THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 1, Issue of January 6, pp. 254–262, 1995
Printed in U.S.A.

Differential Mitotic Phosphorylation of Proteins of the Nuclear Pore Complex*

(Received for publication, August 19, 1994, and in revised form, October 17, 1994)

Colin Macaulay, Eva Meier‡, and Douglass J. Forbes§

From the Department of Biology, University of California at San Diego, La Jolla, California 92093-0347

During each cell cycle, the nucleus of higher eukaryotes undergoes a dramatic assembly and disassembly. These events can be faithfully reproduced *in vitro* using cell-free extracts derived from *Xenopus* eggs. Such extracts contain three major *N*-acetylglucosaminylated proteins, p200, p97, and p60. All three become assembled into reconstituted nuclear pores. Here we show that p200, p97, and p60 exist in eggs in soluble high molecular mass complexes of 1000, 450, and 600 kDA, respectively. The bulk of p60 is stably associated with proteins of 58 and 54 kDa, while p200 is associated with a fraction of p60 in a separate complex lacking p58 and p54. Upon examining the behavior of these proteins in the cell cycle, we find that p200 and p97 are highly phosphorylated at mitosis, both *in vivo* and *in vitro*. Moreover, in extracts that cycle between interphase and mitosis, p200 and p97 are specifically phosphorylated at mitosis. Corresponding with their mitotic phosphorylation, both p200 and p97 are specific substrates for purified mitotic Cdc2 kinase, whereas nucleoporin p60 is not. Analysis indicates that the size of the complexes containing the pore *N*-acetylglucosamine glycoproteins does not change during mitosis, suggesting that such complexes represent stable multicomponent modules into which the nucleus disassembles at mitosis.

The mitotic reorganization of the eukaryotic cell architecture ensures the correct partitioning of the cellular contents to each of the daughter cells. These mitotic changes are seen most vividly in the dramatic alterations of the cell nucleus where the chromosomes condense, the mitotic spindle assembles, and the nuclear envelope breaks down (1–3). There is currently a great deal of evidence to indicate that movement of the cell cycle into mitosis is regulated by the Cdc2 protein kinase (4). The phosphorylation of key cellular proteins by Cdc2 kinase, or activated downstream kinases, is known to correlate with many of the major mitotic rearrangements. Determining the targets of the Cdc2 kinase is, therefore, a means to identify proteins that initiate cell cycle rearrangements (5). For example, one of the non-histone chromosomal scaffold proteins, topoisomerase II, is required for chromosome condensation and shows increased activity during mitosis (6–9). The increase in topoisomerase II activity is associated with an increase in its phosphorylation state as a result of either protein kinase C (10) or casein kinase II (11, 12), the latter of which is a substrate for the Cdc2 kinase. Similarly, a number of proteins that bind to and modulate microtubule dynamics have been isolated, and some of these proteins (MAP4, p220, and p47) are phosphorylated in a cell cycle-dependent fashion (13, 15–18).

Another key mitotic reorganization event seen in higher eukaryotes is the disassembly of the nuclear envelope. Specifically, the nuclear lamina network depolymerizes, the double nuclear membranes vesiculate, and the pore complexes disassemble (19–22). Of all of the mitotic rearrangements that occur, the disassembly of the nuclear lamina is the best understood. The lamina is composed of polymerized intermediate filament-like proteins that are arranged between the inner nuclear membrane and chromatin. The lamin proteins form homo- and heteropolymers through strong coiled/coil interactions both *in vivo* and *in vitro* (23, 24). Despite these strong interactions the lamina is a very dynamic network that both completely disassembles during mitosis and incorporates new lamin proteins during nuclear growth in interphase (20, 25). Lamins have been shown to be phosphorylated during interphase and hyperphosphorylated during mitosis (21). Similarly, lamin-associated proteins of the inner nuclear membrane are also phosphorylated during interphase and mitosis (26–29). Phosphorylation may regulate the dynamics of the lamin network in three ways: 1) by affecting the association between individual lamin monomers (30), 2) by regulating the nuclear import of lamin monomers (31), and 3) by affecting the association between lamins and their putative inner nuclear membrane receptors (26, 29, 32). Purified Cdc2 kinase can phosphorylate and dissociate lamin polymers formed *in vitro* (33). In addition, either purified Cdc2 kinase or protein kinase C will phosphorylate and disassemble the lamin network of detergent-treated nuclei (34, 35), lending support to the hypothesis that mitotic phosphorylation induces disassembly of the nuclear lamina.

The molecular mechanism of disassembly of the other nuclear envelope structures, such as the nuclear membrane and nuclear pore complexes, is much less well understood. A variety of *in vitro* assays have demonstrated that mitotic phosphorylation negatively regulates the binding of membrane vesicles to chromatin (29, 36, 37). It appears that once the nuclear membrane has vesiculated, phosphorylation of membrane components by kinases downstream from Cdc2 prevents the reassociation of vesicles with chromatin until interphase resumes. However, almost nothing is known about the actual mechanism of membrane vesicularization or, indeed, nuclear pore disassembly.

In previous studies we used an *in vitro* nuclear reconstitution system to identify and characterize a family of nuclear pore glycoproteins required for nuclear transport (38, 39). In this reconstitution system, nuclei assemble when sperm chromatin is mixed with the cytosolic and membrane fractions of a

* This work was supported by a fellowship grant from the Medical Research Council of Canada and an independent research allowance from the Alberta Heritage Foundation for Medical Research (to C. M.) and by Grant GM33279 from the National Institutes of Health and a grant from the Pew Memorial Trust (to D. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: The Scripps Research Inst., 10666 N. Torrey Pines Rd., La Jolla, CA 92037.

§ To whom correspondence should be addressed.

Xenopus egg lysate (40, 41). Reconstituted nuclei are fully functional in that they accumulate nuclear proteins (42, 43), replicate their DNA (44, 45), and respond to mitotic signals by condensation of the chromatin and disassembly of the nuclear envelope (46–49). This system has proved to be uniquely useful for biochemically dissecting the various steps in both the assembly and mitotic disassembly of the nucleus (36, 40, 41, 45, 50, 51).

A major open question is the manner in which the nuclear pore complex assembles and disassembles with each cell cycle. In this study we have used interphase and mitotic egg extracts in a first step to investigate whether known nuclear pore proteins are phosphorylated in a cell cycle-dependent manner. The specific proteins examined are the nuclear pore glycoproteins, which are modified with *O*-linked GlcNAc residues. Such proteins can be identified or isolated by their binding to the lectin, wheat germ agglutinin (WGA).[1] Moreover, WGA-coated ferritin binds to both the cytoplasmic and nuclear faces of the nuclear pore, as does a series of anti-pore monoclonal antibodies that react with one or more members of this glycoprotein family (38, 52–57).[2] Previously we had shown that this family of glycoproteins could be depleted from nuclear reconstitution extracts derived from *Xenopus* egg lysates using WGA-Sepharose (39). Nuclei assembled in such depleted extracts are defective in nuclear transport (39, 58, 59). In this study we find that two prominent members of the glycoprotein family, p200 and p97, are specifically phosphorylated during mitosis. They are phosphorylated both *in vivo* when eggs are injected with [γ^{32}P]ATP, and *in vitro* when mitotic egg lysates are labeled with [γ^{32}P]ATP. Moreover, we find that p200 and p97 are specific targets for immunopurified mitotic Cdc2 kinase, while the pore protein p60 is not. The distinct complexes that contain each of these glycoproteins will be discussed.

MATERIALS AND METHODS

Preparation of Nuclear Assembly Extracts—The preparation of cytosol and membrane components from a *Xenopus* egg lysate to reconstitute functional nuclei *in vitro* was done as described previously (60, 61). The egg cytosol was further clarified of residual membranes by recentrifugation (200,000 × *g*, 30 min, 2 °C), distributed into 50-μl aliquots, frozen in liquid nitrogen, and stored at −70 °C. The preparation of an activated egg lysate that cycles between interphase and mitotic states was done as described by Murray and Kirschner (62).

Mitotic Phosphorylation of the WGA-binding Proteins—Egg cytosol (40 μl) was converted to a mitotic state by the addition of a recombinant fusion GST-cyclin-Δ13 protein and incubation at 22 °C for 50 min (63). The mitotic cytosol was then radiolabeled with [γ^{32}P]ATP (ICN 3502010; final concentration, 4 μCi/μl) for 10 min. The phosphorylation reaction was stopped by the addition of 5 μl of 20% SDS, 1 ml of TENP (50 mM Tris·HCl, pH 8, 400 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 10 mM pyrophosphate, 5 mM EDTA), 0.2% Triton X-100 and was then mixed with 20 μl of WGA-Sepharose (4 °C, 2 h). The Sepharose was washed with TENP (8 × 1 ml), TN (0.1 M Tris-HCl pH 6.8, 100 mM NaCl; 4 × 1 ml), and the bound proteins were eluted by boiling in SDS-gel loading buffer.

Alternatively, specific members of the labeled WGA-binding proteins were immunoprecipitated by stopping the reaction with the addition of 5 μl of 20% SDS, 1 ml of HENP (5 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 10 mM pyrophosphate, 5 mM EDTA, 0.5% Triton X-100), 20 μl of protein A-Sepharose (Sigma P9424), and 20 μl of either anti-p200, anti-p97, or anti-rat p62 crude antiserum (64) for 1 h at 4 °C. The Sepharose was washed in HENP (10 × 1 ml), TN (4 × 1 ml), and the bound proteins eluted with SDS-gel loading buffer.

The relative phosphorylation state of the WGA-binding proteins in a cycling extract was monitored every 10 min by pulse labeling an aliquot of the extract with [γ^{32}P]ATP for a 10-min period. Specifically, a cycling extract was prepared and divided into 42-μl aliquots that were all placed at 22 °C at time 0 to initiate cycling, which occurs due to the translation of cyclin from the endogenous mRNA pool (62, 65). Every 10 min, 2 μl of an aliquot was removed and frozen in liquid nitrogen for subsequent H1 kinase assays. The remaining 40 μl was immediately labeled with [γ^{32}P]ATP to a final concentration of 4 mCi/ml. After 10 min, the labeling of the aliquot was stopped, and the WGA-binding proteins were isolated as described above. To monitor the levels of Cdc2 kinase in the cycling extract, histone H1 kinase assays were performed using the frozen aliquots as described by Smythe and Newport (61).

Mobility Shift Assays of the Mitotic and Interphase Glycoproteins—The relative mobility on polyacrylamide gels of the WGA-binding proteins isolated from an interphase egg lysate derived from activated eggs, an egg lysate converted to mitosis by preincubation (50 min) with GST-cyclinΔ13, and a mitotic egg lysate derived from unactivated eggs (prepared using a buffer that preserves the high levels of Cdc2 kinase found in the egg (6)) was determined. Aliquots (100 μl) of each lysate were diluted with 5 μl of SDS (20%), 1 ml of TENP plus 0.1% Triton X-100, and mixed with 20 μl of WGA-Sepharose (4 °C, 6 h). The Sepharose was washed with TENP, 0.1% Triton X-100 (4 × 1 ml), and then potato acid phosphatase buffer (PAP: 40 mM Pipes, pH 5.8, 1 mM dithiotheitol; 3 × 1 ml). PAP buffer (50 μl) with or without 0.1 unit of potato acid phosphatase (Calbiochem 524528), was added to the WGA-Sepharose containing the bound glycoproteins and incubated at 30 °C (1 h). Each reaction was washed in TENP, 0.1% Triton X-100 (3 × 1 ml); the bound proteins were solubilized in SDS-gel loading buffer, and electrophoresed on a long (25 cm) 7.5% polyacrylamide gel. The proteins were transferred to PVDF membrane, probed with horseradish peroxidase-conjugated WGA, and visualized by chemiluminescence (DuPont NEN).

Gel Filtration Chrmatography—The WGA-binding proteins were affinity purified from 4 ml of egg lysate using WGA-Sepharose. The proteins were eluted in 0.2 ml of phosphate-buffered saline containing 0.1% Triton X-100, 0.5 M GlcNAc, and 8 mM trichitotriose. This mixture was applied to a Sephacryl HR S-300 column (50 cm × 1.5 cm) using a flow rate of 0.5 ml/min, and 1-ml fractions were collected. The proteins in each fraction were precipitated with 25% trichloroacetic acid, 0.4% sodium deoxycholate, solubilized in SDS-gel sample buffer, electrophoresed on a 9% polyacrylamide gel, and transferred to PVDF membrane (Millipore). The blot was probed with ^{125}I-WGA and exposed for autoradiography. The same column was calibrated by fractionation of thyroglobulin (670 kDa), β-galactosidase (464 kDa), catalase (240 kDa), bovine serum albumin (67 kDa), and ovalbumin (45 kDa).

Subsequent gel filtration was performed by fast protein liquid chromatography with a Superose 6 column (Pharmacia Biotech Inc.). WGA-binding proteins were purified from 2–4 ml of either interphase cytosol or mitotic cytosol made by the conversion of interphase cytosol with GST-cyclin. These proteins were applied to the column equilibrated with phosphate-buffered saline, 0.05% Triton X-100. Fractions (0.6 ml) were collected at a flow rate of 0.4 ml/min, trichloroacetic acid-precipitated, and resolved on gels as above. The proteins were transferred to PVDF and probed with ^{125}I-WGA. Because iodinated WGA often did not label the minor WGA-binding proteins very well, we routinely stained our protein blots with India ink first, as it labels these proteins more sensitively than does WGA.

In Vivo Phosphorylation of the WGA-Binding Proteins—Freshly laid *Xenopus laevis* eggs were dejellied, sorted, and soaked in 0.2 × MMR (1 × MMR: 5 mM Hepes, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA), plus cycloheximide (50 μg/ml). One portion of the eggs was left untreated, and the other was electrically activated (62). Both the unactivated and activated eggs were transferred into 1 × MMR containing 5% Ficoll, and 50 μg/ml cycloheximide. The eggs (28 each of activated and unactivated) were microinjected with 50 nl of [γ^{32}P]ATP (133 μCi/μl), 20 mM K_2HPO_4, pH 7.2, and 20 mM EGTA. After 10 min, they were transferred to 1 ml of ice-cold phosphatase inhibitor buffer (PIB: 100 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 10 mM pyrophosphate, 5 mM EDTA, 2 mM $NaVO_4$), and the excess buffer was removed. The eggs were homogenized following the addition of 200 μl of PIB containing 0.5% SDS by pipetting up and down with a 200-μl Gilson Pipetman. The yolk and pigment granules were pelleted (13,000 × *g* for 10 min), the supernatant was added to 1 ml of PIB containing 0.1% Triton X-100 plus 0.1% deoxycholate and mixed with 20 μl of WGA-Sepharose (4 °C, 4 h). The Sepharose beads were washed with PIB containing 0.1% Triton X-100, plus 0.1% deoxycholate (5 × 1 ml) and then TN (4 × 1 ml). The labeled WGA-binding proteins were eluted with SDS-gel loading buffer, electrophoresed on an 8% polyacrylamide gel, and processed for autoradiography.

To compare the total amount of the WGA-binding proteins present in mitotic (unactivated) and interphase (activated) eggs, a group (100) of each type of eggs was collected and homogenized as outlined above. The

[1] The abbreviations used are: WGA, wheat germ agglutinin; PVDF, polyvinylidine difluoride.

[2] M. Powers, C. Macaulay, and D. J. Forbes, manuscript in preparation.

crude lysate was mixed with 30 μl of WGA-Sepharose in 1 ml of PIB containing 0.1% SDS, 0.1% Triton X-100, and 0.1% sodium deoxycholate for 6 h at 4 °C. The Sepharose was washed, and the bound proteins were eluted with SDS gel loading buffer. Equal amounts of eluate from the mitotic and activated eggs were electrophoresed on an 8% polyacrylamide gel, blotted to PVDF, probed with horseradish peroxidase-conjugated WGA, and visualized using chemiluminescence.

Phosphorylation of the WGA-binding Proteins with Purified Cdc-2—WGA-binding proteins from interphase egg cytosol (0.6 ml) were isolated by the addition of 0.5 ml of TENT (50 mM Tris·HCl, pH 8, 5 mM EDTA, 500 mM NaCl, 0.1% Triton X-100) and 0.1 ml of WGA-Sepharose to the cytosol followed by mixing at 4 °C for 3 h. The Sepharose was extensively washed first with TENT (8 × 1 ml) and then with HSB_2 (10 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM EGTA, 40 mM β-glycerophosphate; 5 × 1 ml). The bound proteins were eluted with 0.1 ml of HSB_2 containing 0.5 M *N*-acetylglucosamine and 10 mM trichitotriose (1 h 4 °C). The eluted proteins were stored on ice until use later the same day.

Purified Cdc2 kinase was prepared by immunoprecipitation with the highly specific anti-Cdc2 C-terminal peptide antibody D65 (66). This antibody specifically binds to p34 Cdc2 kinase and not the related p32 Cdk2 kinase (66). Protein-A Sepharose (20 μl) was mixed with either D65 (10 μg) or nonspecific rabbit IgG (10 μg) in 1 ml of kinase wash buffer (KWB: 10 mM Hepes, pH 7.4, 80 mM β-glycerophosphate, 250 mM NaCl, 5 mM NaF, 5 mM EGTA, 0.1% Triton X-100). Interphase cytosol (25 μl) or cytosol that had been preincubated (50 min) with GST-cyclin to induce high levels of Cdc2 kinase activity was added, and the samples were mixed at 4 °C for 1.5 h. The Sepharose beads were extensively washed with KWB (5 × 1 ml) and then with the kinase reaction buffer (10 mM Hepes, pH 7.4, 5 mM EGTA, 10 mM $MgCl_2$, 40 mM β-glycerophosphate; 4 × 0.5 ml).

The ability of the immunocomplexes to phosphorylate the WGA-binding proteins was tested by mixing the Cdc2-antibody-Sepharose beads with 20 μl of the eluted WGA-binding proteins and [γ^{32}P]ATP (0.3 mCi/μmol ATP and ATP at 0.4 mM). To determine which phosphorylated proteins originated from the immunoprecipitated Cdc2 complexes themselves, a parallel set of Cdc2-antibody-Sepharose beads were mixed with 20 μl of HSB_2 buffer alone containing [γ^{32}P]ATP (0.3 mCi/μmol ATP and ATP at 0.4 mM). All reactions were incubated for 10 min at 22 °C and stopped by the addition of SDS-gel loading buffer. The samples were electrophoresed on an 8% polyacrylamide gel and processed for autoradiography.

In order to examine the relative Cdc2/H1 kinase levels present on the Cdc2-antibody-Sepharose beads, histone H1 (0.4 mg/ml) was added in place of the WGA-binding proteins in the same reaction buffer (HSB_2, 0.4 mM ATP, [γ^{32}P]ATP at 0.3 mCi/μmol ATP).

RESULTS

Both p200 and p97 Are Mitotically Phosphorylated in Egg Lysates—The cytosolic fraction of a Xenopus egg lysate contains three prominent WGA-binding proteins, designated p200, p97, and p60, as well as a number of minor WGA-binding proteins (38, 58, 59). The three major proteins are assembled into reconstituted nuclei when a chomatin substrate is mixed with the cytosolic and membrane fractions of an egg lysate (38, 58). Protein p60 is a known nuclear pore protein and is the *Xenopus* homolog of the rat nucleoporin, p62 (67). The proteins p200 and p97 have also been localized to the nuclear pore complexes.[2,3] Interestingly, anti-p97 antibodies give both a nuclear pore stain and an intranuclear stain by immunofluorescence.[3] In summary, by nuclear reconstitution and immunolocalization, all three proteins are nuclear pore glycoproteins, although p97 appears to have a more complex localization.

Interphase extracts of *Xenopus* eggs can be made mitotic by the addition of the purified recombinant fusion protein, GST-cyclin-Δ13 (63). Conversion to mitosis is indicated by the induction of H1 kinase activity or can be visualized microscopically by the nuclear envelope breakdown and chomatin condensation of added test nuclei. To examine the nuclear pore glycoproteins described above for evidence of mitotic phosphorylation, the relative phosphorylation states of the WGA-binding proteins from interphase and mitotic lysates was analyzed. Cytosol with and without added GST-cyclin was labeled with [γ^{32}P]ATP for 10 min. The WGA-binding proteins were affinity purified using WGA-Sepharose, eluted with SDS, and electrophoresed on a polyacrylamide gel. An autoradiogram of this gel (Fig. 1) demonstrates that the three major WGA-binding proteins (p200, p97, and p60) are highly phosphorylated in a mitotic extract (*lane 2*), and only lightly so in an interphase extract (*lane 1*). We repeatedly found that p97 and p200 were phosphorylated to similar extents and that p60 was much less phosphorylated. In order to be certain of the identity of these phosphoproteins, they were immunoprecipitated from radiolabeled mitotic extracts using antisera specific for *Xenopus* p200 (*lane 3*), p97 (*lane 4*), or rat p62 (*lane 5*). The latter antisera cross-reacts with the *Xenopus* p60 homolog (64). All three major GlcNAc-containing phosphoproteins were immunoprecipitated by their specific antisera. The total amount of the WGA-binding proteins present in both the interphase and mitotic extracts was found to be identical as determined by Western blotting (not shown), indicating that the increase in phosphorylated protein observed at mitosis was due to increased phosphorylation, not increased protein synthesis.

Whereas the above results indicate that the turnover of phosphate in the mitotic cytosol is much higher than in interphase cytosol, we wished to know if the overall phosphorylation state of the proteins was increased. Posttranslational modifications such as phosphorylation often lead to changes in the mobility of a protein on an SDS-polyacrylamide gel. To examine

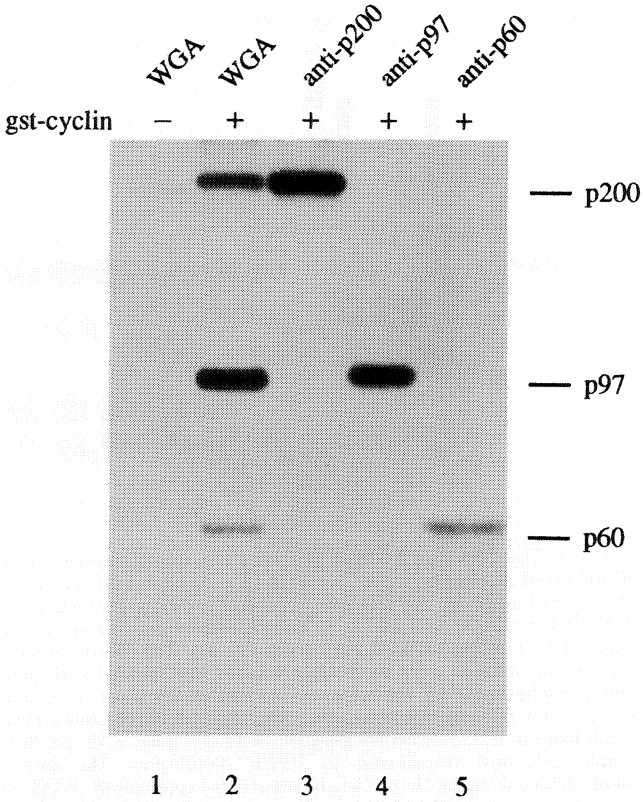


FIG. 1. **The WGA-binding proteins are phosphorylated in mitotic egg extracts.** Interphase cytosol was preincubated with (+) or without (−) GST-cyclin for 50 min at 22 °C. The individual reactions were then pulse-labeled with [γ^{32}P]ATP for 10 min. The labeled cytosol was mixed with WGA-Sepharose (*lanes 1* and *2*) or with protein-A Sepharose containing antisera against p200 (*lane 3*), p97 (*lane 4*), or p60 (*lane 5*). The Sepharose beads were thoroughly washed, and the bound proteins were eluted with SDS, electrophoresed on an 8% polyacrylamide gel, and processed for autoradiography.

[3] M. Powers, C. Macaulay, and D. J. Forbes, submitted for publication.

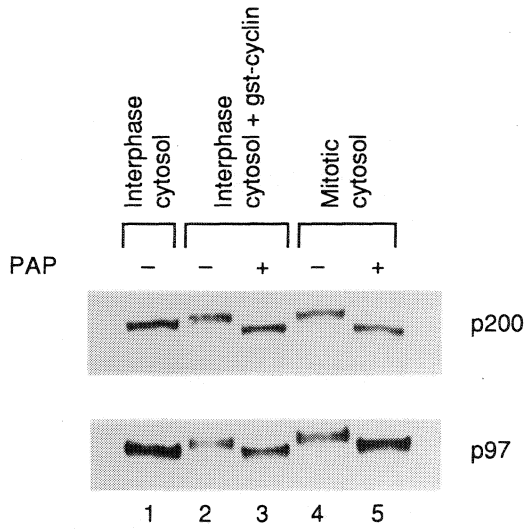


FIG. 2. **The 200- and 97-kDa proteins are quantitatively phosphorylated during mitosis.** The WGA-binding proteins from aliquots (100 μl) of interphase cytosol (*lane 1*), interphase cytosol supplemented with GST-cyclin (*lanes 2* and *3*), or mitotic cytosol (*lanes 4* and *5*) were isolated by binding to WGA-Sepharose (20 μl). The WGA-Sepharose and bound proteins were thoroughly washed and treated with potato acid phosphatase (*PAP*, *lanes 3* and *5*) or PAP buffer (*lanes 1, 2,* and *4*) at 30 °C for 60 min. After washing, the WGA-binding proteins were solubilized in SDS, electrophoresed on a 25-cm long, 7.5% polyacrylamide gel, and transferred to PVDF membrane. The proteins were detected using horseradish peroxidase-conjugated WGA and chemiluminescence.

this possibility, we compared p200 and p97 isolated from a) interphase extracts, b) mitotic extracts made by adding GST-cyclin to an interphase extract, and c) mitotic extracts made using an egg lysis buffer that preserves the naturally high levels of Cdc2 kinase present in mitotically arrested eggs (6). The mobilities were compared both before and after treatment with potato acid phosphatase (Fig. 2). Both p200 and p97 proved to migrate more slowly when isolated from mitotic extracts (*lanes 2* and *4*) than from interphase extracts (*lane 1*). The mobility of p200 and p97 isolated from mitotic extracts could be increased by treatment with potato acid phosphatase (*lanes 3* and *5*), indicating that the change in mobility at mitosis is due to phosphorylation. Note that the mobilities of both p200 and p97 are quantitatively shifted at mitosis, demonstrating that all of the p200 and p97 in the extract becomes mitotically phosphorylated. The mobility of the interphase form of p200 (*lane 1*) appears intermediate between that of the mitotic and phosphatase-treated samples. It is possible that the interphase form is quantitatively phosphorylated on a small number of sites, which is nevertheless below the number of sites phosphorylated at mitosis. In this immunoblot assay, the mobility of p60 did not change at mitosis.

Having observed an alteration in the phosphorylation state of the nucleoporins p200 and p97, we wished to know whether any other biochemical properties of these proteins were altered at mitosis. The rat WGA-binding nucleoporin, p62, can be extracted from pores as a multimeric complex with at least two other nucleoporins, p54 and p58 (64, 68, 69). To determine if the soluble WGA-binding proteins from *Xenopus* egg lysates also exist in large multimeric complexes and, if so, whether these associations change during mitosis, we measured their apparent size by gel filtration. The WGA-binding proteins were affinity purified from egg lysates using WGA-Sepharose and eluted with the competing sugar. This mixture was fractionated on a Sephacryl S300 column; the eluted proteins were precipitated with trichloroacetic acid, electrophoresed on a polyacrylamide gel, transferred to PVDF membrane, and probed with ^{125}I-WGA. By comparing the elution position of the WGA-binding proteins to those of a set of known protein standards, we could estimate the size of these soluble glycoproteins. All three major WGA-binding proteins were seen to exist in distinct high molecular weight complexes (Fig. 3). The peak of p60 elution was in fraction 16, which corresponds to an apparent size of approximately 600 kDa. Like the rat p62 homolog, the *Xenopus* p60 co-chomatographed with WGA-binding proteins of 54 and 58 kDa, although p54 is poorly recognized by ^{125}I-WGA. Note that there is a protein of 52 kDa whose peak of elution is in fractions 19 and 20. On this exposure it is difficult to resolve the 52 and 54 kDa bands, but they are clearly distinguished on lighter exposures.

The elution of p200 peaked in fractions 11 and 12 giving it an estimated molecular mass of 1000 kDa, while the elution of p97 peaked in fractions 19 and 20 giving it an estimated molecular mass of 450 kDa. We noted that a certain amount of the p60 nucleoporin co-chomatographed with the p200 complex. We have consistently seen an association between a small amount of p60 and p200 by fractionation on gel filtration columns, ion exchange columns, and by co-immunoprecipitation with specific antibodies that do not cross-react between the two proteins (not shown). Interestingly, the p54 and p58 proteins that co-elute with the bulk of p60 are not present in the complex containing p60 and p200. Likewise, the p200 protein is seen to co-chomatograph with a number of minor WGA-binding proteins labeled *K* (140 kDa), *L* (125 kDa), and *M* (110 kDa). These proteins do not cofractionate with p200 when examined by either ion exchange chomatography or by immunoprecipitation, so they are not considered to be part of the p200 high molecular weight complex. These same results were obtained when gel filtration was performed using a Superose 6 column.

Given that both p200 and p97 are highly phosphorylated at mitosis, we wished to know if their protein/protein interactions would change during mitosis as indicated by a change in the size of the complexes eluting from the gel filtration column. When the WGA-binding proteins from interphase and mitotic lysates were fractionated on a gel filtration column, there was no apparent difference in the positions at which the proteins eluted, indicating that the size of these complexes did not change significantly during mitosis (Fig. 4). We conclude that the nucleoporins p200, p97, and p60 are contained in complexes with apparent molecular masses of 1000, 450, and 600 kDa, respectively. Most importantly, the soluble interphase form of the complexes does not change upon conversion of cytosol to a mitotic state, indicating that these complexes likely represent the subunits into which the nuclear pore disassembles at mitosis.

Both p200 and p97 Are Mitotically Phosphorylated In Cycling Extracts and in Vivo—The recombinant GST-cyclin fusion protein used to convert interphase extracts to mitosis is not susceptible to the ubiquitin-mediated degradation that normally occurs in cells following the activation of Cdc2 kinase (63, 70). Since these conditions could conceivably represent a "hypermitotic" state, the cell cycle dependence of phosphorylation of the WGA-binding proteins was also examined under more physiological conditions. Cycling egg extracts were prepared which mimic the early *Xenopus* embryo by cycling between the interphase (DNA synthesis) and mitotic states of the cell cycle (62, 65). Such "cycling" extracts start in the interphase state; cyclin B protein accumulates as it is translated from the maternal mRNA pool, whereupon the cyclin combines with preexisting endogenous Cdc2 protein to give a steady increase in H1 kinase activity. When a threshold level of H1 kinase activity is reached, Cdc2 kinase becomes fully activated (62, 71). The

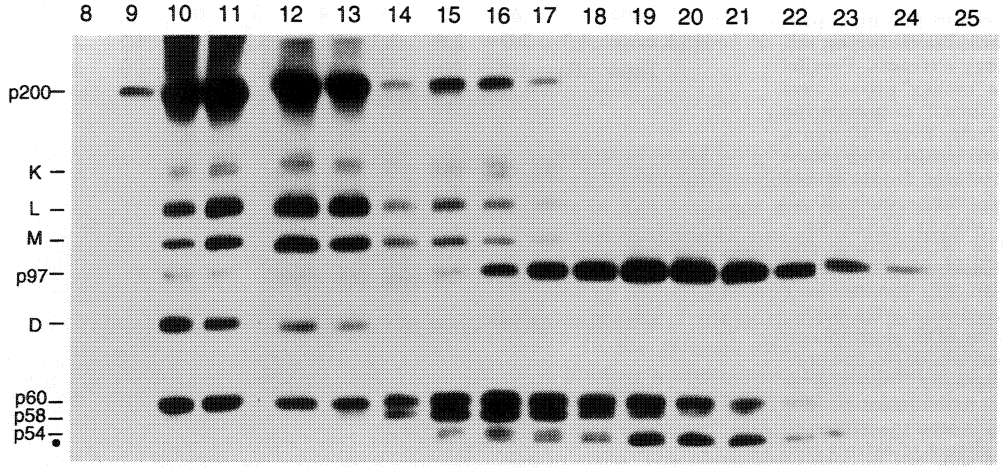
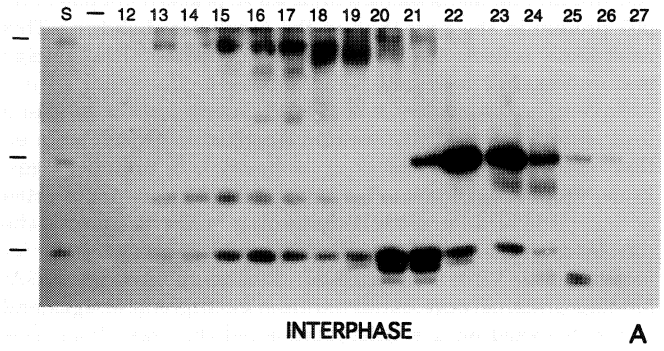


FIG. 3. **The soluble WGA-binding proteins exist as large molecular weight complexes.** The WGA-binding proteins from 4 ml of egg cytosol were applied to a calibrated HR S-300 Sephacryl column. Fractions (1 ml), were collected, trichloroacetic acid-precipitated, electrophoresed on a polyacrylamide gel, transferred to PVDF membrane, and probed with ^{125}I-WGA. Only the column fractions that contain the WGA-binding proteins (*8–25*) are shown. The position of p200, p97, and the p60 complex (p60, p58, and p54), are indicated to the *left* of the gel, as are the positions of the minor WGA-binding proteins *K* (140 kDa), *L* (125 kDa), *M* (110 kDa), and *D* (82 kDa), and a *dot* indicates the position of a 52-kDa protein. The p200 protein elution peak occurs in fractions 11 and 12, the p97 protein elution peak occurs in fractions 19 and 20, while the p60 complex elution peak occurs in fraction 16.

extract then cycles into mitosis, which results in the destruction of the cyclin protein, a corresponding drop in H1 kinase activity, and a return to the interphase state.

When parallel cycling extracts were pulse labeled for successive 10-min intervals with [γ^{32}P]ATP and examined, the phosphorylation state of the WGA-binding proteins clearly varied in a cell cycle-dependent manner (Fig. 5). Phosphorylation of p200 and p97 in the cycling extract peaked at 60 min, corresponding in time to the peak in H1 kinase activity (*lane 6*). This was also visualized to be the time of mitosis by examining parallel samples containing test nuclei which at 60 min underwent chomosome condensation and nuclear envelope breakdown (not shown). After mitosis, the level of H1 kinase activity and the WGA-binding protein kinase activity abruptly dropped (*lane 7*) and then slowly rose during the second interphase period (*lane 9*). The p200 protein is seen to be phosphorylated during the interphase state and is highly phosphorylated during mitosis, whereas p97 phosphorylation is more restricted to the peak of mitosis. In cycling extracts, much less phosphorylated p60 was observed compared with that of p97 and p200. Taken together, the results indicate that *in vitro* both the p200 and p97 WGA-binding proteins become hyperphosphorylated at mitosis, while relatively low levels of p60 phosphorylation are observed.

Xenopus egg extracts seem to accurately represent the *in vivo* conditions found in the intact egg. Nevertheless, we wished to be sure that both p200 and p97 are in fact mitotically phosphorylated *in vivo*. This is typically determined by isolating the proteins from radiolabeled tissue culture cells that have been synchronized and arrested in either mitosis or interphase (72). Although antibodies specific for p200 and p97 (Fig. 1) have been raised and used to characterize the location of these proteins in reconstituted nuclei and tissue culture cells,[2,3] our attempts to synchronize the slow growing *Xenopus* A6 tissue culture cells failed. In addition, the antibodies do not cross-react significantly with other species, so more easily synchronizable cell lines could not be used. Therefore, intact eggs and oocytes were used to look for cell cycle-dependent changes in the phosphorylation state of the WGA-binding proteins *in vivo*. *Xenopus* oocytes are arrested in an interphase state, while eggs are arrested at the second meiotic metaphase of the cell cycle. Oocytes, preloaded with [^{32}P]phosphate, can be matured into eggs *in vitro* by incubation in progesterone (73, 74). When we compared the relative phosphorylation state of the WGA-bind-



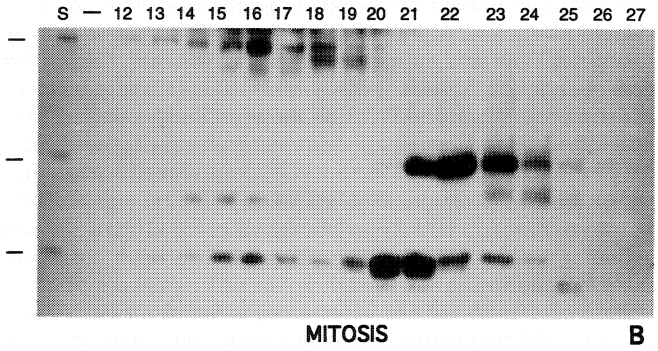


FIG. 4. **The p60, p97, and p200 complexes are maintained in a mitotic extract.** The WGA-binding proteins either from interphase cytosol (*A*) or from cytosol that had been converted to a mitotic state (*B*) were applied to a Superose 6 column. Fractions were collected, subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF, and probed with ^{125}I-WGA as in Fig. 3. The *lanes* marked *S* contain 1% of the material loaded onto the column. Only fractions containing the WGA-binding proteins are shown (12–27). The *bars* denote the positions of p200, p97, and p60. The p97 peaks in fraction 22, and p60 peaks in fractions 20 and 21. Smearing in the high molecular weight region of the WGA blots made it difficult to discern where the peak of elution of the p200 complex was. An India ink stain of the blots (not shown) clearly showed that the peak of elution of p200 (as well as of *K*, *L*, and *M*) was in fraction 16. Both the mitotic and interphase WGA-binding proteins elute from the gel filtration column at the same position.

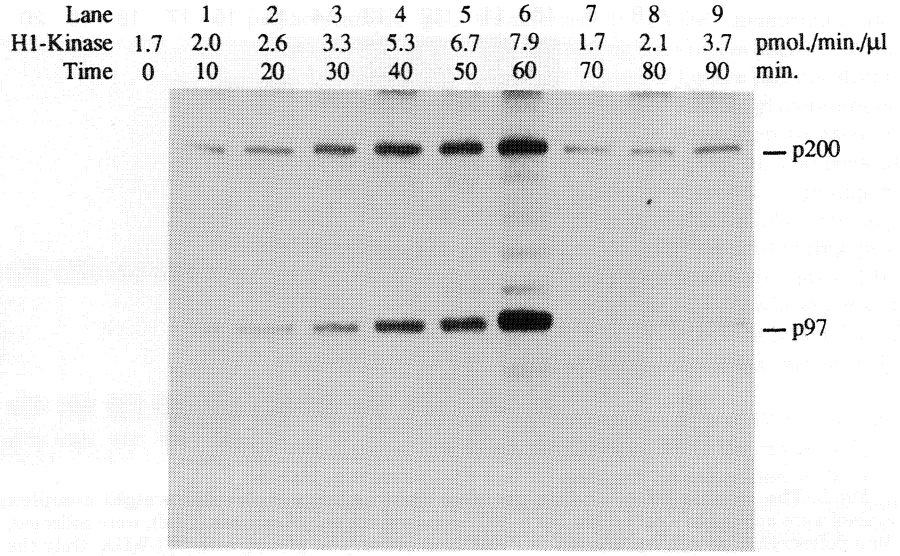


FIG. 5. **The p200 and p97 glycoproteins are phosphorylated during mitosis in cycling egg extracts.** Parallel aliquots (42 μl) of a "cycling" extract were placed at 22 °C at time 0′ to initiate the cycling of the extracts. Every 10 min a different aliquot was treated such that a 2-μl sample was removed for H1 kinase assays, and the remaining sample (40 μl) was pulse-labeled with [γ^{32}P]ATP for a 10-min period. The WGA-binding proteins from each such sequentially labeled aliquot (0′–10′, 10′–20′, 20′–30′, etc.) were affinity purified with WGA-Sepharose, solubilized with SDS, electrophoresed on an 8% polyacrylamide gel, and processed for autoradiography. Time 0′ gives the H1 kinase levels in the extract when it was on ice. Mitosis is seen to occur at 60 min in a parallel aliquot of extract containing test nuclei. This corresponds to the peak in H1 kinase activity and the peak in the phosphorylation of the WGA-binding proteins.

ing proteins present in comparable numbers of preloaded oocytes and eggs, we found that p200 and p97 are much more highly phosphorylated in the egg than in the oocyte (not shown). Immunoblots indicate that there is also more p200 and p97 protein in the eggs than in the oocytes (not shown). This most likely reflects the translation of maternal mRNA during maturation.

To compare equivalent amounts of protein from mitotic and interphase-arrested cells, we microinjected [γ^{32}P]ATP into either activated (interphase) or nonactivated (mitotic) eggs. Equal numbers of activated or nonactivated eggs were injected with [γ^{32}P]ATP in an EGTA-containing buffer to prevent the unwanted activation of the nonactivated egg group by the injection needle (75). After a 10-min labeling period, the WGA-binding proteins were extracted from each group of eggs and electrophoresed on a polyacrylamide gel (Fig. 6*a*). It is clear that WGA-binding proteins from mitotic eggs (*lane 2*) are more highly phosphorylated than those from interphase eggs (*lane 1*). Again as with the cycling extracts, p200 was found to be slightly phosphorylated in the interphase egg and highly phosphorylated in mitosis, while p97 only appears to be phosphorylated at mitosis. A protein migrating at 60 kDa was heavily phosphorylated in the experiment shown; however, the level of phosphorylation for this protein varied substantially between experiments. When the WGA-binding proteins extracted from nonactivated and activated eggs were electrophoresed on a polyacrylamide gel, blotted to PVDF, and probed with horseradish peroxidase-conjugated WGA, equivalent amounts of protein were found in both types of eggs (Fig. 6*b*). Thus p200 and p97 are consistently highly phosphorylated during mitosis both *in vitro* and *in vivo*.

Both p200 and p97 Are Substrates for Purified Cdc2 Kinase in Vitro—Although the degree of mitotic phosphorylation of p200 and p97 correlates with the known activity of Cdc2 kinase, we did not know whether it is Cdc2 or an activated downstream mitotic kinase that directly phosphorylates these proteins. To examine this, a monospecific rabbit antisera produced against a C-terminal peptide of *Xenopus* Cdc2 (66) was used to immunoadsorb Cdc2 kinase from either mitotic or interphase extracts onto protein-A Sepharose beads. The Cdc2 kinase-protein-A Sepharose, or control Sepharose, was extensively washed and then mixed with purified *Xenopus* WGA-binding proteins that had been eluted from WGA-Sepharose by the competing sugar. Control reactions contained buffer alone, WGA-binding proteins alone, kinase beads alone, or combinations of these. The kinase reactions were labeled with

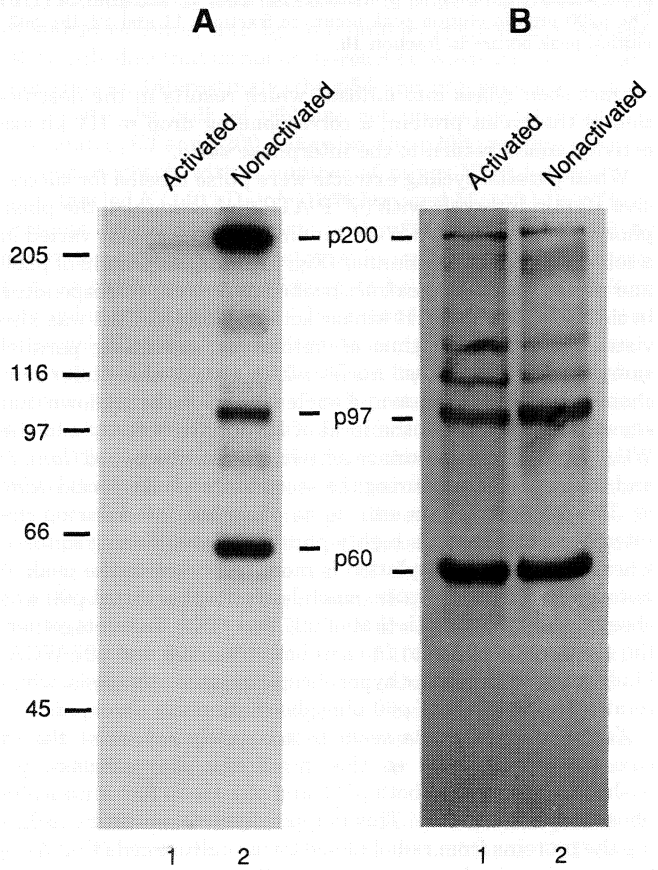


FIG. 6. **The 200- and 97-kDa glycoproteins are mitotically phosphorylated *in vivo*.** *Panel A*, a group (28) of activated (interphase, *lane 1*) and nonactivated (mitotic, *lane 2*) eggs were microinjected with 50 nl of [γ^{32}P]ATP (133 μCi/μl). After 10 min at 22 °C, the eggs were homogenized and the glycoproteins were affinity purified using WGA-Sepharose. The proteins were eluted using SDS, electrophoresed on an 8% polyacrylamide gel, and processed for autoradiography. The position of molecular mass markers are indicated to the *left* of the *panel*, while the position of the three major WGA-binding proteins are indicated between *panels A* and *B*. *Panel B*, a group (100) of activated (interphase, *lane 1*) and nonactivated (mitotic, *lane 2*) eggs were homogenized, and the glycoproteins were affinity purified using WGA-Sepharose. The proteins were eluted with SDS, and equal amounts of eluate from the activated and nonactivated eggs were electrophoresed on an 8% polyacrylamide gel, blotted to PVDF, probed with horseradish peroxidase-conjugated WGA, and visualized by chemiluminescence. The proteins in *A* and *B* were electrophoresed at different times.

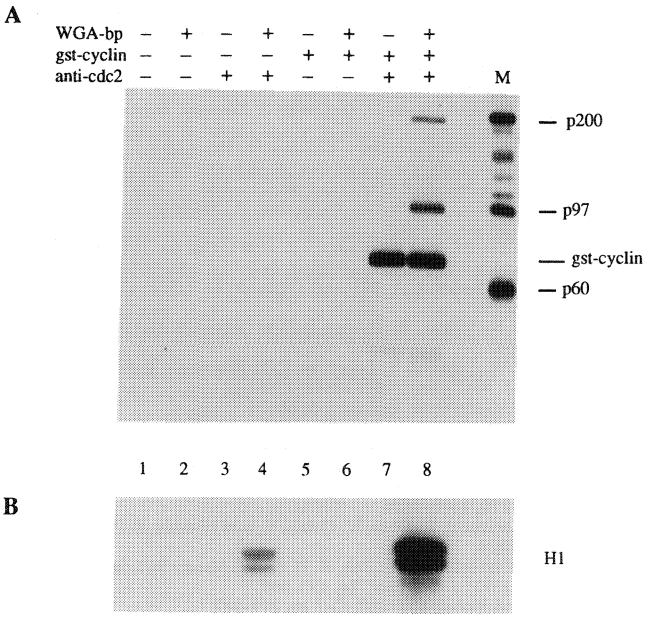


FIG. 7. **Cdc2 kinase specifically phosphorylates the p200 and p97 nuclear pore proteins *in vitro*.** *Panel A*, protein A-Sepharose bound with nonspecific rabbit IgG (*lanes 1, 2, 5,* and *6*), or anti-Cdc2 specific antibodies (*lanes 3, 4, 7,* and *8*) was used to immunoadsorb proteins from interphase egg cytosol (*lanes 1–4*), or egg cytosol that was supplemented with GST-cyclin for 50 min to induce mitotic conversion and high levels of Cdc2 kinase activity (*lanes 5–8*). After extensive washing of the protein A-Sepharose, WGA-binding proteins specifically eluted from WGA-Sepharose with high sugar buffer (HSB_2) were added to the reactions in *lanes 2, 4, 6,* and *8*. No WGA-binding proteins, only the HSB_2 buffer alone, was added to the reactions in *lanes 1, 3, 5,* and *7*. All reactions were then labeled with [γ^{32}P]ATP for 10 min. The reactions were stopped using SDS-gel loading buffer, electrophoresed on an 8% polyacrylamide gel, and processed for autoradiography. Radiolabeled p200 and p97 were observed only when mixed with anti-Cdc2 beads isolated from a mitotically converted extract (*lane 8*). Phosphorylated WGA-binding proteins isolated from mitotic cytosol labeled with [γ^{32}P]ATP in the presence of 2 μM okadaic acid served as markers (*lane M*). *Panel B*, the H1 kinase activity was assayed for each condition in *panel A* by adding histone H1 in HSB_2 (*lanes 2, 4, 6,* and *8*) or HSB_2 buffer alone (*lanes 1, 3, 5,* and *7*) along with [γ^{32}P]ATP to a parallel set of protein-A beads as used in *panel A*. After 10 min, the H1 kinase reactions were solubilized in SDS, electrophoresed on a 10% polyacrylamide gel, and processed for autoradiography. A small amount of H1 kinase activity was seen when Cdc2 was immunoprecipitated from interphase extracts, due to the residual cyclin present in interphase cytosol (Fig. 8*B*, *lane 4*). On much longer exposures, some phosphorylation of p97 can be seen using this kinase (not shown).

[γ^{32}P]ATP for 10 min, stopped with SDS, and the entire reaction mix was electrophoresed on a polyacrylamide gel (Fig. 7*A*). The level of Cdc2 kinase activity present on the Cdc2-protein-A beads or control beads was determined by the addition of the substrate histone H1 (instead of WGA-binding proteins) to a parallel set of beads (Fig. 7*B*). We found that immuno-isolated Cdc2 kinase clearly phosphorylates the p200 and p97 glycoproteins (Fig. 7*A*, *lane 8*). The Cdc2 kinase does not, however, phosphorylate the p60 pore protein (Fig. 7*A*, *lane 8*). The lower heavily phosphorylated protein band, observed even in the absence of WGA-binding proteins, corresponds to GST-cyclin, which is autophosphorylated by the Cdc2-cyclin kinase (*lanes 7* and *8*) (76–78). No autophosphorylation of the WGA-binding proteins was observed (*lanes 2* and *6*), indicating that under these conditions the WGA-binding proteins themselves are neither kinases nor copurify with a kinase. The same results were observed when p13-Sepharose was used to bind the Cdc2 kinase instead of immunoadsorbtion of Cdc2 (not shown). Thus, it is clear from the above results that the mitotic phosphorylation of the p200 and p97 nucleoporins observed *in vivo* and *in vitro* correlates with the activity of the mitotic Cdc2 kinase and that these proteins appear to be major substrates for the Cdc2 kinase *in vitro*.

DISCUSSION

It was previously found that there are three major soluble *N*-acetylglucosamine-modified proteins, p200, p97, and p60, present in *Xenopus* eggs and that each of these proteins becomes associated with nuclei reconstituted *in vitro* (38, 39). Both p200 and p60 have been localized to the nuclear pore complex by immunoelectron microscopy (52, 79),[2] whereas p97 is a component of the nuclear pore, as well as internal structures.[3] In the present study we have found that the major WGA-binding proteins are phosphorylated in a cell cycle-specific manner. The p97 and p200 nucleoporins are highly phosphorylated under all mitotic conditions examined both *in vivo* and *in vitro*. The phosphorylation of the p60 nucleoporin was always less than that of p200 and p97 in mitotic egg lysates, was only occasionally seen in cycling egg extracts, and was inconsistently observed in microinjected eggs. We do not know why p60 phosphorylation was so inconsistent, but because of this we do not consider p60 to be stably mitotically phosphorylated like p200 and p97. Upon further analysis, we find that p200 and p97 are substrates for the mitotic Cdc2 kinase and are the first nucleoporins demonstrated to be mitotically phosphorylated.

In higher eukaryotes the proteins of the nuclear pores are seen by immunofluorescence to be dispersed throughout the cytoplasm during mitosis (52, 80). It is reasonable to expect that, like the other nuclear Cdc2 kinase substrates such as the lamins, mitotic phosphorylation plays a role in the mitotic disassembly of the pore complex (5). We have attempted a number of assays to measure the effects of mitotic phosphorylation, most relevantly the thiophosphorylation of the WGA-binding proteins *in vitro* to determine whether such mitotic phosporylation results in a failure to incorporate into reconstituted nuclei. However, these assays have not yet yielded definitive results.

In analyzing the higher order structures into which the glycoproteins assemble, we found that each of the three major WGA-binding proteins exist in the egg cytosol as part of distinct high molecular weight complexes. Like the rat p62 nucleoporin complex (64, 68, 69), the bulk of *Xenopus* p60 is complexed with proteins of 58 and 54 kDa. This is interesting considering that the *Xenopus* complex exists in the egg cytosol as part of the soluble disassembled pore components derived from the mitotically disassembled oocyte nucleus, while the rat complex was biochemically extracted from intact interphase nuclei using mild detergents. The p200 nucleoporin exists as a higher molecular mass complex of ~1000 kDa, as judged by gel filtration, which contains p200, p60, and possibly other unknown components. The p97 protein is contained in a 450-kDa complex; other protein components of this complex, if they exist, are unknown. The size of the complexes are the same in interphase and mitotic extracts, suggesting that they are in their most disassembled state and represent stable subunits into which the nuclear pores disassemble. With respect to other such complexes, Dabauvalle *et al.* (59) reported a 254-kDa complex in *Xenopus* containing p62, which we did not observe. It has also been reported that the NUP153 pore protein is part of a separate large (16 S) complex in *Xenopus* egg cytosol (81). Together, these results suggest that the pore mitotically disassembles into discrete multicomponent subunits. Further analysis of such subunits should prove greatly informative both in identifying new pore proteins, as well as in elucidating the nearest neighbor interactions among the ~100 different proteins present in the nuclear pore.

When the WGA-binding proteins from a *Xenopus* egg lysate are electrophoresed on a polyacrylamide gel and stained with Coomassie Blue, the p60 complex, p97, and p200 appear in roughly equimolar amounts and are among the most prominent proteins present.[4] However, in addition to these major soluble glycoproteins, there are a number of less abundant WGA-binding proteins present in the egg lysates (Fig. 3). Proteins other than nucleoporins are also modified by GlcNAc additions. In fact, the interior of the nucleus is a major location for GlcNAc-modified proteins (82). For example, RNA polymerase II and many RNA polymerase II transcription factors contain GlcNAc modifications (83, 84), as do chomosomal proteins (85) and a nuclear tyrosine phosphatase (86, 87). Indeed, certain monoclonal antibodies with a broad specificity for GlcNAc-containing proteins exhibit strong intranuclear staining (53). When long autoradiographic exposures of labeled WGA-binding proteins isolated from mitotic extracts were examined, the less abundant WGA-binding proteins were also seen to be phosphorylated. The localization and extent of phosphorylation of these proteins will be a future area to be explored.

It is also interesting to note that a number of the minor WGA-binding proteins are revealed to be phosphorylated when egg lysates are treated with the phosphatase inhibitor, okadaic acid (Fig. 7, *lane M*). Under these conditions, highly phosphorylated forms of the three major WGA-binding proteins, as well as a number of the minor WGA-binding proteins, accumulate. The kinase or kinases responsible for this phosphorylation are not known, but it is not likely to be the Cdc2 kinase since okadaic acid alone does not increase H1 kinase activity when added to the lysates (not shown). Moreover, p60, which is heavily phosphorylated in okadaic acid-treated lysates, is not a substrate for Cdc2 kinase *in vitro* (Fig. 7). The role of the phosphorylation events revealed by okadaic acid is not known, but this finding demonstrates that the phosphorylation of pore proteins and other WGA-binding proteins may also occur during interphase. It is possible that okadaic acid acts to stabilize normally transient interphase phosphorylation that is important for the function of these proteins. There exists precedent for such phosphorylation events playing a crucial role in regulating the nuclear import of both lamins and the SV40 T antigen (31, 88, 89). Other transient interphase phosphorylation events are also known to transmit growth regulatory signals from the cell surface to the nucleus of a cell (90). Indeed, it has been found that the maximum size of nuclear transport probes that can be transported though the pore increases as cells are stimulated to divide (91), indicating that the nuclear pore complex is regulated by growth controls. The mechanism of this regulation is not yet known, but may well involve phosphorylation. In assessing other putative roles for pore protein phosphorylation, it is known that nuclear pores are not only assembled into the nuclear envelope at telophase during postmitotic reassembly of the nucleus, but also can insert into existing nuclear envelopes during S phase, when both the size of the nucleus and the number of pores doubles. It is possible therefore that transient interphase phosphorylation of pore proteins not only regulates the function of these proteins in nuclear transport but also their assembly. Clearly, this is a preliminary discussion of such hypotheses. It will be interesting to examine the specific roles of interphase and mitotic phosphorylation in future work.

Acknowledgment—We thank Fang Fang for the gift of the anti-Cdc2 antibody D65.

[4] C. Macaulay, unpublished results.

REFERENCES

1. Robbins, E., and Gonatas, N. K. (1964) *J. Cell Biol.* **21,** 429–463
2. Steinert, G., Baltus, E., Hanocq-Quertier, J., and Brachet, J. (1974) *J. Ultrastruct. Res.* **49,** 188–210
3. Bement, W. M., and Capco, D. G. (1990) *J. Electron. Microsc. Technol.* **16,** 202–234
4. Solomon, M. J. (1993) *Curr. Opin. Cell Biol.* **5,** 180–186
5. Nigg, E. A. (1993) *Curr. Opin. Cell Biol.* **5,** 187–193
6. Newport, J., and Spann, T. (1987) *Cell* **48,** 219–230
7. Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., and Yanagida, M.(1987) *Cell* **50,** 917–925
8. Adachi, Y., Luke, M., and Laemmli, U. K. (1991) *Cell* **64,** 137–148
9. Hirano, T., and Mitchison, T. J. (1993) *J. Cell Biol.* **120,** 601–612
10. Corbett, A. H., Fernald, A. W., and Osheroff, N. (1993) *Biochemistry* **32,** 2090–2097
11. Cardenas, M. E., Walter, R., Hanna, D., and Gasser, S. M. (1993) *J. Cell Sci.* **104,** 533–543
12. Litchfield, D. W., Luscher, B., Lozeman, F. J., Eisenman, R. N., and Krebs, E. G. (1992) *J. Biol. Chem.* **267,** 13943–13951
13. Lee, G. (1993) *Curr. Opin. Cell Biol.* **5,** 88–94
14. Deleted in proof
15. Hirokawa, N. (1994) *Curr. Opin. Cell Biol.* **6,** 74–81
16. Aizawa, H., Kamijo, M., Ohba, Y., Mori, A., Okuhara, K., Kawasaki, H., Murofushi, H., Suzuki, K., and Yasuda, H. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1620–1626
17. Shiina, N., Moriguchi, T., Ohta, K., Gotoh, Y., and Nishida, E. (1992) *EMBO J.* **11,** 3977–3984
18. Yeo, J. P., Alderuccio, F., and Toh, B. H. (1994) *Nature* **367,** 288–291
19. Maul, G. G. (1977) *J. Cell Biol.* **74,** 492–500
20. Gerace, L., and Blobel, G. (1980) *Cell* **19,** 277–287
21. Ottaviano, Y., and Gerace, L. (1985) *J. Biol. Chem.* **260,** 624–632
22. Gerace, L., and Foisner, R. (1994) *Trends Cell Biol.* **4,** 127–131
23. Bloemendal, H., and Peiper, F. R. (1989) *Biochim. Biophys. Acta* **1007,** 245–253
24. Robson, R. M. (1989) *Curr. Opin. Cell Biol.* **1,** 36–43
25. Goldman, A. E., Moir, R. D., Montag-Lowy, M., Stewart, M., and Goldman, R. D. (1992) *J. Cell Biol.* **119,** 725–735
26. Appelbaum, J., Blobel, G., and Georgatos, S. D. (1990) *J. Biol. Chem.* **265,** 4181–4184
27. Courvalin, J. C., Segil, N., Blobel, G., and Worman, H. J. (1992) *J. Biol. Chem.* **267,** 19035–19038
28. Simos, G., and Georgatos, S. D. (1992) *EMBO J.* **11,** 4027–4036
29. Foisner, R., and Gerace, L. (1993) *Cell* **73,** 1267–1279
30. Moir, R. D., and Goldman, R. D. (1993) *Curr. Opin. Cell Biol.* **5,** 408–411
31. Hennekes, H., Peter, M., Weber, K., and Nigg, E. A. (1993) *J. Cell Biol.* **120,** 1293–1304
32. Meier, J., and Georgatos, S. D. (1994) *EMBO J.* **13,** 1888–1898
33. Peter, M., Heitlinger, E., Haner, M., Aebi, U., and Nigg, E. A. (1991) *EMBO J.* **10,** 1535–1544
34. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) *Cell* **61,** 591–602
35. Hocevar, B. A., Burns, D. J., and Fields, A. P. (1993) *J. Biol. Chem.* **268,** 7545–7552
36. Pfaller, R., Smythe, C., and Newport, J. W. (1991) *Cell* **65,** 209–217
37. Vigers, G. P., and Lohka, M. J. (1992) *J. Cell Sci.* **102,** 273–284
38. Finlay, D. R., Newmeyer, D. D., Price, T. M., and Forbes, D. J. (1987) *J. Cell Biol.* **104,** 189–200
39. Finlay, D. R., and Forbes, D. J. (1990) *Cell* **60,** 17–29
40. Lohka, M. J., and Masui, Y. (1984) *J. Cell Biol.* **98,** 1222–1230
41. Lohka, M. J., and Masui, Y. (1983) *Science* **220,** 719–721
42. Newmeyer, D. D., Lucocq, J. M., Burglin, T. R., and DeRobertis, E. M. (1986) *EMBO J.* **5,** 501–510
43. Newmeyer, D. D., Finlay, D. R., and Forbes, D. J. (1986) *J. Cell Biol.* **103,** 2091–2102
44. Blow, J. J., and Laskey, R. A. (1986) *Cell* **47,** 577–587
45. Newport, J. (1987) *Cell* **48,** 205–217
46. Lohka, M. J., and Maller, J. L. (1985) *J. Cell Biol.* **101,** 518–523
47. Miake-Lye, R., and Kirschner, M. W. (1985) *Cell* **41,** 165–175
48. Dunphy, W. G., and Newport, J. W. (1988) *Cell* **55,** 925–928
49. Lohka, M. J., Hayes, M. K., and Maller, J. L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 3009–3013
50. Sheehan, M. A., Mills, A. D., Sleeman, A. M., Laskey, R. A., and Blow, J. J. (1988) *J. Cell Biol.* **106,** 1–12
51. Wiese, C., and Wilson, K. L. (1993) *Curr. Opin. Cell Biol.* **5,** 387–394
52. Davis, L. I., and Blobel, G. (1986) *Cell* **45,** 699–709
53. Davis, L. I., and Blobel, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7552–7556
54. Hanover, J. A., Cohen, C. K., Willingham, M. C., and Park, M. K. (1987) *J. Biol. Chem.* **262,** 9887–9894
55. Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., and Hart, G. W. (1987) *J. Cell Biol.* **104,** 1157–1164
56. Park, M. K., D'Onofrio, M., Willingham, M. C., and Hanover, J. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 6462–6466
57. Hart, G. W., Haltiwanger, R. S., Holt, G. D., and Kelly, W. G. (1989) *Annu. Rev. Biochem.* **58,** 841–874
58. Miller, M. W., and Hanover, J. A. (1994) *J. Biol. Chem.* **269,** 9289–9297
59. Dabauvalle, M. C., Loos, K., and Scheer, U. (1990) *Chromosoma (Berl.)* **100,** 56–66
60. Newmeyer, D. D., and Wilson, K. L. (1991) *Methods Cell Biol.* **36,** 607–634
61. Smythe, C., and Newport, J. W. (1991) *Methods Cell Biol.* **35,** 449–468
62. Murray, A. W., and Kirschner, M. W. (1989) *Nature* **339,** 275–280
63. Solomon, M. J., Glotzer, M., Lee, T. H., Philippe, M., and Kirschner, M. W. (1990) *Cell* **63,** 1013–1024
64. Finlay, D. R., Meier, E., Bradley, P., Horecka, J., and Forbes, D. J. (1991)

J. Cell Biol. **114,** 169–183
65. Murray, A. W., Solomon, M. J., and Kirschner, M. W. (1989) *Nature* **339,** 280–286
66. Fang, F., and Newport, J. W. (1991) *Cell* **66,** 731–742
67. Cordes, V. C., Waizenegger, I., and Krohne, G. (1991) *Eur. J. Cell Biol.* **55,** 31–47
68. Pante, N., Bastos, R., McMorrow, I., Burke, B., and Aebi, U. (1994) *J. Cell Biol.* **126,** 603–617
69. Kita, K., Omata, S., and Horigome, T. (1993) *J. Biochem. (Tokyo)* **113,** 377–382
70. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) *Nature* **349,** 132–138
71. Dasso, M., and Newport, J. W. (1990) *Cell* **61,** 811–823
72. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) *Cell* **60,** 791–801
73. Maller, J. L., and Smith, D. S. (1985) *Dev. Biol.* **109,** 150–156
74. Capony, J. P., Picard, A., Peaucellier, G., Labbe, J. C., and Doree, M. (1986) *Dev. Biol.* **117,** 1–12
75. Karsenti, E., Newport, J. W., Hubble, R., and Kirschner, M. (1984) *J. Cell Biol.* **98,** 1730–1745
76. Gautier, J., Minshull, J., Lohka, M., Glotzer, M., Hunt, T., and Maller, J. L. (1990) *Cell* **60,** 487–494
77. Gautier, J., and Maller, J. L. (1991) *EMBO J.* **10,** 177–182
78. Izumi, T., and Maller, J. L. (1991) *Mol. Cell. Biol.* **11,** 3860–3867
79. Dabauvalle, M. C., Benavente, R., and Chaly, N. (1988) *Chromosoma (Berl.)* **97,** 193–197
80. Harel, A., Zlotkin, E., Nainudel-Epszteyn, S., Feinstein, N., and Fisher, P. A. (1989) *J. Cell Sci.* **94,** 463–470
81. Cordes, V. C., Reidenbach, S., Kohler, A., Stuurman, N., van Driel, R., and Franke, W. W. (1993) *J. Cell Biol.* **123,** 1333–1344
82. Holt, G. D., and Hart, G. W. (1986) *J. Biol. Chem.* **261,** 8049–8057
83. Jackson, S. P., and Tijan, R. (1988) *Cell* **55,** 125–133
84. Jackson, S. P., and Tijan, R. (1989) *Proc. Nat. Acad. Sci. U. S. A.* **86,** 1781–1785
85. Kelly, W. G., Dahmus, M. E., and Hart, G. W. (1993) *J. Biol. Chem.* **268,** 10416–10424
86. Meikrantz, W., Smith, D. M., Sladicka, R. A., and Schlegal, R. A. (1991) *J. Cell Sci.* **98,** 303–307
87. Hart, G. W. (1992) *Curr. Opin. Cell Biol.* **4,** 1017–1023
88. Rih, H. P., Jans, D. A., Fan, H., and Peters, R. (1991) *EMBO J.* **10,** 633–639
89. Da, J., Ackermann, J. J., Bischoff, J. R., Beach, D. H., and Peters, R. (1991) *J. Cell Biol.* **115,** 1203–1212
90. Karin, M. (1994) *Curr. Opin. Cell Biol.* **6,** 415–424
91. Feldherr, C. M., and Akin, D. (1991) *J. Cell Biol.* **115,** 933–939