Identification of Protein Phosphatase 1 as a Mitotic Lamin Phosphatase*

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In higher eukaryotes, mitosis is characterized by a dramatic structural reorganization of the cell. Mitotic changes include chromosome condensation, mitotic spindle formation, nuclear lamina disassembly, vesiculation of the Golgi and endoplasmic reticulum, and nuclear envelope breakdown (1). Nuclear lamina disassembly is the best characterized of these mitotic processes (2). The nuclear lamina is a proteinaceous scaffold underlying the inner nuclear membrane and is an anchoring site for interphase chromatin (3). The major constituents of the nuclear lamina are the A-, B-, and C-type lamins, which are members of the intermediate filament protein family (3). HL60 cells, a human promyelocytic leukemia cell line, express only lamin B, making them a convenient system for the study of lamin dynamics (4). The lamins contain a central α-helical domain that mediates coiled-coil dimerization (5). Head-to-tail dimer interactions form lamin polymers that associate laterally to form the supramolecular meshwork that constitutes the nuclear lamina (3).

At the onset of mitosis, the nuclear lamins are hyperphosphorylated leading to nuclear lamina disassembly, a process required for nuclear envelope breakdown and entry into mitosis. Multiple lamin kinases have been identified, including protein kinase C, that mediate mitotic lamin phosphorylation and mitotic nuclear lamina disassembly. Conversely, lamin dephosphorylation is required for nuclear lamina reassembly at the completion of mitosis. However, the protein phosphatase(s) responsible for the removal of mitotic phosphates from the lamins is unknown. In this study, we use human lamin B phosphorylated at mitosis-specific sites as a substrate to identify and characterize a lamin phosphatase activity from mitotic human cells. Several lines of evidence demonstrate that the mitotic lamin phosphatase corresponds to type 1 protein phosphatase (PP1). First, mitotic lamin phosphatase activity is inhibited by high nanomolar concentrations of okadaic acid and the specific PP1 peptide inhibitor, inhibitor-2. Second, mitotic lamin phosphatase activity cofractionates with PP1 after ion exchange chromatography. Third, microcystin-agarose depletes mitotic extracts of both PP1 and lamin phosphatase activity. Our results demonstrate that PP1 is the major mitotic lamin phosphatase responsible for removal of mitotic phosphates from lamin B, a process required for nuclear lamina reassembly.

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† The abbreviations used are: PKC, protein kinase C; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A.
assayed for protein phosphatase activity using phosphorylated human lamin B as substrate. To prepare lamin B phosphorylated at mitotic sites, nuclear envelopes were isolated from unsynchronized HL60 cells as described previously (4, 8, 9, 12). Purified nuclear envelopes were phosphorylated using recombinant, baculovirus-expressed human PKC \( \beta_1 \) in a standard PKC reaction buffer containing \( [\gamma-\text{32P}]\text{ATP} \) (8, 9, 12). 1 x 10⁸ 32P-labeled nuclear envelopes were used per reaction and incubated at 25 °C in the presence of late mitotic cell lysate prepared as described above. Routinely, 5 x 10⁶ cell equivalents of late mitotic cell lysate were used in 15-min reactions, conditions under which phosphatase activity was linear. In some cases, the protein phosphatase inhibitors okadaic acid (LC Laboratories) or inhibitor-2 peptide (Calbiochem) were included in the phosphatase assay at the concentrations indicated in the figure legends. Reactions were stopped by the addition of 3 x Laemmli sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis analysis. 32P-Labeled lamin B was visualized by autoradiography, and lamin B phosphorylation was quantitated using a PhosphorImager (Molecular Dynamics).

**Immunoblot Analysis**—The presence of lamin B and PP1 in cell lysates and column fractions was determined by immunoblot analysis. After electrophoresis in either 8% (lamin B) or 12% (PP1) acrylamide gels, resolved proteins were electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell) as described previously (9). Immunoblot analysis was conducted as described previously using either a monoclonal antibody to human lamin B (Matritech), rabbit polyclonal antibodies to the carboxyl-terminal residues 317–330 (RPIT-), antibodies to the carboxyl-terminal residues 296–309 (GEPHVTRRTP-), or monoclonal antibody to human lamin B (Matritech), rabbit polyclonal antibodies to the carboxyl-terminal residues 296–309 (RPIT-PPRNSAKAKK) of rat PP1, or rabbit polyclonal antibodies to the carboxyl-terminal residues 317–330 (RPTTPRNTAVTSL) of the catalytic subunit of PP2A (Upstate Biotechnology), rabbit polyclonal antibodies to the carboxyl-terminal residues 317–330 (RPIT-PPRNSAKAKK) of rat PP1, or rabbit polyclonal antibodies to the carboxyl-terminal residues 317–330 (RPTTPRNTAVTSL) of the catalytic subunit of PP2A (Upstate Biotechnology).

**Fractionation of PP1 by Anion Exchange Fast Protein Liquid Chromatography**—Mitotic cell lysate from 1 x 10⁶ cells was loaded onto a Bio-Scale Q5 column (Bio-Rad) at a flow rate of 4.0 ml/min. The column was washed with 50 mM triethanolamine buffer, pH 7.5, and the column was developed using a linear gradient from 0 to 1.0 M NaCl in 50 mM triethanolamine buffer, pH 7.5. 2-ml fractions were collected, and protein elution profiles were monitored by absorbance at 280 nm. 10 μl of each fraction was analyzed for lamin B phosphatase activity as described above. 50 μl of each fraction was subjected to immunoblot analysis for PP1 content.

**Microcystin-Agarose Affinity Depletion of PP1 and Lamin Phosphatase Activity**—HL60 cells were harvested and lysed in late mitosis as described above. Cell lysates were incubated with microcystin-agarose beads (Upstate Biotechnology) for 10 min at 4 °C. Beads were washed twice with buffer A (50 mM triethanolamine, pH 7.5, 0.1 mM EGTA, 5% glycerol, 0.5 M NaCl, 0.1% β-mercaptoethanol, and protease inhibitors) to remove unbound proteins prior to elution with 3 M NaSCN in buffer A to remove affinity-purified PP1 along with associated subunits. The onput lysate, unbound fraction, and bound eluates were subjected to immunoblot analysis for PP1 as described above. In addition, output lysate and unbound fraction were assayed for lamin B phosphatase activity as described above.

**RESULTS**

**Detection of Lamin B Phosphatase Activity in Mitotic Cells**—Previous studies from our laboratory have examined the role of site-specific phosphorylation of lamin B in the process of mitotic nuclear lamina disassembly (8, 9). We demonstrated that PKC \( \beta_1 \) mediates direct phosphorylation of lamin B at the major mitosis-specific site, Ser405, within the carboxyl-terminal domain of lamin B just prior to metaphase (8, 9, 12). PKC-mediated phosphorylation of lamin B leads to nuclear lamina disassembly in vitro (13), and inhibition of PKC \( \beta_1 \)-mediated phosphorylation of lamin B leads to cell cycle arrest in the G1 phase (9), demonstrating the importance of this phosphorylation event in mitotic nuclear lamina disassembly and cell cycle progression.

In the present study, we wished to identify the cellular phosphatase responsible for removing mitotic phosphate residues from lamin B. For this purpose, we developed an *in vitro* assay system consisting of human lamin B phosphorylated at mitotic sites by recombinant human PKC \( \beta_1 \) as a substrate for phosphatases derived from extracts of cells in late mitosis. When mitotically phosphorylated lamin B is incubated with mitotic cell lysate, a time-dependent loss of [32P]-phosphate label in lamin B is observed (Fig. 1A). Dephosphorylation is not due to an endogenous phosphatase activity within the nuclear envelope because dephosphorylation is not seen upon incubation with diluent alone (Fig. 1A). Loss of phosphate on lamin B is dose-dependent, because addition of increasing amounts of mitotic cell lysate leads to a corresponding decrease in phosphate on lamin B (Fig. 1B). Lamin B is readily dephosphorylated by crude mitotic lysate in a time- and dose-dependent fashion (Fig. 1A and C, respectively), characteristic of an enzymatic activity. Because proteolysis of lamin B could mimic dephosphorylation in this assay, immunoblot analysis for lamin B was performed to establish that loss of 32P-labeled lamin B is not attributable to protein degradation (Fig. 1C). As can be seen, no loss of lamin B mass is observed following incubation with mitotic lysates. Taken together, these results demonstrate that loss of phosphate from lamin B is due to the presence of a protein phosphatase activity in mitotic cell
lysates.

Lamin B Phosphatase Is PP1—Although hundreds of serine/threonine protein kinases phosphorylate substrates within a cell, most dephosphorylation is mediated by only four major classes of serine/threonine protein phosphatases (PP1, PP2A, PP2B, and PP2C) (14). Differences in substrate specificity and inhibitor sensitivity can distinguish between these classes (15). Inhibitor studies with microcystin and okadaic acid have demonstrated that most of the serine/threonine phosphatase activity within a cell is attributable to PP1 and PP2A (14). Interestingly, both of these phosphatase classes have been linked to mitotic events (14, 16–18). Therefore, we suspected that our lamin B phosphatase activity could be one of these two classes, and inhibitor studies were conducted to distinguish between them.

Okadaic acid (LC Laboratories), a potent inhibitor of serine/threonine protein phosphatases, inhibits PP1 with an IC$_{50}$ of 60–200 nM and PP2A with an IC$_{50}$ of 1–2 nM (16, 17, 19). Therefore, we used okadaic acid to characterize the lamin B phosphatase activity (Fig. 2A). Okadaic acid was found to inhibit lamin B phosphatase activity in a dose-dependent fashion with an apparent IC$_{50}$ of 80 nM, consistent with the published IC$_{50}$ of okadaic acid for PP1.

In addition to okadaic acid, inhibitory proteins can also be used to distinguish these two classes of protein phosphatase. Several inhibitory polypeptides (inhibitor-1, inhibitor-2, NIPP-1, and DARPP-32) exist in the cell that are highly selective for PP1, whereas PP2A remains completely insensitive to inhibition by these proteins (15). For this assay, we used inhibitor-2, which can completely abolish PP1 activity at nanomolar concentrations (20). Purified inhibitor-2 potently inhibits lamin B phosphatase activity in a dose-dependent fashion with an apparent IC$_{50}$ of 1.2 nM (Fig. 2B). These studies indicate that the mitotic lamin B phosphatase is PP1.

Anion Exchange Chromatography Distinguishes between PP1 and PP2A—To explore the possibility that multiple lamin B phosphatases are present in mitotic cells, late mitotic cell extracts were fractionated by anion exchange chromatography using a gradient from 0 to 1.0 M NaCl (Fig. 3A). Protein elution profile was monitored by following absorbance at 280 nm (solid line). Column fractions were assayed for lamin phosphatase activity as described under “Experimental Procedures” (12). Immunoblot analyses of the peak fractions with an antibody to PP1 demonstrate that PP1 and lamin phosphatase activity cofractionate (inset). B, anion exchange column fractions were assayed for the presence of PP1 and PP2A by immunoblot analysis as described under “Experimental Procedures.” Immunoblots were quantitated by densitometry, and the levels of PP1 (■) and PP2A (●) were plotted versus fraction number.

Fig. 2. Mitotic lamin phosphatase is inhibited by okadaic acid and inhibitor-2. A, 32P-labeled lamin B was incubated with 5 × 10$^5$ cell equivalents of late mitotic cell lysate for 15 min in the presence of increasing concentrations of okadaic acid. Lamin B phosphorylation was quantitated to determine relative phosphatase activity as described under “Experimental Procedures.” Data represent the means of triplicate experiments ± S.E. B, 32P-labeled lamin B was incubated with 5 × 10$^5$ cell equivalents of late mitotic cell lysate for 15 min in the presence of increasing concentrations of inhibitor-2. Lamin B phosphatase activity was quantitated as described above. Data represent the means of triplicate experiments ± S.E.

Fig. 3. Lamin B phosphatase activity cofractionates with PP1 on anion exchange chromatography. A, mitotic cell lysate was fractionated by anion exchange chromatography using a gradient from 0 to 1.0 M NaCl (■) as described under “Experimental Procedures.” Protein elution profile was monitored by following absorbance at 280 nm (solid line). Column fractions were assayed for lamin phosphatase activity as described under “Experimental Procedures” (12). Immunoblot analyses of the peak fractions with an antibody to PP1 demonstrate that PP1 and lamin phosphatase activity cofractionate (inset). B, anion exchange column fractions were assayed for the presence of PP1 and PP2A by immunoblot analysis as described under “Experimental Procedures.” Immunoblots were quantitated by densitometry, and the levels of PP1 (■) and PP2A (●) were plotted versus fraction number.
Protein Phosphatase 1 Dephosphorylates Lamin B

**DISCUSSION**

PP1 and PP2A are potential mediators of lamin B dephosphorylation because they have both been implicated in mitotic processes (15, 17, 18, 24, 25). The catalytic subunits of these two phosphatases share approximately 40% sequence identity (26). These two phosphatase families are distinguished by their sensitivity to selective phosphatase inhibitors as well as to inhibitory polypeptide subunits (17, 26). In this study we demonstrate that PP1 mediates lamin B dephosphorylation in late mitotic cells.

Genetic studies in yeast, *Aspergillus* and *Drosophila* implicate a role for PP1 in cell cycle regulation (15, 26). In these organisms, mutation of their PP1 homologs leads to mid-miotic arrest or other mitotic defects (24, 27–29). Hence, PP1 activity seems to be required for the completion of mitosis. Several lines of evidence indicate that PP1 plays a role in mitosis in mammalian cells as well. First, exposure of HL60 cells to okadaic acid, a potent inhibitor of PP1, leads to cell cycle arrest at G2/M over a 24-h period (19). Second, microinjection of an inhibitory PP1 antibody into rat embryo fibroblasts at mitosis arrests cells in metaphase (30). Finally, microinjection of active PP1 during anaphase accelerates cytokinesis and reflattening of the injected cell (30).

Immunolocalization studies in rat embryo fibroblasts reveal that PP1 is mainly localized in the cytoplasm of G2 and S phase cells, accumulates in the nucleus during G2 phase, and intensely colocalizes with the nuclear chromatin at mitosis (30). This colocalization suggests the presence of a nuclear targeting subunit as well as an important role in nuclear processes (31). Although there is genetic and biochemical evidence of a role for PP1 in nuclear function and mitosis, little is known about the physiologic mitotic substrates for PP1 (32). To date, only one potential mitotic substrate for PP1, histone H1, has been identified (33).

In this study we demonstrate that mitotically phosphorylated lamin B is a substrate for PP1 isolated from late mitotic cells. We propose that PP1 mediates nuclear lamina reassembly, in part by dephosphorylation of lamin B, based on several lines of evidence. First, HL60 cells treated with a high concentration of okadaic acid enter a mitosis-like state characterized by nuclear lamina disassembly, suggesting that PP1 activity is required to maintain an intact nuclear lamina (19). Second, dephosphorylation of chicken lamin B2 by purified PP1 in vitro allows reassembly of lamin head-to-tail polymers (6). Finally, immunolocalization studies in mitotic rat fibroblasts reveal PP1 translocation to the nuclear chromatin (30), which is the site of nuclear lamina reassembly.

PP1 is a family of serine/threonine phosphatases that play a role in many cellular processes including muscle contraction, protein synthesis, glycogen metabolism, and cell cycle control (27). The activity, substrate specificity, and subcellular localization of PP1 are regulated through association of the PP1 catalytic subunit (PP1C) with one or more regulatory subunits that function in an inhibitory, regulatory, or targeting manner (15, 24, 25, 34, 35). In preliminary studies using microcin affinity chromatography, we have identified at least six proteins associated with mitotic PP1C that are candidates for regulatory or targeting subunits for PP1. Further studies will be required to determine if any of these PP1C-binding proteins convey lamin B specificity. To date, the only known regulator of PP1 that has been implicated in the control of mitosis is sds22. In yeast, sds22 acts as a positive regulator of PP1 and is required for the completion of anaphase (24, 36). Homologs of sds22 have recently been identified as regulatory subunits of PP1 in both rat and human (18, 37). However, immunoblot analysis of the lamin B phosphatase after purification on microcin-agarose using an antibody to sds22 did not reveal its presence (data not shown). Likewise, the major nuclear regulator of PP1, NIPP-1, was not detected by immunoblot analysis of affinity-purified lamin phosphatase (data not shown). Therefore, the polypeptide(s) associated with mitotic PP1C appear to be novel, because they do not correspond to these known PP1 regulators. Future studies will focus on the further identification and biochemical characterization of these PP1-binding proteins and analysis of their role in the mitotic function of PP1C.

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**Fig. 4.** Microcystin-agarose depletes mitotic cell lysates of immunoreactive PP1 and lamin phosphatase activity. A, affinity dephosphorylation of lamin phosphatase activity by microcin agarose. Mitotic cell lysates were assayed for lamin phosphatase activity before and after incubation with microcin-agarose as described under “Experimental Procedures.” Data represent the means of triplicate experiments ± S.E. B, affinity dephosphorylation of PP1 by microcin-agarose. Immunoblot analysis was performed on mitotic cell lysates before incubation with microcin-agarose (lysate), on microcin-depleted lysate (unbound), and on eluates from microcin-agarose (bound) as described under “Experimental Procedures.”

Microcystin-Agarose Depletes PP1 and Lamin B Phosphatase Activity from Late Mitotic Lysate—Microcystin is a cyclic hep-tapeptide produced by most strains of the blue-green algae *Microcystis aeruginosa* and is a potent inhibitor of PP1 (21–23). Microcystin is routinely coupled to agarose beads for rapid affinity purification of these phosphatases along with associated regulatory subunits (14, 22, 23). The activity, substrate specificity, and subcellular localization of PP1 are regulated through association of the PP1 catalytic subunit (PP1C) with one or more regulatory subunits that function in an inhibitory, regulatory, or targeting manner (15, 24, 25, 34, 35). In preliminary studies using microcin affinity chromatography, we have identified at least six proteins associated with mitotic PP1C that are candidates for regulatory or targeting subunits for PP1. Further studies will be required to determine if any of these PP1C-binding proteins convey lamin B specificity. To date, the only known regulator of PP1 that has been implicated in the control of mitosis is sds22. In yeast, sds22 acts as a positive regulator of PP1 and is required for the completion of anaphase (24, 36). Homologs of sds22 have recently been identified as regulatory subunits of PP1 in both rat and human (18, 37). However, immunoblot analysis of the lamin B phosphatase after purification on microcin-agarose using an antibody to sds22 did not reveal its presence (data not shown). Likewise, the major nuclear regulator of PP1, NIPP-1, was not detected by immunoblot analysis of affinity-purified lamin phosphatase (data not shown). Therefore, the polypeptide(s) associated with mitotic PP1C appear to be novel, because they do not correspond to these known PP1 regulators. Future studies will focus on the further identification and biochemical characterization of these PP1-binding proteins and analysis of their role in the mitotic function of PP1C.

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2 L. J. Thompson and A. P. Fields, unpublished results.
the immunoblot analysis of the lamin phosphatase for the presence of sds22 and NIPP-1.

REFERENCES
1. Nigg, E. A. (1993) Curr. Opin. Cell Biol. 5, 187–193
2. Nigg, E. A. (1992) Curr. Opin. Cell Biol. 4, 105–109
3. Eggert, M., Radomski, N., Linder, D., Tripier, D., Traub, P., and Jost, E. (1993) Eur. J. Biochem. 213, 659–671
4. Fields, A. P., Pettit, G. R., and May, W. S. (1988) J. Biol. Chem. 263, 8253–8260
5. McKeon, F. (1991) Curr. Opin. Cell Biol. 3, 82–86
6. Peter, M., Heitlinger, E., Haner, M., Aebi, U., and Nigg, E. A. (1991) EMBO J. 10, 1535–1544
7. Meir, R. D., and Goldman, R. D. (1993) Curr. Opin. Cell Biol. 5, 408–411
8. Goss, V. L., Hocevar, B. A., Thompson, L. J., Stratton, C. A., Burns, D. J., and Fields, A. P. (1994) J. Biol. Chem. 269, 19074–19080
9. Thompson, L. J., and Fields, A. P. (1996) J. Biol. Chem. 271, 15045–15053
10. Murray, N. R., Baumgardner, G. P., Burns, D. J., and Fields, A. P. (1993) J. Biol. Chem. 268, 15847–15853
11. Hoesch, B. A., Burns, D. J., and Fields, A. P. (1993) J. Biol. Chem. 268, 7545–7552
12. Campos, M., Fadden, P., Alms, G., Qian, Z., and Haystead, T. A. J. (1996) J. Biol. Chem. 271, 28478–28484
13. Mubny, M. C., and Walter, G. (1993) Physiol. Rev. 73, 673–699
14. Ajbo, K., Yoda, K., Utsumi, K., and Nishikawa, Y. (1996) J. Biol. Chem. 271, 13197–13201
15. Johnston, J. A., Sloboza, R. D., and Silver, R. V. (1994) Cell Motil. Cytoskeleton 29, 280–290
16. Diniischiotu, A., Beullens, M., Stalmans, W., and Bollen, M. (1997) FEBS Lett. 402, 141–144
17. Ishida, Y., Furukawa, Y., Decaprio, J. A., Saito, M., and Griffin, J. D. (1992) J. Cell. Physiol. 150, 484–492
18. Helpp, N. R., Street, A. J., Elledge, S. J., and Cohen, P. T. W. (1994) FEBS Lett. 340, 93–98
19. Honkanen, R. E., Zwiller, J., Moore, R. E., Dally, S. L., Khatra, B. S., Dukelew, M., and Boynton, A. L. (1996) J. Biol. Chem. 265, 19401–19404
20. Moorhead, G., Mackintosh, R. W., Morrice, N., Gallagher, T., and Mackintosh, C. (1994) FEBS Lett. 356, 46–50
21. Mackintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T. W., Cohen, P., and Mackintosh, C. (1995) FEBS Lett. 371, 236–240
22. Mackelvie, S. H., Andrews, P. D., and Stark, M. J. R. (1995) Mol. Cell. Biol. 15, 3777–3785
23. Ludlow, J. W., and Nelson, D. A. (1995) Semin. Cancer Biol. 6, 195–202
24. Egloff, M. P., Cohen, P. T. W., Reinemer, P., and Barford, D. (1995) J. Mol. Biol. 254, 942–959
25. Tu, J., Song, W., and Carlson, M. (1996) Mol. Cell. Biol. 16, 4199–4206
26. Doonan, J. H., and Morris, N. R. (1989) Cell 57, 987–996
27. Axton, J. M., Dombradi, V., Cohen, P. T. W., and Glover, D. M. (1996) Cell 63, 33–46
28. Fernandez, A., Brautigan, D. L., and Lamb, N. J. C. (1992) J. Cell Biol. 116, 1421–1430
29. Hubbard, M. J., and Cohen, P. (1993) TIBS 18, 172–177
30. Jagiello, I., Beullens, M., Stalmans, W., and Bollen, M. (1995) J. Biol. Chem. 270, 17257–17263
31. Paulson, J. R., Patzlaff, J. S., and Vallis, A. J. (1996) J. Cell Sci. 109, 1457–1447
32. Bollen, M., and Stalmans, W. (1989) Crit. Rev. Biochem. Mol. Biol. 27, 227–281
33. Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508
34. Ohkura, H., and Yanagida, M. (1991) Cell 64, 149–157
35. Renouf, S., Beullens, M., Wera, S., Van Eynde, A., Sikela, J., Stalmans, W., and Bollen, M. (1995) FEBS Lett. 375, 75–78