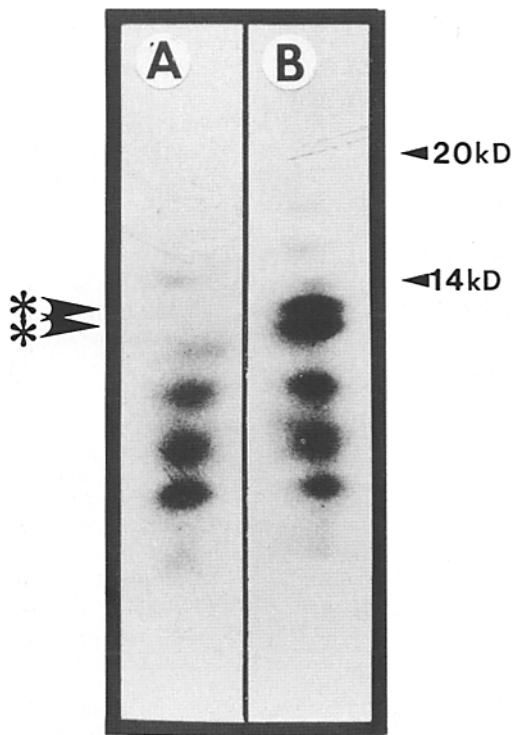


**Figure 6.** Increased phosphorylation of vimentin after A-kinase injection does not lead to an increase in vimentin solubility. To examine for possible changes in vimentin solubility, REF-52 cells were injected with either injection buffer or a solution of the purified catalytic subunit of A-kinase. 30–60 min after injection the cells were labeled with [<sup>32</sup>P]-H<sub>3</sub>PO<sub>4</sub> as described for Fig. 5. Alternatively cells were steady-state labeled with 250 μCi of [<sup>35</sup>S]methionine for 24 h before the cells were injected with injection buffer or A-kinase solution. 1 h after injection [<sup>35</sup>S]methionine or [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> labeled cells were subjected to three cycles of Triton X-100 high salt extraction as described in Materials and Methods. After extraction, 5× boiling

sample buffer was added to the pooled soluble protein fractions while 2× boiling sample buffer was used to solubilize the residual Triton high salt-insoluble fraction. Equal proportions of the soluble (*S*) and insoluble (*F*) cytoskeletal fractions were then analyzed by one-dimensional SDS-PAGE. Shown on the autoradiogram of the gels from <sup>32</sup>P-labeled cells (*A* and *B*) are the position of vimentin (*V*) and the 28-kD heat-shock proteins (*28K*), and on the autoradiograms from <sup>35</sup>S-labeled cells (*C* and *D*) are actin (*A*) and vimentin (*V*) as identified by immunoblotting. (*A*) Phosphoproteins from cells injected with IB and labeled with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> 30–60 min afterward. (*B*) Phosphoproteins from cells injected with A-kinase and labeled with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub> 30–60 min afterward. (*C*) [<sup>35</sup>S]methionine-labeled proteins from cells injected with buffer and extracted after 60 min. (*D*) [<sup>35</sup>S]methionine-labeled proteins from cells injected with the catalytic subunit and extracted after 60 min.

phorylate vimentin in vitro (19, 28–30, 37), we have investigated the phosphorylation sites after A-kinase injection by peptide mapping phosphorylated vimentin excised from two dimensional gels. The phosphorylated vimentin was subjected to limited proteolytic cleavage with V8 protease and one-dimensional gel analysis as described by Cleveland et al., 1977 (10). Fig. 7 *A* shows the proteolytic cleavage products from vimentin derived from control cells injected with buffer alone. Clearly, V8 cleaves vimentin into three major phosphopeptides with a molecular mass ranging between 6 and 10 kD. We have also consistently observed these three major labeled peptides in vimentin isolated from uninjected cells (data not shown). When a similar analysis was carried out on vimentin from cells injected with A-kinase, the same three peptides were found, albeit with a slightly elevated level of phosphorylation (note that the vimentin from twice as many cells was loaded in the control sample). However, together with these three peptides, two additional and heavily phosphorylated peptides of molecular mass 10–14 kD were also apparent (Fig. 7 *B*). These same two additional phosphopeptides were found in vimentin from in situ-labeled cells or vimentin which has been phosphorylated in vitro with A-kinase, while they were not present in vimentin isolated from cells treated with TPA or reacted with purified C-kinase in vitro (data not shown). To make a better estimate of the number of sites phosphorylated by A-kinase, we carried out a more stringent analysis of the phosphopeptides from A-kinase-labeled vimentin using two-dimensional peptide mapping (first dimension electrophoresis at pH 1.9) of vimentin after digestion with trypsin. Fig. 8 *A* shows the

tryptic vimentin phosphopeptides from ~500 control cells injected with buffer alone (such a high number of cells is required to obtain sufficient signal). Clearly, five major tryptic phosphopeptides could be discerned and are designated as *a*, *b*, *d*, *e*, and *f*. Under these conditions, peptides *a* and *b*, as well as peptides *e* and *f*, resolve as a pair of phosphopeptides whereas *d* resolves as a single spot. However, when extracted filamentous vimentin, labeled in vitro with A-kinase, was analyzed (Fig. 8 *B*), we observed, together with the same peptides as seen in vimentin from control cells (*a*, *b*, *d*, *e*, and *f*), the appearance of one new phosphopeptide (arrowheaded with an asterisk and designated as *c*), and the increased phosphorylation of another peptide (arrowheaded with an asterisk only). Furthermore, analysis of the vimentin phosphopeptides from cells injected with A-kinase (Fig. 8 *C*), reveals the major phosphorylation of peptide *c* and the strongly increased phosphorylation of peptide \*, together with the other peptides (*a*, *b*, *d*, *e*, and *f*), showing a similar pattern of phosphorylation as vimentin from control cells (the levels of all these peptides is also increased since there were only 200 injected cells in the A-kinase sample as opposed to 500 cells in the control sample). The same pattern of five major phosphopeptides, closely resembling that obtained in A-kinase injected cells, was found upon analysis of vimentin from extracted cells phosphorylated in situ by the addition of A-kinase (data not shown). These results clearly show that two additional vimentin peptides become specifically phosphorylated upon injection of A-kinase, strongly suggesting that the phosphorylation of these two vimentin peptides by A-kinase is involved in the reorganization of in-



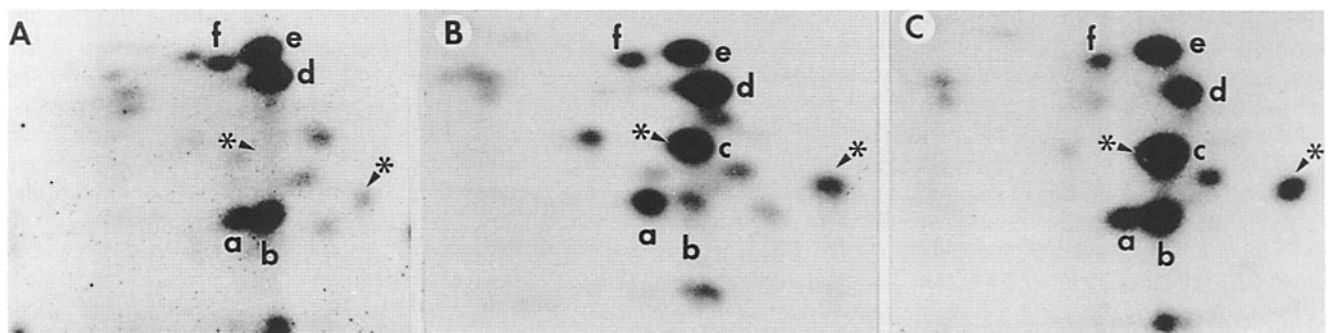
**Figure 7.** Appearance of two additional phosphopeptides in cells injected with purified A-kinase. REF-52 cells were injected with either injection buffer or a solution of the purified catalytic subunit of A-kinase and labeled 30–60 min after injection with  $[^{32}\text{P}]\text{H}_3\text{PO}_4$ . After labeling, the cells were harvested and the phosphoproteins separated by two-dimensional electrophoresis. Phosphovimentin was excised from the dried gels and subjected to peptide mapping using the technique of Cleveland et al. (10). Arrows (A and B) show the position of the two additional phosphopeptides present only in cells injected with A-kinase. Also shown are molecular mass markers on the right. (A) V8 phosphovimentin peptides derived from 250 cells injected with microinjection buffer alone and labeled 30–60 min after injection. (B) V8 phosphovimentin peptides derived from 150 cells injected with A-kinase and labeled 30–60 min after injection.

intermediate filaments which takes place after A-kinase microinjection in living cells.

## Discussion

Intermediate filaments are thought to play a role in maintaining the organization of cytoplasmic space (52) and, although markedly insoluble under physiological conditions, their organization can change dramatically as cells divide (1, 3, 16, 17, 27, 32, 58), in response to cellular stress (48, 55) or in cells treated with drugs which ultimately lead to the depolymerization of the microtubules (25). However, the mechanisms that regulate such changes remain poorly understood (22). While there is increasing evidence that phosphorylation of IF proteins is involved in the reorganization of the IF, a number of different hypotheses can be advanced to account for those changes. For example, changes in filament dynamics could result from modulating the assembly/disassembly equilibrium between the filament and its associated pool of tetrameric subunits, or alternatively they could occur through changes in the higher order structure of the IF such as by increased bundling, and these two possibilities are not mutually exclusive.

While many reports have described changes in the distribution and/or phosphorylation of intermediate filaments under various physiological conditions, our data provide the first direct *in vivo* evidence that phosphorylation of vimentin by A-kinase results in a marked (but fully reversible) redistribution of the vimentin intermediate filaments. This reorganization involves the collapse of the filaments into dense bundles which align along the cell axis and surround the nucleus in a tight cap and, in some cell lines, a further reorganization of the IF into a complex nonfibrillar staining pattern comprised of punctate clusters throughout the cytoplasm. In neither case, however, are these effects accompanied by an apparent disassembly of the IF nor an increased solubility of vimentin. Further electron microscopic analysis revealed, that the bundles of filaments contained 25 or more individual fibers in REF-52 cells or CCL-146 cells whereas analysis of



**Figure 8.** Two additional vimentin phosphopeptides are found in cells injected with A-kinase or in partially purified vimentin labeled *in vitro* with A-kinase. Phosphovimentin was derived from living cells injected with either injection buffer or purified A-kinase and labeled from 30–60 min after injection with  $[^{32}\text{P}]\text{H}_3\text{PO}_4$ . Alternatively, filamentous vimentin obtained after three cycles of Triton high salt extraction was labeled with 25 ng/ml of the purified catalytic subunit *in vitro* with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (specific activity 3,000 Ci/mmol) as described in Materials and Methods. After labeling and two-dimensional electrophoresis, phosphovimentin was excised from the dried gels and subjected to tryptic mapping. Shown are the autoradiograms of the silica thin layer plates. The major vimentin phosphopeptides are labeled a–f; c and \* mark sites of new A-kinase dependent phosphorylation. Electrophoresis was from right to left (anode to cathode). (A) Tryptic vimentin phosphopeptides from 500 cells injected with microinjection buffer alone. (B) Tryptic phosphopeptides from purified vimentin labeled *in vitro* by A-kinase. (C) Tryptic vimentin phosphopeptides from 200 cells injected with A-kinase and labeled 30–60 min afterwards.

the punctate structures identified in CCL-146, revealed them to contain either densely staining amorphous material or short fiber bundles. Both the punctate and bundled patterns of IF redistribution have been described previously in studies of the behavior of IF in mitotic cells (1, 3, 16, 17, 27, 32, 58), although the molecular basis by which such changes occurs remains to be elucidated.

By following the changes in protein phosphorylation in living cells after injection of A-kinase, we have observed that these changes in IF distribution are accompanied by a specific increase in the levels of phosphorylated vimentin. The close temporal coincidence which we have observed between the changes in vimentin phosphorylation and the reorganization of the IF induced after A-kinase injection, strongly suggests that vimentin phosphorylation by A-kinase is responsible for the changes observed in IF distribution. That A-kinase itself phosphorylates vimentin was additionally supported by our observations that in heavily extracted cells, addition of A-kinase resulted in both an increased phosphorylation of vimentin and a corresponding collapse of the IF. A number of reports have recently described the direct phosphorylation of vimentin and desmin by either A-kinase or the calcium phospholipid-dependent protein kinase (C-kinase) under *in vitro* conditions (28–30). The authors suggested that the assembly of purified vimentin and desmin into filaments could be directly modulated by changes in phosphorylation, with an increased phosphorylation inducing disassembly of the filaments (29). We have observed no similar effect in living cells: Indeed, analyzing the insoluble and soluble protein pools after injection of A-kinase revealed that, although there was an 8–10-fold increase in vimentin phosphorylation, we found no increased solubility of the vimentin after the injection of the kinase or in extracted cells after addition of exogenous A-kinase. Moreover, when analyzed by electron microscopy the IF were clearly bundled together after the injection of A-kinase as opposed to showing any filament fragmentation. From our data, it appears that the reorganization of the IF after microinjection of A-kinase involves a change in the higher order structure of the filament, with no obvious disassembly of the filaments or increase in soluble vimentin. These conclusions reveal a clear contradiction with the recent *in vitro* studies described above. The reason for these discrepancies remains unclear. Possibly, in living cells the phosphorylation of vimentin by A-kinase is indirectly modulated by a number of associated proteins not present in the purified fraction, since the *in vivo* phosphorylation peak occurs 60 min after injection of the kinase and the *in vitro* phosphorylation reaction requires 2–3 h to reach equilibrium. Alternatively, the nature of the purified vimentin (after cycling in urea) differs from that of the protein in living cells, allowing for the more promiscuous phosphorylation of the protein *in vitro*. We are currently investigating the basis for these discrepancies through microinjection of purified protein phosphatases to establish the effect of vimentin dephosphorylation or increased phosphate turnover on the soluble and filamentous vimentin pools.

To further characterize the potential role of A-kinase in the reorganization of vimentin containing intermediate filaments, we have compared the sites of phosphorylation on vimentin from control or A-kinase-injected cells. Analysis of the phosphopeptides using V8 protease revealed five phosphopeptides of which two (the major phosphopeptides) were

observed only after A-kinase injection. We obtained a similar result under more stringent analysis using two-dimensional peptide mapping after tryptic digestion, which revealed five peptides continuously phosphorylated in control cells and two additional major phosphopeptides appearing after A-kinase injection. The occurrence of these phosphopeptides indicate that several sites of phosphorylation on vimentin do not appear to be specifically involved in the changes in IF organization, a finding in agreement with a number of other reports (11, 13, 20, 37). For example, between 6 and 14 vimentin phosphopeptides have been described in interphase mouse L929 (13) cells or rabbit and human ciliary epithelium, respectively (11), whereas Gard and Lazarides (19, 20, 37), identified in chicken smooth muscle, at least 11 vimentin phosphopeptides (and 5 major desmin phosphopeptides). Our observation that five to six peptides are continuously phosphorylated in control uninjected cells or cells injected with buffer implies similarly that these phosphopeptides are not involved in the changes in IF structure seen after A-kinase injection. However, as is clear from Fig. 8, all five control peptides are also phosphorylated *in vitro* by A-kinase (although one of them, peptide *b*, seems to be phosphorylated to a lesser extent). The implication that these sites can be phosphorylated by A-kinase is further supported by *in vivo* increased phosphorylation after the injection of the kinase (note that vimentin from three times as many cells was analyzed in control sample). However, this suggestion is difficult to reconcile with the observation that at least one of the two major phosphopeptides induced after A-kinase injection or *in vitro* (Fig. 8 C), is absent in control cells (where presumably at least some of the cells will contain active A-kinase). An alternative, is that these five control phosphopeptides contain sites of high phosphate turnover (possibly involving A-kinase), which are differentially regulated by phosphatases in control cells. However, we cannot exclude the alternative possibility that they are also sites for other kinases. Another question which also remains concerns the potential function of these other peptide sites? Since the levels of soluble vimentin in living cells is postulated to be very low (0.18–0.39% [43]), one possibility is that these continuously phosphorylated sites are involved in the regulation of new filament assembly by helping to modulate the polymer monomer equilibrium. However, this latter report identified no significant effect on the assembly properties of soluble tetrameric vimentin with regard to its phosphorylation state (43).

In addition to these five continuously phosphorylated sites, it is clear that A-kinase can induce the specific phosphorylation of two new sites (Fig. 8, C and \*) both *in vitro* and after injection in living cells. These findings suggest that the changes in intermediate filament organization observed after A-kinase injection result from the increase phosphorylation of these two additional sites on vimentin, and that in living cells, these same sites may become phosphorylated at other times when the intermediate filaments become heavily bundled. Such a reorganization of vimentin filaments occurs in cells before mitosis (1, 3, 8, 16, 17, 27, 32, 51, 58) or after heat-shock (48, 55), and was also associated with increased vimentin phosphorylation (7, 11, 13–15, 57, 58). Moreover, a number of reports have detailed a site-specific increase in vimentin phosphorylation associated with these changes in vimentin structure especially with respect to mitosis (13, 14,

57). For example, the capping of the intermediate filaments before mitosis has been correlated with the increased phosphorylation of two vimentin tryptic peptides in both hamster (57) and mouse L929 fibroblasts (13). Although not demonstrated directly, the implicit role of A-kinase in mediating these changes has been suggested previously: the increased phosphorylation of two major vimentin phosphopeptides after treatment with agents which activate A-kinase *in vivo* was reported in muscle cells (20) (these authors also detail the increased phosphorylation of two sites on desmin when A-kinase is activated *in vivo* while they observed four sites could be phosphorylated by A-kinase *in vitro*). In an alternative study, three additional phosphopeptides were identified when rabbit and human cells were exposed to a variety of A-kinase activating agents, including hormones (11). In the latter case a similar collapse of the vimentin filaments around the nuclei also accompanied  $\beta$ -adrenergic activation. Taken together with the findings we have reported here, there is now quite strong evidence that the A-kinase dependent phosphorylation of vimentin at two (or possible more) sites is involved in the pronounced reorganization of the intermediate filaments during a number of different cellular events. In this respect, it is clear that the mechanisms which modulate the equilibrium between soluble vimentin and vimentin filaments remain to be determined, and the involvement of A-kinase, C-kinase, or other cyclic nucleotide-independent protein kinases (for example calcium/calmodulin dependent kinase [44]) and vimentin subunit phosphorylation in this process will require further investigation (14, 43). An alternative hypothesis, involving the calcium-dependent activation of a neutral protease which specifically cleaves an amino-terminal fragment essential for IF assembly has also been advanced to account for the changes in IF organization (36, 52-54). However, it is unlikely to be involved in the effects described here since we did not observe cleavage products in either the soluble or insoluble fractions after A-kinase injection (they would be visible in either  $^{35}\text{S}$  or  $^{32}\text{P}$  gels in Fig. 6, because the protease cleaves at the amino terminus (53), which also appears to contain the bulk of the A-kinase dependent potential phosphorylation sites [14]). With respect to these other potential mechanisms, the data presented here clearly demonstrate that the site-specific phosphorylation of vimentin in living cells by A-kinase induces a marked reorganization of the bulk of the IF network without any detectable filament disassembly. However, many interesting questions still remain. For example, how does increasing the phosphorylation of the vimentin subunit bring about such changes in filament structure. Different possibilities could be advanced: direct phosphorylation of vimentin may induce changes in the molecular interactions between vimentin subunits within the filament, and/or alter the interaction between these subunits and intermediate filament-associated proteins. Direct or indirect phosphorylation of an intermediate filament associated protein (by A-kinase or other kinases) could also modulate its interaction with the filament ultimately leading to filament disorganization. In this respect, previous reports have shown that injection of antibodies directed against vimentin or a 95-kD vimentin-associated protein leads to the bundling and collapse of vimentin IF in fibroblasts (34, 50), similarly to that described here. Alternatively, the reorganization of the IF after A-kinase injection could result from a

perturbed interaction between IF and the other cytoskeletal networks, since it has been reported that microinjection of antibodies which bind to tubulin *in vivo* (3) or drugs which induce the disassembly of the microtubules also lead to the dramatic bundling and capping of the IF (25). However, we have found no significant effect on the microtubule distribution after injection of the kinase (Lamb, N. J. C., and A. Fernandez, manuscript in preparation), although we have previously identified that injecting A-kinase prominently alters the distribution of the actin microfilaments (31). Since the microfilament network is also closely associated with the IF (26), it can be conceived that the collapse of the IF would result indirectly from the disassembly of the microfilaments. This is however unlikely since we could induce microfilament disassembly after injection of antibodies directed against myosin light chain kinase without affecting the IF networks (31). Finally, we have also examined the distribution of A-kinase in cells after injection to ensure that the kinase does not localize on the filaments, in turn inducing their collapse. In injected cells the kinase appears to be evenly distributed throughout the cytoplasm and does not show any association to the cytoskeleton as has been previously suggested (6) (data not shown).

A number of physiological situations involve a reorganization of intermediate filaments similar to that described in the present study, after injection of A-kinase. For example, the collapse and tight bundling of IF around the nucleus has been described after heat-shock (55), where it accompanies the overall shutdown of protein synthesis (48) (with the exception of the heat-shock proteins). Since IF can bind RNA, proteins and a number of other components of the protein synthesis machinery of cells (52-54) it is possible that the collapse of the IF has a functional role in the heat-shock response, and in this respect A-kinase-mediated phosphorylation of the IF may be involved. Since we have found two additional phosphopeptides specifically phosphorylated by A-kinase *in vivo*, it will be of interest to identify the amino acid residues and sites phosphorylated. By investigating these sites further we may probe the potential role of A-kinase in the reorganization of the IF especially during the cell cycle and mitosis. Indeed, the phosphorylation of vimentin on two tryptic peptides in mitotic cells has been observed previously (13), and several studies have implied a role for A-kinase in the events which accompany mitosis (5, 7, 13-15, 33, 44). Our previous work has detailed the effects of A-kinase injection on cell morphology and the organization of microfilament (31). These effects together with the changes described here on the distribution and phosphorylation of the IF closely mimic the events described at mitosis. We are currently further investigating the potential role of A-kinase in mediating these changes in the living cell.

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