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Identification of mitosis-specific p65 dimer as a component of human M phase-promoting factor

(p34^{cdc2}/cyclin B/histone H1 kinase/sulfhydryl cycle)

WILLIAM MEIKRANTZ*, FRANK A. SUPRYNOWICZ†, MARGARET S. HALLECK*‡, AND ROBERT A. SCHLEGEL*§

*Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, PA 16802; and †Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **Antisera raised against two mitosis-specific protein kinases from human cells recognized a single 65-kDa polypeptide (p65) that is present in similar amounts in interphase and mitotic cell extracts. Immunoblot analysis of reduced and unreduced extracts revealed that p65 exists as a 65-kDa monomer during interphase but forms a 130-kDa disulfide-linked homodimer during mitosis. Several different antibodies recognizing the p34^{cdc2} protein kinase and cyclin B components of M phase-promoting factor (MPF) coprecipitated p65 from mitotic but not from interphase extracts. In addition, an anti-p65 immunoaffinity column substantially depleted mitotic extracts of histone H1 kinase activity assayed under conditions diagnostic for MPF. These results suggest that active human MPF may be a complex of p34^{cdc2}, cyclin B, and dimeric p65. A sulfhydryl cycle, proposed in the earlier literature on the biochemistry of mitosis, might underlie the dimerization of p65 and formation of active MPF.**

Analysis of cell-division cycle (*cdc*) mutants in yeast has revealed a hierarchy of proteins directing the onset of mitosis, which converges on the 34-kDa protein kinase (p34) encoded by the fission yeast *cdc2* locus (1, 2). A human homolog to p34 identified by DNA complementation (3) and immunoprecipitation (4) is present throughout the cell cycle and is active as a casein kinase (5). At mitosis, a fraction of human p34 is isolated as part of a high molecular mass complex (5, 6) that also includes cyclin B, a protein that accumulates during interphase and is rapidly degraded at anaphase onset (7, 8). Unlike monomeric p34, this complex exhibits histone H1 kinase activity (6) and has been identified as the human equivalent of maturation or M phase-promoting factor (MPF), an activity containing p34 and cyclin B (9–11) that rapidly induces entry into mitosis or meiosis in the absence of protein synthesis in species ranging from starfish to man (12–15).

Examination of histone kinase profiles of human cells, generated by overlaying native gels of cell extracts with [γ-^{32}P]ATP and substrate, has led to the identification of several distinct species whose activities are maximal at mitosis (16, 17). In the present work, polyclonal antibodies raised against two such mitosis-specific protein kinases (A2 and B) have been used to determine their relation to MPF.

MATERIALS AND METHODS

Preparation of Cell Extracts. Human D98/AH-2 cells (ATCC CCL 18.3) were cultured as described (17). Mitotic cells (85–95% mitotic index) were collected by "shake-off" after 18 hr in the presence of 10 ng of nocodazole per ml. Interphase cells were collected by trypsin treatment of monolayers after vigorous washing to remove all mitotic cells. Synchronized cell populations were prepared, and cell cycle distributions were confirmed by flow cytometry exactly as reported (16). Cells were washed; resuspended to 2×10^7 per ml in 15 mM Hepes, pH 7.4/150 mM KCl/5 mM $MgCl_2$/1 mM EGTA/18% (wt/vol) sucrose/1 mM ATP containing phosphatase inhibitors (5 mM each $ZnCl_2$, NaF, 2-glycerophosphate, and Na_3VO_4) and protease inhibitors [1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine chloride, and 10 μg each of aprotinin, leupeptin, pepstatin A, and *o*-phenanthroline per ml (homogenization buffer)]; and lysed by using a Dounce homogenizer with a tight-fitting pestle. Homogenates were centrifuged for 2 hr at 150,000 $\times$ *g*, and the resulting supernatants were stored at −80°C. For the experiment shown in Fig. 3, cells were extracted for 20 min on ice by gentle pipetting in homogenization buffer containing 0.15% bovine serum albumin and 0.2% Triton X-100 (2×10^7 cells per ml) and were used immediately after ultracentrifugation.

Preparation of Antibodies. Mitotic cell extracts were fractionated by native PAGE and overlaid with histone and [γ-^{32}P]ATP to identify the position of protein kinases (16). Regions of the gel corresponding to protein kinase activities A2 or B (16) were macerated and emulsified in complete Freund's adjuvant and injected into female New Zealand White rabbits in a series of intradermal dorsal sites. After 30 days, each rabbit was administered a booster with half the original amount of immunogen; subsequent boosters were given intramuscularly at 6 and 12 months. Screening of sera by ELISA vs. 50 ng of immobilized mitotic extract indicated titers of ≈10,000. IgG was purified from serum by using Affi-Gel protein A (Bio-Rad) according to the manufacturer's protocol.

Immunoblot Analysis. Extracts [either unreduced or reduced with 20 mM dithiothreitol and alkylated with 80 mM iodoacetamide (18)] were separated by SDS/PAGE (19) on 7.5% or 10% gels, transferred to Immobilon-P membranes (Millipore), and dried completely at 37°C. After rehydrating in methanol and rinsing with 50 mM triethanolamine, pH 7.4/0.9% NaCl/2 mM EDTA/0.5% Tween 20/0.1% SDS (wash buffer), membranes were precoated for 20 min with wash buffer containing 3% bovine serum albumin (blocking buffer) and labeled for 2 hr with anti-A2 or anti-B protein kinase antiserum or with preimmune serum (diluted 1:1000 in blocking buffer) for 2 hr. The membranes were then washed, blocked for 10 min, and incubated for 2 hr with ^{125}I-labeled protein A (NEN) diluted to 10^6 cpm/ml in blocking buffer. After labeling, immunoblots were washed, allowed to dry, and visualized with Kodak XAR film. High titers of the A2 and B antisera permitted their use at relatively high dilution

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Abbreviation: MPF, maturation-promoting factor (M phase-promoting factor).

‡Present address: Department of Biochemistry, University of Utah Medical Center, Salt Lake City, UT 84132.
§To whom reprint requests should be addressed.

(up to 1:10,000). At lower dilutions (e.g., 1:100), p65 remained the sole immunoreactive species, with occasional background bands appearing from experiment to experiment with little consistency.

Immunoprecipitation. Extracts were incubated with an excess of anti-A2 or anti-B IgG (2 μg of IgG per 100 μg of crude extract) for 1 hr on ice, followed by adsorption onto fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem). Bound material was washed five times with wash buffer and released by incubation in SDS sample buffer (18). JP4 and J4 ascites (4) and anti-PSK-J3 antiserum (20) were used at 2 μl of ascites or antiserum per 100 μl of cell extract. Affinity-purified antibody against surf clam cyclin B (21) was used at 1 μg per 100 μg of crude extract. For immunoprecipitation with JP4 and J4 ascites, protein G-bearing cells (Omnisorb, Calbiochem) were used in place of Pansorbin, and EDTA and SDS were omitted from the washing buffer, which contained 50 mM NaF and 10 mM 2-glycerophosphate.

Immunoaffinity Chromatography. Periodate-oxidized anti-p65 or control IgG was coupled to Affi-Gel Hz (Bio-Rad) according to the manufacturer's recommendations. Mitotic extract was precipitated with 20–60% $(NH_4)_2SO_4$ to remove cAMP-dependent protein kinase (22) and was applied to the antibody columns in 100 mM Tris chloride, pH 7.4/1 mM EDTA/500 mM NaCl/0.5 mM phenylmethylsulfonyl fluoride/0.1 mM dithiothreitol/50 mM NaF/10 mM 2-glycerophosphate containing 10 μg of leupeptin per ml. The columns were washed with 20 vol of the same buffer without leupeptin and dithiothreitol, followed by 20 vol of buffer containing 1.0 M NaCl. Bound material was eluted sequentially with 0.2 M citrate (pH 3.0) followed by 50% ethylene glycol (pH 8.0). Fractions were assayed for histone H1 kinase activity as described (17, 23, 24) or were separated by SDS/PAGE and transferred to Immobilon membranes. Protein was detected on Immobilon blots by biotinylation using 150 μM *N*-hydroxysuccinimidobiotin (Pierce) in 50 mM borate, pH 9.5/500 mM NaCl/0.2% Tween 20 followed by incubation with 0.1 μCi (1 μCi = 37 kBq) of ^{125}I-labeled streptavidin (Amersham) per ml in 50 mM Tris chloride, pH 7.4/500 mM NaCl/0.2% Tween 20.

RESULTS

Polyclonal antisera against two human mitotic protein kinases (A2 and B described in ref. 16) were used to probe Western blots of unreduced extracts prepared from interphase and mitotic D98/AH-2 cells. In mitotic extract, both the A2 and B antisera recognized 130-kDa polypeptides (Fig. 1, mitosis lanes A2 and B), whereas in interphase extract, both recognized 65-kDa polypeptides (interph lanes A2 and B). Reduction and alkylation of mitotic extract prior to electrophoresis eliminated the 130-kDa immunoreactive species, concomitant with the appearance of 65-kDa forms (mitosis lanes A2′ and B′). No change in immunoreactivity was seen after similar treatment of interphase extracts, where the 65-kDa species persisted (interph lanes A2′ and B′). No signal resulted from probing either reduced or unreduced samples with preimmune serum (mitosis lane P and interph lane P). Identical results were obtained if cells were extracted directly in boiling SDS (data not shown), eliminating the possibility that the higher molecular mass species are artefacts of preparation. Thus, both the A2 and B antisera recognize one or more 65-kDa polypeptides that exist as part of 130-kDa, disulfide-linked species at mitosis.

To ascertain whether the A2 and B antisera recognized the same 65-kDa polypeptide, Western blots of reduced and alkylated A2 and B immunoprecipitates were probed with A2 and B antisera. The 65-kDa protein immunoprecipitated by

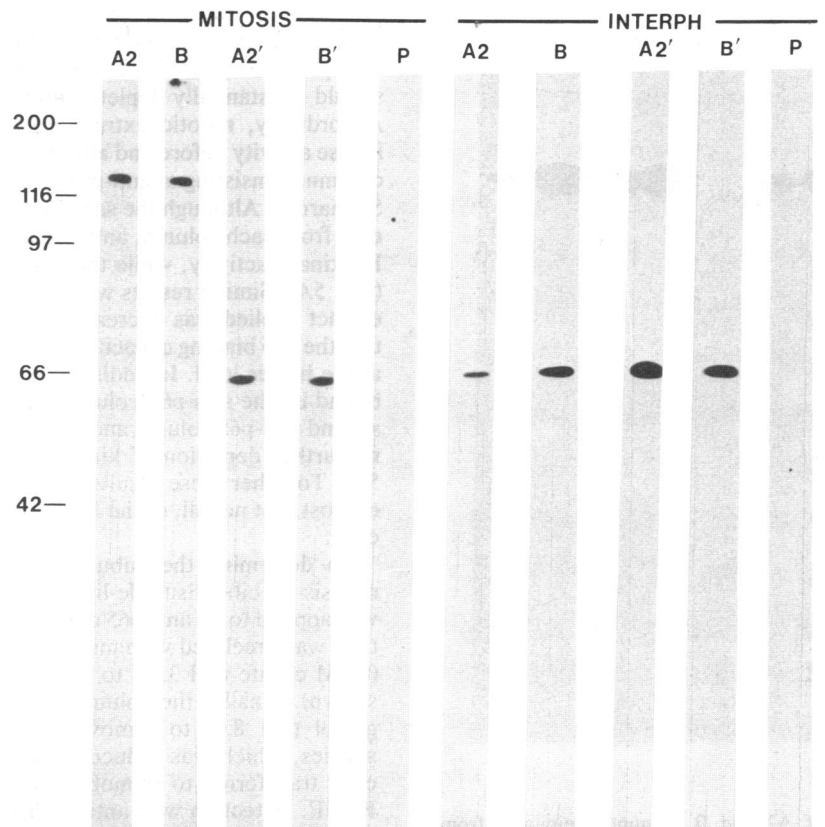

FIG. 1. Immunoblot (Western blot) of interphase (Interph) and mitotic extracts probed with anti-A2 or anti-B protein kinase antiserum or with preimmune serum (lanes A2, B, and P). The prime indicates samples that were reduced and alkylated prior to electrophoresis. Molecular mass markers are indicated in kDa.

A2 antibodies from interphase or mitotic cells was recognized by A2 and B antisera and, similarly, the 65-kDa protein immunoprecipitated by B antibodies from interphase or mitotic extracts was recognized by both B and A2 antisera (Fig. 2). Thus, the antisera recognize a common 65-kDa protein present during interphase and mitosis, referred to now as p65. To determine whether p65 is present throughout interphase, anti-p65 immunoprecipitates prepared from cells synchronized in early G_1, early S, or G_2 phase were reduced, alkylated and analyzed on Western blots with anti-p65. As shown (Fig. 3), no dramatic difference in immunoprecipitated p65 was observed over the course of the cell cycle, in contrast to the cyclins (7, 8).

Since the protein kinases used as immunogens and MPF exhibit mitosis-specific histone kinase activity (6, 16), association of p65 with the p34 and cyclin B components of human MPF was tested. To detect p65 associated with p34 homologs, two monoclonal antibodies (JP4 and J4; ref. 4) and a polyclonal antiserum raised against a bacterial fusion protein containing the C terminus of a human cdc2-related protein (clone PSK-J3; ref. 20) were used to immunoprecipitate p34 from interphase and mitotic cells. The immunoprecipitates were then reduced, alkylated, and analyzed on Western blots probed with anti-p65 antiserum. All three immunoprecipitates from mitotic extracts contained a 65-kDa polypeptide reactive with anti-p65 antiserum (Fig. 4). In contrast, p65 was not coprecipitated from interphase extracts, even though it is present in similar amounts (compare Figs. 1 and 3). Affinity-purified antibodies against surf clam cyclin B (21) also coprecipitated p65, but only from mitotic extracts (Fig. 4). Thus during mitosis, when human cyclin B and a fraction of p34 are present in the high molecular weight complex

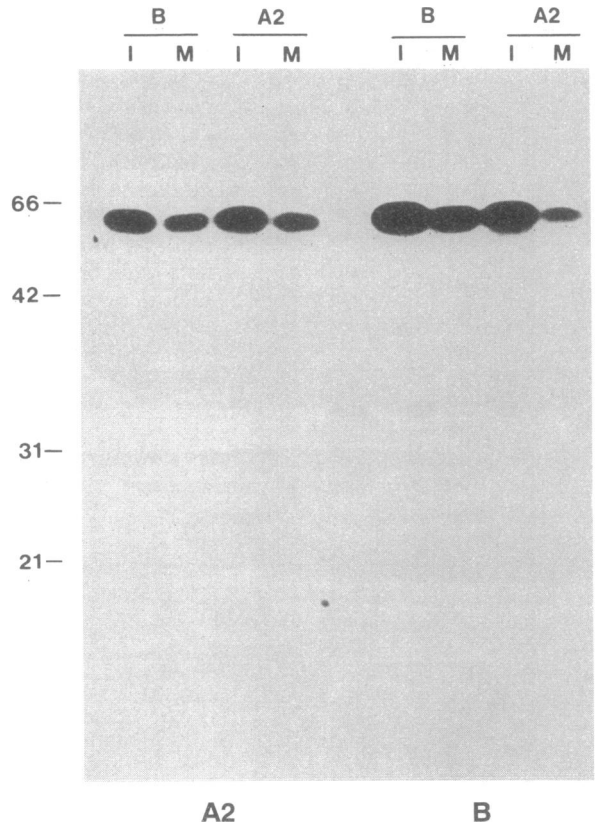

FIG. 2. Western blot of A2 and B immunoprecipitates from interphase (lanes I) and mitotic (lanes M) extracts. Reduced and alkylated immunoprecipitates were separated on 10% gels. The designations above the blot indicate the antibody used for precipitation. Molecular mass is shown in kDa.

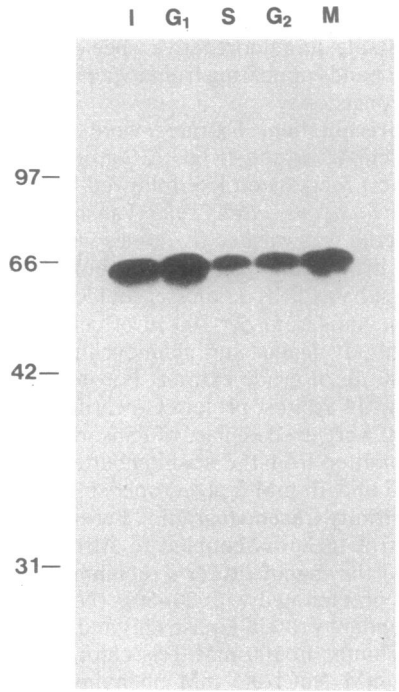

FIG. 3. Western blot of p65 immunoprecipitates from synchronized cells. Reduced and alkylated immunoprecipitates were separated on 10% gels, transferred to Immobilon membranes, and labeled with anti-p65 IgG. Each lane was loaded with 10^5 cell equivalents of p65 immunoprecipitate. Molecular mass is shown in kDa.

characteristic of MPF (5, 6), at least a fraction of these proteins also are associated with p65.

In a variety of cells (10–14), including human cells (6), MPF accounts for most of the Ca^{2+}- and cAMP-independent histone H1 kinase activity during mitosis. If p65 is associated with p34 and cyclin B that are present in MPF, anti-p65 IgG should substantially deplete mitotic extract of this activity. Accordingly, mitotic extract was assayed for histone H1 kinase activity before and after adsorption to immunoaffinity columns consisting of anti-p65 or a control IgG conjugated to Sepharose. Although the same amount of protein was recovered from each column, anti-p65 depleted 60% of the histone H1 kinase activity, while the control IgG removed only 8% (Fig. 5*A*). Similar results were obtained when the amount of extract applied was decreased by 60% (Fig. 5*B*), indicating that the p65 binding capacity of the column was not exceeded at the higher load. In addition, when half of the protein not bound by the anti-p65 column was rechromatographed on a second anti-p65 column and half on a second control column, no further depletion of kinase activity was observed (Fig. 5*C*). Together these results suggest that p65 is a component of most, but not all, of the MPF present in extracts of human cells.

To determine the subunit composition of the 130-kDa mitosis-specific disulfide-linked form of p65, mitotic extract was applied to an anti-p65 column and washed as before, but then was preeluted with mild acid [0.1 M glycine (pH 3.0) or 0.2 M citrate (pH 3.0)] to remove p34 and cyclin (data not shown). Finally, the column was eluted with 50% ethylene glycol (pH 8.0) to remove the 130-kDa immunoreactive species, which was reduced, alkylated, and electrophoretically transferred to Immobilon membranes following SDS/PAGE. Detection with anti-p65 IgG confirmed the presence of p65 in the eluant (Fig. 6, lane 1). Total protein in the fraction, revealed by probing with ^{125}I-labeled streptavidin following biotinylation of blotted proteins, consisted of a single species comigrating exactly with immunoreactive p65

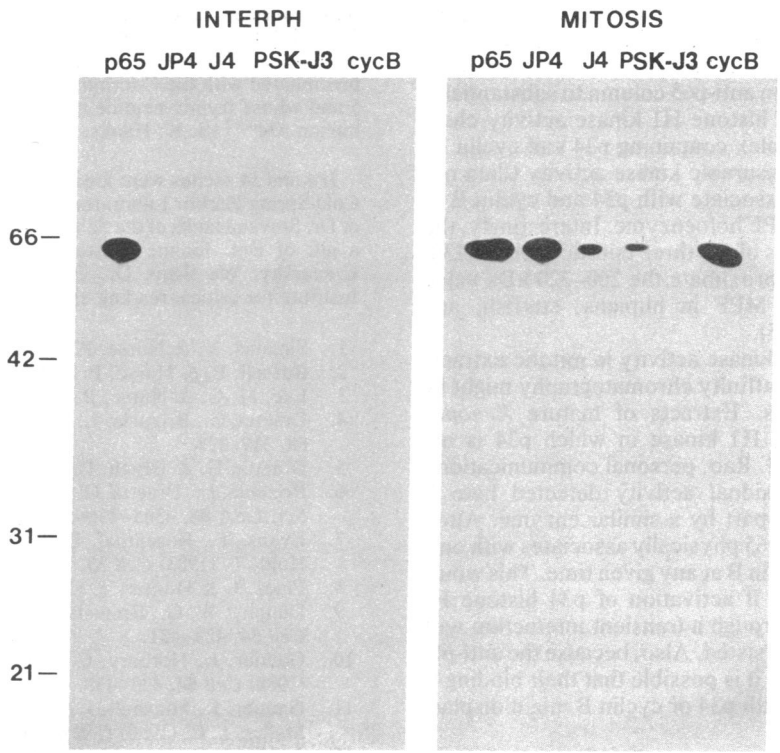

FIG. 4. Western blots of anti-p34 and anti-cyclin B immunoprecipitates from interphase (Interph) and mitotic extracts probed with anti-p65 antibody. The precipitating antibody used is indicated above each lane (*cycB* = cyclin B). Molecular mass is shown in kDa.

(Fig. 6, lane 2). Thus, the 130-kDa form of p65 found at mitosis is a disulfide-linked homodimer.

DISCUSSION

p65 differs in several significant respects from a 62-kDa component of the human mitotic p34 complex (5) subsequently identified as cyclin B (8). In addition to the difference in electrophoretic mobility, metabolic labeling of cells with [^{35}S]methionine does not lead to significant incorporation of radioactivity into p65 under conditions where the 62-kDa species is readily labeled (data not shown), perhaps explaining why it has not been identified previously in anti-p34 immunoprecipitates from extracts of metabolically labeled cells (5). Moreover, unlike cyclin B, levels of p65 do not vary dramatically with the cell cycle.

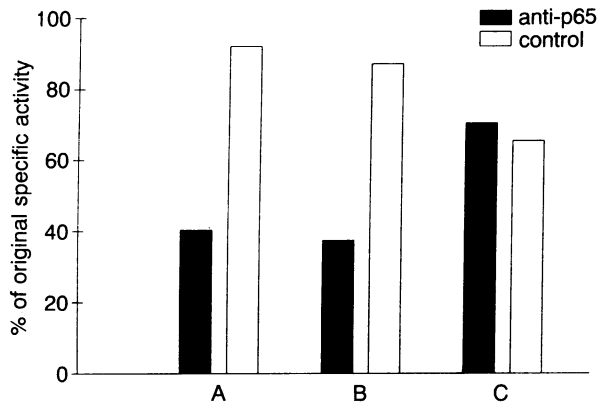

FIG. 5. Depletion of histone H1 kinase activity from mitotic extract by anti-p65 or control IgG. Bars: A, 21 mg of protein was applied; B, 8.5 mg of protein was applied; C, the nonbinding fraction from the anti-p65 column in bar B was divided in two and rechromatographed. Equal amounts of protein were eluted from both the anti-p65 and control IgG columns in each case.

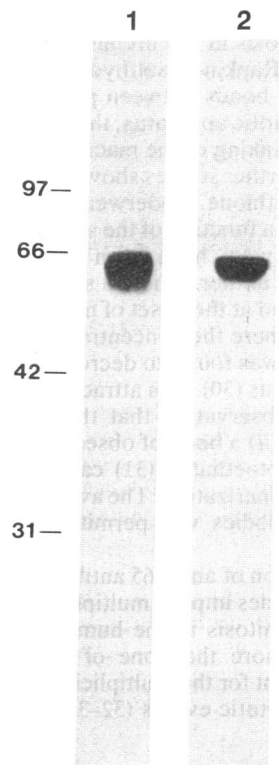

FIG. 6. Blot of anti-p65 column eluant. Reduced and alkylated samples were separated on a 10% gel and transferred to Immobilon membranes. Lanes: 1, blot was probed with anti-p65 antibody; 2, blotted protein was biotinylated with *N*-hydroxysuccinimidobiotin and detected with ^{125}I-labeled streptavidin.

Coprecipitation of p65 by anti-p34 and anti-cyclin B antibodies suggests that a fraction of these molecules might exist as a trimolecular complex during mitosis. This notion is supported by the ability of an anti-p65 column to substantially deplete mitotic extracts of histone H1 kinase activity characteristic of the MPF complex containing p34 and cyclin B. Since p65 itself has no measurable kinase activity (data not shown), some or all may associate with p34 and cyclin B at mitosis to form an active MPF holoenzyme. Interestingly, the combined molecular masses of the three mitotic species (130, 62, and 34 kDa) closely approximate the 200–220 kDa value obtained for the size of MPF in humans, starfish, and *Xenopus* oocytes (6, 13, 25).

The residual histone H1 kinase activity in mitotic extracts following anti-p65 immunoaffinity chromatography might be explained in several ways. Extracts of mature *Xenopus* oocytes contain a histone H1 kinase in which p34 is not present (J. Kuang and P. N. Rao, personal communication); it is possible that the residual activity detected here is accounted for in whole or part by a similar enzyme. Alternatively, it is possible that p65 physically associates with only a fraction of p34 or p34-cyclin B at any given time. This would be the case, for example, if activation of p34 histone H1 kinase activity occurred through a transient interaction with p65, after which activity persisted. Also, because the anti-p65 IgG species are polyclonal, it is possible that their binding to sites on p65 that interact with p34 or cyclin B might displace these molecules.

At mitosis, p65 is converted to a disulfide-linked dimer concomitant with its association with p34 and cyclin B in an active histone H1 kinase complex characteristic of MPF. Thus, it is conceivable that cyclic oxidation and reduction of disulfide bonds linking p65 monomers may drive cycles of MPF assembly and activation. Such a hypothesis draws on the earliest biochemical studies of cell division. Sixty years ago Rapkine (26) observed a decrease in the concentration of soluble sulfhydryl (SH) groups (presumed to be glutathione) at the onset of mitosis in sea urchin embryos. Later, Mazia (27) proposed that Rapkine's sulfhydryl cycle was driving the formation of S—S bonds between protein molecules in the assembly of the mitotic apparatus, the decrease in soluble SH corresponding to linking of the macromolecules by disulfide bridges. Indeed, further studies showed that a soluble protein fraction, not glutathione, underwent cyclic fluctuations in free SH content as a function of the sea urchin cell cycle (28). Similar Rapkine cycles have been reported for tissues as diverse as *Trillium* anther, where a sharp increase in protein disulfides was found at the onset of meiotic metaphase I (29), and HeLa cells, where the concentration of exposed protein sulfhydryl groups was found to decrease during formation of the mitotic apparatus (30). It is attractive to speculate that (*i*) Rapkine's early observation that thiol reagents reversibly block mitosis and (*ii*) a body of observations on the arrest of mitosis by mercaptoethanol (31) can be interpreted as an inhibition of p65 dimerization. The availability of purified p65 and anti-p65 antibodies will permit this hypothesis to be tested directly.

Finally, derivation of anti-p65 antibodies from two distinct protein kinase species implies multiple complexes containing p65 exist during mitosis in the human cell. Should p34 be associated with more than one of these complexes, this finding may account for the multiplicity of effects ascribed to p34 in directing mitotic events (32–34).

Note Added in Proof. Anti-PSK-J3 antiserum immunoprecipitates from HeLa cell lysates a polypeptide that has a slightly different mobility on SDS/polyacrylamide gels than that of $p34^{cdc2}$ immunoprecipitated with the C-terminal peptide antiserum described in ref. 5 and whose tryptic peptide map does not closely resemble that of human $p34^{cdc2}$ (S. K. Hanks, personal communication).

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