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M-Phase–specific Phosphorylation and Structural Rearrangement of the Cytoplasmic Cross-linking Protein Plectin Involve $p34^{cdc2}$ Kinase

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Plectin, a widespread and abundant cytoskeletal cross-linking protein, serves as a target for protein kinases throughout the cell cycle, without any significant variation in overall phosphorylation level. One of the various phosphorylation sites of the molecule was found to be phosphorylated preferentially during mitosis. By in vivo phosphorylation of ectopically expressed plectin domains in stably transfected Chinese hamster ovary cells, this site was mapped to the C-terminal repeat 6 domain of the polypeptide. The same site has been identified as an in vitro target for $p34^{cdc2}$ kinase. Mitosis-specific phosphorylation of plectin was accompanied by a rearrangement of plectin structures, changing from a filamentous, largely vimentin-associated state in interphase to a diffuse vimentin-independent distribution in mitosis as visualized by immunofluorescence microscopy. Subcellular fractionation studies showed that in interphase cells up to 80% of cellular plectin was found associated with an insoluble cell fraction mostly consisting of intermediate filaments, while during mitosis the majority of plectin (> 75%) became soluble. Furthermore, phosphorylation of purified plectin by $p34^{cdc2}$ kinase decreased plectin's ability to interact with preassembled vimentin filaments in vitro. Together, our data suggest that a mitosis-specific phosphorylation involving $p34^{cdc2}$ kinase regulates plectin's cross-linking activities and association with intermediate filaments during the cell cycle.

INTRODUCTION

In eukaryotic cells entry into mitosis is characterized by a profound reorganization of the cytoskeleton, such as the formation of the mitotic spindle (McIntosh and Koonce, 1989), the reorganization of microfilaments to the contractile ring (Satterwhite and Pollard, 1992), the structural rearrangement of the cytoplasmic intermediate filament (IF) network to cage-like bundles or aggregates (for a review see Georgatos, 1993), and the breakdown of the nuclear envelope, accompanied by a disassembly of the nuclear IF structure, the nuclear lamina (Gerace and Burke, 1988).

Over the past few years all of these events have been suggested to be regulated through cyclin-dependent kinases (cdks),[1] particularly $p34^{cdc2}$ kinase, which together with its regulatory subunit cyclin B has been identified as the master regulator of the cell cycle machinery at the G2/M transition and as a tightly controlled "workhorse" phosphorylating structural proteins (Norbury and Nurse, 1992; Nigg, 1993a,b). However, due to overlapping specificities of related kinases, such as MAP kinase and glycogen synthase kinase, and of other probably functionally redundant cdks, many $p34^{cdc2}$ substrates identified in vitro so far remain to be verified as in vivo targets and as regulators of cytoskeletal structure.

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[1] Abbreviations used: cdk, cyclin dependent kinase; IF, intermediate filament.

Microtubule dynamics and stability is controlled by $p34^{cdc2}$ kinase (Verde *et al.*, 1990, 1992) probably due to a phosphorylation-dependent dissociation of microtubule-associated protein (mammalian MAP 4, MAP 1B, and *Xenopus* p220) from the microtubule surface (Tombes *et al.*, 1991; Shiina *et al.*, 1992). $p34^{cdc2}$-dependent phosphorylation of the actin-binding protein caldesmon releases the protein from actin filaments and is likely to cause microfilament disassembly (Yashimiro *et al.*, 1990, 1991; Mak *et al.*, 1991; Hosoya *et al.*, 1993), whereas phosphorylation of myosin II regulatory light chain decreases actomyosin ATPase activity and seems to inhibit cytokinesis until completion of mitosis (Satterwhite *et al.*, 1992; Yamakita *et al.*, 1994). Best understood among $p34^{cdc2}$-dependent events involving the reorganization of structural components is the breakdown of the nuclear envelope at the onset of mitosis. Several lines of evidence, including in vivo and in vitro studies, indicate that direct phosphorylation of lamins by $p34^{cdc2}$/cyclin B causes the disassembly of the nuclear lamina (Heald and McKeon, 1990; Peter *et al.*, 1990, 1991; Ward and Kirschner, 1990; Dessev *et al.*, 1991; Enoch *et al.*, 1991; Lüscher *et al.*, 1991). Also, cytoplasmic IFs have been described as substrates for $p34^{cdc2}$kinase. The use of antibodies specific to phosphorylated epitopes of vimentin and glial fibrillary acidic protein (Matsuoka *et al.*, 1992; Tsujimura *et al.*, 1994) as well as cell cycle–dependent in vivo phosphorylation studies paired with in vitro assays (Chou *et al.*, 1990, 1991) have suggested a role of $p34^{cdc2}$ phosphorylation of vimentin and glial fibrillary acidic protein for mitotic IF reorganization. IF disassembly has mainly been described to be caused by phosphorylation of IF-subunit proteins themselves, which make up the core of the filaments, rather than by phosphorylation of filament-associated components as in the case of the microtubule and microfilament systems.

Any structural element of the cytomatrix that is involved in the cross-linking and anchorage of IFs within the cytoplasmic space would seem to be an excellent candidate for a protein regulating filament reorganization by modulating its cross-linking activities and is likely to be a target of $p34^{cdc2}$ kinase, as well. One of the few well characterized proteins that seems to have all the potential of being a general cross-linking element of the cytoplasm is plectin (for reviews see Wiche, 1989; Foisner and Wiche, 1991), an abundant and widespread cytoskeletal protein. Plectin's interaction partners identified on the molecular level encompass nuclear and various cytoplasmic IF-type proteins, the microtubule–associated proteins MAP1 and MAP2, α-spectrin–type molecules, and plectin itself, the latter giving rise to filamentous network-type arrays (Foisner and Wiche, 1987; Herrmann and Wiche, 1987; Foisner *et al.*, 1988, 1991b; Wiche *et al.*, 1993). Plectin is an in vivo and in vitro target of various kinases (Herrmann and Wiche, 1983; Foisner *et al.*, 1991b), and its phosphorylation by protein kinases A and C has been shown to have differential effects on its interaction with vimentin and lamin B (Foisner *et al.*, 1991b). Here we demonstrate that plectin is a phosphoprotein throughout the cell cycle and, although the overall phosphorylation level of the protein stays nearly constant, plectin is phosphorylated at one site within the polypeptide chain in a mitosis-specific manner. Furthermore, we show that the mitosis-specific phosphorylation site is also targeted by $p34^{cdc2}$ kinase in vitro and that in vitro phosphorylation influences plectin's interaction with preassembled IFs. We also describe a rearrangement of plectin structures during mitosis, suggesting that $p34^{cdc2}$-specific phosphorylation of plectin regulates its structural organization and its molecular interactions in vivo.

MATERIALS AND METHODS

Cell Culture, Synchronization, and Metabolic Labeling

Chinese hamster ovary cells (clone CHO K1, passage numbers between 8 and 25) were grown to 70–90% confluency in plastic petri dishes or in roller bottles in DMEM supplemented with 10% fetal calf serum (FCS; Life Technologies, Paisley, UK) and 50 μg/ml penicillin and streptomycin (both Life Technologies). For synchronization of cell growth, the cultures were arrested at G1/S by an overnight incubation in Joklik's modified minimum essential medium (S-MEM, Life Technologies) supplemented with 10% FCS, nonessential amino acid mix (1:100, Life Technologies), 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)/NaOH, pH 7.4, and penicillin and streptomycin, in the presence of 2 mM thymidine (Terasima and Tolmach, 1963). To obtain cultures at various sequential stages through one cell cycle, cells were released from the thymidine block in complete S-MEM medium without thymidine for 10–12 h, subjected to a second overnight block in thymidine-containing medium, and released from the second block for various time periods between 3 and 24 h at 3-h intervals. The progress through the cell cycle was followed by FACS analyses. For the collection of mitotic cells, cultures were released from the first overnight thymidine block for 4–5 h in complete S-MEM and cultivated for an additional 4 h in growth medium containing 0.2 μg/ml nocodazole. The mitotic cells that round up and detach from the petri dish were harvested by mechanical shake off [fraction M (Noc)], whereas the attached interphase cells were collected by scraping them off [fraction I (Noc)]. To select for S-phase cells, cultures were released from one thymidine block in complete S-MEM for 4 h and cells were collected by scraping them off.

Cultures were metabolically labeled with 0.2 mCi/ml [^{32}P]orthophosphate (carrier free, Dupont NEN, Dreieich, Germany) for 2 h before harvest in phosphate-free DMEM (Sigma, Deisenhofen, Germany) supplemented with 25 mM HEPES/NaOH, pH 7.4, and 10% FCS dialyzed against 25 mM HEPES/NaOH (pH 7.4), or with 100 μCi/ml EXPRE [^{35}S]-protein labeling mix (>1000 Ci/mmol, Dupont NEN) for 3 h in methionine-free DMEM (Sigma) supplemented with 25 mM HEPES/NaOH, pH 7.4, and 10% dialyzed FCS.

Stable Transfection of Chinese Hamster Ovary (CHO) Cells

cDNA corresponding to repeat 6 of the plectin molecule (amino acid residue 4368–4620; numbers are according to the revised plectin sequence starting 1635 bp upstream of the originally published

ATG; Wiche *et al.*, 1991) was amplified by polymerase chain reaction, and the product was subcloned into pGW15-derived (Wiche *et al.*, 1993) pAD 29, an expression vector encoding a 10-amino acid sequence of the human c-myc oncogene (residues 410–420; Evan *et al.*, 1985), fused to the C-terminus of the recombinant protein. The section of the plasmid containing the initiation codon, the repeat 6, and the myc tag cDNA was cloned into the CMV promoter-driven expression vector pBc/CMV (Invitrogen, San Diego, CA) via *Hin*dIII and *Xba*I restriction sites (Vector pBN 48). Ten micrograms of purified plasmid DNA was transfected into CHO cells grown to 50% confluency on a 10-cm petri dish using the calcium precipitation method (Graham and Van der Eb, 1973). G418-selected single clones were picked at day 17 and transferred to 96-well plates in medium plus 0.4 mg/ml G418. At day 22 cells were transferred to larger plates and frozen at day 35.

Preparation of Cell Lysates and Triton X-100/High Salt-soluble and -insoluble Cell Fractions and Immunoprecipitation of Plectin

Interphase cells collected from one petri dish or mitotic cells collected from three dishes were centrifuged for 5 min at 500 × *g*, washed in cold PBS, and lysed in 300 μl of ice-cold 50 mM HEPES/NaOH, pH 7.0, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 100 mM NaCl, 0.1 mM dithiothreitol (buffer A) supplemented with the following: protease inhibitors, such as 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin; phosphatase inhibitors, such as 10 μM microcystin, 1 μM okadaic acid, 100 nM calyculin (all Life Technologies), 10 mM NaF, 10 mM sodium pyrophosphate, 5 mM β-glycerophosphate, and 1 mM sodium orthovanadate; and kinase inhibitors such as 300 μM H7 and 2 μM staurosporin (both Sigma). Cell lysates were then incubated for 10 min with 500 μg/ml and 200 μg/ml of DNAse and RNAse, respectively (both Boehringer Mannheim, Germany). The total cell lysates were supplemented with Triton X-100 and NaCl to a final concentration of 1% and 500 mM, respectively, and samples were centrifuged for 10 min in an Eppendorf centrifuge at maximum speed. Supernatant fractions were transferred to a new tube; pellet fractions were suspended in buffer A containing 1% Triton and 500 mM NaCl. Cell lysates, supernatant fractions, and pellet fractions were then either mixed with SDS sample buffer (Laemmli, 1970) and subjected to SDS-PAGE and immunoblot analyses, or supplemented with 1% SDS and 5 mM EDTA, diluted 1:10 in immunoprecipitation buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA), and processed for immunoprecipitation. One milliliter samples were incubated with 100 μl protein A-Sepharose (Pharmacia Biotech, Brussels, Belgium; 10% (w/v) in immunoprecipitation buffer) for 1 h, briefly centrifuged in an Eppendorf centrifuge, and supernatants were incubated with 10 μl of plectin antiserum (Wiche and Baker, 1982), or with 10 μg/ml purified monoclonal antibodies to c-myc (Evan *et al.*, 1985; ATCC CRL 1229), or with 100 μl of Sepharose beads coupled to monoclonal antibody to plectin 7A8 (0.25 mg/ml beads; Foisner *et al.*, 1991a, 1994) or to the myc antibody (1–2 mg/ml) overnight at 4°C. After addition of 10% protein A Sepharose to the former two samples and 4 h of incubation, all samples were centrifuged for 5 min in an Eppendorf centrifuge and pellets were washed three times in immunoprecipitation buffer and solubilized in SDS-PAGE sample buffer (Laemmli, 1970).

Preparation of Protein Kinases and Purification of Proteins

For the isolation of $p34^{cdc2}$ kinase, cells were grown in 900 cm^2 roller bottles, and nocodazole-arrested mitotic cells collected from one bottle were lysed for 10 s in 1 ml of buffer A plus protease inhibitors at 4°C using an ultra turrax at maximum speed (20,000 rpm), incubated with 50 μg/ml DNAse and 20 μg/ml RNAse for 10 min, and centrifuged for 20 min at 35,000 rpm in a Beckman 65 rotor (Beckman Instruments, Palo Alto, CA). Cdk-type kinases were isolated from the supernatants by incubation with 100 μl p13-Sepharose beads (5 mg p13/ml Sepharose beads; Brizuela *et al.*, 1987) overnight at 4°C. The beads were precipitated, washed in kinase buffer (20 mM HEPES/NaOH, pH 7.0, 10 mM $MgCl_2$), and frozen in the same buffer containing 16% glycerol. For the isolation of $p34^{cdc2}$ kinase, 1 ml of the supernatants of extracted cells was mixed with 100 μl of 10 times concentrated immunoprecipitation buffer containing 10% SDS and incubated with 100 μl Protein A-Sepharose beads (Pharmacia; 10% (w/v) in immunoprecipitation buffer) for 1 h at 4°C. Following the removal of the beads by brief centrifugation in an Eppendorf centrifuge, the supernatants were incubated overnight at 4°C with 10 μl of a rabbit antiserum generated against a synthetic peptide sequence representing the C-terminus of human $p34^{cdc2}$ kinase (kindly provided by L. Gerace). The immunocomplex was precipitated by a 2-h incubation with 200 μl 10% protein A-Sepharose, washed in kinase buffer, and frozen in kinase buffer plus 16% glycerol. Protein kinase C was isolated from hog brain as described in Foisner *et al.* (1991b); plectin and vimentin were purified from rat glioma C6 cells as described in Foisner and Wiche (1987) and Foisner *et al.* (1988).

In Vitro Phosphorylation and Two-dimensional Tryptic Peptide Mapping

Plectin was desalted into 10 mM sodium borate/NaOH, pH 8.9, 1 mM PMSF using Biogel P10 (Bio-Rad, Hercules, CA), and 30 μl of the sample (0.1–0.2 mg/ml plectin) was mixed with 20 μl immunoprecipitated or $p13^{suc1}$-Sepharose-precipitated kinase fractions (beads:buffer, 1:1) and 5 μl of a 10 times concentrated kinase buffer. After addition of 50 μM [γ-^{32}P]ATP (200–500 mCi/mmol) the samples were incubated for 30–90 min at 30°C and the reaction was terminated by addition of three times concentrated SDS sample buffer (Laemmli, 1970). Phosphorylation with protein kinase C was carried out as described elsewhere (Foisner *et al.*, 1991b), except that 50 ng/ml phorbol 12-myristate 13-acetate was used as kinase activator.

For two-dimensional tryptic phosphopeptide mapping, plectin samples immunoprecipitated from [^{32}P]-labeled cell lysates or samples phosphorylated in vitro, were separated on SDS-PAGE and processed according to the method of Boyle *et al.* (1991) and Dessev *et al.* (1991). Briefly, gels were washed twice in 50% methanol, twice in water, and dried between sheets of cellophane. Plectin bands, localized by autoradiography, were cut out, washed in 50% and 100% methanol for 2 h each, and dried in a Speed-Vac concentrator. The dried residues were oxidized in cold performic acid for 1 h, lyophilized, and washed several times in water. Lyophilized samples were dissolved in 200 μl of 50 mM $(NH_4)HCO_3$, pH 7.9, 2 mM dithiothreitol, 100 μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Sigma) and digested for 20–24 h at 37°C, with additions of equal volumes of fresh trypsin after 4 and 16–20 h. Samples were dried in a Speed-Vac concentrator and washed by two cycles of resuspension in water and vacuum drying. The dried material was solubilized in ~50 μl of acetic acid, formic acid, and H_2O (45/20/435 v/v), and aliquots (500–1000 cpm) of the supernatant were subjected to electrophoresis on thin layer (100 μm) cellulose plates (Merck, Darmstadt, Germany) for 60 min at 900 V at 8°C. The second dimension was ascending chromatography in *n*-butanol, acetic acid, pyridine, H_2O (60/12/40/48 v/v). Phosphopeptides were detected by autoradiography.

Immunofluorescence Microscopy

Synchronized CHO cells grown in the absence or presence of nocodazole were either fixed in 4% formaldehyde for 20 min and permeabilized in 0.1% Triton in PBS for 5 min at room temperature, or fixed in −20°C cold methanol for 5 min. After several washes in PBS, samples were incubated with the primary antibodies in PBS/0.2% gelatin for 1 h at room temperature, washed three times in PBS,

and incubated with secondary antibodies in PBS/gelatin for 1 h at room temperature. After three washes, samples were counterstained with 10 μg/ml Hoechst 33258 (Calbiochem, La Jolla, CA) in PBS for 1 min, washed in water, and mounted in Moviol (Hoechst, Frankfurt, Germany). Samples were viewed in a Zeiss Axiophot microscope, or with the MRC 600 confocal microscope (Bio-Rad). The following primary antibodies were used: antiserum to plectin (Wiche and Baker, 1982), diluted 1:40; ascites fluids containing monoclonal antibody to plectin (7A8) (Foisner *et al.*, 1991a; 1994), diluted 1:10; monoclonal antibody to vimentin (Dakopatts, Glostrup, Denmark), diluted 1:10; antiserum to vimentin (Wiche and Baker, 1982), diluted 1:40; and hybridoma supernatants containing monoclonal antibodies to c-myc (Evan *et al.*, 1985; ATCC CRL 1229), undiluted. Secondary antibodies used included the following: Texas Red-conjugated goat anti-rabbit IgG (Accurate Chemical & Scientific, Westbury, NY), diluted 1:80; and fluorescein isothiocyanate-conjugated affinity-purified goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), diluted 1:50.

In Vitro Cosedimentation Assay

Plectin was incubated with immunoprecipitated kinase in the absence or presence of ATP as described above and mixed with half the volume of vimentin filaments (~1 mg/ml), preassembled by dialysis against PBS containing 5 mM $MgCl_2$, 2 mM EGTA, 1 mM PMSF for 3 h at 37°C, or with buffer alone. Samples were incubated for 30 min at 30°C and centrifuged through a cushion of 30% sucrose in PBS in an Eppendorf centrifuge. Supernatant and pellet fractions were solubilized in SDS sample buffer (Laemmli, 1970), and analyzed by SDS-PAGE and autoradiography. The amount of plectin was measured either by densitometric scanning of Coomassie-stained gels or by Cerenkov counting of excised bands.

SDS-PAGE and Immunoblotting

Cell fractions were separated on 7.5% or 10% polyacrylamide gels according to the method of Laemmli (1970). Plectin was transferred onto nitrocellulose sheets (Schleicher & Schüll, Dassel, Germany) in 40 mM glycine, 48 mM Tris using the Bio-Rad Mini Trans-blot system (Bio-Rad). Immunological detection of the proteins was done using the Protoblot Immunoscreening System (Promega, Madison, WI) or the ECL detection system (Amersham, Buckinghamshire, UK) with the following antibodies and dilutions: antiserum to plectin, 1:2000; hybridoma supernatants containing monoclonal antibody 7A8 to plectin, or hybridoma supernatants containing monoclonal antibody to c-myc, undiluted; or a monoclonal antibody to vimentin (Dakopatts), 1:100.

Other Procedures

Protein $p13^{suc1}$ was purified from a strain of *Escherichia coli* expressing high levels of the fission yeast protein in a soluble form (Brizuela *et al.*, 1987; Moreno *et al.*, 1989) and purified by chromatography on Sepharose CL-6B (Pharmacia) in 100 mM $NaHCO_3$, pH 8.3. p13 was coupled to CNBr-activated Sepharose 4B (Pharmacia) at 5 mg/ml gel according to the instructions of the manufacturer.

Monoclonal antibodies were precipitated from serum-free hybridoma supernatants (Ultra Doma, BioWhittaker, Walkersville, MD) by addition of 55% ammonium sulfate, solubilized in one-tenth the original volume of PBS, dialyzed against PBS, and purified by fast performance liquid chromatography using a Protein G-Superose HR column (Pharmacia). The antibodies were eluted with 100 mM glycine/HCl, pH 2.7, mixed with 1 M NH_4HCO_3, pH 8.9 (1:10), and dialyzed against 100 mM ammonium carbonat buffer before coupling to CNBr-activated Sepharose.

For FACS analyses, 1 ml of cell suspension was slowly added to 4 ml of −20°C cold 85% ethanol and precipitated cells were briefly treated with a few drops of 0.05% aqueous pepsin, mixed with 100 mM Tris/HCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 2 μg/ml 4,6-diamidino-2-phenylindole and 15 μg/ml sulforhodamine, and analyzed with a Partec PAS-II flow cytometer (Partec, Münster, Germany).

RESULTS

Phosphorylation of Plectin during Different Cell Cycle Stages

To obtain cells at various cell cycle stages, CHO cell cultures were arrested at G1/S by an overnight incubation in the presence of thymidine (Terasima and Tolmach, 1963). A release from the growth arrest in complete medium in the absence of thymidine for 4 h yielded mainly S-phase cells, as determined by FACS analysis (Figure 1A, S). After a further 3- to 5-h incubation in the presence of the microtubule-depolymerizing drug nocodazole, 30–50% of the cells were arrested in mitosis and could easily be selected by mechanical shake off. The majority of the collected cells, which gave rise to a typical G2/M profile in FACS analysis [Figure 1A, M (Noc)], were found to be in prometaphase by fluorescence microscopy using the DNA specific dye Hoechst (see Figure 7). The nocodazole-treated cells that were still attached to the petri dishes were mostly in G2 [Figure 1A, I (Noc)].

To address the cell cycle–specific phosphorylation of plectin, cells at these defined cell cycle stages were labeled with [^{32}P]orthophosphate, and plectin was immunoprecipitated from cell lysates using an antiserum (Figure 1B, lane l) or a monoclonal antibody (Figure 1C, lane l) to plectin. The protein was found to be a phosphoprotein at all cell cycle stages tested (Figure 1, B and C), showing hardly any cell cycle–specific variation in the extent of phosphorylation. To cover the sequential stages of one complete cell cycle, we followed the phosphorylation of plectin in synchronized cell cultures in 3-h intervals for 24 h after a double thymidine block. Unlike the nocodazole selection, this approach did not yield cells at clearly defined narrow cell cycle stages, but the progress through the cycle could be clearly monitored by FACS analysis (Figure 1D). The cells started to grow out of the block at G1/early S phase after 3 h of cultivation in complete medium, went through S phase between 3 and 7 h, entered mitosis between 8 and 10 h after release, and started a new cycle at around 18–20 h. The specific plectin phosphorylation, again, did not show any significant variance over this time period (Figure 1E). Even in the presence of okadaic acid in the labeling medium, a potent phosphatase inhibitor that shifts the balance between protein kinases and phosphatases in favor of the kinase activities within the cell, we could not detect any cell cycle–dependent variation in plectin's phosphorylation state, although the overall amount of incorporated phosphate was increased two- to threefold (our unpublished results).

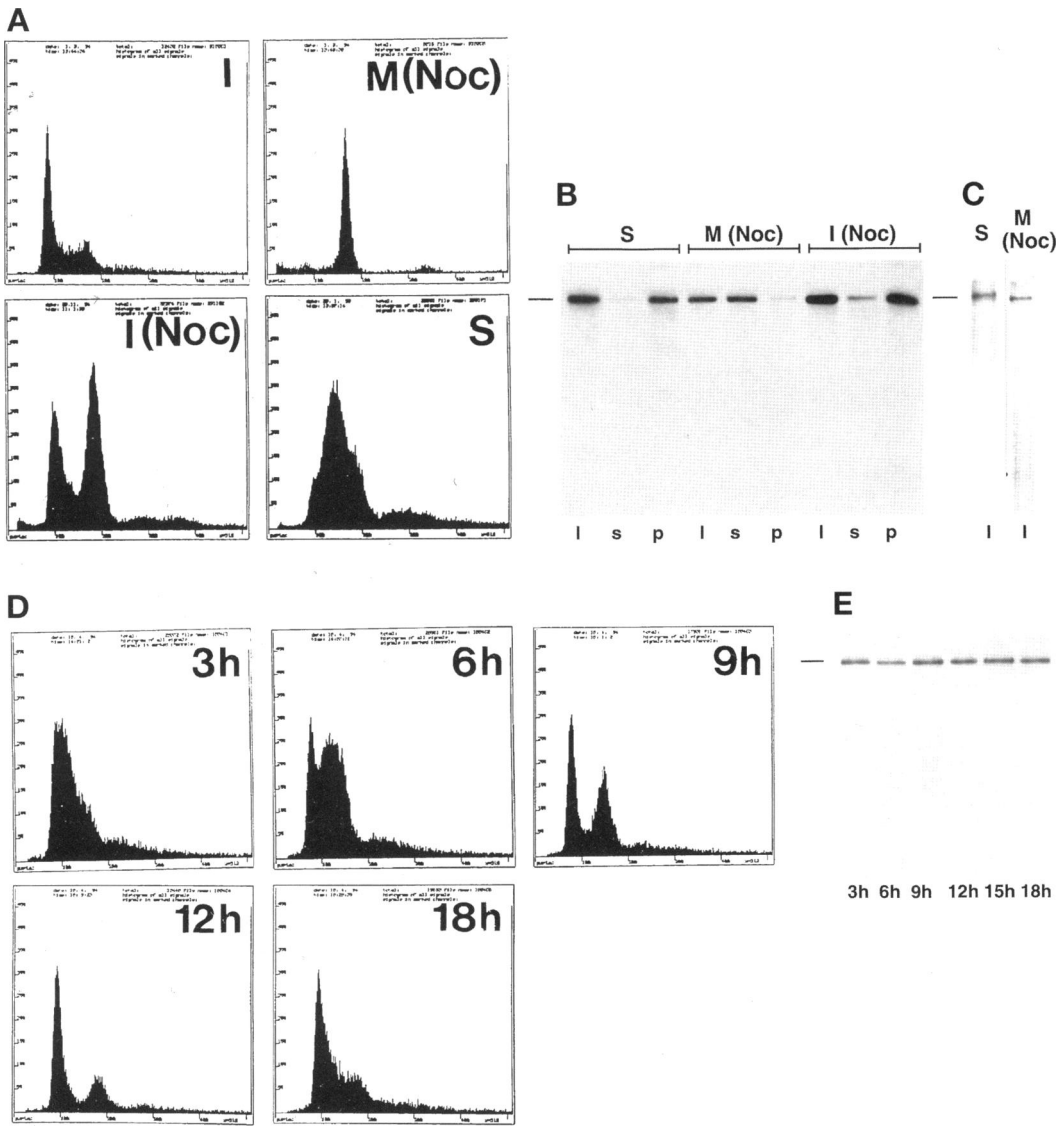

Figure 1. In vivo phosphorylation of plectin at various stages of the cell cycle. CHO cell cultures enriched in S phase (S), or nocodazole-treated mitotic [M (Noc)] or interphase [I(Noc)] cells (FACS profiles shown in A; I represents an asynchronously growing culture), or cells synchronized by a double thymidine block and released from the second block for the time periods indicated (FACS profiles shown in D), were metabolically labeled with [^{32}P]orthophosphate. Plectin was immunoprecipitated from total cell lysates (lanes l in B and C, and lanes 3 h through 18 h in E) or from Triton X-100/high salt-soluble (lanes s) or -insoluble (lanes p) fractions using an antiserum (B and E) or a monoclonal antibody 7A8 (C) to plectin. Bars mark the position of plectin.

To test whether only a subset of cellular plectin is differentially phosphorylated in the course of the cell cycle, the total cellular protein was separated into an IF-associated insoluble fraction and an IF-independent soluble portion by Triton X-100/high salt extraction of cells. Although in mitotic cells the majority of cellular plectin was found in the soluble fraction, unlike in S-phase and in nocodazole-treated interphase cells (Figure 1B, lanes s and p; see also Figure 6), the amount of phosphate incorporated per mole of soluble and insoluble plectin was similar at all stages. Thus, it may be concluded that plectin is a major phosphoprotein without any detectable changes in the overall extent of phosphorylation throughout the cell cycle.

Two-dimensional tryptic peptide mapping of plectin samples immunoprecipitated from mitotic or interphase (S-phase) cells was performed to compare the sites on the plectin polypeptide chain that were phosphorylated at these cell cycle stages. Mitotic plectin gave rise to three major phosphopeptides [Figure 2, M (in vivo), spots 1–3] and several

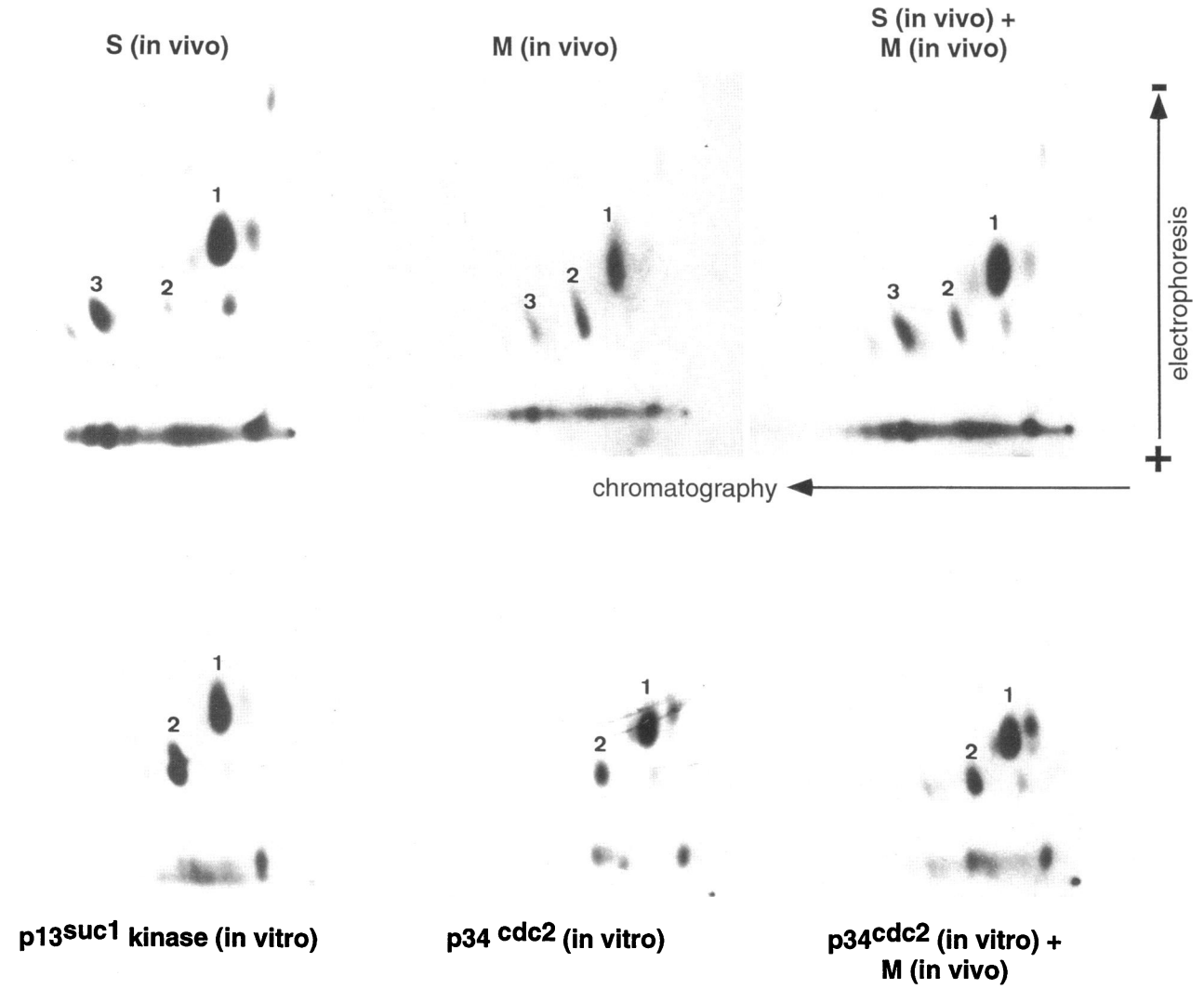

Figure 2. Two-dimensional tryptic peptide maps of phosphorylated plectin samples. Plectin was immunoprecipitated from metabolically labeled S phase cells [S (in vivo)] or nocodazole-arrested mitotic cells [M (in vivo)]; or purified plectin was phosphorylated in vitro using $p13^{suc1}$-affinity–purified [$p13^{suc1}$ kinase (in vitro)] or immunoprecipitated p34 kinase [$p34^{cdc2}$ (in vitro)]. Samples were separated by SDS-PAGE and excised bands were processed for tryptic peptide mapping. Mixtures of tryptic peptide were run for direct comparison (right panels). Numbers indicate corresponding phosphopeptides. Starting points were on the lower right corners of panels and separations in first (electrophoresis) and second (chromatography) dimension were as indicated.

minor ones. Two major peptides detected in interphase samples [Figure 2, S (in vivo), spots 1 and 3] were confirmed to be identical to peptides 1 and 3 of the mitotic sample by running peptide maps of mixed samples [Figure 2, S (in vivo) + M (in vivo)]. Peptide 2 of mitotic samples, on the other hand, was labeled only very weakly in interphase samples, suggesting that it contained a site that was phosphorylated preferentially during mitosis. These findings were confirmed by analyzing labeled plectin samples obtained from synchronized cell cultures after 9 and 18 h of release from the thymidine block, when cell cultures were enriched in mitotic and S-phase cells, respectively (our unpublished results). Phosphopeptide mapping of plectin after in vitro phosphorylation using mitotic cell extracts as a source for plectin kinase activity also yielded peptides 1 and 2 [Figure 3, M (in vitro)], while samples phosphorylated by S-phase extracts yielded mainly peptide 1 [Figure 3, S (in vitro)]. Altogether, it may be concluded that at least one site in the plectin molecule that is located in peptide 2, is phosphorylated in a cell cycle–dependent manner, preferentially during mitosis. Peptide 1 on the other hand seemed to be phosphorylated throughout the cell cycle, although one cannot exclude the possibility

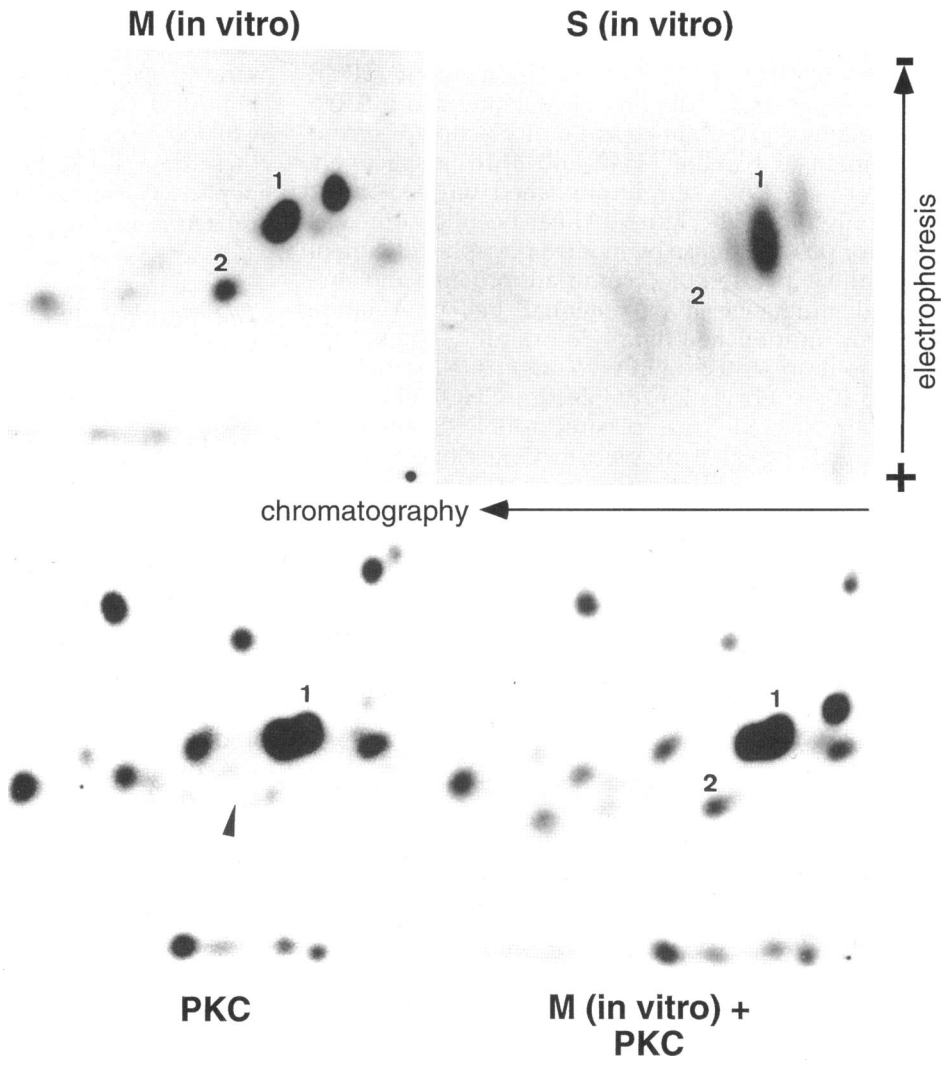

Figure 3. Two-dimensional tryptic peptide maps of in vitro–phosphorylated plectin. Plectin samples were phosphorylated using total cell lysates of mitotic [M (in vitro)] or S-phase [S (in vitro)] cells, or purified kinase C (PKC) and processed for tryptic peptide mapping. Autoradiographs of samples alone and of a mixed sample are shown. Numbers indicate corresponding phosphopeptides; arrowhead in PKC denotes the position of missing peptide 2. Starting points were on the lower right hand corners of panels, separations in first (electrophoresis) and second (chromatography) dimension were as indicated.

that mitotic and interphase phosphopeptides 1 are phosphorylated at different sites or comprise different peptides migrating at the same position.

$p34^{cdc2}$ Kinase Phosphorylates Plectin at a Mitosis-specific Site

In an effort to identify kinases involved in the mitosis-specific phosphorylation of plectin, we prepared $p34^{cdc2}$ kinase from mitotic cells by immunoprecipitation using an antiserum directed against the C-terminus of human $p34^{cdc2}$, or by affinity chromatography using $p13^{suc1}$ coupled to Sepharose beads. Both kinase preparations were confirmed to contain $p34^{cdc2}$ kinase activity by several criteria, including their ability to phosphorylate histone H1, their dependence on a $p34^{cdc2}$-specific inhibitor (olumucine; Vesely *et al.*, 1994), and the presence of a 34-kDa band in immunoblot analysis using anti-cdc2 antiserum (our unpublished results). Purified plectin was found to be a substrate for $p34^{cdc2}$ kinase preparations in vitro incorporating ~0.5 to 1 mol phosphate per mol plectin depending on the kinase preparations. Two-dimensional tryptic peptide maps generated from plectin phosphorylated in vitro by immunoprecipitated $p34^{cdc2}$ kinase gave rise to two major peptides, which comigrated with the two major peptides obtained from metabolically labeled mitotic plectin samples (Figure 2, lower row, spots 1 and 2). This indicated that the phosphorylation site(s) of plectin recognized by $p34^{cdc2}$ in vitro was also a target in vivo. Whereas peptide 2 was phosphorylated exclusively by mitotic kinase preparations [Figure 2 and Figure 3, M (in vitro)], peptide 1 was also targeted by protein kinase C, which has not been reported to be under cell cycle control (Figure 3, lower panels). This suggested that peptide 2, rather than peptide 1, contains a site whose

phosphorylation might be relevant for mitosis-specific properties of the molecule.

One consensus $p34^{cdc2}$ recognition motif TPGR (Nigg, 1993a) and a slightly degenerate motif SPYS have been found within repeat 6 of plectin's C-terminal domain at residues 4542 and 4616, respectively (Wiche *et al.*, 1991; our unpublished data). Among these, only thr 4542 (Figure 4A) has been identified as target for $p34^{cdc2}$ kinase by in vitro phosphorylation of bacterially expressed plectin mutant proteins and site-directed mutagenesis of potential phosphorylation sites (our unpublished data). To examine whether thr 4542 also represented an in vivo target site for mitosis-specific phosphorylation of plectin, CHO cells were stably transfected with plasmid pBN 48 encoding myc-tagged repeat 6 of plectin. One clone isolated efficiently expressed the fusion protein, as demonstrated by immunoblot analyses of total cell lysates (Figure 4B, lysate) and immunofluorescence microscopy (Figure 4C, myc). Double immunofluorescence microscopy of stably transfected CHO cells using antibodies to myc and to plectin or vimentin revealed a largely diffuse distribution of the ectopically expressed protein. Limited codistribution with endogenous plectin structures was observed in some areas (Figure 4C, upper row), but none with vimentin networks and vimentin bundles in interphase or mitotic cells (Figure 4C).

A protein of ~26 kDa comigrating with the immunoreactive mutant protein was efficiently immunoprecipitated from total cell lysates using anti-myc antibodies followed by protein A-Sepharose, or anti-myc antibodies coupled to Sepharose beads (Figure 4B). Immunoprecipitation of the ectopically expressed protein from S-phase cells or from nocodazole-treated mitotic and interphase cells (see Figure 1D) after metabolic labeling with [^{32}P]orthophosphate yielded strongly labeled protein bands of ~26 kDa (Figure 5A). The label associated with the ectopically expressed 26-kDa protein in S-phase and nocodazole-treated interphase cells was ~10 times higher than that found in endogenous plectin samples immunoprecipitated in parallel, and it was even 30-fold increased in mitotic cells (Figure 5B). Since the plectin associated label was found to remain nearly unchanged during the cell cycle (see Figure 1), this observation implied that the specific phosphorylation of ectopically expressed repeat 6 of plectin was increased threefold in mitotic versus interphase cells.

To test whether plectin's repeat 6 domain was phosphorylated at the same site(s) that was also affected in intact plectin, we performed two-dimensional tryptic peptide mapping of in vivo–labeled recombinant proteins. Mitotic samples gave rise to two major peptides (Figure 5C, a, spots 1 and 2), one of which (peptide 2) comigrated with the mitosis-specific peptide generated by $p34^{cdc2}$-phosphorylated plectin (Figure 5C, c, spot 2; see also Figure 2), as confirmed by mixing both samples (Figure 5C, d). Furthermore, in the mitotic sample, peptide 2 was more strongly labeled than peptide 1 (Figure 5C, a), and was missing in samples isolated from S-phase cells (Figure 5C, b). Based on these data, it can be concluded that the site(s) within peptide 2 was indeed the preferred target in mitotic cells and was derived from plectin's repeat 6 domain.

The Morphology of Plectin Structures and Their Subcellular Distribution Are Altered during Mitosis

The observation that phosphorylated cellular plectin was predominantly found in Triton X-100/high salt-soluble, IF-deficient fractions of mitotic cells, whereas it was mostly insoluble in S-phase and nocodazole-treated interphase cells (Figure 1B, lanes s and p; and Figure 6C, ^{32}P), indicated a major structural reorganization of plectin structures in the course of the cell cycle. To analyze the distribution of total protein rather than of phosphoproteins, we determined the relative amount of soluble versus total plectin by densitometric scanning of Coomassie-stained protein bands from immunoprecipitated samples (Figure 6, B and C, Coomassie). Alternatively, we prepared Triton/high salt-soluble and -insoluble fractions from [^{35}S]methionine-labeled cells, immunoprecipitated plectin from the cell fractions, and measured its amount by scintillation counting (Figure 6B, ^{35}S). In both cases we found that in mitotic cells, similar to the phosphoprotein, over 75% of total plectin were soluble in Triton X-100/high salt, compared with only 10–30% in S-phase cells. The fact that nocodazole-treated interphase cells showed a distribution of plectin between insoluble and soluble cell fractions similar to S-phase cells, suggested that the increase in plectin's solubility in nocodazole-treated mitotic cells was not simply due to a drug effect on the cytoskeleton, but rather reflected a specific change in the polymerization state of plectin or in its interactions with the Triton X-100/high salt-insoluble IF network.

The mitosis-dependent decrease in the relative amount of IF-associated plectin and the increased solubility of the protein was demonstrated also by quantitative probing of transblotted cell fractions with monoclonal antibodies to plectin followed by chemiluminiscence detection of bands on x-ray film and densitometric scanning of the bands (Figure 6, A and C, Immunoblot). Again, the solubility of plectin increased three- to fourfold in mitotic versus interphase cells. In contrast, the cellular distribution of vimentin was nearly unchanged in mitotic versus interphase cells, with over 90% of the protein being insoluble. Thus, the increased solubility of plectin during mitosis seemed not to be a consequence of a higher solubility and/or disassembly of vimentin filaments.

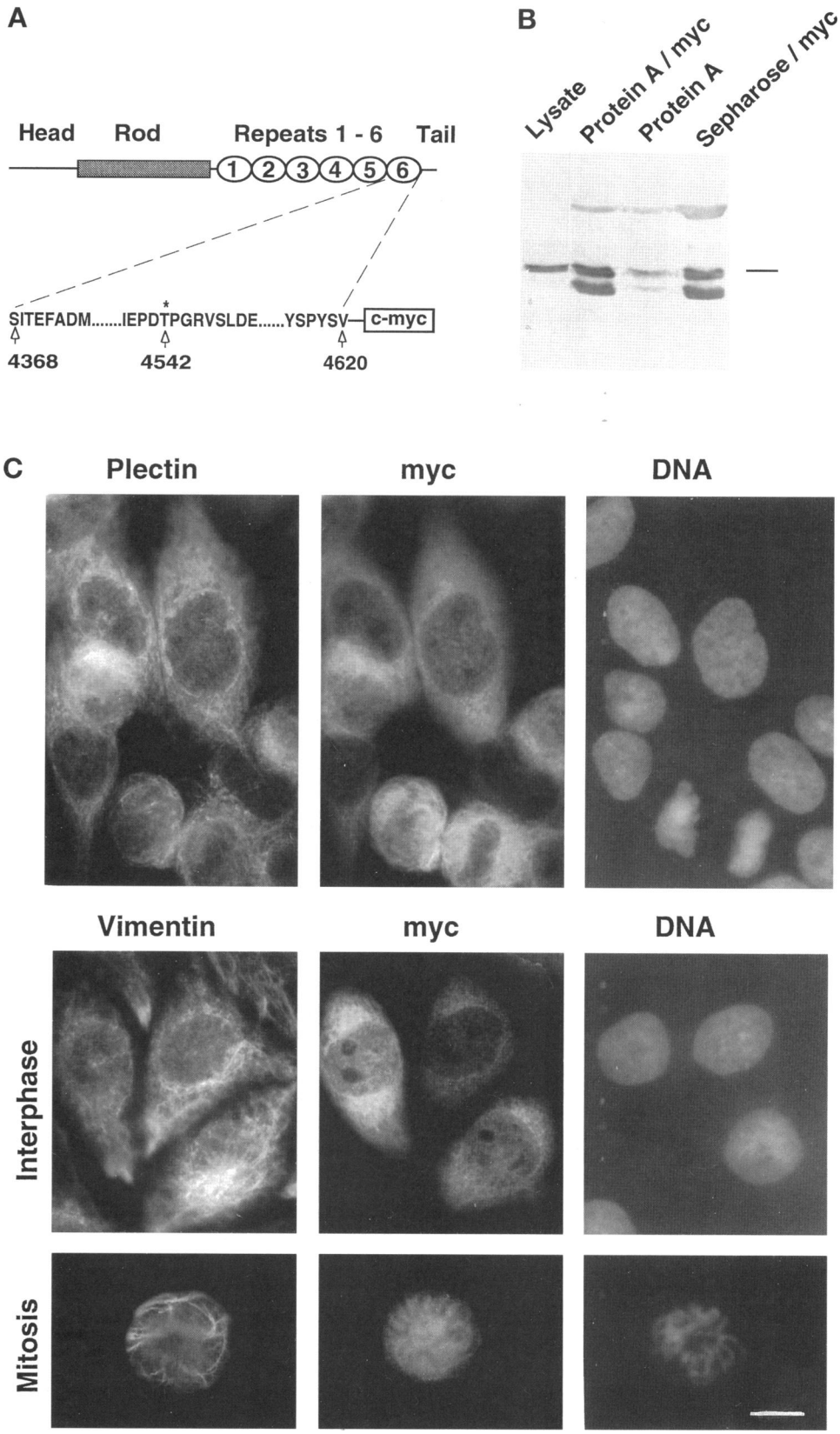

Figure 4. Stable transfection of CHO cells with a myc-tagged repeat 6 domain of plectin. CHO cells were stably transfected with a vector containing the repeat 6 domain of plectin tagged with a c-myc immunoreactive peptide at its C-terminus. (A) Domain organization of plectin and partial amino acid sequence of the repeat 6 c-myc peptide fusion protein is shown. Numbers indicate the position of respective amino acid residues within the plectin sequence. Note that the numbering system refers to a newly identified ATG start codon 1635 bp upstream of the formerly published presumptive start codon (Wiche *et al.*, 1991). A star denotes the potential $p34^{cdc2}$ phosphorylation site indentified by in vitro analysis. (B) Immunoblotting using anti-myc antibodies. Lanes contained cell lysates or samples immunoprecipitated with anti-myc antibodies/protein A, protein A alone, or Sepharose-immobilized myc antibodies. (C) Double immunofluorescence microscopy of transfected cells stained with anti-myc, anti-plectin, or anti-vimentin antibodies, or with Hoechst dye to visualize DNA. Bar, 5 μm.

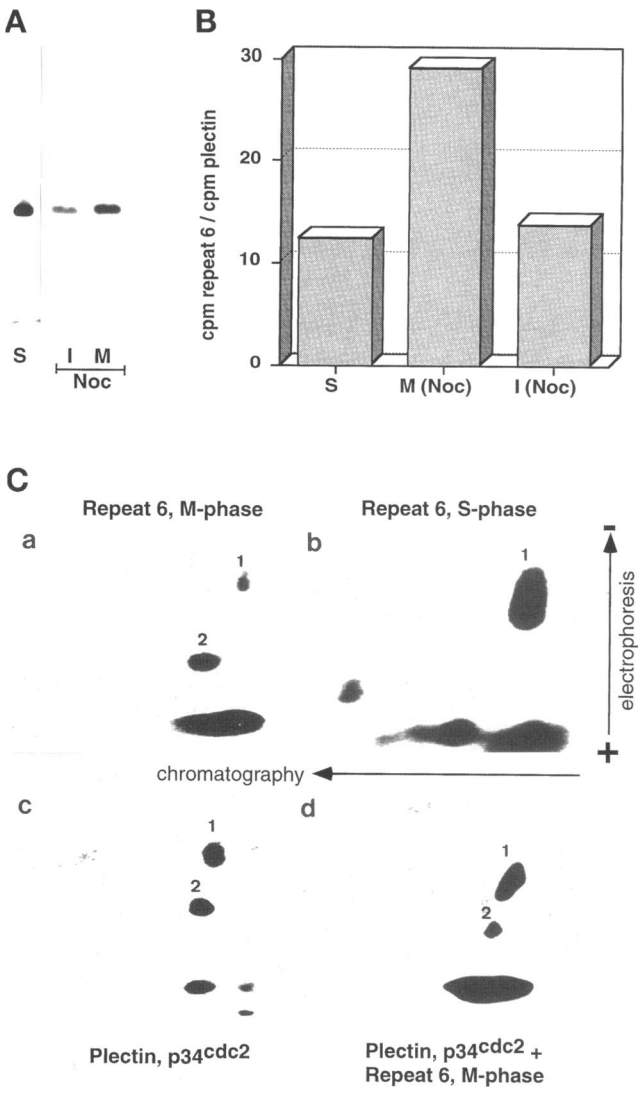

Figure 5. In vivo phosphorylation of ectopically expressed repeat 6 domain of plectin. Stably transfected cells expressing repeat 6/myc fusion proteins were synchronized by an overnight thymidine block to obtain S-phase cells or by nocodazole arrest to yield mitotic (M) or interphase (I) cells, and metabolically labeled with [^{32}P]orthophosphate. (A) Autoradiogram of samples immunoprecipitated from cell lysates using Sepharose-immobilized myc antibodies. (B) Relative intensities of repeat 6-bound label compared with label bound to plectin immunoprecipitated from the same cell lysates in parallel. (C) Autoradiograms of tryptic peptide maps of samples shown in panel A, and of plectin phosphorylated in vitro using p34^{cdc2} kinase, and mixtures thereof as indicated. Numbers indicate corresponding phosphopeptides. Starting points were in the lower right hand corners of panels, separations in first (electrophoresis) and second (chromatography) dimension were as indicated.

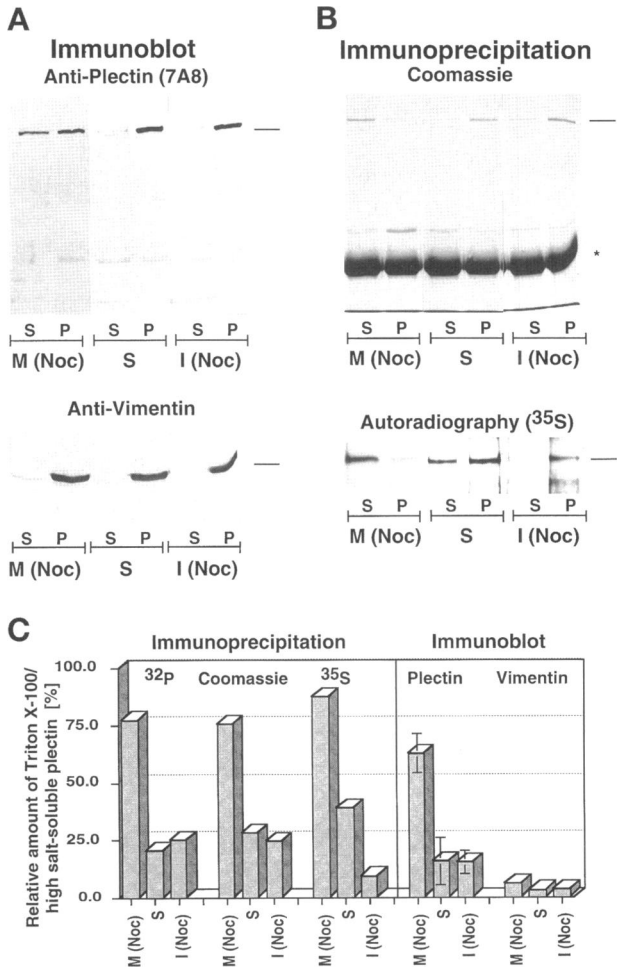

Figure 6. Subcellular distribution of plectin in mitotic and interphase cells. S-phase and nocodazole-arrested mitotic [M (Noc)] and interphase [I(Noc)] cells were obtained as described in MATERIAL AND METHODS and cell lysates were separated into Triton X-100/high salt-soluble (lanes S) or insoluble (lanes P) fractions. (A) Immunoblots of cell fractions using monoclonal antibodies 7A8 to plectin or monoclonal antibodies to vimentin and chemiluminescence detection. (B) Plectin was immunoprecipitated from cell fractions obtained from untreated cells or [^{35}S]methionine-labeled cells using antiserum to plectin and analyzed by SDS-PAGE and Coomassie staining or autoradiography. Bars in panels A and B indicate the position of stained proteins; star in panel B indicates the position of the IgG heavy chain. (C) Quantitation of the relative amount of Triton X-100/high salt soluble plectin determined by densitometric scanning of bands on immunoblots and in Coomassie-stained gels or by scintillation counting of ^{35}S-labeled or Cerenkov counting of ^{32}P-labeled proteins (see Figure 1).

To examine whether the interaction of plectin with vimentin structures is altered during mitosis, we performed double immunofluorescence microscopy of CHO cells at various stages of the cell cycle using antibodies to plectin and to vimentin. Interphase cells contained throughout their cytoplasm a dense, filamentous network of plectin structures, which largely colocalized with vimentin filaments (Figure 7, I). In contrast, mitotic cells, characterized by their round shape and condensed chromosomes showed a rather diffuse plectin-specific staining throughout their cyto-

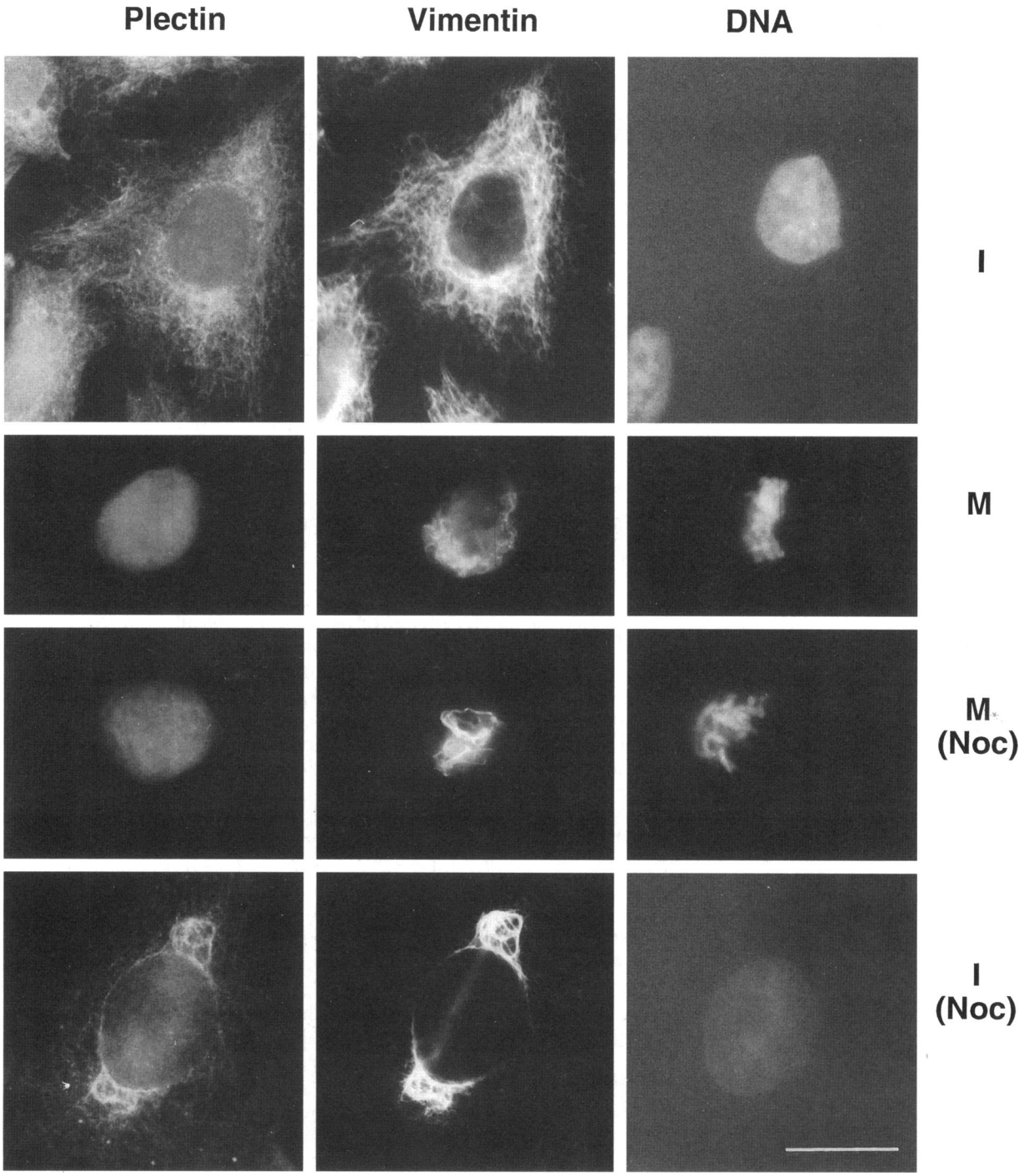

Figure 7. Double immunofluorescence microscopy of CHO cells. I, interphase cells; M, mitotic cells; [M (Noc)], nocodazole-treated mitotic cells; and [I (Noc)], nocodazole-treated interphase cells. Antiserum to plectin, a monoclonal antibody to vimentin, and DNA Hoechst dye were used. Bar, 10 μm.

plasm, whereas vimentin filaments were visualized in such cells mainly in the form of thick bundles (Figure 7, M). The IF-independent diffuse distribution of plectin was seen more clearly in nocodazole-arrested mitotic cells, in which the IF-network displayed extensive bundling [Figure 7, M (Noc)]. On the other hand, in nocodazole-treated interphase cells, which also displayed thick vimentin bundles collapsed onto the

surface of the nucleus, plectin was predominantly codistributed with these IF structures [Figure 7, I (Noc)]. Optical sectioning through a labeled mitotic cell using confocal laser scanning microscopy revealed extensive bundling of IFs and diffuse staining of plectin throughout the cell (our unpublished results).

Mitosis-specific Phosphorylation of Plectin Influences the In Vitro Interaction with IFs

To investigate whether the timely coordinated events of site-specific phosphorylation and structural reorganization of cytoplasmic plectin structures were functionally linked, we analyzed the effect of plectin phosphorylation by $p34^{cdc2}$ kinase on plectin-vimentin filament interaction in vitro. When unphosphorylated plectin samples were sedimented in the absence of preassembled vimentin filaments, only ~20% of plectin were found in the pellet fraction compared with 70–90% in the presence of preassembled vimentin filaments (Figure 8 and Table 1), suggesting that the majority of plectin associated with vimentin filaments. However, upon addition of vimentin filaments to plectin samples phosphorylated by $p34^{cdc2}$ kinase, the amount of sedimentable plectin was decreased two- to threefold to only ~40% of total plectin (Figure 8 and

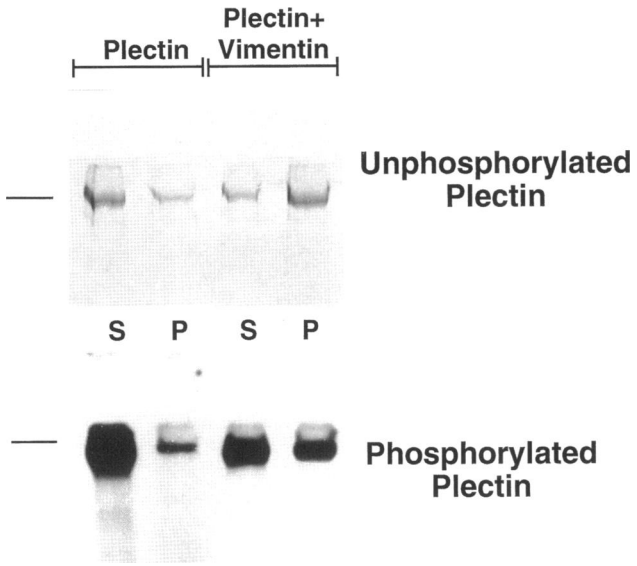

Figure 8. Influence of $p34^{cdc2}$ phosphorylation of plectin on its in vitro interaction with vimentin filaments. Purified plectin was mixed with immunoprecipitated $p34^{cdc2}$ kinase and incubated in the absence (unphosphorylated plectin) or presence (phosphorylated plectin) of [γ-^{32}P]ATP and mixed either with buffer alone (Plectin) or with preassembled vimentin (Plectin + Vimentin) and centrifuged through a sucrose cushion. Supernatant (lanes S) and pellet (lanes P) fractions were analyzed by SDS-PAGE and bands were visualized by Coomassie staining (upper panel) or autoradiography (lower panel). Bar denotes the position of plectin.

Table 1. Relative amount of sedimented plectin

Sample	n	Mean (%)	Min. (%)	Max. (%)	SD
Plectin	5	19.8	11	26	5.2
Phosphorylated plectin	7	18.3	11	29	5.2
Plectin + vimentin	6	77.3	70	89	5.9
Phosphorylated plectin + vimentin	7	43.1	30	52	8.7

Table 1). Since the amounts of phosphorylated and unphosphorylated plectin samples sedimenting in the absence of vimentin filaments were roughly comparable (Table 1), it can be concluded that $p34^{cdc2}$ kinase-specific phosphorylation decreased the plectin-vimentin interaction. Considering that mitosis-specific phosphorylation of plectin occurs in vivo at the same site(s) as in vitro, the structural rearrangements of plectin seen during mitosis might indeed be regulated by phosphorylation.

DISCUSSION

Here we propose a molecular mechanism that may be of importance for regulating dynamic properties of the cytomatrix, in particular the rearrangement of cytoskeletal filaments in the course of the cell cycle. The proposed principle of this mechanism is a phosphorylation-dependent control of plectin's cross-linking activity, particularly its interaction with IFs. We show that plectin structures become dramatically reorganized from a mostly IF-associated form in interphase cells to a soluble, IF-independent, and diffusely distributed form in mitosis. This reorganization is accompanied by phosphorylation of the protein at a mitosis-specific site within its C-terminal repeat 6. The proposed mechanism is further corroborated by in vitro analyses, revealing that $p34^{cdc2}$ kinase phosphorylation of plectin at its mitosis-specific site(s) reduces its interaction with vimentin filaments.

Given this mechanism, the mitosis-dependent reorganization of IFs could be a two-step process. First, it could involve the release of the IF network from anchorage sites at the membrane or the nucleus and the loosening of filament cross-links by a phosphorylation-dependent dissociation of IF-associated or IF-interacting proteins. Second, it could effect the assembly state of the filament itself by phosphorylation of its core subunit proteins. Unlike the microtubule and microfilament systems, where the phosphorylation of filament-associated proteins has been suggested as a major mechanism for structural rearrangements (Mak *et al.*, 1991; Tombes *et al.*, 1991; Yashimiro *et al.*, 1991; Shiina *et al.*, 1992; Verde *et al.*, 1992; Hosoya *et al.*, 1993), the regulation of IF assembly and its structural

organization has mostly been reported to involve phosphorylation of the core subunits (for review see Skalli *et al.*, 1992b). Only a few studies have also addressed the fate of IF-associated proteins during mitosis. IFAP 300, a protein that is immunologically related to plectin (Herrmann and Wiche, 1987; Skalli *et al.*, 1994) and possibly represents a member of the plectin, desmoplakin, and bullous pemphigoid antigen gene family (Wiche *et al.*, 1991; Green *et al.*, 1992), similar to plectin, has been shown to become phosphorylated in mitosis at a unique site, which also serves as target site for purified $p34^{cdc2}$ kinase. However, because IFAP 300 has been shown to remain closely associated with IFs during mitosis (Skalli *et al.*, 1992a), the function of mitosis-specific phosphorylation of IFAP 300 is not yet clear. The resemblance of tryptic phosphopeptide maps of mitotic plectin and IFAP-300, on the other hand, suggested that the phosphorylation domain is conserved in these proteins and might serve important functions during mitosis (Skalli *et al.*, 1992a). Proteins associated with the nuclear IF system, the nuclear lamina (for reviews see Georgatos *et al.*, 1994; Gerace and Foisner, 1994), have also been shown to become phosphorylated at the onset of nuclear envelope breakdown (Bailer *et al.*, 1991; Courvalin *et al.*, 1992; Foisner and Gerace, 1993).

Due to the overlapping substrate specificities of various kinases and the existence of various cdk/cyclin complexes (Nigg, 1993; Pines, 1993) it is difficult to verify a potential substrate as an authentic target of $p34^{cdc2}$ kinase. By the criteria currently applied (Nigg, 1993), plectin qualifies as an authentic $p34^{cdc2}$ substrate: 1) It is phosphorylated by immunoprecipitated and $p13^{suc1}$-Sepharose precipitated $p34^{cdc2}$ kinase fractions in vitro. 2) In vitro and in vivo phosphorylation occur at common sites. 3) At least one of these sites showed a cell cycle–dependent phosphorylation, correlating with the activation of $p34^{cdc2}$/cyclin B complexes. 4) Phosphorylation of the protein at the mitosis-specific site changes its properties in a cell cycle–specific manner, because $p34^{cdc2}$-dependent phosphorylation affects plectin's association with IFs, consistent with the observed dissociation of plectin structures from IF bundles during mitosis.

Regarding the number of $p34^{cdc2}$-specific phosphorylation sites of plectin molecules, the appearance of two major phosphopeptides in two-dimensional tryptic peptide maps of in vitro–phosphorylated plectin argues for two such sites. However, only one of these sites is phosphorylated in a cell cycle–dependent manner, the other one being phosphorylated throughout the cell cycle. Thus, we cannot exclude the possibility that phosphorylation of this second site by purified $p34^{cdc2}$ kinase could be an in vitro artifact. Alternatively, other kinases showing overlapping substrate specificities might be responsible for the phosphorylation of this site in vivo. The phosphorylation of interphase-specific sites by purified $p34^{cdc2}$ kinase has previously been observed with chicken lamin B_2 (Lüscher *et al.*, 1991), without evidence for in vivo relevance. An intriguing recent finding has revealed that neurofilament proteins in nonproliferating neurons are extensively phosphorylated by cdks that apparently are not cell cyle–regulated (Hisanaga *et al.*, 1991; Lew *et al.*, 1992; Shetty *et al.*, 1993). The phosphorylation of neurofilament proteins at their C-terminus by cdks occurs during transport of newly synthesized proteins along axons and the mature phosphorylation state is assumed to influence axon caliber and lateral spacing between neurofilaments (Gotow *et al.*, 1994; Nixon *et al.*, 1994). Probably the same kinase has been implicated in the abnormal Alzheimer-like phosphorylation of tau-protein (Baumann *et al.*, 1993; Hisanaga *et al.*, 1993; Kobayashi *et al.*, 1993; Paudel *et al.*, 1993). Also titin, a structural protein in striated muscle, has been shown to be phosphorylated in a differentiation-specific manner at KSP motifs (Gautel *et al.*, 1993). This suggests that cdk-activities are not necessarily cell cycle–dependent, but may serve important functions in differentiated cells and tissues, as well. Thus, it is possible that the second $p34^{cdc2}$ phosphorylation site of plectin detected is a physiological target, important for cell cycle–independent properties of plectin, rather than an in vitro artifact.

Two $p34^{cdc2}$ kinase consensus sites of the type S/T-P-X-K/R were detected in plectin's polypeptide chain, one in the rod domain at position 1438 and one in plectin's C-terminal repeat 6 domain at residue 4542 (Wiche *et al.*, 1991; our unpublished data). In addition, repeat 6 contains a slightly degenerate motif (SPYS) at residue 4616. Metabolic labeling of transfected CHO cells ectopically expressing repeat 6, coupled with phosphopeptide analyses, suggested that this mutant protein indeed contained the $p34^{cdc2}$ kinase-specific site identified in the intact molecule. In vitro phosphorylation analysis involving site-directed mutagenesis confirmed thr 4542 as the only site targeted by $p34^{cdc2}$ kinase in vitro (our unpublished data). The generation of another minor phosphopeptide from ectopically expressed mitotic repeat 6 protein suggested that this plectin domain contained a second phosphorylation site serving, although less efficiently, as a target site during mitosis. Due to a smear often appearing at the position of this second peptide, it is unclear whether it corresponds to the cell cycle–independent peptide seen in metabolically labeled or $p34^{cdc2}$ kinase-phosphorylated intact plectin. Interestingly, this peptide was also detected in S-phase cells. However, it is unknown whether the same site or sites located close to each other were phosphorylated in mitosis and S-phase.

Because the interaction domain of plectin with vimentin has been mapped to a C-terminal domain of plectin comprising repeats 4 to 6 and the tail section

(Wiche *et al.*, 1993), one could easily envisage that phosphorylation of plectin by $p34^{cdc2}$ kinase within repeat 6 regulates plectin's interaction with IFs. Considering the molecular structure of plectin, consisting of a rod flanked by two globular domains (Foisner and Wiche, 1987), phosphorylation of plectin anywhere in its C-terminal globular domain may affect even distant interaction sites within this domain due to global conformational changes.

We have previously shown that phosphorylation of plectin by protein kinases A and C influences plectin's interaction with IF proteins in vitro (Foisner *et al.*, 1991b). This type of phosphorylation could be of importance for differentiation-dependent events but it might have little relevance for actively growing cells. This hypothesis would be consistent with the observation that protein kinases A and C phosphorylated plectin molecules at sites clearly different from those affected during the cell cycle. In contrast, phosphorylation of plectin by $p34^{cdc2}$-related kinase(s) could be a common mechanism regulating plectin-IF interaction during cell growth. It is therefore tempting to speculate that a phosphorylation-dependent mechanism involving $p34^{cdc2}$ kinase influences plectin's ability to interact with its partners during mitosis and thereby regulates the rearrangement of IFs and of the cytoskeleton as a whole.

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REFERENCES

Bailer, S.M., Eppenberger, H.M., Griffiths, G., and Nigg, E.A. (1991). Characterization of a 54-kD protein of the inner nuclear membrane: evidence for cell cycle-dependent interaction with the nuclear lamina. J. Cell Biol. *114*, 389–400.

Baumann, K., Mandelkow, E.-M., Biernat, J., Piwnica-Worms, H., and Mandelkow, E. (1993). Abnormal Alzheimer-like phosphorylation of tau-protein by cyclin-dependent kinases cdk2 and cdk5. FEBS Lett. *336*, 417–424.

Boyle, W.J., Van der Geer, P., and Hunter, T. (1991). Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods Enzymol. *201*, 110–149.

Brizuela, L., Draetta, G., and Beach, D. (1987). $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase. EMBO J. *6*, 3507–3514.

Chou, Y.-H., Bischoff, J.R., Beach, D., and Goldman, R.D. (1990). Intermediate filament reorganization during mitosis is mediated by p34cdc2 phosphorylation of vimentin. Cell *62*, 1063–1071.

Chou, Y.-H., Ngai, K.-L., and Goldman, R.D. (1991). The regulation of intermediate filament reorganization in mitosis. J. Biol. Chem. *266*, 7325–7328.

Courvalin, J.-C., Segil, N., Blobel, G., and Worman, H.J. (1992). The lamin B receptor of the inner nuclear membrane undergoes mitosis-specific phosphorylation and is a substrate for p34cdc2-type protein kinase. J. Biol. Chem. *267*, 19035–19038.

Dessev, G., Iovcheva-Dessev, C., Bischoff, J.R., Beach, D., and Goldman, R. (1991). A complex containing p34cdc2 and cyclin B phosphorylates the nuclear lamin and disassembles nuclei of calm oocytes in vitro. J. Cell Biol. *112*, 523–533.

Enoch, T., Peter, M., Nurse, P., and Nigg, E.A. (1991). p34cdc2 acts as a lamin kinase in fission yeast. J. Cell Biol. *112*, 797–807.

Evan, G.I., Lewis, G.K., Ramsay, G., and Bishop, J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Foisner, R., Feldman, B., Sander, L., Seifert, G., Artlieb, U., and Wiche, G. (1994). A panel of monoclonal antibodies to rat plectin: distinction by epitope mapping and immunoreactivity with different tissues and cell lines. Acta Histochem. *96*, 421–438.

Foisner, R., Feldman, B., Sander, L., and Wiche, G. (1991a). Monoclonal antibody mapping of structural and functional plectin epitopes. J. Cell Biol. *112*, 397–405.

Foisner, R., and Gerace, L. (1993). Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. Cell *73*, 1267–1279.

Foisner, R., Leichtfried, F.E., Herrmann, H., Small, J.V., Lawson, D., and Wiche, G. (1988). Cytoskeleton-associated plectin: in situ localization, in vitro reconstitution, and binding to immobilized intermediate filament proteins. J. Cell Biol. *106*, 723–733.

Foisner, R., Traub, P., and Wiche, G. (1991b). Protein kinase A- and protein kinase C-regulated interaction of plectin with lamin B and vimentin. Proc. Natl. Acad. Sci. USA *88*, 3812–3816.

Foisner, R., and Wiche, G. (1987). Structure and hydrodynamic properties of plectin molecules. J. Mol. Biol. *198*, 515–531.

Foisner, R., and Wiche, G. (1991). Intermediate filament-associated proteins. Curr. Opin. Cell Biol. *3*, 75–81.

Gautel, M., Leonard, K., and Labeit, S. (1993). Phosphorylation of KSP motifs in the C-terminal region of titin in differentiating myoblasts. EMBO J. *12*, 3827–3834.

Georgatos, S.D. (1993). Dynamics of intermediate filaments: recent progress and unanswered questions. FEBS Lett. *318*, 101–107.

Georgatos, S.D., Meier, J., and Simos, G. (1994). Lamins and lamin-associated proteins. Curr. Opin. Cell Biol. *6*, 347–353.

Gerace, L., and Burke, B. (1988). Functional organization of the nuclear envelope. Annu. Rev. Cell Biol. *4*, 335–374.

Gerace, L., and Foisner, R. (1994). Integral membrane proteins and dynamic organization of the nuclear envelope. Trends Cell Biol. *4*, 127–131.

Gotow, T., Toshihisa, T., Nakamura, Y., and Takeda, M. (1994). Dephosphorylation of the largest neurofilament subunit protein influences the structure of crossbridges in reassembled neurofilaments. J. Cell Sci. *107*, 1949–1957.

Graham, F.L., and Van der Eb, E. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology *52*, 456–467.

Green, K.J., Virata, M.L., Elgart, G.W., Stanley, J.R., and Parry, D.A.D. (1992). Comparative structural analyses of desmoplakin, bullous pemphigoid antigen and plectin: members of a new gene family involved in organization of intermediate filaments. Int. J. Biol. Macromol. *14*, 145–153.

Heald, R., and McKeon, F. (1990). Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly. Cell *61*, 579–589.

Herrmann, H., and Wiche, G. (1983). Specific in situ phosphorylation of plectin in detergent-resistant cytoskeletons from cultured Chinese hamster ovary cells. J. Biol. Chem. *258*, 14610–14618.

Herrmann, H., and Wiche, G. (1987). Plectin and IFAP-300K are homologous proteins binding to microtubule-associated proteins 1 and 2 and to the 240-kilodalton subunit of spectrin. J. Biol. Chem. *262*, 1320–1325.

Hisanaga, S.i., Ishiguro, K., Uchida, T., Okumura, E., Okano, T., and Kishimoto, T. (1993). Tau protein kinase II has a similar characteristic to cdc2 kinase for phosphorylating neurofilament proteins. J. Biol. Chem. *268*, 15056–15060.

Hisanaga, S.-i., Kusubata, M., Okumura, E., and Kishimoto, T. (1991). Phosphorylation of neurofilament H subunit at the tail domain by cdc2 kinase dissociates the association to microtubules. J. Biol. Chem. *266*, 21798–21803.

Hosoya, N., Hosoya, H., Yashimiro, S., Mohri, H., and Matsumura, F. (1993). Localization of caldesmon and its dephosphorylation during the cell division. J. Cell Biol. 1075–1082.

Kobayashi, S., Ishiguro, K., Omori, A., Takamatsu, M., Arioka, M., Imahori, K., and Uchida, T. (1993). A cdc2-related kinase PSSALRE/cdk5 is homologous with the 30-kDa subunit of tau protein kinase II, a proline-directed protein kinase associated with microtubule. FEBS Lett. *335*, 171–175.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Lew, J., Winkfein, R.J., Paudel, H.K., and Wang, J.H. (1992). Brain proline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. J. Biol. Chem. *267*, 25922–25926.

Lüscher, B., Brizuela, L., Beach, D., and Eisenmann, R.N. (1991). A role for the p34cdc2 kinase and phosphatases in the regulation of phosphorylation and disassembly of lamin B2 during the cell cycle. EMBO J. *10*, 865–875.

Mak, A.S., Carpenter, M., Smillie, B., and Wang, J.H. (1991). Phosphorylation of caldesmon by p34cdc2 kinase. J. Biol. Chem. *266*, 19971–19975.

Matsuoka, Y., Nishizawa, K., Yano, T., Shibata, M., Ando, S., Takahashi, T., and Inagaki, M. (1992). Two different protein kinases act on a different time schedule as glial filament kinases during mitosis. EMBO J. *11*, 2895–2902.

McIntosh, J.R., and Koonce, M.P. (1989). Mitosis. Science *246*, 622–628.

Moreno, S., Hayles, J., and Nurse, P. (1989). Regulation of $p34^{cdc2}$ protein kinase during mitosis. Cell *58*, 361–372.

Nigg, E. (1993a). Cellular substrates of $p34^{cdc2}$ and its companion cyclin-dependent kinases. Trends Cell Biol. *3*, 296–301.

Nigg, E.A. (1993b). Targets of cyclin-dependent protein kinases. Curr. Opin. Cell Biol. *5*, 187–193.

Nixon, R.A., Paskevich, P.A., Sihag, R.K., and Thayer, C.Y. (1994). Phosphorylation on carboxyl terminus domains of neurofilament proteins in retinal ganglion cell neurons in vivo: influences on regional neurofilament accumulation, interneurofilament spacing and axon caliber. J. Cell Biol. *126*, 1031–1046.

Norbury, C., and Nurse, P. (1992). Animal cell cycle and their control. Annu. Rev. Biochem. *61*, 441–470.

Paudel, H.K., Lew, J., Ali, Z., and Wang, J.H. (1993). Brain proline-directed protein kinase phosphorylates tau on sites that are abnormally phosphorylated in tau associated with Alzheimer's paired helical filaments. J. Biol. Chem. *268*, 23512–23518.

Peter, M., Heitlinger, E., Häner, M., Aebi, U., and Nigg, E.A. (1991). Disassembly of in vitro-formed lamin head-to-tail polymers by cdc2 kinase. EMBO J. *10*, 1535–1544.

Peter, M., Nkagawa, J., Doree, M., Labbe, J.C., and Nigg, E.A. (1990). In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. Cell *61*, 591–602.

Pines, J. (1993). Cyclins and cyclin-dependent kinases: take your partners. Trends Biochem. Sci. *18*, 195–197.

Satterwhite, L.L., Lohka, M.J., Wilson, K.L., Scherson, T.Y., Cisek, L.J., Corden, J.L., and Pollard, T.D. (1992). Phosphorylation of myosin-II regulatory light chain by cyclin-p34cdc2: a mechanism for timing of cytokinesis. J. Cell Biol. *118*, 595–605.

Satterwhite, L.L., and Pollard, T.D. (1992). Cytokinesis. Curr. Opin. Cell Biol. *4*, 43–52.

Shetty, K.T., Link, W.T., and Pant, H.C. (1993). cdc2-like kinase from rat spinal cord specifically phosphorylates KSPXK motifs in neurofilament proteins: isolation and characterization. Proc. Natl. Acad. Sci. USA *90*, 6844–6848.

Shiina, N., Moriguchi, T., Ohta, K., Gotoh, Y., and Nishida, E. (1992). Regulation of a major microtubule-associated protein by MPF and MAP kinase. EMBO J. *11*, 3977–3984.

Skalli, O., Chou, Y.-H., and Goldman, R.D. (1992a). Cell cycle-dependent changes in the organization of an intermediate filament-associated protein: correlation with phosphorylation by p34cdc2. Proc. Natl. Acad. Sci. USA *89*, 11959–11963.

Skalli, O., Chou, Y.-H., and Goldman, R.D. (1992b). Intermediate filaments: not so tough after all. Trends Cell Biol. *2*, 308–312.

Skalli, O., Jones, J.C.R., Gagescu, R., and Goldman, R.D. (1994). IFAP 300 is common to desmosomes and hemidesmosomes and is a possible linker of intermediate filaments to these junctions. J. Cell Biol. *125*, 159–170.

Terasima, T., and Tolmach, L.J. (1963). Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. Exp. Cell Res. *30*, 344–362.

Tombes, R.M., Peloquin, J.G., and Borisy, G.G. (1991). Specific association of an M-phase kinase with isolated mitotic spindles and identification of two of its substrates as MAP 4 and MAP1B. Cell Regul. *2*, 861–874.

Tsujimura, K., Ogawara, M., Takeuchi, Y., Imajoh-Ohmi, S., Ha, M., and Inagaki, M. (1994). Visualization and function of vimentin phosphorylation by cdc2 kinase during mitosis. J. Cell Biol. *269*, 31097–31106.

Verde, F., Dogterom, M., Stelzer, E., Karsenti, E., and Leibler S. (1992). Control of microtubule dynamics and length by cyclin A- and cyclin B-dependent kinases in *Xenopus* egg extracts. J. Cell Biol. *118*, 1097–1108.

Verde, F., Labbe, J.-C., Doree, M., and Karsenti, E. (1990). Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of *Xenopus* eggs. Nature *343*, 233–238.

Vesely, J., Havlicek, L., Strnad, M., Blow, J.J., Donella-Deana, A., Pinna, L., Letham, D.S., Kato, J., Detivaud, L., and Leclerc, S. (1994). Inhibition of cyclin-dependent kinases by purine analogues. Eur. J. Biochem. *224*, 771–86.

Ward, G.E., and Kirschner, M.W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. Cell *61*, 561–577.

Wiche, G. (1989). Plectin: general overview and appraisal of its potential role as a subunit protein of the cytomatrix. Crit. Rev. Biochem. Mol. Biol. *24*, 41–67.

Wiche, G., and Baker, M.A. (1982). Cytoplasmic network arrays demonstrated by immunolocalization using antibodies to a high molecular weight protein present in cytoskeletal preparations from cultured cells. Exp. Cell Res. *138*, 15–29.

Wiche, G., Becker, B., Luber, K., Weitzer, G., Castañon, M.J., Hauptmann, R., Stratowa, C., and Stewart, M. (1991). Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. J. Cell Biol. *114*, 83–99.

Wiche, G., Gromov, D., Donovan, A., Castañon, M.J., and Fuchs, E. (1993). Expression of plectin mutant cDNA in cultured cells indicates a role of COOH-terminal domain in intermediate filament association. J. Cell Biol. *121*, 607–619.

Yamakita, Y., Yamashiro, S., and Matsumura, F. (1994). In vivo phosphorylation of regulatory light chain of myosin II during mitosis of cultured cells. J. Cell Biol. *124*, 129–137.

Yashimiro, S., Yamakita, Y., Hosoya, H., and Matsumura, F. (1991). Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. Nature *349*, 169–172.

Yashimiro, S., Yamakita, Y., Ishikawa, R., and Matsumura, F. (1990). Mitosis-specific phosphorylation causes 83K non-muscle caldesmon to dissociate from microfilaments. Nature *344*, 675–678.