Cell Cycle-Dependent Phosphorylation of Nucleoporins and Nuclear Pore Membrane Protein Gp210†

Catherine Favreau,‡ Howard J. Worman,§ Richard W. Wozniak,|| Thierry Frappier,‡ and Jean-Claude Courvalin*,‡

Département de Biologie Cellulaire, Institut Jacques Monod, CNRS, Université Paris 7, 75251 Paris Cedex 05, France, Departments of Medicine and of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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ABSTRACT: During mitosis in higher eukaryotic cells, the nuclear envelope membranes break down into distinct populations of vesicles and the proteins of the nuclear lamina and the nuclear pore complexes disperse in the cytoplasm. Since phosphorylation can alter protein−protein interactions and membrane traffic, we have examined the cell cycle-dependent phosphorylation of nuclear pore complex proteins. Nonmembrane nucleoporins Nup153, Nup214, and Nup358 that are modified by O-linked *N*-acetylglucosamine and recognized by a monoclonal antibody were phosphorylated throughout the cell cycle and hyperphosphorylated during M phase. Pore membrane glycoprotein gp210, that has a cytoplasmic, carboxyl-terminal domain facing the pore, was not phosphorylated in interphase but specifically phosphorylated in mitosis. Mutant and wild-type fusion proteins containing the cytoplasmic domain of gp210 were phosphorylated *in vitro* and their phosphopeptide maps compared to that of mitotic gp210. This analysis showed that Ser^{1880} of gp210 was phosphorylated in mitosis, possibly by cyclin B-$p34^{cdc2}$ or a related kinase. Several nuclear pore complex proteins are therefore differentially phosphorylated during mitosis when pore complexes disassemble and reassemble.

The nuclear envelope of higher eukaryotic cells is composed of the nuclear membranes, a meshwork of intermediate filament proteins termed the nuclear lamina, and the nuclear pore complexes. The nuclear envelope constitutes a mechanical barrier to exchange between the cytoplasmic and the nuclear compartments of eukaryotic cells. Bidirectional traffic between these two cell compartments is mediated by the nuclear pore complexes, supramolecular structures with diameters of approximately 120 nm and estimated molecular masses of approximately 124×10^6 Da (Dingwall & Laskey, 1992; Panté & Aebi, 1993). The pore complexes form transcisternal channels across the nuclear envelope in regions where the inner and outer nuclear membranes fuse to form the pore membrane domain (Franke et al., 1981; Reichelt et al., 1990).

Although the nuclear pore complex consists of 80−100 gene products, in multiple copies making a total of about 1000 polypeptides, only several of these have been identified and characterized in vertebrates or yeast (Rout & Wente, 1994; Wu et al., 1995; Yokoyama et al., 1995). Many of the nonmembrane nucleoporins that have been characterized contain repeat motifs of five (XFXFG), four (GLFG), and/or two (FG) amino acids. In vertebrates, these proteins have numerous sites that are modified by the posttranslational addition of O-linked *N*-acetylglucosamine (GlcNAc)[1] residues. Four GlcNAc-containing nucleoporins are recognized by a monoclonal antibody called mab414 that was first used to identify nucleoporin p62 (Davis & Blobel, 1986). Nucleoporin p62 has since been characterized by cDNA cloning and sequencing in several vertebrate species (Starr et al., 1990; Wente & Rout, 1994). Mab414 also recognizes Nup153 (Sukegawa & Blobel, 1993; McMorrow et al., 1994), Nup214 (von Lindern et al., 1992; Kraemer et al., 1994), and the Ran-binding protein Nup358 (Wu et al., 1995; Yokoyama et al., 1995) that have all been characterized by cDNA cloning and sequencing in mammalian species. In addition to containing multiple sites for the addition of O-linked GlcNAc residues, each of the nucleoporins also contains several putative phosphorylation sites.

Several integral membrane proteins have also been identified that are components of the nuclear pore complex (Wozniak et al., 1989; Hallberg et al., 1993; Wozniak et al., 1994). Among these integral membrane proteins, two proteins with single transmembrane domains, gp210 and POM121, have been characterized by cDNA cloning and sequencing in the rat (Wozniak et al., 1989; Hallberg et al., 1993). Both gp210 and POM121 are type I integral

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* Author to whom correspondence should be addressed: Département de Biologie Cellulaire, Institut Jacques Monod, CNRS, Université Paris 7, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France. Tel.: 33 1 44 27 77 65; FAX: 33 1 44 27 59 94; e-mail: courvalin@ijm.jussieu.fr.

‡ Institut Jacques Monod.

§ Columbia University.

|| University of Alberta.

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[1] Abbreviations: GlcNAc, *N*-acetylglucosamine; DMEM, Dulbecco modified Eagle's medium; FACS, fluorescence activated cell sorter; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; PCR, polymerase chain reaction.

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membrane proteins with cytoplasmic carboxyl-terminal domains and amino-terminal domains located in the lumen of the perinuclear space (Greber et al., 1990; Söderqvist & Hallberg, 1994). Gp210 has a cytoplasmic, carboxyl-terminal domain of 58 amino acids and a luminal domain of 1808 amino acids (including the signal sequence) with several N-linked high mannose sugar groups (Wozniak et al., 1989; Greber et al., 1990).

The nonmembrane nucleoporins and nuclear pore membrane proteins contribute to a pore complex structure that contains cytoplasmic filaments, nuclear filaments, and an octahedral core structure (Panté & Aebi, 1993; Rout & Wente, 1994). On the basis of its location and relative abundance (25 copies per nuclear pore, totaling ~5 MDa), gp210 may be a major component of the outer spoke ring of the nuclear pore (Wozniak et al., 1989) and play a role in nuclear pore complex assembly by anchoring the octahedral core structure of the pore complex to the membrane. However, the identities of the putative ligands of gp210 in the nuclear pore complex remain unknown. Nonmembrane nucleoporins are the predominant structural components of the filaments and octahedral core. The highly organized pore complex structure functions as the docking site and transport channel for the machinery responsible for the active nuclear import and export of macromolecules. Two of the best known components of the import machinery are the GTPase Ran and a protein that binds to the nuclear localization signal or NLS of actively imported protein. This NLS-binding protein has been given several names in different species including karyopherin-α, importin 60, Srp1p, and the NLS receptor (Rexach & Blobel, 1995).

During cell division, the nuclear envelope of higher eukaryotic cells undergoes dramatic structural changes (McKeon, 1991; Wiese & Wilson, 1993). In prophase and metaphase, the lamina depolymerizes, the nuclear membranes break down into vesicles, and the nonmembrane nucleoporins disassemble into soluble complexes and disperse throughout the cytoplasm (Heald & McKeon, 1990; Peter et al., 1990; Dabauvalle et al., 1990, 1991; Chaudhary & Courvalin, 1993; Macaulay et al., 1995). Membrane disassembly is domain-specific with integral membrane proteins of the pore and of the inner nuclear membrane segregating into different populations of mitotic vesicles (Chaudhary & Courvalin, 1993). As mitosis nears completion, the preexisting pools of nuclear envelope components are used in a stepwise reassembly characterized by the early targeting of membrane vesicles that contain inner nuclear membrane proteins and the subsequent targeting of gp210-containing pore membrane vesicles (Chaudhary & Courvalin, 1993). The nuclear pore complexes also sequentially reassemble from the dispersed cytosolic components (Davis & Blobel, 1986; Sheehan et al., 1988; Vigers & Lohka, 1991; Chaudhary & Courvalin, 1993; Meier et al., 1995).

Many of the structural modifications of the cellular architecture that take place in mitosis, including nuclear envelope breakdown, occur concurrently with protein phosphorylation (Nurse, 1990). Nuclear lamins, the intermediate filament proteins of the nuclear lamina (McKeon, 1991), and LBR, an integral protein that attaches the lamina to the inner nuclear membrane (Worman et al., 1988, 1990; Ye & Worman, 1994), have been shown to be mitotic substrates for cyclin B-p34^{cdc2} protein kinase, and the locations of the phosphorylated residues have been identified (Heald & McKeon, 1990; Peter et al., 1990; Courvalin et al., 1992). In the present report, we have examined the cell cycle-dependent phosphorylation of a group of GlcNAc-containing nucleoporins that are recognized by a single monoclonal antibody and have characterized in detail the mitosis-specific phosphorylation of nuclear pore membrane protein gp210.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture. HeLa D cells were grown in suspension in Joklik's modified Eagle's medium supplemented with 5% fetal calf serum (Life Technologies). HeLa suspension cells (3×10^9) were fractionated by counterflow centrifugal elutriation using a Beckman JE5.0 rotor at 20 °C with Joklik's medium to collect and wash the cells (LaBella et al., 1989). Aliquots of each cell fraction were analyzed with a FACScan flow cytometer (Becton Dickinson) according to Roberts et al. (1991). Cells in G1, S, and G2/M were pooled, and since the G2/M fraction contained less than 5% mitotic cells as assessed by fluorescence microscopic analysis of an aliquot of fixed cells stained with the DNA-binding dye 4,6-diamidino-2-phenylindole, it was referred to as G2. An aliquot of cells in S phase of the cell cycle was blocked in mitosis with nocodazole (40 ng/mL), and after 12 h, the mitotic index was greater than 80%. For ^{35}S-labeling of interphase cells, a G1 fraction was grown for 5 h in the presence of [^{35}S]methionine (1 mCi/mL). For ^{35}S-labeling of mitotic cells, nocodazole (40 ng/mL) was added to an S fraction of cells which was then grown for 12 h with [^{35}S]methionine (1 mCi/mL) present for the final 5 h (mitotic index = 80%).

Rat hepatocellular carcinoma cell line N1S1 was grown in suspension in DMEM (Life Technologies) supplemented with 10% fetal calf serum. Mitotic N1S1 cells were produced by a single thymidine block followed by a nocodazole accumulation after the release (Zieve et al., 1980). In brief, cells at a density of 6×10^5/mL were grown for 15 h in the presence of 2 mM thymidine, washed, and then grown in DMEM medium for 3 h. Nocodazole (100 ng/mL) was then added for an additional 4 h. Mitotic indices of 70−75% were obtained.

Isolation of ^{32}P-Labeled Pore Proteins from Cells. Human autoantibodies that recognize gp210 (Courvalin et al., 1990) were coupled to protein A−Sepharose (Pierce), and monoclonal antibody mab414 (Davis & Blobel, 1986) was coupled to protein G−Sepharose (Pierce) according to Schneider et al. (1982). Rabbit antibodies against rat gp210 were produced in the following fashion. A 25 amino acid polypeptide corresponding to residues 1862−1886 of rat gp210 (Wozniak et al., 1989) was synthesized on a Milligen 9050 Pepsynthesizer (Biosearch, Framingham, MA). The polypeptide was coupled to keyhole limpet hemocyanin using 0.2% glutaraldehyde and the conjugated complex injected into rabbits as described (Harlow & Lane, 1988). Antibodies were affinity purified from rabbit serum on a column consisting of 2 mg of the synthetic polypeptide that was coupled to *N*-hydroxysuccinimide-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Procedures for antibody affinity purification have been described (Harlow & Lane, 1988).

HeLa cells in G1, S, G2, and M of the cell cycle and N1S1 cells, either unsynchronized or mitotic, were collected by centrifugation and resuspended in phosphate-free DMEM

supplemented with 5% dialyzed serum for 1 h. Cells at a density of 5×10^6/mL in a volume of 2 mL were labeled at 37 °C with 2 mCi of [^{32}P]orthophosphate (NEN) for 1 h. Labeling of mitotic cells was performed in the presence of nocodazole (40−100 ng/mL), and the mitotic index did not change during this time period. After labeling, cells were washed in cold PBS and lysed at 4 °C in an immunoprecipitation medium containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 2% bovine serum albumin, and mixtures of protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 100 μg/mL aprotinin, 10 μg/mL antipain, 10 μg/mL chymostatin, 10 μg/mL leupeptin, and 10 μg/mL pepstatin A) and phosphatase inhibitors (50 mM NaF, 5 mM sodium pyrophosphate, and 0.1 mM sodium orthovanadate).

Cell lysates were incubated with 30 μL of Sepharose beads bearing human antibodies at 4 °C for 2 h with gentle agitation. When soluble affinity-purified rabbit antibodies were used, they were incubated with lysates at a 1:50 dilution for 2 h at 4 °C prior to incubation with the protein A−Sepharose. After incubation, Sepharose beads were washed three times with the lysate medium without protease or phosphatases inhibitors, once in the same medium without bovine serum albumin, once in 100 mM Tris-HCl (pH 6.8) containing 0.5 M NaCl, and finally in 100 mM Tris-HCl (pH 6.8). Bound antigens were eluted from Sepharose beads by incubating the beads for 15 min at 70 °C in Laemmli sample buffer (Laemmli, 1970) containing 100 mM DTT. The eluted proteins were separated by SDS−PAGE under reducing conditions as described (Laemmli, 1970). The ^{32}P-labeled proteins were detected either by autoradiography of the dried polyacrylamide slab gels at −70 °C using BioMax MR film (Kodak) and an intensifying screen or by using a PhosphorImager (Molecular Dynamics).

Phosphatase Treatment of Nucleoporins. For preparation of nuclei, HeLa cells in suspension were incubated for 30 min at 37 °C in culture medium containing 1 μg/mL cytochalasin B (Sigma), which was also present in all solutions used before homogenization. After washing in cold PBS, cells were swollen for 6 min in 1 mM Tris-HCl (pH 8) at 4 °C and then transferred to 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1.5 mM $MgCl_2$, and 1 mM DTT containing protease inhibitors. Further steps of nuclei preparation were performed as described (Chaudhary & Courvalin, 1993). Nuclear envelopes were prepared according to Dwyer and Blobel (1976). Mitotic membranes were prepared from attached HeLa cells treated overnight with 1 μg/mL nocodazole. Mitotic cells, isolated by shaking loosely adherent cells from culture dishes (95% mitotic), were fractionated as described (Chaudhary & Courvalin, 1993) except that the centrifugation of the postchromosomal supernatant was performed at 200000*g*. Proteins present in the 200000*g* supernatant were precipitated using 10% trichloroacetic acid and washed with 90% ethanol. Incubations with alkaline phosphatase were performed for 30 min at 30 °C in 0.25 mL of buffer containing 50 mM Tris-HCl (pH 8.4), 5 mM $MgCl_2$, 1 mM DTT, protease inhibitors, 15 units of enzyme (Boehringer Mannheim, Mannheim, Germany), and nuclear envelopes, mitotic membranes, or proteins precipitated from the 200000*g* mitotic supernatant each obtained from 3×10^6 cells. Alakaline phosphatase was omitted from control reactions. Reactions were stopped by the addition of 5× denaturing Laemmli electrophoresis sample buffer (Laemmli, 1970). Electrophoresis and transfer of proteins to nitrocellulose were performed as described (Courvalin et al., 1990). Nitrocellulose blots were probed with mab414 or gp210 polyclonal antibodies, then with species-specific secondary antibodies linked to horseradish peroxidase (Promega) for detection by enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.).

Construction of Plasmids and Expression of Fusion Proteins. Plasmids encoding GST fusion proteins of the carboxyl-terminal domain of gp210 and smaller portions of this domain were constructed in pGEX-2T (Pharmacia) and have been described previously (Nickowitz & Worman, 1993). *Escherichia coli* strain TG1 was transformed with these plasmids by standard methods (Sambrook et al., 1989), and the transformed recombinant plasmids were analyzed by restriction endonuclease digestion and examination of ethidium bromide-stained agarose gels.

To amplify cDNAs encoding polypeptides containing the last 33 amino acids of the rat gp210 with Ser^{1873}, Ser^{1876}, and Ser^{1880} mutated to alanines, an oligonucleotide of sequence 5′CGTGGATCCCCCCACTATCT3′ was used as the sense primer and oligonucleotides of sequences 5′ATCGAATTCTAGTGAGAGGCATAGGCTGGGCTCCAGAGCCCTGAGGGAGGTGCGGCTTTGCGATCAGAAG3′, 5′ATCGAATTCTAGTGAGAGGCATAGGCTGGGCTCCAGAGCCCTGCGGGAGGGCTGGCTTTGCGAT3′, and 5′ATCGAATTCTAGTGAGAGGCATAGGCTGGGGCCCAGAGCCCTGAGGGAG3′ were used as the antisense primers, respectively. The sense primer contained a *Bam*HI restriction endonuclease site preceded by CGT at its 5′ end, and the antisense primers contained *Eco*RI restriction endonuclease sites preceded by ATC at their 5′ ends to facilitate oriented cloning in pGEX-2T. PCR (Saiki et al., 1987) was performed using the GeneAmp Kit (Perkin-Elmer), and the reaction products were ligated in pGEX-2T by standard methods (Sambrook et al., 1989). Recombinant plasmids were sequenced by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase Kit Version 2.0 (United States Biochemical Corp.).

GST fusion proteins were expressed in transformed *E. coli* and purified on glutathione−Sepharose (Pharmacia) as described (Smith & Johnson, 1988). Fractions containing purified fusion proteins were analyzed by SDS−PAGE and Coomassie blue staining.

In Vitro Phosphorylation. Cyclin B-p34^{cdc2} protein kinase purified from starfish oocytes (Labbé et al., 1991) was a gift from Drs. J.-C. Labbé and M. Dorée (CNRS, Montpellier, France). Purified MAP kinase was a gift from Dr. George Thomas (Friedrich Miescher Institute, Basel, Switzerland). Kinase reactions were performed in 20 mM Na-Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mM ATP. Kinase assays were carried out at 30 °C for 20 min in a volume of 20 μL containing 250 ng of fusion protein, 20 μCi of [γ-^{32}P]-ATP with a specific activity of 6000 Ci/mmol (NEN), and 0.4 μL of the enzyme preparation. Reactions were stopped by the addition of 5 μL of Laemmli 5× sample buffer (Laemmli, 1970) and heating at 80 °C for 15 min in the presence of 100 mM DTT. Control assays were performed using histone H1 (Boehringer-Mannheim) as substrate. Phosphorylated polypeptides were separated by SDS−PAGE on 15% polyacrylamide slab gels. After electrophoresis, gels were fixed in 3 volumes of methanol, 1 volume of acetic acid, and 6 volumes of water, washed extensively with the

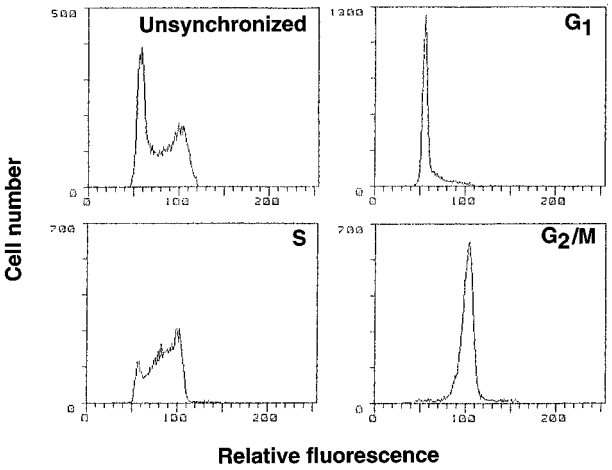

FIGURE 1: FACS analysis of Hela cells. Representative graphs from FACS analysis show the relative DNA content of unsynchronized, G1, S, and G2/M populations of Hela cells. Abscissa shows the relative fluorescence and the ordinate the cell number.

same solution, dried and subjected to autoradiography as described above.

Thermolysin Phosphopeptide Analysis and Phosphoamino Acid Analysis. Phosphopeptide and phosphoamino acid analyses were carried out essentially as described by Hemmings et al. (1984). In brief, ^{32}P-labeled proteins which were detected by autoradiography were excised from dried SDS−polyacrylamide slab gels and digested with thermolysin (Boehringer-Mannheim). The digested peptides were collected, transferred to thin-layer cellulose plates (Kodak), and separated by electrophoresis (pH 3.5) in the first dimension followed by chromatography in 5 volumes of pyridine, 7.5 volumes of 1-butanol, 1.5 volumes of acetic acid, and 6 volumes of water.

Phosphoamino acid analysis was performed on phosphopeptides eluted from SDS−polyacrylamide slab gels or on phosphopeptides scraped from chromatograms. Phosphopeptides were hydrolyzed in 6 M HCl, applied to thin-layer cellulose plates, and fractionated by electrophoresis (pH 1.9). Phosphoserine, phosphothreonine, and phosphotyrosine (20 μg/sample) were applied to the same plates and visualized with ninhydrin. Chromatograms were revealed either by standard autoradiography as described above or by using a PhosphorImager.

Materials. Unless otherwise specified, routine chemical reagents were obtained from Sigma or Fisher. Restriction endonucleases and DNA ligase were from New England Biolabs.

RESULTS

Cell Cycle-Dependent Differences in Phosphorylation of Nuclear Pore Complex Proteins. Cultures of HeLa D cells growing in suspension were fractionated into populations of cells in the G1, S, and G2/M phases of the cell cycle by centrifugal elutriation (Figure 1). The G2/M fraction had a mitotic index of less than 5% and was operationally referred to as G2. A population of cells in M phase (mitotic index >80%) was obtained by culturing elutriated S-phase cells in the presence of nocodazole for 12 h.

All four populations of cells were labeled for 1 h with [^{32}P]orthophosphate and then lysed, the mitotic population being maintained in the presence of nocodazole. The resulting lysates were used for immunoprecipitation with two different antibodies, and the precipitated polypeptides were separated by SDS−PAGE (8% acrylamide) and visualized by autoradiography. One of the antibodies used for immunoprecipitation was the mouse monoclonal antibody mab414 which recognizes several nucleoporins. In vertebrates, mab414 recognizes polypeptides with apparent molecular masses of 62, 180, 210, and 270 kDa on polyacrylamide gels that correspond to p62, Nup153, Nup214, and Nup358. Figure 2A shows that, among the proteins immunoprecipitated with mab414 from Hela cell lysates in G1, S, G2, and M, only the three higher molecular mass proteins were phosphorylated while p62 was not. These nucleoporins were phosphorylated at all stages of the cell cycle but were hyperphosphorylated roughly twice as much in S phase and mitosis (M) compared to G1 and G2 (Figure 2A). M-phase hyperphosphorylation was accompanied by shifts of these nucleoporins toward higher apparent molecular masses, shifts that were not observed in S phase (Figure 2A,B). These data demonstrate that three of four nucleoporins recognized by mab414 show cell cycle-dependent changes in phosphorylation state and that different sites may be phosphorylated

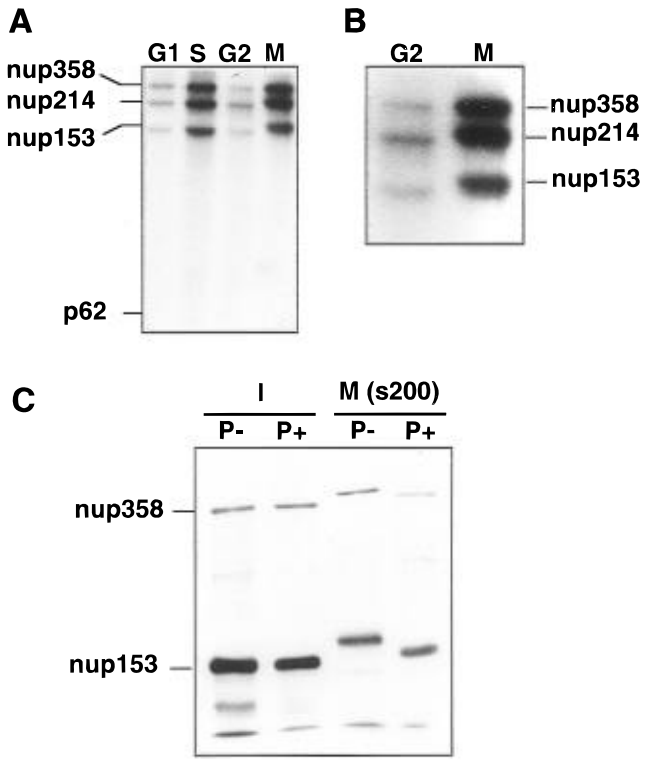

FIGURE 2: Cell cycle-dependent phosphorylation of nucleoporins in HeLa cells. Populations of HeLa cells in the G1, S, G2, and M phase of the cell cycle, obtained by centrifugal elutriation, were metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated by monoclonal antibody mab414 (A, B), and then separated by SDS−PAGE (8% acrylamide). (A) Autoradiogram showing that Nup153, Nup 214, and Nup358 are phosphorylated throughout the cell cycle while p62 is not. (B) Autoradiogram of an enlargement of panel A shows that phosphorylated proteins have a slower migration in mitosis (M) compared to interphase (G2). (C) Interphase nuclear envelopes (I) and a 200000*g* supernatant fraction of mitotic HeLa cells (M (s200)) were treated with alkaline phosphatase (P+) or buffer alone (P−), then subjected to SDS−PAGE (4% acrylamide) followed by transfer to nitrocellulose and revealed by mab414. Note that the signal for Nup214 was almost invisible in the immunoblotting experiment (lower panel), while it was highly visible in the immunoprecipitation experiment (higher panels).

in mitosis, while additional sites may also be phosphorylated in S phase.

Since we were mainly interested in comparing phosphorylation of nucleoporins in interphase and mitosis, further experiments on these proteins were performed using mitotic cells and unfractionated interphasic cells. Mitotic HeLa cells were homogenized, and the 2000*g* postchromosomal supernatant was centrifuged at 200000*g* to obtain a cytosol supernatant (s200) and a pellet enriched in membranes and other particles (p200). Proteins contained in these two fractions and in nuclear envelopes prepared from unsynchronized HeLa cells were treated with alkaline phosphatase, fractionated by SDS−PAGE (4% acrylamide), and analyzed by immunoblotting, using mab414 (Figure 2C). The shift of Nup 153 and Nup358 toward higher apparent molecular masses in mitosis (Figure 2C, M, P−) compared to interphase (I, P−) was again observed; however, the signal obtained for Nup214 was too weak to detect. When interphasic nucleoporins (Figure 2C, I) were treated with alkaline phosphatase (Figure 2C, P+), there was no shift toward a lower apparent molecular mass (Figure 2C, compare I, P− to I, P+), suggesting a low level of phosphorylation for these proteins in interphase. By contrast, when the mitotic nucleoporins (Figure 2C, M) were treated with alkaline phosphatase (Figure 2C, P+), the signals for these proteins were shifted to a lower apparent molecular mass, similar if not identical to that of their interphasic counterparts (Figure 2C, compare M, P+ to M, P− and I). Figure 2C only shows data obtained with the fraction of mitotic nucleoporins that were soluble at 200000*g* (s200); however, identical data were obtained with the fraction of mitotic nucleoporins that sedimented at this speed (data not shown). These results further show that some nucleoporins are hyperphosphorylated in mitosis.

The other antibody used for immunoprecipitation from [^{32}P]orthophosphate metabolically labeled Hela cells was directed against the nuclear pore membrane glycoprotein gp210. The cell cycle phosphorylation pattern of gp210 was different from that of the nonmembrane nucleoporins. Gp210 was exclusively phosphorylated in mitosis but not in the G1, S, or G2 phases of the cell cycle (Figure 3A). The absence of a signal in interphase was not due to lack of protein synthesis, failure of immunoprecipitation, or protein degradation as gp210 was detected by autoradiography when immunoprecipitated from HeLa cells in interphase or mitosis when cells were grown in the presence of [^{35}S]methionine (Figure 3B). In addition, when immunoblots of whole cell extracts corresponding to an identical number of HeLa cells in interphase (Figure 3C, WCE, I) and in mitosis (Figure 3C, WCE, M) were analyzed using antibodies to gp210, signals of similar intensity were obtained. In these two types of experiments, the apparent molecular mass of gp210 in interphase and mitosis was identical, suggesting a low level of gp210 phosphorylation in mitosis. Accordingly, when proteins of nuclear envelopes and mitotic membranes were treated by alkaline phosphatase before analysis by immunoblotting (Figure 3C), the apparent molecular mass of gp210 in mitotic membranes (M) and nuclear envelopes (I), either treated by the enzyme (P+) or untreated (P−), was identical. ^{32}P-Labeled gp210 from mitotic cells was further analyzed by phosphopeptide mapping after digestion with thermolysin (Figure 3D), which cleaves before hydrophobic amino acids, and by phosphoamino acid analysis (Figure 3E). Gp210 was

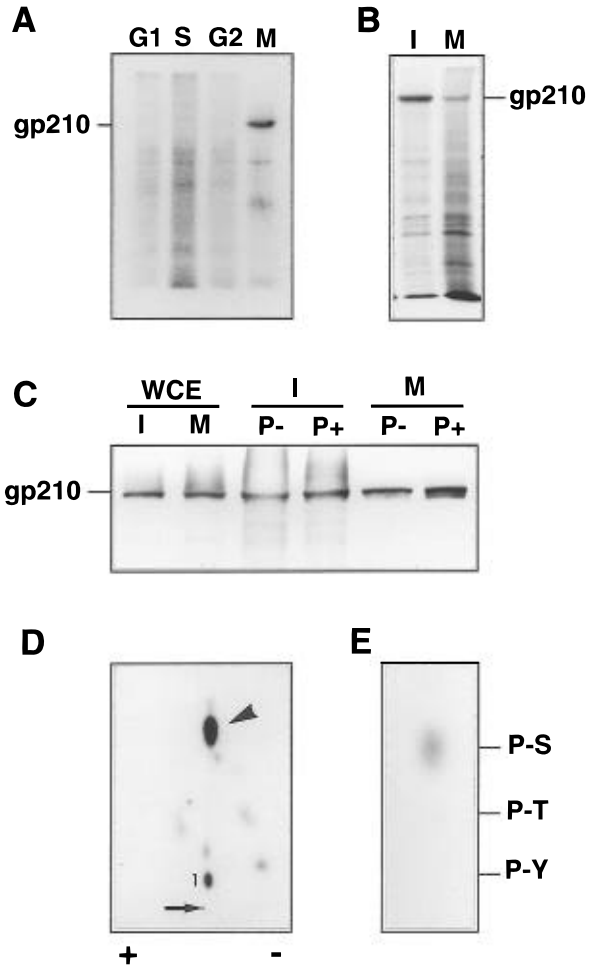

FIGURE 3: Cell cycle-dependent phosphorylation of gp210 in HeLa cells. Populations of HeLa cells in the G1, S, G2, and M phase of the cell cycle, obtained by centrifugal elutriation, were metabolically labeled with [^{32}P]orthophosphate, immunoprecipitated by polyclonal antibodies to gp210, and then separated by SDS−PAGE (8% acrylamide). (A) Autoradiogram showing that gp210 is only phosphorylated in mitosis (M). (B) Autoradiogram showing ^{35}S-labeled gp210 immunoprecipitated from interphase (I) and mitotic (M) cells. (C) Proteins from whole extracts of interphasic (WCE, I) and mitotic (WCE, M) Hela cells (10^5 cells/lane) and proteins from interphase nuclear envelopes (I) and mitotic membranes (M) treated with either alkaline phosphatase (P+) or buffer alone (P−) were subjected to SDS−PAGE (6% acrylamide) and transferred to nitrocellulose sheets, and gp210 was detected using polyclonal antibodies in the immunoblot shown. (D) Autoradiogram showing a two-dimensional phosphopeptide analysis of ^{32}P-labeled gp210 eluted from a SDS−polyacrylamide gel as in panel A. Arrowhead indicates a major phosphopeptide and number 1 a minor phosphopeptide present in the preparation. The arrow indicates the origin; the anode is on the left, and the cathode on the right. (E) Phosphoamino acid analysis of ^{32}P-labeled gp210 from mitotic cells. Migration of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards is shown at right.

phosphorylated on a serine residue, and thermolysin digestion produced a prominent single phosphopeptide (arrowhead in Figure 3D). Another phosphopeptide that migrated near the origin of the chromatogram (labeled 1 in Figure 3D) was inconsistently observed and likely due to incomplete thermolysin digestion. These data demonstrate that gp210 is exclusively phosphorylated in mitosis on either a unique serine residue or a few nearby serine residues.

The Peptide of Gp210 That Is Phosphorylated in Mitosis Can Be Phosphorylated in Vitro by Cyclin B-p34^{cdc2} Protein Kinase. We decided to further characterize the mechanism

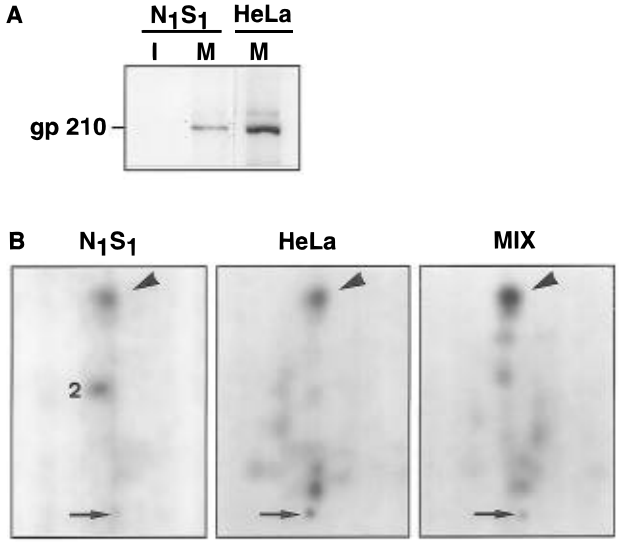

FIGURE 4: Cell cycle-dependent phosphorylation of gp210 in rat cells N1S1 and human HeLa cells. (A) Rat N1S1 cells, either unsynchronized (N1S1, I) or arrested in mitosis by nocodazole (N1S1, M), were metabolically labeled with [^{32}P]orthophosphate, and cell lysates were immunoprecipitated with antibodies against gp210 and the immunoprecipitated proteins separated by SDS−PAGE. Autoradiogram shows that rat gp210 is only phosphorylated in mitosis. ^{32}P-Labeled gp210 obtained form HeLa cells as in Figure 3 was run in parallel (HeLa, M). (B) PhosphorImager printouts showing two-dimensional phosphopeptide analysis of ^{32}P-labeled gp210 immunoprecipitated from mitotic N1S1 cells, or mitotic HeLa cells, and a mixture of the phosphopeptides of these two preparations (equal amounts of radioactivity from each) run on the same chromatogram (MIX). Arrowheads indicate the major phosphopeptides common to gp210 in both cell types, and number 2 indicates a phosphopeptide unique to N1S1 cells. Arrows indicate origin. Anode is on the left and cathode on the right.

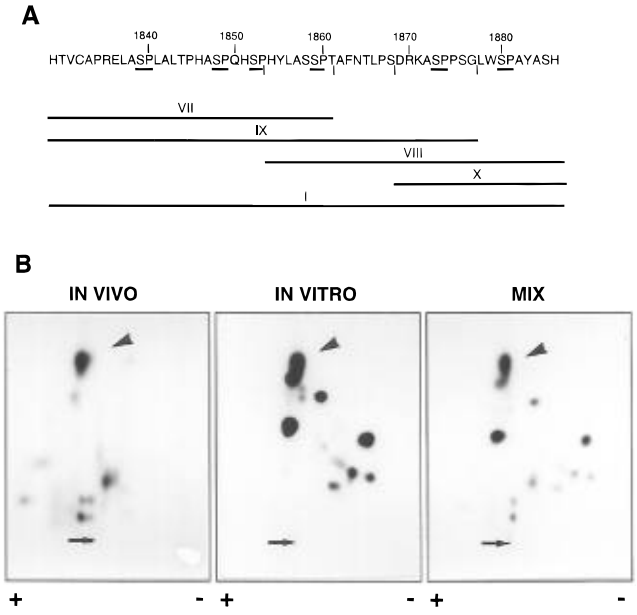

FIGURE 5: *In vitro* phosphorylation of GST fusion protein containing the carboxyl-terminal domain of gp210 by cyclin B-p34^{cdc2} protein kinase generates a phosphopeptide that comigrates with a mitosis-specific phosphopeptide. (A) Amino acid sequence of the cytoplasmic tail of gp210. Regions labeled VII, IX, VIII, X, and I correspond to the regions of the cytoplasmic tail of gp210 that were expressed from plasmid clones as GST-fusion proteins. The small vertical bars below the amino acid sequence indicate the limits of the fusion proteins. The underlined amino acids correspond to the six Ser-Pro motifs. (B) Autoradiograms showing two-dimensional phosphopeptide analysis of ^{32}P-labeled gp210 immunoprecipitated from mitotic HeLa cells (IN VIVO), of ^{32}P-labeled fusion protein expressed from clone I that was phosphorylated *in vitro* by cyclin B-p34^{cdc2} protein kinase (IN VITRO), and of a mixture of the phosphopeptides of these two preparations (equal amounts of radioactivity from each) run on the same chromatogram (MIX). Arrowheads indicate the major phosphopeptide common to gp210 immunoprecipitated from mitotic cells and the fusion protein expressed from clone I. Arrows indicate origin. Anode is on the left and cathode on the right.

of phosphorylation of nuclear pore membrane glycoprotein gp210 in mitosis. However, a drawback was that *in vivo* data were obtained in human HeLa cells while the available gp210 fusion proteins that were the putative substrates for *in vitro* phosphorylation originated from rat cDNA (Wozniak et al., 1989; Nickowitz & Worman, 1993). Since the human sequence was not elucidated, we decided to check the conservation of the mitotic phosphopeptide of gp210 by comparing the phosphopeptide maps of *in vivo* phosphorylated human and rat gp210 isolated from mitotic cells.

Rat N1S1 cells, growing in suspension, were chosen for these experiments because the cDNA library used for the sequencing of gp210 was obtained from this cell line (Wozniak et al., 1989). Figure 4A shows autoradiograms of the ^{32}P-labeled polypeptides of gp210 immunoprecipitated from unsynchronized (I) and mitotic (M) N1S1 cells. In both rat N1S1 cells and human HeLa cells, gp210 was only phosphorylated in mitosis. The major thermolysin phosphopeptide derived from both rat and human gp210 demonstrated similar migrations on two-dimensional maps (Figure 4B, left and middle panels, arrowheads), although rat gp210 contained an additional phosphopeptide (labeled 2 in Figure 4B). When phosphopeptides from both the human and the rat preparations were run on the same chromatogram, the two predominant phosphopeptides were shown to comigrate (Figure 4B, panel labeled MIX). Because this phosphopeptide was likely identical in both species, we decided to use *in vivo* phosphorylated human gp210 in further experiments. The reasons for the choice of human cells for *in vivo* experiments were that significantly greater amounts of radioactive phosphoprotein were obtained when immunoprecipitation was performed in HeLa cells compared to N1S1 cells (see Figure 4A) and that it was much easier to arrest Hela cells in mitosis. Difficulties in the synchronization of rodent cell lines have also been documented by others (Johnson et al., 1993).

Gp210 contains six Ser-Pro motifs (Figure 5A) that represent consensus sites for phosphorylation by proline-directed protein kinases. Since cyclin B-p34^{cdc2} is the major mitotic protein kinase (Nurse, 1990) with the motif Ser-Pro within its consensus sites (Labbé et al., 1991; Moreno & Nurse, 1990), it was chosen as a putative kinase for *in vitro* phosphorylation of the carboxyl-terminal domain of gp210. An additional reason for this choice was that cyclin B-p34^{cdc2} protein kinase catalyzes the mitosis-specific phosphorylation of LBR, another integral membrane protein of the nuclear envelope (Courvalin et al., 1992). A protein consisting of GST fused to the 58-amino acid cytoplasmic domain of rat gp210 was expressed from pGEX-2T clone I (Figure 5A). The purified fusion protein was phosphorylated by cyclin B-p34^{cdc2} protein kinase *in vitro* and then separated from other components of the reaction mixture by SDS−PAGE. After autoradiography of the dried gel, the ^{32}P-labeled fusion protein was analyzed by phosphopeptide mapping. Phosphopeptide maps showed a major phosphopeptide (Figure

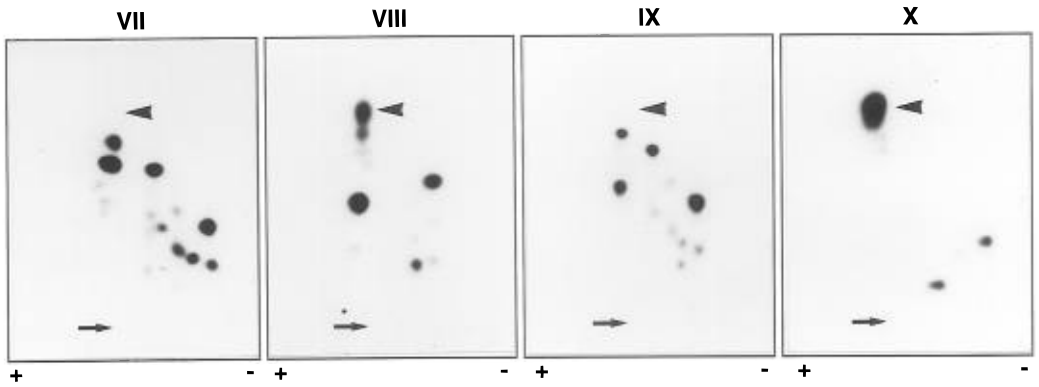

FIGURE 6: Mitosis-specific phosphorylation site is located at the extreme carboxyl-terminal end of the cytoplasmic domain of gp210. Autoradiograms showing two-dimensional phosphopeptide maps of ^{32}P-labeled fusion proteins containing different domains of the C-terminus of gp210. Fusion proteins expressed from clones VII, VIII, IX, and X (see Figure 5) were phosphorylated *in vitro* by cyclin B-p34^{cdc2} protein kinase, and phosphopeptides were analyzed in two dimensions. Each phosphopeptide preparation was mixed with phosphopeptides obtained from clone I as in Figure 5 and analyzed in parallel (data not shown) to localize the migration of the mitosis-specific phosphopeptide (arrowheads). Note that this phosphopeptide is present in phosphorylated fusion proteins expressed from clones VIII and X and absent from phosphorylated fusion proteins expressed from clones VII and IX. Arrows indicate origin. Anode is on the left and cathode on the right.

5B, arrowhead in panel labeled IN VITRO) that was similar to the major phosphopeptide derived from mitotic gp210 *in vivo* (Figure 5B, arrowhead in panel labeled IN VIVO). These major phosphopeptides of both the *in vitro* and *in vivo* preparations comigrated when separated on the same chromatogram (Figure 5B, arrowhead in panel labeled MIX). The major phosphopeptide derived from the fusion protein that colocalized with the mitosis-specific phosphopeptide of gp210 was scraped from the chromatography plate and shown by phosphoamino acid analysis to contain phosphoserine (data not shown). These data showed that the same peptide of gp210 that was phosphorylated during mitosis was phosphorylated *in vitro* by the cyclin B-p34^{cdc2} protein kinase complex. This protein kinase was quite promiscuous *in vitro* as several peptides of the fusion protein were phosphorylated. Modifications in the respective concentrations of the enzyme, the fusion protein, and ATP did not modify the pattern of phosphorylation of the different peptides. When MAP kinase, another proline-directed protein kinase, was substituted for cyclin B-p34^{cdc2} in the *in vitro* assay, the thermolysin-generated phosphopeptide corresponding to the *in vivo* phosphorylated peptide was also detected (data not shown).

Ser^{1880} Is the Mitotic Phosphorylation Site of Gp210. To identify the serine residue(s) in the carboxyl-terminal domain of gp210 that was phosphorylated by cyclin B-p34^{cdc2} protein kinase, GST fusion proteins were expressed from pGEX-2T clones VII, VIII, IX, and X (Figure 5A). These fusion proteins contain, in different combinations, all the Ser-Pro motifs in the carboxyl-terminal domain of gp210. Purified fusion proteins were phosphorylated by cyclin B-p34^{cdc2} protein kinase and then analyzed by two-dimensional phosphopeptide mapping (Figure 6). Phosphopeptides derived from each of these fusion proteins were also analyzed on the same chromatogram with phosphopeptides obtained from the entire 58-amino acid carboxyl-terminal domain fusion protein to clearly identify the one that corresponded to the mitosis-specific phosphopeptide (data not shown). The migration of the mitosis-specific phosphopeptide observed in these mixing experiments is indicated by the arrowheads in Figure 6. This mitosis-specific phosphopeptide was present in the phosphorylated thermolysin-digested fusion proteins expressed from clones VIII and X but absent from those expressed from clones VII and IX. The presence of this phosphopeptide in the fusion protein expressed from clone VIII and its absence from the fusion protein expressed from clone VII indicated that the phosphorylation site(s) was located in the second half of the carboxyl-terminal domain of gp210 and eliminates Ser^{1839} and Ser^{1848} as candidates. Furthermore, since the mitosis-specific phosphopeptide was absent from the phosphorylated fusion protein expressed from clone IX but present in that expressed from clone X, we deduced that Ser^{1880} was the most probable phosphorylation site. Even though Ser^{1873} was only four amino acids away from the carboxyl-terminus of the fusion protein expressed from clone IX, this serine residue was still considered as a putative candidate for the mitosis-specific phosphorylation site.

To unequivocally identify the serine residue phosphorylated *in vitro* by cyclin B-p34^{cdc2} that corresponds to the site phosphorylated *in vivo*, three fusion proteins identical to that expressed from clone VIII were produced in which Ser^{1873}, Ser^{1876}, and Ser^{1880} were each independently mutated to alanines. Wild-type and mutated fusion proteins were phosphorylated *in vitro* by cyclin B-p34^{cdc2} protein kinase. Figure 7 shows that the mitosis-specific phosphopeptide (Figure 7, left panel, arrowhead) was absent from the fusion protein where Ser^{1880} was mutated to alanine (Figure 7, middle panel). This was confirmed by mixing phosphopeptides of wild-type fusion protein (VIII^{S1880}) and mutated fusion protein (VIII^{A1880}) and analyzing them on the same chromatogram (Figure 7, panel labeled MIX). Other phosphopeptides derived from *in vitro* phosphorylated fusion proteins (Figure 7, numbers 3 and 4) that were not present in *in vivo* phosphorylated gp210 (see Figure 5B) were not affected by this mutation. Such sites are not likely to be physiologically relevant. Phosphopeptide maps obtained from fusion proteins with Ser^{1873} and Ser^{1876} mutated to alanines were identical to those obtained from the wild-type fusion protein (data not shown). The phosphopeptide containing Ser^{1880} that results from digestion with thermolysin is predicted to contain only this serine residue and to be distinct from the other potential phosphopeptides generated by thermolysin digestion of the carboxyl-terminal domain of gp210. These results demonstrate that gp210 is phosphorylated *in vivo* during mitosis on Ser^{1880}. Of note, a TBLASTN search of the XREF Database at the National

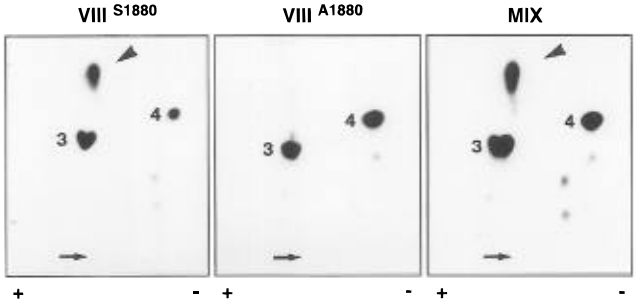

FIGURE 7: Identification of the serine residue on gp210 that is phosphorylated *in vitro* by cyclin B-p34^{cdc2} protein kinase. Autoradiograms showing two-dimensional phosphopeptide maps of ^{32}P-labeled fusion proteins expressed from clone VIII, either wild-type (VIII^{S1880}) or mutagenized (VIII^{A1880}), and mixture of both (MIX). Equal amounts of each fusion protein were loaded in the experiment shown in MIX. Arrowheads indicate the ^{32}P-labeled phosphopeptide of VIII^{S1880} that corresponds to the *in vivo* phosphorylated peptide. This phosphopeptide is absent from fusion protein VIII^{A1880} in which Ser^{1880} is mutated to Ala. Numbers 3 and 4 indicate ^{32}P-labeled phosphopeptides that are present in both *in vitro* phosphorylated fusion proteins but not present in gp210 immunoprecipitated from HeLa or N1S1 cells (see Figure 4). Arrows indicate origin. Anode is on the left and cathode on the right.

Center for Biotechnology Information (World Wide Web URL http://www.ncbi.nlm.nih.gov/XREFdb/), performed after these experiments were completed, identified a human EST (GenBank Accession Number N55034, entry date February 20, 1996) that encoded an amino acid sequence identical to rat gp210 from amino acids 1870 to 1886.

DISCUSSION

In the present report, we have examined the cell cycle-dependent phosphorylation of five nuclear pore complex proteins. Three nonmembrane nucleoporins modified by O-linked GlcNAc residues, Nup153, Nup214, and Nup358, were phosphorylated during interphase and hyperphosphorylated during mitosis. By contrast, nucleoporin p62 that bears the same glycosylation modification was not phosphorylated at any stage of the cell cycle. Phosphorylation of NLS-binding proteins in the mobile import machinery appears to be necessary for nuclear import to occur (Stochaj & Silver, 1992; Azuma et al., 1995; Mishra & Parnaik, 1995). In *Saccharomyces cerevisiae*, a nuclear pore complex-associated protein kinase has been shown to phosphorylate Srp1p, an NLS-binding protein essential for nuclear import (Azuma et al., 1995). Phosphorylation of structural proteins of the nuclear pore complex also appears to be necessary for nuclear import (Feldherr & Akin, 1995), and the kinases responsible for the phosphorylation may be cAMP-dependent protein kinase (Vandromme et al., 1994; Gauthier-Rouviere et al., 1995), protein kinase C (Feldherr & Akin, 1995), or both (Mishra & Parnaik, 1995). Except for the NLS-binding proteins, however, specific pore complex or import machinery substrates for protein kinases have not been identified. Thus, the interphasic phosphorylation shown in this paper of Nup 153, Nup 214, and especially Nup 358, a Ran-binding protein known to function in nucleocytoplasmic transport (Wu et al., 1995; Yokoyama et al., 1995), further suggests that this modification may be necessary for effective nuclear import.

In contrast to the nonmembrane nucleoporins, pore membrane glycoprotein gp210 is not phosphorylated in interphase but is specifically phosphorylated during mitosis. In mitosis, gp210 is phosphorylated on Ser^{1880}, a serine residue located within a Ser-Pro site in the cytoplasmic domain seven amino acid residues away from the carboxyl-terminus. This same site is phosphorylated *in vitro* by the proline-directed protein kinases p34^{cdc2} and MAP kinase. Because cyclin B-p34^{cdc2} is the major mitotic protein kinase (Nurse, 1990), and because MAP kinase is inactivated in metaphase-arrested cells (Tamemoto et al., 1992), we hypothesize that cyclin B-p34^{cdc2}, or a related protein kinase, is the enzyme that catalyzes the phosphorylation of gp210 during mitosis. Several other serine or threonine residues in the carboxyl-terminal domain of gp210 are also phosphorylated by p34^{cdc2} or MAP kinases *in vitro*, but because they are not observed to be phosphorylated *in vivo*, they have unclear physiological significance (Moreno & Nurse, 1990). Although we favor mitosis-specific phosphorylation of gp210, we cannot rigorously exclude the alternative possibility that cell cycle differences in protein phosphorylation result from a fluctuating phosphatase activity (Ferrigno et al., 1993). The nonmembrane nucleoporins examined in this study also have high densities of Ser/Thr-Pro motifs with frequencies of one per 40−70 amino acids (Starr et al., 1990; von Lindern et al., 1992; Sukegawa & Blobel, 1993; Kraemer et al., 1994; McMorrow et al., 1994; Wu et al., 1995; Yokoyama et al., 1995), suggesting that the mitotic phosphorylation of these nucleoporins may also be catalyzed by cyclin B-p34^{cdc2} or a related protein kinase.

The cell cycle-dependent phosphorylation of O-linked GlcNAc-containing nucleoporins has recently been studied in *Xenopus* oocytes both *in vivo* and in cell-free extracts (Macaulay et al., 1995). Protein p60, the *Xenopus* equivalent of mammalian p62, was not phosphorylated while a protein with an apparent molecular mass of 200 kDa was phosphorylated in interphase and hyperphosphorylated in mitosis. The 200 kDa glycoprotein was further shown to be an *in vitro* substrate for Cdc2 kinase, although the *in vivo* and *in vitro* phosphorylation sites were not identified. Although performed in two different systems, mammalian somatic cells versus amphibian germinal cells, data from this study and our study reach the similar conclusion that mitotic phosphorylation of pore complex proteins parallels pore complex disassembly and that pore complex proteins are substrates for cyclin B-p34^{cdc2} protein kinase or a related protein kinase.

What can be the role of phosphorylation of nuclear pore complex proteins during mitosis? A possible role could be the prevention of protein−protein interactions in the pore complex as has been proposed for lamin−lamin interactions during mitosis (Heald & McKeon, 1990; Peter et al., 1990). However, identification of the ligands of the O-linked GlcNAc-containing nucleoporins and of gp210 is a prerequisite to further studies on this subject. Interaction of gp210 with another component of the pore complex has been suggested by transfection experiments showing that a reporter integral membrane protein containing the carboxyl-terminal domain of gp210 is targeted to the pore complex (Wozniak & Blobel, 1992). The same reporter protein from which the last 20 amino acids of gp210 was deleted was not targeted to the pore membrane, showing that this portion of gp210 is involved in proper localization at the pore. We suggest that this subdomain of the carboxyl terminus of gp210 that contains Ser^{1880} interacts in interphase with a component of the octahedral core structure of the nuclear pore complex and contributes to its anchoring to the membrane. During

mitosis, phosphorylation of Ser^{1880} would destabilize the interaction and allow the detachment, or prevent the reattachment of such components from the membrane.

The carboxyl-terminal end of gp210 contains a low amount of hydrophobic (19%) and a high amount of proline residues (17%) that prevents any accurate prediction of secondary structure. However, the linear sequence shows that the phosphorylated serine residue (Ser^{1880}) is close to a tryptophan (Trp^{1879}) and a tyrosine (Tyr^{1883}) residue. In several studies where protein−protein interactions were analyzed by crystallography, aromatic amino acid residues were present in the core of the binding site and were shown to be major contributors of binding free energy (Clackson & Wells, 1995). If Trp^{1879} and Tyr^{1883} belong to the binding interface of gp210 with its ligand, the introduction of a phosphate group at Ser^{1880} could dramatically alter the strength of these hydrophobic interactions.

Gp210 is targeted to the nuclear pore complex by two different mechanisms at different times during the cell cycle. In interphase, gp210 likely diffuses laterally from its site of synthesis on the endoplasmic reticulum membrane to the interconnected nuclear membranes until it reaches its location in the pore membrane domain (Wozniak & Blobel, 1992). In mitosis, gp210-containing vesicles are targeted to the chromatin of daughter nuclei where they subsequently fuse with inner nuclear membrane-derived vesicles and presumably outer nuclear membrane-derived vesicles to reform a functional nuclear envelope (Chaudhary & Courvalin, 1993). It is not known if the target for gp210-containing mitotic vesicles is chromatin or previously targeted chromatin-associated vesicles derived from the inner nuclear membrane. It is also not known if gp210 functions in this targeting or if phosphorylation plays a role at this step. The fusion of membranes has been shown to be inhibited in prophase and restored after metaphase, and this process depends upon protein phosphorylation and dephosphorylation (Tuomikoski et al., 1989; Woodman et al., 1992). Whether the cell cycle-dependent phosphorylation of gp210 plays a role in the nuclear pore membrane fission and fusion that occurs in mitosis is an open question. Current attempts to isolate domain-specific populations of mitotic nuclear envelope membranes (Buendia and Courvalin, in submission) may enable us to answer this question.

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