Inhibitor-2 (I-2) is a regulator of protein phosphatase type-1 (PP1), known to be phosphorylated in vitro by multiple kinases. In particular Thr72 is a Thr-Pro phosphorylation site conserved from yeast to human, but there is no evidence that this phosphorylation responds to any physiological signals. Here, we used electrophoretic mobility shift and immunoblotting with a site-specific phospho-Thr72 antibody to establish Thr72 phosphorylation in HeLa cells and show a 25-fold increase in phosphorylation during mitosis. Mass spectrometry demonstrated I-2 in actively growing HeLa cells was also phosphorylated at three other sites, Ser120, Ser121, and an additional Ser located between residues 70 and 90. In vitro kinase assays using recombinant I-2 as a substrate showed that the Thr72 kinase(s) was activated during mitosis and sensitivity to kinase inhibitors indicated that the principal I-2-Thr72 kinase was not GSK3 but instead a member of the cyclin-dependent protein kinase family. Immunocytochemistry confirmed Thr72 phosphorylation of I-2 during mitosis, with peak intensity at prophase, and revealed subcellular concentration of the phospho-Thr72 I-2 at centrosomes. Together, the data show dynamic changes in I-2 phosphorylation during mitosis and localization of phosphorylated I-2 at centrosomes, suggesting involvement in mammalian cell division.

Inhibitor-2 (I-2)\(^1\) was first identified as a heat stable protein capable of selectively inhibiting the phosphorylase phosphatase activity of the PP1 catalytic subunit (1). Subsequent studies purified a heterodimer containing PP1 and I-2, termed MgATP-dependent phosphatase, and showed that I-2 phosphorylation at Thr72 by GSK3 resulted in transient activation of this complex (2–4). Phosphorylation of I-2 at Ser86, Ser120, and Ser121 by casein kinase II (CKII) promoted phosphorylation at Thr72 by GSK3, but CKII phosphorylation alone had little effect on the activity of the complex. More recent studies suggest that I-2 is subject to phosphorylation by multiple protein kinases, including CKII, CKI, cdc2, mitogen-activated protein kinase, and GSK3 (2–7). A subset of these kinases, also known as Pro-directed kinases, namely GSK3, cdc2, and mitogen-activated protein kinase, specifically phosphorylate I-2 in the conserved Thr72-Pro site and therefore may modulate PP1 function in cells.

Phosphorylation of I-2 in animal tissues has been studied. Depaoli-Roach and Lee (8) immunoprecipitated I-2 from \(^{32}\)P metabolically labeled mouse diaphragm. Phospho-amino acid analysis showed that I-2 was phosphorylated on both serine and threonine, with nine times more \(^{32}\)Pphosphoserine than \(^{32}\)Pphosphothreonine. Holmes et al. (9) purified I-2 from rabbit skeletal muscle and using mass spectrometry showed that Ser96 (0.7 mol/mol), Ser120, and Ser121 (0.3 mol/mol each) were phosphorylated, but no phosphorylation of Thr72 was detected. Lawrence et al. (10) immunoprecipitated I-2 from \(^{32}\)P-labeled mouse fat cells and showed the presence of both \(^{32}\)Pphosphoserine and \(^{32}\)Pphosphothreonine, with the phosphothreonine level being less than 10% of phosphoserine. Moreover, despite the fact that GSK3 is inactivated in response to insulin, threonine phosphorylation of I-2 was unchanged by insulin treatment of the fat cells (10). This suggested that phosphorylation of Thr72 in vivo was most likely not regulated by GSK3. Physiological regulation of Thr72 phosphorylation in I-2 has not been demonstrated.

To analyze the phosphorylation of I-2 in living cells we generated a phospho-site-specific antibody that uniquely recognized I-2 phosphorylated at Thr72. Using this antibody, we show here that I-2 is indeed phosphorylated on Thr72 in cells, and this modification is enhanced nearly 25-fold during mitosis. Using kinase inhibitors we tested whether Thr72 phosphorylation of I-2 in mitotic cells was catalyzed by GSK3 or cyclin-dependent protein kinases. Immunostaining showed that phosphorylation of I-2 at Thr72 in mitotic cells was concentrated at centrosomes. The data suggested that reversible phosphorylation of I-2 may control centrosome-associated PP1 activity, which could play a role in mammalian cell division.

Experimental Procedures

**General Reagents—**Tissue culture media and sera were obtained from In Vitrogen. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% neonatal calf serum at 37 °C in 5% CO\(_2\). Roscovitine and GSK3 inhibitor (3-(3-carboxy-4-chloroanilino)-4-(3-nitrophenyl)maleimide) were purchased from Calbiochem. Chemicals were from Sigma unless otherwise noted. Recombinant I-2 was purified as previously described by Eto et al. (11).

**Cell Synchronization and Cell Lysate Preparation—**HeLa cells were synchronized by incubating with 400 μM nocodazole for 24 h or with 200 ng/ml nucodazole for 16 h. Cells were rinsed with PBS and scraped into SDS-containing sample buffer.

**Inhibitor 2 Immunoprecipitation—**HeLa cells were rinsed with room temperature PBS, and the dish was plated on a mixture of acetone and dry ice, and 1 ml of ice-cold 5% trichloroacetic acid was added for 10
min. Cells were scraped from the dish using a Costar cell lifter. The slurry was transferred to a 1.7-ml microcentrifuge tube and centrifuged for 10 min at 10,000 \( \times \) g. The supernatant was discarded, and the pellet was washed twice with cold acetone and resuspended in urea resolubilization buffer (9 mM urea, 20 mM Tris, 23 mM glycine, 1 mM EDTA, and 10 mM \( \beta \)-glycerophosphate). This suspension was incubated at 30 \( ^\circ \) C for 1 h until the protein pellet was dissolved. The solution was diluted 10-fold with immunoprecipitation buffer (1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris, pH 8, 1 mM EDTA, 1 mM EGTA, 0.5% deoxycholate, 20 mM \( \beta \)-glycerophosphate, 1 mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 0.4% Pefabloc, 1\% Microcystin-LR). After centrifugation for 10 min at 10,000 \( \times \) g the supernatant was used for I-2 immunoprecipitation. Briefly, the lysate was incubated at 4 \( ^\circ \) C for 2 h with I-2 antibody coupled to protein G beads. The beads were pelleted and washed once with wash buffer (0.1% Nonidet P-40, 50 mM MOPS, pH 7.4, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 1\% Microcystin-LR, 20 mM \( \beta \)-glycerophosphate, 1 mM sodium orthovanadate, 0.1\% 2-mercaptoethanol) three times. The beads were resuspended in SDS-containing sample buffer, and the proteins were resolved by SDS-PAGE.

**Mass Spectrometric Analysis**—The protein band corresponding to I-2 was excised from the gel and destained with 0.5\% of 50\% methanol/5\% acetic acid overnight at room temperature before dehydation in 200\% of acetonitrile and drying in a vacuum centrifuge. The proteins were reduced by addition of 50\% of 10\% dithiothreitol and alkylated by addition of 50\% of 100\% iodoacetamide (each for 30 min at room temperature). The gel slices were dehydrated in 200\% of acetonitrile, hydrated in 200\% of 100\% ammonium bicarbonate, and dehydrated again in 200\% of acetonitrile. The dehydrated gel pieces were again dried in a vacuum centrifuge and rehydrated in 50\% of 20\% ice-cold, sequencing-grade modified porcine trypsin (Promega) for 5 min on ice. Excess trypsin was removed, and digestion was carried out overnight at 37 \( ^\circ \) C. The peptides released by the digestion were collected by successive extractions with 50\% of 50\% ammonium bicarbonate and 50\% of 50\% acetonitrile (both for 30 min) at room temperature. The combined extract was concentrated in a vacuum centrifuge to 20\% prior to an analysis on a Finnigan LCQ (ThermoQuest) ion-trap mass spectrometer with a Protea nanospray ion source that was interfaced to a self-packed 8-cm C18 reverse-phase capillary column. The peptides (0.5\% \( \mu \)l) were injected and eluted from the column with an acetonitrile/0.1\% acetic acid gradient (2–85\% acetonitrile at 30 min) at a flow rate of 0.25 \( \mu \)l min\(^{-1}\). The microspray ion source was set at 2.8 kV, and the data were analyzed using full data-dependent acquisition routine with the amino acid sequence acquired in the four scans before the cycle repeats. This produced ~500 tandem mass spectrometry spectra of peptides ranging in abundance and captured with a Hamamatsu C4742 digital camera operated by OpenLab software (Improvision) and processed in Adobe Photoshop.

**RESULTS**

**Specificity of Phospho-site Antibody against P-Thr\(^{72}\) of I-2**—To examine I-2 phosphorylation at Thr\(^{72}\) in HeLa cells we developed an phospho-site-specific antibody using a phosphorylated peptide corresponding to the amino acid sequence surrounding this site in human I-2. The antibody was affinity purified using differential binding between unphospho- and phospho-I-2. Specificity was confirmed with recombinant I-2 (rI-2) that had been phosphorylated in vitro using purified protein kinases. As seen in Fig. 1, the antibody did not cross-react with the unphosphorylated rI-2 or rI-2 phosphorylated by CKII alone (i.e., phosphorylated at Ser\(^{86}\), Ser\(^{120}\), and Ser\(^{121}\)). However, the phospho-Thr\(^{72}\) antibody recognized rI-2 that had been phosphorylated by the combination of GSK3 and CKII, which is well known to produce I-2 phosphorylation at Thr\(^{72}\), in addition to Ser\(^{86}\), Ser\(^{120}\), and Ser\(^{121}\). Thus, the antibody only recognized I-2 that was phosphorylated at Thr\(^{72}\).

**Phosphorylation of I-2 in Living Cells**—HeLa cells were prepared at different phases of the cell cycle, either unsynchronized (U, mostly interphase; see Fig. 2) or synchronized by addition of mimosine (to block at S phase) or nocodazole (to block at mitosis). Cell lysates were subjected to SDS-PAGE and blotted by Western blotting (Fig. 2A). I-2 migrated as a single band in either unsynchronized or S-phase cells and was recognized by anti-I-2 antibody. This form of I-2 was not reactive with phospho-Thr\(^{72}\) antibody, suggesting that Thr\(^{72}\) in I-2 was predominantly unphosphorylated in these cells. However, the I-2 recovered from nocodazole-treated cells displayed an additional band of I-2 with decreased mobility. This lower mobility form of I-2 was readily recognized by the anti-phospho-Thr\(^{72}\) antibody, showing phosphorylation of I-2 at Thr\(^{72}\) in cells during mitosis.

We immunoblotted total cell proteins from unsynchronized and mitotic HeLa cells with the phospho-Thr\(^{72}\) site-specific antibody (Fig. 2B). In proteins from mitotic cells only one band corresponding to phospho-Thr\(^{72}\) I-2 was specifically stained near 35 kDa, and this was not detected in unsynchronized cells. One nonspecific band at 50 kDa was stained in both samples with equal intensity. These results further demonstrated the high degree of specificity of the phospho-Thr\(^{72}\) antibodies and confirmed that phosphorylation of I-2 at the Thr\(^{72}\) site occurred in particular during mitosis.

To directly determine the phosphorylation state of all the other sites in I-2 in unsynchronized HeLa cells, we employed the trichloroacetic acid fixation method. This protocol was first...
Fig. 2. Endogenous I-2 in different phases of the cell cycle. A, HeLa cells were unsynchronized (U) or synchronized in S phase using mimosine (S) or in mitosis with nocodazole (M). Cells were lysed directly into an SDS-containing sample buffer, separated by SDS-PAGE, and immunoblotted. The upper panel shows staining with anti-I-2, and the lower panel shows staining with anti-phospho-Thr\(^{72}\) I-2. Arrows show position of phospho-Thr\(^{72}\) I-2 (upper) and I-2 (lower). B, immunoblotted total HeLa cell proteins with phospho-Thr\(^{72}\) site-specific antibodies. Unsynchronized (U) or mitotic (M) HeLa cells were lysed into sample buffer and immunoblotted with anti-phospho-Thr\(^{72}\) I-2 antibodies. Arrow indicates position of I-2.

developed for immunohistochemistry, to preserve the phosphorylation state of proteins that were otherwise rapidly dephosphorylated (12). We adapted this method to purify phosphorylated I-2. Cells were directly treated with trichloroacetic acid, scraped, and centrifuged, and the trichloroacetic acid-precipitated proteins were resuspended in a saturated urea solution. Following the dilution of the urea solution, I-2 was immunoprecipitated, resolved by SDS-PAGE, and visualized by Coomassie blue staining. The I-2 protein was excised from the gel and digested with trypsin. Mass spectrometry revealed that Ser\(^{120}\) and Ser\(^{121}\) were phosphorylated to a stoichiometry of 0.9 mol/mol each. An additional phosphate was also detected in a peptide corresponding to residues 70–90 (which contains a single serine residue, Ser\(^{86}\)), but this could not be further analyzed because of low yield of this peptide (Fig. 3). Alkaline phosphatase treatment of HeLa cell lysates resulted in conversion of I-2 to a single band that migrated faster than all of the forms seen in Fig. 2A, consistent with Ser phosphorylation of I-2 in cells throughout the cell cycle (data not shown).

Thr\(^{72}\) Phosphorylation of I-2 during Mitosis—To examine in more detail the phosphorylation of I-2 on Thr\(^{72}\) during mitosis, we employed immunoblotting with the phospho-Thr\(^{72}\) site-specific antibody using unsynchronized (U) and mitotic cells (M) (see Fig. 4) isolated by shaking off cells from nocodazole-treated cultures. The shake-off procedure greatly enriched the population of mitotic cells, relative to nocodazole treatment alone. Using approximately the same amount of total I-2 protein in the samples, immunoblotting with anti-phospho-Thr\(^{72}\) demonstrated a 25-fold increase in I-2 phosphorylation at Thr\(^{72}\) in mitotic HeLa cells (Fig. 4).

We prepared lysates from unsynchronized, mimosine-synchronized, or nocodazole-synchronized HeLa cells to determine whether increased I-2 (Thr\(^{72}\)) phosphorylation during mitosis corresponded to an increase in kinase specific activity. These lysates were used as source of I-2 kinase activity. The same amount of lysate protein was incubated with recombinant I-2 plus MgATP. The results of the phosphorylation reactions were analyzed by Western blotting with the anti-phospho-Thr\(^{72}\) antibody (Fig. 5A). Unsynchronized cells displayed a low level of I-2 (Thr\(^{72}\)) kinase activity. In contrast, little or no I-2 (Thr\(^{72}\)) kinase activity was observed in lysates of mimosine-synchronized cells. Lysates from nocodazole-treated cells displayed significantly higher I-2 (Thr\(^{72}\)) kinase activity, indicating that increased I-2 (Thr\(^{72}\)) phosphorylation observed during mitosis resulted from the activation of a mitotic I-2 (Thr\(^{72}\)) kinase.

Studies with purified protein kinases have suggested that GSK3 is the most effective I-2 (Thr\(^{72}\)) kinase. To test whether GSK3 was the major I-2 kinase in mitotic cells we assayed I-2 phosphorylation by mitotic cell lysates in the presence of kinase inhibitors. Addition of a known GSK3 inhibitor (3-(3-carboxy-4-chloroanilino)-4-(3-nitrophenyl)maleimide) had no effect on the I-2 Thr\(^{72}\) kinase activity of the mitotic cell lysates (Fig. 5B). Another GSK3 inhibitor (2-thio(3-iodobenzyl)-5-(1-pyridyl)-(1,3,4)-oxadiazole) likewise had no effect (not shown). In contrast, roscovitine, an inhibitor of cyclin-dependent protein kinases (CDK), decreased I-2 Thr\(^{72}\) phosphorylation by 60% (Fig. 5B). The results show that in mitotic cells phosphorylation of Thr\(^{72}\) in I-2 was not because of GSK3, but probably a CDK.

Analysis of I-2 (Thr\(^{72}\)) Phosphorylation in Cells—HeLa cells were treated using immunohistochemistry with the phospho-Thr\(^{72}\) site-specific I-2 antibody (Fig. 6A). We demonstrated the specificity of this antibody in this protocol, in addition to the results seen in Fig. 2. Pre-mixing the antibody with 1 μM

![Fig. 3. Mass spectrometry shows multiple sites of Ser phosphorylation in endogenous HeLa cell I-2. I-2 was purified from mimosine-synchronized HeLa cells by direct trichloroacetic acid precipitation followed by immunoprecipitation and resolved by SDS-PAGE. The sample was subjected to mass spectrometry as described under "Experimental Procedures." The top part of the figure shows the I-2 sequence with the phosphorylated residues as enlarged letters and the recovered peptides indicated by lines. The chart shows the observed and predicted masses and the sequences of the recovered peptides.](image-url)
phospho-Thr72 I-2 abolished all the immunostaining (Fig. 6C), in contrast with pre-mixing with 1 μM unphosphorylated I-2, which had little effect on staining (Fig. 6E). DNA was stained using DAPI (shown in Fig. 6B, D, and F) to show the alignment of condensed chromosomes at the metaphase plate, establishing that the stained cells were at the same stage of mitosis. Staining of HeLa cells with an I-2 antibody (Fig. 7A) and with the phospho-Thr72 antibody (Fig. 7B) revealed the most intense staining for phospho-Thr72 occurred at the centrosomes. This is highlighted in the merged images of total plus phospho-I-2 (panel C) and in the phospho-Thr72 minus total I-2 images (panel D). Quantitation of the phospho-Thr72 staining relative to total I-2 revealed that I-2 (Thr72) phosphorylation peaked in prophase cells and decreased as cells progressed through mitosis (Fig. 7).

DISCUSSION

Mammalian PP1 activity is controlled by multiple regulatory subunits and inhibitor proteins. The most ancient of the PP1 regulators is I-2, called Glc8 in yeast, and the most highly conserved region of the protein surrounds the Thr-Pro phosphorylation site, with the sequence IDEPXYTPY. Mutation of the corresponding residue Thr118 in yeast Glc8 prevents rescue of temperature-sensitive mutants of Ipl1 protein kinase, indicating that this phosphorylation site is important for function (13). Numerous biochemical studies established that I-2 forms a 1:1 complex with the PP1 catalytic subunit to form a heterodimer termed MgATP-dependent phosphatase (2, 3). The activity of this latent heterodimer was transiently increased following the phosphorylation of I-2 at Thr72 by GSK3 (2–4). This proposed regulatory mechanism has remained controversial in part because previous studies have failed to demonstrate regulation of I-2 (Thr72) phosphorylation in vivo.

In this study we produced a new experimental tool, namely an anti-phospho-Thr72 antibody, and showed I-2 becomes phosphorylated on Thr72 in living cells during mitosis. We also utilized gel mobility shifts and mass spectrometry to demonstrate that I-2 was phosphorylated throughout the cell cycle on three Ser residues. Our tests of kinase inhibitors indicate that GSK3, the best known I-2 kinase, contributes little to phosphorylation of I-2 during mitosis. Instead, the data point to other mitotic protein kinases. Roscovitine, a broad spectrum CDK inhibitor, blocked 60% of the I-2 (Thr72) kinase activity when used at a dose more than 10 times the IC50 for the purified cyclinB/cdc2 kinase complex. Thus, we conclude that one or more CDKs account for the majority of the mitotic I-2 (Thr72) kinase activity. Consistent with this finding, recent biochemical and genetic studies in yeast implicate PHO85, a cyclin-dependent protein kinase homologous to mammalian CDK5, as the sole kinase that phosphorylates Glc8 at the Thr118 site that is homologous to Thr72 (14). In addition, in HeLa cells there is another fraction of mitotic I-2 (Thr72) kinase that is resistant to roscovitine and as such is presumably distinct from the CDK family. Further work is required to establish the identity of this mitotic I-2 kinase.
Timely changes in phosphorylation of mitotic substrates require precise coordination of protein kinase and phosphatase activities. Genetic evidence with yeast, other fungi, and fruit flies points to a critical role for PP1 in various aspects of mitosis (15–19). Inhibition of PP1 activity leads to premature mitotic entry (20–22). In fungi and in mammals, PP1 activity appears particularly important for the metaphase-anaphase transition (16, 23, 24). However, the mechanisms that regulate PP1 activity during various stages of mitosis remain largely unknown.

Interestingly, we found phosphorylation of I-2 (Thr 72) rose and fell during stages of mitosis with a peak at prophase. This suggests a highly dynamic regulation of I-2 and its associated PP1. These results are consistent with prior studies that showed PP1 activity is suppressed at entry into mitosis but required for the metaphase-anaphase transition (24). PP1 activity in mammalian cells also seems to be suppressed during telophase, to promote cell spreading (24). Thus, the cell cycle-dependent phosphorylation and dephosphorylation of I-2 on Thr 72 may play a role in fluctuations in PP1 activity during the cell division cycle (2, 3, 16, 20–23, 25). Measuring the activity of any one of the many forms of PP1 present in cells (associated with I-2 or not) is a daunting task that would require physical separation, as well as specific substrates.

The physiological function(s) of I-2 in mammalian cells is not well understood, and the presence of multiple pseudogenes has made gene deletion problematic. By demonstrating that I-2 (Thr 72) phosphorylation at mitosis is highly focused at centrosomes, our data at least begin to point to a potential physiological role for this conserved modification. Our own recent evidence showed that I-2 is concentrated at centrosomes and associates in a complex with PP1 and the kinase Nek2. Overexpression of I-2 produced premature centrosome separation, consistent with activation of the Nek2 kinase (26). Nek2 is of specific interest, because it is localized at centrosomes, and prior studies have implicated a role for the NEK2/PP1 complex in the coordinate control of the phosphorylation of the associated substrate, cNAP (27). However, this is not the only target for I-2 action in cells, because I-2 also associates with neurabin/...
spinnophilin, as well as a novel transmembrane Ser/Thr kinase called KPI-2 (28, 29). Work of a decade ago (30) documented changes in the levels of I-2 during the cell cycle, with peak expression during mitosis, indicating a possible role for I-2 in mitosis. We speculate that phosphorylation of I-2 at Thr\(^{72}\) during mitosis plays a role in the control of centrosomes and maybe in other events at this critical stage of mammalian cell division.

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