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Cell cycle-dependent changes in the organization of an intermediate filament-associated protein: Correlation with phosphorylation by p34^{cdc2}

(mitosis/p34^{cdc2} protein kinase/cytoskeleton)

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ABSTRACT **During mitosis in BHK-21 baby hamster kidney cells the hyperphosphorylation of the type III intermediate filament (IF) protein vimentin is accompanied by the disruption of the IF network into punctate, protofilamentous structures. In this study, the morphological and biochemical changes of IFAP 300, a 300-kDa IF-crossbridging protein, are examined during mitosis. Double-label immunofluorescence shows that the distribution of IFAP 300 coincides with the typical filamentous pattern displayed by vimentin in interphase cells, whereas in mitotic cells it is reorganized into a punctate, nonfilamentous pattern. Accompanying these latter morphological changes, IFAP 300 is phosphorylated at a unique, mitosis-specific site. Comparison of the sites phosphorylated in cultured cells with those phosphorylated *in vitro* by various kinases suggests that IFAP 300 is phosphorylated by the same two kinases that phosphorylate vimentin during mitosis. One of these is p34^{cdc2} protein kinase, which appears to be responsible for the phosphorylation of the mitosis-specific site. The other kinase phosphorylates IFAP 300 *in vitro* at a site that is also found in the protein immunoprecipitated from either mitotic or interphase cells. In contrast to vimentin, the phosphorylation levels of IFAP 300 are not obviously altered between interphase and mitosis. Our results show that IFAP 300 is a physiological substrate for p34^{cdc2} and that this kinase may be involved in the mitotic reorganization of IFAP 300 by phosphorylating a mitosis-specific site. Taken together with our previous results, this study suggests that the activation of p34^{cdc2} coordinates the mitotic reorganization of the vimentin IF network both by severing IF–IF connections mediated by IFAP 300 and by disassembling individual IFs into protofilaments.**

Intermediate filament (IF)-associated proteins (IFAPs) and phosphorylation have emerged as two important regulators of the supramolecular organization of IFs in different cell types (for reviews see refs. 1 and 2). By bundling or crosslinking, IFAPs increase the degree of lateral association between IFs to form various types of cytoplasmic networks, whereas phosphorylation of IF subunits frequently leads to the disassembly of the IF network. This latter phenomenon has been well characterized during mitosis, where hyperphosphorylation of IF subunits (3–6) temporally correlates with changes in the organization of the IF network, such as the disassembly of IF and the formation of protofilamentous aggregates (7–12). It has been established that in BHK-21 cells the mitotic disassembly of the IF network is due to the activity of two protein kinases (3, 13). One of these activities, vimentin kinase II, has been purified as a complex consisting of three proteins of 110, 67, and 34 kDa (13). The 34-kDa component has been identified as p34^{cdc2} (13), the catalytic subunit of a kinase promoting the entry of cells into mitosis (for review see ref. 14). The other kinase has been partially purified and is referred to as vimentin kinase I (3).

IFAP 300, a 300-kDa protein originally described in BHK-21 cells, has been shown by double-label immunofluorescence to have a distribution similar to that of IFs, and by immunogold electron microscopy is often seen at junctions between IFs (15, 16). *In vitro*, IFAP 300 remains associated with IFs through several cycles of polymerization and depolymerization (15, 16), and in reconstitution assays, purified IFAP 300 forms crossbridges between IFs (15). IFAP 300 appears to be biochemically and immunologically similar to a protein called plectin, which is present in a number of cell lines and tissues (17–19). However, there are differences in the staining pattern obtained with antibodies against these two proteins. While immunofluorescence with IFAP 300 antibodies results in staining similar to that of vimentin (16), plectin antibodies stain a network of short fibers and dots that is clearly distinguishable from the vimentin IF network (20). It remains to be shown whether these discrepancies are due to differences in the reactivity of the antibodies used or to differences in the primary sequence between IFAP 300 and plectin.

It has not been determined whether the close association between IFAP 300 and vimentin is maintained during the mitotic reorganization of the IF network. Therefore, we have examined the localization of IFAP 300 during mitosis and have found that overall it parallels that of vimentin. We further demonstrate that, like vimentin (13), IFAP 300 is phosphorylated in cultured cells by p34^{cdc2}, which most likely contributes to the coordinated reorganization of these two closely associated proteins during mitosis.

MATERIAL AND METHODS

Cell Culture and Metabolic Labeling with [^{32}P]Orthophosphate. BHK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 10% tryptose phosphate, penicillin (50 units/ml), and streptomycin (50 μg/ml). For metabolic labeling experiments, cells were grown in phosphate-free DMEM supplemented with 10% calf serum and 0.2 mCi (7.4 MBq) of [^{32}P]orthophosphate/ml (^{32}P-medium). Interphase cells were obtained by maintaining cells for 36 hr in isoleucine-free F10 medium (21) or in DMEM containing only 0.5% calf serum (22). The cells were then switched first to DMEM with 10% calf serum for 5 hr and then to ^{32}P-medium for an additional 3 hr. Mitotic cells were collected by mechanical agitation after a 3-hr incubation in ^{32}P-medium containing nocodazole at 0.4 μg/ml (3).

Antibodies, Immunofluorescence, and Confocal Microscopy. Rabbit polyclonal and mouse monoclonal antibodies raised against IFAP 300 from BHK cells have been reported

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Abbreviations: IF, intermediate filament; IFAP, IF-associated protein.

(16). An IgG fraction was prepared from the polyclonal anti-IFAP 300 by precipitation with ammonium sulfate at 40% saturation; this IgG fraction was then adsorbed against glutaraldehyde-crosslinked (23) vimentin purified from BHK cells (15). The resulting antibodies reacted only with IFAP 300 when tested by immunoblotting against a BHK cytoskeletal preparation. A monoclonal vimentin antibody was purchased from Amersham. Single immunofluorescence with IFAP 300 antibodies and double immunofluorescence with IFAP 300 and vimentin antibodies were carried out on mitotic and interphase cells according to published procedures (12).

Confocal microscopic observations were carried out on a Zeiss LSM instrument (Zeiss) using a He/Ne laser at 514 nm for rhodamine and an Ar laser at 488 nm for fluorescein.

Determination of IFAP 300 Phosphorylation Levels. The phosphorylation levels of IFAP 300 were determined in BHK cells incubated for 3 hr in the appropriate ^{32}P-medium. The specific activity of IFAP 300 was measured either from immunoprecipitates of cells lysed in 20 mM Tris/HCl, pH 7.4/1% (vol/vol) Nonidet P-40/0.2% (wt/vol) SDS/140 mM NaCl/5 mM EDTA/20 mM sodium pyrophosphate/40 mM β-glycerophosphate or from the insoluble fraction of cells extracted with 5 mM sodium phosphate, pH 7.2/1% (vol/vol) Triton X-100/0.6 M KCl/5 mM EDTA/20 mM sodium pyrophosphate/40 mM β-glycerophosphate. Both procedures yielded similar results. When the latter procedure was performed, vimentin was used as an internal standard, since cell cycle-dependent changes in the phosphorylation levels of this protein have been documented (3–6). After the separation of IFAP 300 and vimentin by SDS/6.5% PAGE (24), their specific activities were measured as described (3).

***In Vitro* Phosphorylation.** The substrate used for *in vitro* phosphorylation experiments was a Triton/high-salt-insoluble BHK IF cytoskeletal preparation subjected to one or two cycles of disassembly–reassembly (15, 25). Several kinases were used to phosphorylate this preparation. These included vimentin kinase I (3), $p34^{cdc2}$ purified from mitotic BHK cells (13), the catalytic subunit of bovine heart cAMP-dependent kinase (kinase A) (Sigma), and protein kinase C from porcine brain (Calbiochem). For all kinases tested, phosphorylation was carried out in 2 mM Hepes, pH 7.2/100 mM NaCl/2 mM $MgSO_4$ containing 0.1 mM [γ-^{32}P]ATP (specific activity, 10 mCi/mmol) and 5 mM EGTA. For phosphorylation with kinase C, 5 mM EGTA was replaced by 0.5 mM $CaCl_2$, diacylglycerol (1-oleoyl-2-acetyl-*rac*-glycerol, Sigma catalogue no. O-8130, 0.04 mg/ml), and phosphatidylserine (Sigma catalogue no. P-7769, 0.1 mg/ml). The phosphorylation reaction was carried out for 30 min at 20°C and was terminated by the addition of 0.625 M Tris/HCl, pH 6.8/2% SDS/8 M urea/2% 2-mercaptoethanol/20% glyc-

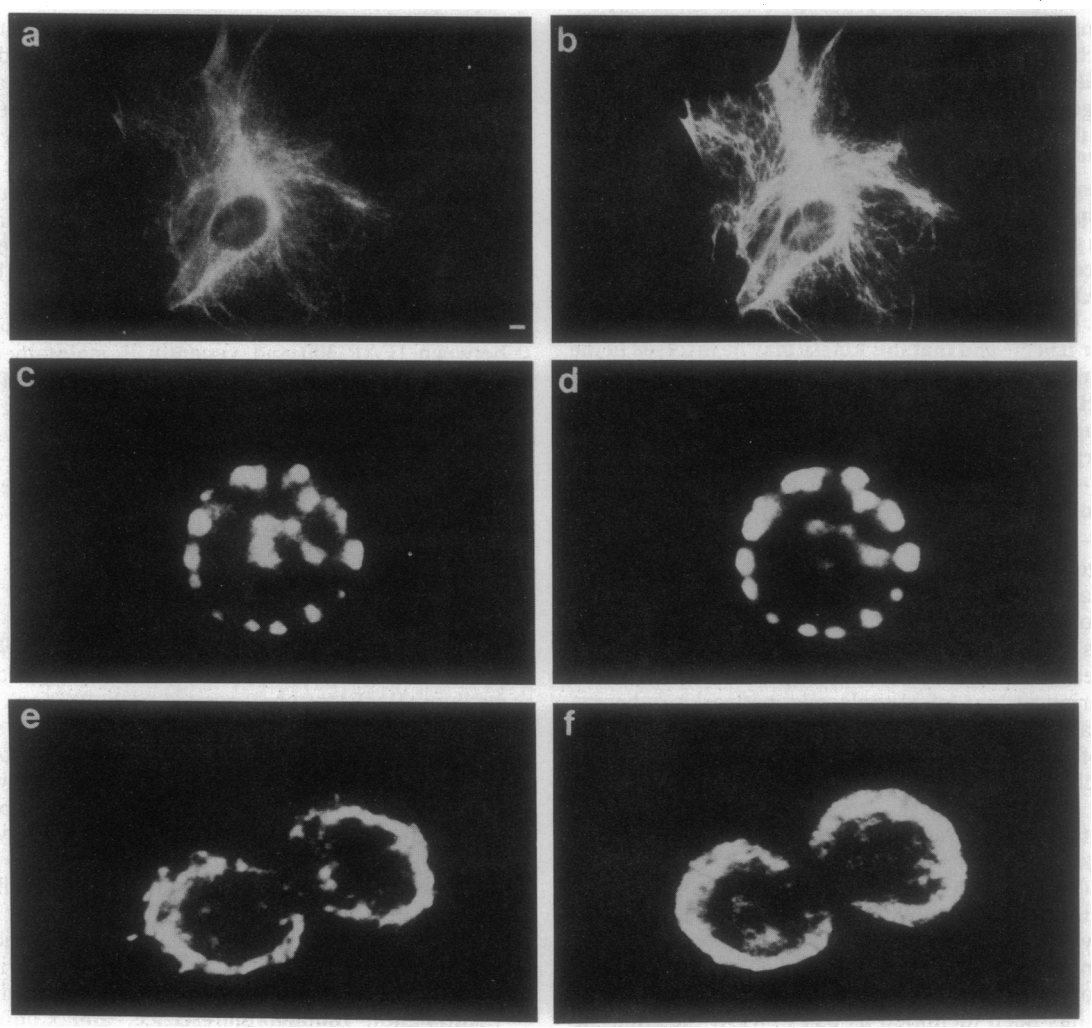

FIG. 1. Double immunofluorescence staining with IFAP 300 (*a*, *c*, and *e*) and vimentin (*b*, *d*, and *f*) antibodies on BHK cells during interphase (*a* and *b*), mitosis (*c* and *d*), and cytokinesis (*e* and *f*). In interphase cells, the staining pattern of IFAP 300 (*a*) is filamentous and similar to that of vimentin (*b*). In mitotic cells, there is a striking redistribution of IFAP 300 (*c*), giving a pattern that consists of spots of various sizes and shapes. These spots are also stained with anti-vimentin (*d*). During cytokinesis, anti-IFAP 300 (*e*) and anti-vimentin (*f*) stain a crescent-shaped region where daughter nuclei form. Images are from nonconfocal (*a* and *b*) or confocal (*c–f*) immunofluorescence microscopy. (Bar = 1 μm.)

erol. Under the different conditions used for *in vitro* phosphorylation, no endogenous kinase activity could be detected in the substrate preparation.

Two-Dimensional Phosphopeptide Mapping. IFAP 300 was immunoprecipitated (26) from cells metabolically labeled with [^{32}P]orthophosphate or from cytoskeletal preparations phosphorylated *in vitro* with different kinases. IFAP 300 was separated from other proteins by SDS/6.5% PAGE (24). The gel was vacuum dried between two cellophane sheets and autoradiographed, and IFAP 300 bands were excised from the gel by using the autoradiograph as a template. Following rehydration, the gel slices were washed for 4 hr in 50% methanol and for an additional 4 hr in absolute methanol. Proteolytic digestion was carried out overnight at 37°C by incubating the gel slices in 0.5 ml of 50 mM ammonium bicarbonate containing 10 μg of trypsin (Sigma). A fresh aliquot of 10 μg of trypsin was added for an additional 4-hr digestion. The eluted tryptic peptides were recovered by lyophilization. About 2000 cpm of each sample was loaded on thin-layer cellulose plates (Kodak) and the tryptic peptides were separated by high-voltage electrophoresis followed by ascending chromatography (27). ^{32}P-labeled phosphopeptides were identified by autoradiography on Kodak X-Omat films.

Phospho Amino Acid Determination. To determine which amino acid is phosphorylated by p34^{cdc2}, the major IFAP 300 tryptic peptide phosphorylated by this kinase was eluted from a thin-layer cellulose plate with 0.1% trifluoroacetic acid in water. Acid hydrolysis was performed with 6 M HCl at 110°C for 3 hr. Separation of the phospho amino acids was carried out by electrophoresis on thin-layer cellulose plates in a pH 3.5 buffer (10:100:1890 pyridine/acetic acid/water). Phospho amino acid standards (Sigma) were stained with 0.2% ninhydrin in acetone and the labeled phospho amino acids were identified by autoradiography.

RESULTS AND DISCUSSION

The mitotic process involves the remodeling of all cytoskeletal systems, including cytoplasmic and nuclear IFs (for review see ref. 1). Since IFAPs may be critical in regulating the organization of IFs and in mediating their linkage to different cellular components (2, 17), we have examined the mitotic distribution of IFAP 300.

Double immunofluorescence staining shows that the distributions of IFAP 300 and vimentin are very similar in interphase BHK cells (Fig. 1 *a* and *b*) (16). Similar observations of mitotic BHK cells with either monoclonal or polyclonal IFAP 300 antibodies reveal a significant reorganization of IFAP 300. This consists of fluorescent spots of various sizes and shapes, occasionally surrounded by a diffuse fluorescent background (Fig. 1*c*). These spots are also stained with vimentin antibodies (Fig. 1*d*). During cytokinesis, IFAP 300 staining is concentrated in the region of the former mitotic spindle poles as daughter cell nuclei reform (Fig. 1*e*). This staining pattern is very similar to that of vimentin (Fig. 1*f*) (also see ref. 12). These observations suggest that vimentin and IFAP 300 remain closely associated during the mitotic process.

The mitotic reorganization of cytoplasmic and nuclear IFs (the nuclear lamins) is accompanied by a marked increase in their phosphorylation levels (3–6, 28–30). However, this is not the case for IFAP 300, as its phosphorylation level remains constant when samples from interphase and mitotic cells are compared with each other (Fig. 2). As a control, we have also determined the specific activity of vimentin in the same preparations and found an ≈6-fold increase in samples derived from mitotic cells as compared with interphase cells (Fig. 2). This value agrees with previously published data (3).

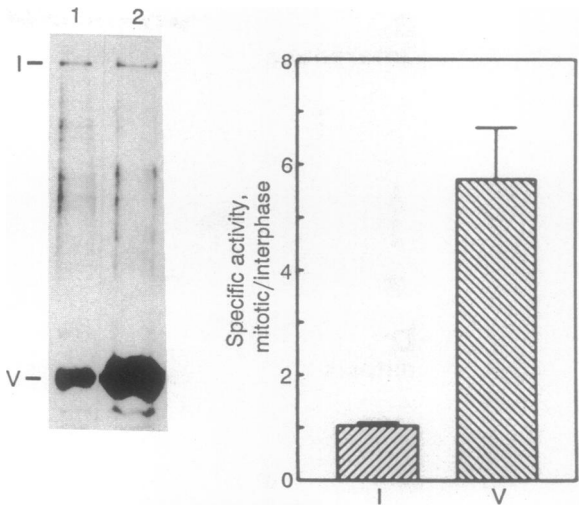

FIG. 2. (*Left*) Autoradiograph of an SDS/6.5% polyacrylamide gel loaded with a Triton/high-salt-insoluble fraction prepared from interphase (lane 1) and mitotic (lane 2) BHK cells metabolically labeled with [^{32}P]orthophosphate for 3 hr. The same amount of protein was loaded in each lane. (*Right*) There is no significant change in the specific activity of IFAP 300 when samples from interphase cells are compared with those from mitotic cells. This contrasts with the 5.7-fold increase in the specific activity of vimentin observed when mitotic cells are compared with interphase cells. Bars represent mean ± SD from four samples. I, IFAP 300; V, vimentin.

To further investigate whether phosphorylation is involved in the mitotic reorganization of IFAP 300, we have compared phosphopeptide maps of this protein from interphase and mitotic cells. Two-dimensional phosphopeptide maps of IFAP 300 immunoprecipitated from interphase cells display one major and one minor labeled phosphopeptide (Fig. 3*a*). Similar maps of IFAP 300 immunoprecipitated from mitotic cells show two major phosphopeptides (Fig. 3*b*). One of these is mitosis-specific, and the other comigrates with the major phosphopeptide seen in IFAP 300 derived from interphase cells (Fig. 3*c*). The minor spot in maps of IFAP 300 from interphase cells (Fig. 3*a*) is not seen in maps of IFAP 300 from mitotic cells (Fig. 3 *b* and *c*).

Since the kinases phosphorylating IFAP 300 *in vivo* are unknown, we have examined whether or not its mitotic reorganization is mediated by known protein kinases. For this purpose, preparations of BHK cytoskeletons were first phosphorylated *in vitro* by two previously identified physiological vimentin kinases (p34^{cdc2} and vimentin kinase I; ref. 13) and by kinase A and kinase C. Two-dimensional phosphopeptide maps of IFAP 300 phosphorylated with each kinase were then compared with those derived from IFAP 300 metabolically labeled with ^{32}P. After phosphorylation with p34^{cdc2}, IFAP 300 tryptic digests yielded a single major phosphopeptide, and in some experiments a few minor phosphopeptides (Fig. 4*a*). This major phosphopeptide comigrated with the mitosis-specific peptide found in cultured cells (Fig. 4*b*). The same major phosphopeptide was obtained with p34^{cdc2}/cyclin B complexes (maturation-promoting factor, MPF) purified from surf clam oocytes (26) (data not shown). The occurrence of minor phosphopeptides found only after *in vitro* phosphorylation probably results from the phosphorylation of sites not accessible *in vivo*. Phosphorylation of the major phosphopeptide takes place on a threonine residue (Fig. 5), which is consistent with the fact that p34^{cdc2} is a serine/threonine kinase (32, 33).

Two-dimensional phosphopeptide maps derived from IFAP 300 phosphorylated *in vitro* with vimentin kinase I reveal one phosphopeptide (Fig. 4*c*), which comigrates with the phosphopeptide common to IFAP 300 from both mitotic

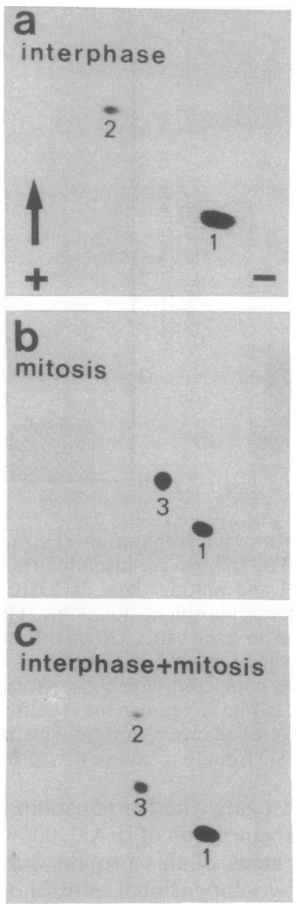

FIG. 3. Two-dimensional phosphopeptide maps of IFAP 300 immunoprecipitated from interphase (*a*) and mitotic (*b*) BHK cells, and comigration of interphase and mitotic samples (*c*). Interphase IFAP 300 displays one major and one minor phosphopeptide (phosphopeptides 1 and 2, respectively) (*a*). Mitotic IFAP 300 exhibits two major phosphopeptides, one of which (phosphopeptide 3) is mitosis-specific; the other comigrates with phosphopeptide 1 of interphase IFAP 300 (*c*). Phosphopeptide 2 is not found in mitotic cells. Anode (+) and cathode (−) in electrophoresis and direction of the ascending chromatography (arrow) are indicated.

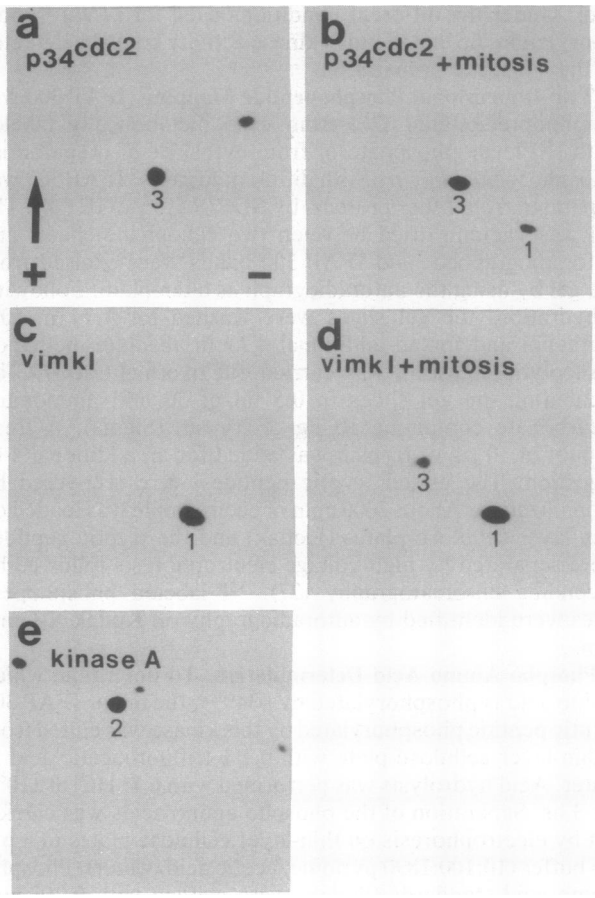

FIG. 4. Two-dimensional phosphopeptide maps of IFAP 300 phosphorylated *in vitro* with $p34^{cdc2}$ (*a*), vimentin kinase I (*c*) and kinase A (*e*), and comigration of the tryptic peptides derived from metabolically labeled IFAP 300 and from IFAP 300 phosphorylated *in vitro* with $p34^{cdc2}$ (*b*) and vimentin kinase I (*d*). *In vitro* phosphorylation of IFAP 300 by $p34^{cdc2}$ results in the phosphorylation of a major peptide (phosphopeptide 3) and, occasionally, of a number of minor peptides. Phosphopeptide 3 comigrates with the mitosis-specific phosphopeptide (*d*). A single peptide can be derived from IFAP 300 phosphorylated *in vitro* with vimentin kinase I (*c*) (phosphopeptide 1); this phosphopeptide comigrates with the phosphopeptide common to mitotic and interphase IFAP 300 (*d*). The major tryptic peptide derived from IFAP 300 tryptic peptide phosphorylated *in vitro* with kinase A (phosphopeptide 2) does not comigrate with any major metabolically labeled peptide, but with a minor phosphopeptide present in maps of interphase IFAP 300. Anode (+) and cathode (−) in electrophoresis and direction of the ascending chromatography (arrow) are indicated. vimk I, Vimentin kinase I.

and interphase cells (Fig. 4*d*). Four phosphopeptides are derived from IFAP 300 phosphorylated *in vitro* with the catalytic subunit of the cAMP-dependent kinase (kinase A) (Fig. 4*e*). These phosphopeptides do not comigrate with the major phosphopeptides found in cells during mitosis. However, a minor peptide found in interphase cells (Fig. 3*a*) does comigrate with one of the kinase A sites (data not shown). Maps of IFAP 300 phosphorylated with kinase C disclose one major phosphopeptide, which comigrates with a minor phosphopeptide found in some preparations obtained from interphase cells (data not shown).

These results identify IFAP 300 as a physiological substrate for $p34^{cdc2}$ and suggest that this kinase is involved in the mitotic reorganization of IFAP 300. This conclusion is based on the findings that (*i*) IFAP 300 is an *in vitro* substrate for $p34^{cdc2}$; (*ii*) the major IFAP 300 peptide phosphorylated *in vitro* by $p34^{cdc2}$ comigrates with a metabolically labeled phosphopeptide found only in IFAP 300 derived from mitotic cells; (*iii*) the major mitosis-specific phosphopeptide is not phosphorylated by vimentin kinase I, kinase A, or kinase C; and (*iv*) the phosphorylation of the mitosis-specific peptide temporally correlates with the mitotic reorganization of IFAP 300. In further support of this conclusion, plectin, a protein shown by various biochemical criteria to be homologous to IFAP 300 (18), has eight Ser/Thr-Pro motifs (31), which are recognition sites for $p34^{cdc2}$ kinase (32). Of these only one is a Thr-Pro motif, and it is located in the C-terminal region of the molecule, which might be involved in the binding of plectin to IFs (31).

The determination of the physiological substrates for $p34^{cdc2}$ is critical to our understanding of how this kinase

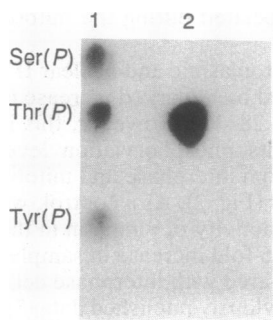

FIG. 5. Separation of the phospho amino acids derived from acid hydrolysis of the major IFAP 300 peptide phosphorylated by $p34^{cdc2}$. Lane 1, phospho amino acid standards stained with ninhydrin; lane 2, corresponding autoradiograph of the amino acids derived from the hydrolyzed peptide. Phosphorylation occurs at a threonine residue.

triggers the remodeling of the cytoplasm and the nucleus during mitosis. A number of cytoskeletal proteins have been identified as endogenous substrates (for review see ref. 14 and 33), including two types of IF proteins, the nuclear lamins (26, 34–36), and vimentin (13). In addition, neurofilament proteins have also been identified as *in vitro* substrates for $p34^{cdc2}$ (37). The mitotic disassembly of the nuclear lamina, which is coincident with nuclear envelope breakdown in many eukaryotic cell types, has been directly related to both phosphorylation of the type V IF proteins, the nuclear lamins at $p34^{cdc2}$-specific sites (34–36), and to increased nuclear lamin phosphate content (28–30). The mitotic disassembly of the vimentin IF network in BHK cells follows similar principles (13). However, our results with IFAP 300 suggest that the mitotic reorganization of this protein is accompanied by $p34^{cdc2}$-mediated phosphorylation at a mitosis-specific site, without a corresponding increase in total phosphate content, probably because of the down regulation of interphase sites (see below).

Interestingly, phosphopeptide maps of IFAP 300 reveal the presence of a major phosphopeptide common to both interphase and mitotic cells. This peptide comigrates with the only peptide phosphorylated *in vitro* with vimentin kinase I, a kinase activity originally identified in mitotic BHK cell lysates (3). In contrast, the minor phosphopeptide found in interphase cells, which is likely to be phosphorylated by kinase A, is not detected in mitotic cells. This is in keeping with the finding that kinase A is downregulated before cells enter mitosis (38). It further suggests that phosphorylation of IFAP 300 by kinase A is important for the interphase organization of IFAP 300, as has been suggested for plectin (39).

The identification of IFAP 300 as a physiological substrate for $p34^{cdc2}$ is important because this protein, in addition to its IF-crossbridging function, has the potential to connect IFs to other cytoskeletal elements or cellular structures, which has been shown for the related protein called plectin (17). As shown here, IFAP 300 remains associated with vimentin in BHK cells during mitosis. Therefore, the effect of phosphorylation by $p34^{cdc2}$ on IFAP 300 organization is probably due not to changes in its capacity to bind to $p34^{cdc2}$-phosphorylated vimentin but to its function as a crossbridging element between IF polymers, and possibly also between IFs and different cellular elements. This is supported by preliminary biochemical experiments suggesting that the binding of IFAP 300 to vimentin is not significantly altered when these two proteins are phosphorylated by $p34^{cdc2}$. Further work is necessary to determine whether phosphorylation by $p34^{cdc2}$ may affect the crossbridging function of IFAP 300, perhaps through the disruption of some oligomeric state(s) known to exist *in vitro* (15, 40). The findings that cytoplasmic and nuclear IF proteins (13, 26, 34–36) and, as is shown here, an IFAP are all physiological substrates for $p34^{cdc2}$ place this kinase at the core of the regulatory mechanism(s) leading to the mitotic remodeling of IF polymers and their associations with other cellular components.

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