Polo-like kinases (Plks) control multiple important events during M phase progression, but little is known about their activation during the cell cycle. The activities of both mammalian Plk1 and Xenopus Plx1 peak during M phase, and this activation has been attributed to phosphorylation. However, no phosphorylation sites have previously been identified in any member of the Plk family. Here we have combined tryptic phosphopeptide mapping with mass spectrometry to identify four major phosphorylation sites in Xenopus Plx1. All four sites appear to be phosphorylated in a cell cycle-dependent manner. Phosphorylation at two sites (Ser-260 and Ser-326) most likely represent autophosphorylation events, whereas two other sites (Thr-201 and Ser-340) are targeted by upstream kinases. Several recombinant kinases were tested for their ability to phosphorylate Plx1 in vitro. Whereas xPlkk1 phosphorylated primarily Thr-10, Thr-201 was readily phosphorylated by protein kinase A, and Cdk1/cyclin B was identified as a likely kinase acting on Ser-340. Phosphorylation of Ser-340 was shown to be responsible for the retarded electrophoretic mobility of Plx1 during M phase, and phosphorylation of Thr-201 was identified as a major activating event.

Over the past few years, Polo-like kinases (Plks) have emerged as major regulators of mitotic progression (1–4). The most extensively studied members of this family comprise Cdc5p (Saccharomyces cerevisiae), Polo1p (Schizosaccharomyces pombe), Polo (Drosophila melanogaster), Plk1 (mammals), and Plx1 (Xenopus laevis). At the G2/M transition and during early stages of mitosis, Plks have been implicated in the maturation of centrosomes (5–7), the activation of Cdk1/cyclin B (8–12), the disassembly of the Golgi complex (13, 14), and the removal of cohesin subunits from chromatin (15). At later stages, Plks also play important roles in the regulation of mitotic exit (16–22) and cytokinesis (23–27). Although not all of the above functions have been demonstrated in all species, there is no doubt that Plks are important regulators of cell division in all eukaryotes. Furthermore, human Plk1 is overexpressed in many tumors (e.g. Refs. 28, 29), and constitutive expression of Plk1 is able to transform mouse NIH-3T3 cells (30).

Both Plk levels and activities are regulated during the cell cycle. In mammalian cells, Plk1 protein peaks at the G2/M transition, at least in part as a result of transcriptional regulation (31). Upon exit from M phase Plk1 levels drop, apparently due to ubiquitin-dependent proteolysis (17, 22, 32, 33). Most important in the present context, variations in Plk1 protein levels are not sufficient to explain the sharp increase in Plk1 activity that is observed at the G2/M transition (34). Instead, Plk activation is accompanied by phosphorylation (34, 35), and phosphatase treatment of mitotic Plk1 reduces kinase activity to interphase levels (26, 36, 37). These findings demonstrate that phosphorylation represents an important mechanism for Plk activation. Moreover, recent evidence indicates that the DNA damage checkpoint interferes with this activating mechanism (38).

To better understand the signaling pathways converging onto Plks, it will be important to map the physiologically relevant phosphorylation sites in these kinases and to identify the enzymes (kinases and phosphatases) acting upon individual sites. A first attempt at identifying relevant phosphorylation sites focused on a number of highly conserved serine and threonine residues within the catalytic domains of Plks, and site-directed mutagenesis of Xenopus Plx1 pointed to serine 128 and threonine 201 as potential activating phosphorylation sites (25, 39). Mutation of either one of these residues to aspartic acid caused a partial activation of Plx1, and strong activation was seen upon simultaneous mutation of both residues (39). However, whether Ser-128 or Thr-201 are actually phosphorylated has not been determined.

Most interestingly, a putative regulatory kinase, termed xPlkk1, has been purified and cloned from Xenopus egg extracts (40). xPlkk1 is itself regulated during the cell cycle, and the available evidence suggests that xPlkk1 acts upstream of Plx1 (40). A related mammalian kinase, termed SLK, was also shown to phosphorylate mouse Plk1 in vitro (41). Both xPlkk1 and SLK belong to a subfamily of Ste20-related enzymes, indicating that they could be orthologs.

Here we have performed a detailed molecular analysis of the phosphorylation state of Plx1 in both interphase and M phase Xenopus egg extracts. In parallel, we have analyzed the okadaic acid (OA)-induced phosphorylation of Plx1 expressed from a recombinant baculovirus in insect cells. Our studies identify four physiological phosphorylation sites in Plx1. The identification of Thr-201, a highly conserved residue within the so-called T-loop (or activation loop) of Plx1, confirms the prediction that this residue might represent an important
Phosphorylation site (39). The three other sites identified here (serines 260, 326, and 340) were not previously predicted. Finally, we show that Cdk1/cyclin B readily phosphorylates Plx1 on Ser-340, whereas PKA can be used to activate Plx1 through phosphorylation of Thr-201.

EXPERIMENTAL PROCEDURES

Mutagenesis and in Vitro Synthesis of Plx1—Site-directed mutagenesis of Plx1 (42) was performed using a transformer mutagenesis kit (CLONTECH Laboratories) for the T201A and T201D mutations, and the QuikChange kit (Stratagene) was used for all other mutations. All mutagenesis reactions were performed by sequencing. Mutated Plx1 constructs were generated as described previously (42), and the corresponding proteins were expressed by transcription-translation, using the TNT coupled reticulocyte lysate system (Promega).

Preparation of Baculoviruses, SF9 Cell Culture, and Purification of Recombinant Proteins—Baculovirus coding for His6-Plx1 (WT) and His6-Plx1 (N172A) have been described previously (9). Other His6-Plx1 mutants were produced with appropriate pVL1393 constructs, using the BaculoGold transfection kit (BD PharMingen). The viruses for human cyclin B1 and Cdk1-His6 were obtained from J. Pines (Wellcome/ Cancer Research Campaign Institute, Cambridge), and the virus coding for xPlk1-His6 (40) was kindly provided by J. Miller (University of Colorado, Denver, CO). Sf9 cells (Invitrogen) were grown in T.C.100 medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin (Invitrogen) at 27 °C prior to infection with baculoviruses. To study the phosphorylation of Plx1 proteins in SF9 cells, the medium was changed 42 h post infection to SF-1 medium without phosphate (Amimed). (This medium was supplemented with fetal calf serum that had been dialyzed extensively against 150 mM NaCl). One hour later, cells were placed into SF-1 medium containing 200 μM carrier-free [32P]orthophosphoric acid solution (Amersham Biosciences, Hartmann Analytic), and incubation was continued for another 5 h. Where indicated, cells were treated with 100 μM OA (Sigma Chemical Co.) in MeSO, 3 h prior to harvesting (MeSO alone was added to control cultures). 48 h post infection Plx1 proteins were purified as described previously (9) and subjected to SDS-PAGE or used for kinase assays.

Xenopus Egg Extract Preparation and 32P-Labeling of Plx1—Interphase extracts were prepared from eggs of Xenopus laevis (Nasco) as described (18), except that 100 μg/ml cycloheximide (Sigma Chemical Co.) was added to block protein translation. M phase extracts were prepared by adding 10 μg/ml non-degradable (49) cyclin B to interphase extract (43). CSF extracts were prepared by a previous study (44), and they were released into the first mitotic cell cycle by addition of 0.4 mM CaCl₂. For labeling of endogenous Plx1, 100 μl of interphase extract was first incubated for 30 min at 22 °C with 200 μCi of carrier-free [32P]orthophosphoric acid solution (Amersham Biosciences, Hartmann Analytic). To analyze interphase phosphorylation of Plx1, extracts were incubated for a further 90 min at 22 °C. To analyze M phase phosphorylation, 390 cyclin B was added and incubations were performed under the same conditions. Endogenous Plx1 protein was recovered by immunoprecipitation.

For labeling of recombinant Plx1 in Xenopus egg extracts, 2 μg of purified His6-Plx1 was added to 50 μl of extract (resulting in an excess of ~4-fold over endogenous protein). All extracts had been preincubated with 100 μCi of [32P]orthophosphoric acid, and M phase extracts were prepared by the addition of 390 cyclin B, as described above. Recombinant proteins were then re-purified using 10 μl of nickel-nitrotriacetic acid-agarose beads (Qiagen). For extensive washing of the beads, bound Plx1 proteins were released by boiling in gel sample buffer and analyzed by SDS-PAGE.

Immunoblotting, Kinase Assays, and Immunoprecipitations—Immunoblotting and kinase assays with mutant Plx1 proteins were performed essentially as described (18), except that kinase assays were carried out at 30 °C for 15 min. For immunoprecipitation of endogenous Plx1 from 32P-labeled extracts, a rabbit anti-Plx1 antibody (18) was bound for 1 h at 4 °C to pre-washed protein A-Sepharose (Amersham Biosciences) at an antibody to bead ratio of 2:1 (v/v). The beads were then washed twice with phosphate-buffered saline, 0.05% Tween 20, and 0.5% wt/vol BSA (all reagents were used with ES buffer (80 mM β-glycerophosphate, 20 mM EGTA, 50 mM NaF, 0.1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, pepstatin, and chymostatin) (18). Then, 25 μl of beads was incubated with 100 μl of 32P-labeled Xenopus egg extract for 1 h at 4 °C, before they were washed twice with ES buffer, twice with ES buffer containing 250 mM NaCl, and twice with Plx1 kinase buffer (18). Finally, beads were boiled in gel sample buffer and the released Plx1 proteins analyzed by SDS-PAGE.

For in vitro phosphorylation reactions with recombinant kinases, xPlk1 and Cdk1/cyclin B were purified as described (40, 45), except that for Cdk1/cyclin B the gel filtration was omitted. PKA was obtained from Sigma Chemical Co. Reactions were carried out with xPlk1 kinase buffer (9), and with Plx1 kinase buffer (18) in the presence or absence of 250 μM roscovitin (Calbiochem). For analysis of activation, Hist6-Plx1 (WT) and Hist6-Plx1 (N172A) were incubated for the times indicated with xPlk1 or PKA or with Cdk1/cyclin B. In the latter case, incubation was carried out in the presence or absence of 250 μM roscovitin (Calbiochem). Then, Plx1 was immunoprecipitated as described above and analyzed for activity in the presence of [γ-32P]ATP, with a constant incubation time of 15 min and casein as an exogenous substrate.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis—Following the localization of 32P-labeled Plx1 proteins by autoradiography, the corresponding bands were excised from Coomassie Blue-stained gels and macerated. Gel pieces were incubated for 5 min under vigorous shaking at 37 °C, first in 40% isopropanol, then in 50% methanol/50% 50 mM NH₄HCO₃. Then the gel pieces were completely dried in a Speedvac (Savant) before they were resuspended in 100 μl of 50 mM NH₄HCO₃ containing 10 μg of modified trypsin (Promega). After 12 h of incubation with fresh shaking at 37 °C, 10 μg of additional trypsin was added and the incubation continued for 12 h. To recover the tryptic peptides, 500 μl of water was added to the gel pieces and the mixture was shaken for 5 min at 37 °C. Then, samples were spun in an Eppendorf tabletop centrifuge, and the supernatants were recovered. Subsequently, the gel pieces were incubated for 5 min under shaking with 50% acetonitrile, and the supernatant was again recovered. Both supernatants were pooled and completely dried in the Speedvac. The dried pellets were resuspended in 20 μl of pH 1.9 buffer (46), and the samples were spotted onto cellulose thin layer plates (Merck). In general, samples were compared by analyzing the same amounts of tryptic peptides (rather than the same amounts of radioactive activity). However, for comparing the tryptic phosphopeptide map of recombinant Plx1 (WT) with that of the same amount of Plx1 (N172A), the same amount of 32P-labeled peptide was loaded. First, dimension electrophoresis was performed in pH 1.9 buffer at 1600 V for 30 min, using an HTLE 7000 thin-layer peptide mapping electrophoresis system (CBS Scientific). Second-dimension ascending chromatography was carried out for 8 h, using phospho-chromatography buffer (46). Finally, plates were exposed to film or phosphoimaging (Fuji Corp.). For predicting the theoretical mobility of tryptic peptides, the online version of Mobility 5.0 was used (available at acrux.igh.cnrs.fr/gs/mobility5.html). Phosphoamino acid analysis was performed on phosphopeptides recovered from thin-layer chromatography plates as described previously (46).

Mass Spectrometry—Mass spectrometry was performed as described elsewhere (56). In short, protein bands were digested in-gel using a standard protocol (47), including reduction by dithiothreitol and alkylation by iodoacetamide. Peptides were extracted, dried down under nitrogen, and redissolved in 2% formic acid. This solution was either directly analyzed by LC-ESI-MS or first desalted with reversed phase C₁₈ material (ZipTip, Millipore) and then analyzed by static nano-ESI tandem MS. The mass spectrometric measurements were performed using a hybrid Q-TOF instrument (Q-TOF 2, Micromass) equipped with a nanospray ionization device or with a static nano-ESI source. Argon was used as the collision gas for tandem MS.

Peptide Synthesis—Phosphopeptides were synthesized using standard protocols based on the N-9-fluorenylmethoxycarbonyl-L-tryptophanyl-L-leucine strategy (43A 1-synthetiser, Applied Biosystems). All peptides were characterized by reverse phase-high performance liquid chromatography (Alliance, Waters) and ESI-MS (API 165, PerkinElmer Life Sciences).

RESULTS

Cell Cycle-dependent Phosphorylation of Xenopus Plx1—As a first step toward exploring the complexity of Plx1 phosphorylation, Plx1 was labeled with 32P in Xenopus egg extracts and then immunoprecipitated and subjected to tryptic phosphopeptide mapping (46). As revealed by Coomassie Blue staining, equal amounts of Plx1 were recovered from interphase (I) and M phase (M) extracts (Fig. 1A, left panel). However, as shown by autoradiography, M phase Plx1 incorporated much more 32P

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Phosphorylation of Xenopus Polo-like Kinase Plx1

**Fig. 1. Cell cycle-dependent phosphorylation of Plx1 in Xenopus egg extracts.** A, interphase (I) and M phase (M) extracts were incubated with \[^{32}P\]orthophosphate, then endogenous Plx1 was immunoprecipitated and subjected to SDS-PAGE. The gel was stained with Coomassie Blue (left panel and data not shown), and exposed for autoradiography (right panel). Molecular weight markers are indicated on the left. The arrows show the migration of Plx1; the asterisk denotes IgG heavy chain. B, phosphorylated Plx1 proteins were excised from the gel shown in A and subjected to tryptic phosphopeptide mapping. The arrows indicate the origins of sample loading. Electrophoresis was performed in the first dimension (eletr.) and ascending chromatography in the second dimension (chr.).

**Fig. 2. Phosphorylation of recombinant Plx1 in M phase Xenopus egg extracts.** A, recombinant His\(_6\)-Plx1 (N172A) (left panel) and His\(_6\)-Plx1 (WT) (right panel) were purified from SF9 insect cells, incubated in M phase Xenopus egg extract in the presence of \[^{32}P\]orthophosphate, repurified, and subjected to tryptic phosphopeptide mapping. B, peptides corresponding to spots A, B, c, e, and f were recovered from thin-layer plates (as shown in A, right-hand panel) and hydrolyzed with HCl. Phosphoamino acid analyses were performed by two-dimensional electrophoresis on thin-layer plates, and phosphorylated amino acids were identified by comparison to the migration of ninhydrin-stained phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards (circles).

**Text:**

than interphase Plx1 (Fig. 1A, right panel). Concomitantly, M phase Plx1 showed a retarded electrophoretic mobility and a 3- to 5-fold increased specific activity (Fig. 1A, left panel and data not shown), in agreement with previous results (8–10, 18). As revealed by tryptic phosphopeptide mapping, Plx1 was phosphorylated on several distinct sites, and the extent of phosphorylation on individual sites was cell cycle dependent (Fig. 1B).

The analysis of Plx1 isolated from the M phase extract revealed two major spots (labeled A and B) and several minor spots (labeled c through h; c denotes a group of spots). Throughout this study, the lettering on this M phase map will be used as a reference. In the map prepared from interphase Plx1, spots B, c, e, and f–h were virtually undetectable, and spots A and d were very weak, indicating that the corresponding phosphopeptides were produced mostly as a result of M phase-specific phosphorylation.

**Phosphorylation of Recombinant Plx1 in Xenopus Extracts**—In the next series of experiments, recombinant wild-type (WT) and catalytically inactive (N172A) mutant His\(_6\)-Plx1 proteins were purified from baculovirus-infected SF9 insect cells and incubated in the presence of \[^{32}P\]orthophosphate in M phase Xenopus extracts prepared by the addition of 390 cyclin B. Then, His\(_6\)-Plx1 proteins were re-isolated on nickel-nitrilotriacetic acid-agarose and subjected to both tryptic phosphopeptide mapping and phosphoamino acid analysis. Exogenous WT Plx1 was phosphorylated at both major sites A and B, as well as on most of the minor sites (c, e, and f) seen in the reference map (Fig. 2A, right panel). In contrast, the N172A mutant Plx1 was phosphorylated only on site B and on the minor sites f and c, but not on sites A and e (Fig. 2A, left panel). This indicates that phosphorylations on sites B, f, and c almost certainly reflect the activities of upstream kinases. Conversely, phosphorylations on sites A and e require Plx1 activity, suggesting that they probably arise through autophosphorylation or phosphorylation by downstream kinases. This was investigated by incubating purified His\(_6\)-Plx1 (WT) in the presence of \[^{32}P\]orthophosphate, in a cytoytic factor (CSF)-arrested M phase extract and then released this extract by the addition of calcium (Fig. 3). At different times after the release, tryptic phosphopeptide maps were prepared from recombinant Plx1. In parallel, Cdk1 activity was monitored, using aliquots of the same extract for assaying histone H1 kinase activity at each time point. The Cdk1 activity profile shows that the extract was in an interphase state within 5 min after calcium addition, entered the first M phase by 30–40 min, and returned toward interphase by 50 min (Fig. 3A). When no calcium was added to the extract, Cdk1 activity in the CSF extract remained high throughout the experiment (data not shown). Concomitantly, the phosphopeptide maps obtained from Plx1 at 5 and 50 min resembled interphase maps, whereas those obtained at 30–40 min resembled typical M phasic maps (Fig. 3B). Careful inspection of the maps...
either WT His6-Plx1 or catalytically inactive His6-Plx1.

"mitotic state"

"in various experimental systems (48, 49), we observed that OA-induced hyperphosphorylation of recombinant Plx1 in insect cells might recapitulate M phase-specific phosphorylation of endogenous Plx1 in Xenopus extracts. Sf9 cells were infected with baculovirus, Plx1 can be activated by treatment of Sf9 insect cells with OA (9). Because this phosphatase inhibitor has been used to induce a "pseudo-mitotic state" in various experimental systems (48, 49), we asked to what extent OA-induced hyperphosphorylation of recombinant Plx1 in insect cells might recapitulate M phase-specific phosphorylation of endogenous Plx1 in Xenopus extracts. Sf9 cells were infected with baculoviruses encoding either WT His6-Plx1 or catalytically inactive His6-Plx1 (N172A) (9) and labeled with [32P]orthophosphate in the presence or absence of OA. Then the recombinant Plx1 proteins were purified, analyzed by SDS-PAGE, and subjected to tryptic phosphopeptide mapping. After isolation from OA-treated Sf9 cells, both WT and N172A Plx1 showed a retarded electrophoretic mobility (Fig. 4A, upper panel), in line with previous findings (9, 18, 48). Autoradiography confirmed that this altered mobility was accompanied by extensive phosphorylation (Fig. 4A, lower panel). Tryptic phosphopeptide maps prepared from these samples revealed patterns of spots that were remarkably similar to those seen in the reference map, i.e. the map prepared from endogenous Plx1 labeled in M phase Xenopus extracts (compare Fig. 4B, lower right panel, with Fig. 1B, right panel). Specifically, the map of WT Plx1 isolated from OA-treated cells showed most of the previously observed peptides, that is the two major spots A and B, as well as the minor spots c, e, and f–h. Minor differences concern spot d, which was barely visible, and the presence of two spurious phosphopeptides (marked by the asterisk), which had not been seen in the reference map. Spots A and e could only be seen in maps prepared from WT Plx1, whereas spot B was strongly enhanced upon OA treatment of either WT or catalytically inactive Plx1. This confirms that phosphopeptides A and e arise most likely from OA treatment. In contrast, the appearance of spot B is strongly stimulated by OA treatment and almost certainly reflects the action of an upstream kinase.

Identification of Serines 260, 326, and 340 as Major Phosphorylation Sites in Plx1—To identify phosphorylation sites in recombinant Plx1 proteins, these were purified from Sf9 insect cells, digested with trypsin, and analyzed by mass spectrometry. Specifically, samples were subjected sequentially to inductively coupled plasma mass spectrometry with phosphorous detection (ICP-MS), capillary liquid chromatography electrospray mass spectrometry (LC-ESI), and electrospray tandem mass spectrometry (ESI-MS/MS), as described in detail elsewhere (56). Analysis of relevant LC-ESI spectra revealed two tryptic peptides, designated pT47 and pT49, that showed a characteristic difference of 80 m/z to their calculated masses (Fig. 5A). Subsequent analysis of the two phosphate-containing peptides by nano-ESI-MS/MS identified serine 326 and serine 340 as phosphorylation sites (Fig. 5B).

To confirm the identity of these sites, the two predicted tryptic phosphopeptides (FSIAPSTIDQ-pS-LR and GQD-pS-PLVEK) were synthesized and mixed with a tryptic digest of recombinant Plx1 that had been labeled with [32P] in an M phase Xenopus egg extract. Then, the samples were subjected to electrophoresis and chromatography on thin layer plates. As shown by comparison of ninhydrin staining with autoradiography, the two synthetic peptides comigrated exactly with spots A and B, respectively (Fig. 6, upper and middle panels). These data demonstrate that the major putative autocatalytic phosphorylation site in Plx1 (spot A) corresponds to serine 326, whereas the major site phosphorylated by an as yet unidentified upstream kinase corresponds to serine 340.

Although mass spectrometry has not yet allowed us to identify the phosphopeptide corresponding to spot e, the available evidence points to serine 260 as the relevant phosphorylation site. In particular, a phosphopeptide comigrating exactly with spot e could be generated upon digestion of Plx1 with Lys-C protease (data not shown), indicating that this peptide arose from cleavage at two lysine residues. Conceptual cleavage of Plx1 by Lys-C yielded 10 candidate phosphopeptides, and theoretical predictions of their electrophoretic and chromatographic mobilities (available at acrux.igh.cnrs.fr/gs/mobility5.html) identified the phosphopeptide NEY-pS-IPK as the most likely candidate for spot e. This peptide was therefore synthesized and examined by comigration with a tryptic digest of [32P]-labeled Plx1. As shown in Fig. 6 (bottom panel), the synthetic phosphopeptide comigrated exactly with radiolabeled spot e, indicating that this spot almost certainly represents phosphorylation of serine 260. This interpretation is supported further by the fact that the EXS motif within this peptide matches the emerging consensus for a Plx1 phosphorylation site (see “Discussion”).
Are Thr-201 and Ser-128 Phosphorylated in Plx1?—Although mutation of Thr-201 and/or Ser-128 to aspartic acid results in substantial activation of Plx1 (39), the studies described so far did not provide any evidence for phosphorylation of either Thr-201 or Ser-128. A priori, Thr-201 appeared to be a likely phosphorylation site, because this residue is located within the so-called T-loop (or activation loop), and corresponding residues constitute activating phosphorylation sites in many serine-threonine kinases (reviewed in Ref. 50). To more directly address this issue, Thr-201 of Plx1 was therefore mutated to alanine, and phosphorylation of the corresponding recombinant protein was studied in an M phase Xenopus egg extract. Compared with WT Plx1, the phosphopeptide map derived from the T201A mutant showed a conspicuous lack of spots c and f (Fig. 7, top panels), indicating that these phosphopeptides depended on Thr-201. Next, we synthesized predicted phosphopeptides comprising Thr-201. Because Thr-201 is preceded by one arginine and two lysine residues, and trypsin cleavage is known to be inefficient when multiple Arg and Lys residues appear in tandem (46), peptides carrying zero, one, or two lysines N-terminal to Thr-201 were analyzed. Of these, the peptide KK-pT-LCGTPNYIAPEVLGK was found to comigrate exactly with spot f (Fig. 7, lower left panel), but the other two peptides did not show any comigration with 32P-labeled phosphopeptides. This shows that spot f is almost certainly generated by trypsin cleavage of a pThr-201-containing peptide at position —2 and implies that the T-loop is indeed phosphorylated in Plks. Most of the c peptides are also likely to

**Fig. 4.** Phosphorylation of recombinant Plx1 proteins expressed in SF9 insect cells. SF9 cells were infected with baculoviruses encoding His6-Plx1 (N172A) or His6-Plx1 (WT) and incubated with [32P]orthophosphate in the presence or absence of OA. A, recombinant 32P-labeled Plx1 proteins were purified and subjected to SDS-PAGE. Coomassie Blue staining (upper panel) shows His6-Plx1 (N172A) (lanes 1 and 2) and His6-Plx1 (WT) (lanes 3 and 4) purified from SF9 cells. The addition of OA is indicated by a plus sign (+). The same gel was also exposed for autoradiography (lower panel). The arrows indicate the migration of His6-Plx1 (N172A/WT). B, tryptic phosphopeptide mapping was performed on Plx1 protein bands excised from A. The upper panels show the maps derived from His6-Plx1 (N172A); the lower panel shows those from His6-Plx1 (WT). Mock-treated samples are shown on the left (−OA); OA-treated (+OA) samples are on the right. Annotations of tryptic phosphopeptides are as described in Fig. 1. Two minor spots that were not reproducibly seen in these maps are marked by asterisks.

**Fig. 5.** Identification of two major Plx1 phosphorylation sites by mass spectrometry. His6-Plx1 (WT) was overexpressed in SF9 cells, treated with OA, and purified. Then the sample was digested with trypsin and analyzed with LC-ESI-MS and nano-ESI tandem MS. A, mass spectra extracted from the LC-ESI-MS runs show doubly charged phosphorylated peptides pT49 (upper panel) and pT47 (lower panel). B, pT49 (upper panel) and pT47 (lower panel) were analyzed by nano-ESI Tandem MS. The observed masses are indicative of phosphate groups on serine 340 for pT49 and on serine 326 for pT47. The corresponding peptides are shown with the phosphorylated serines indicated as pS.
contain pT201, because they disappear upon mutation of Thr-201 to alanine (Fig. 7, upper right panel). Their identities have not yet been established, but they most probably represent larger peptides arising through incomplete trypsin digestion.

We have also used a similar approach to seek evidence in support of phosphorylation at serine 128. This potential phosphorylation site is bounded by several basic residues and thus might give rise to multiple peptides with staggered trypsin cuts. Two phosphopeptides encompassing Ser-128, but differing in the presence or absence of an arginine N-terminal to Ser-128, were synthesized and subjected to analysis on thin-layer plates. Neither of these peptides comigrated with any Plx1-derived radiolabeled phosphopeptide (Fig. 7, lower right panel, and data not shown). Furthermore, we note that any peptide with additional positive charges would be expected to migrate even further to the cathode. Thus, at least under the conditions explored so far, we did not find evidence to support the idea that Ser-128 is a phosphorylation site in Plx1. We cannot rigorously exclude that phosphorylation of this residue may occur under as yet unknown conditions or might have escaped detection. However, it is also possible that the S128D mutation may directly cause an activating conformational change.

**Discussion**

In exploring the functional significance of the phosphorylation sites identified here, we have mutated individual sites to either alanine (to mimic a de-phosphorylated state) or aspartic acid (to mimic a phosphorylated state) and then tested recombinant mutants for altered kinase activity. Upon mutation of Thr-201 to aspartic acid, Plx1 activity was significantly enhanced (Fig. 9), consistent with previous results (39). Most interestingly, OA treatment of a T201D mutant did not cause further activation. Conversely, mutation of Thr-201 to alanine impaired Plx1 activity and made the kinase unresponsive to OA. These results suggest that the activating influence of OA is mediated mostly through phosphorylation of Thr-201. So far, we have not been able to detect major effects on Plx1 activity in response to mutation of other phosphorylation sites. However, because of the multiplicity of sites and the possibility of combinatorial interactions, it would be premature to exclude that other phosphorylation sites may contribute to the regulation of Plx1 activity.

Upon phosphorylation of Plx1 in either M phase Xenopus egg extracts or OA-treated Sf9 insect cells, the protein shows a strikingly retarded electrophoretic mobility (e.g. see Figs. 1A and 4A). To determine which phosphorylation site is involved in causing this upshift, appropriate Plx1 mutants were produced by coupled in vitro transcription translation and then incubated in both interphase and M phase Xenopus egg extracts (Fig. 10A and data not shown). Although both WT Plx1 and the N172A and T201A mutants showed a conspicuous upshift in M phase extracts (lanes 2, 6, and 8), no such upshift was seen following mutation of Ser-340 to alanine (compare lanes 3 and 4). When Plx1 mutants were immunoprecipitated from reticulocyte lysates and their activities were assayed in
Casein was monitored by autoradiography (arrowhead in the middle panel), Drosophila phosphorylation sites with corresponding regions in human Plk1, Recombinant WT and mutant His<sub>6</sub>-Plx1 proteins were expressed in Sf9 cells. Following purification from cells that had been treated with (+) or without (−) OA, Plx1 proteins were assayed for in vitro kinase activity, using casein as a substrate. The recovery of recombinant proteins was assessed by immunoblotting with an anti-Plx1 antibody (upper panel, arrow points to Plx1), and <sup>32</sup>P incorporation into casein was monitored by autoradiography (middle panel, arrowhead points to casein). The histogram shows a quantification of kinase activity, as determined by densitometric scanning of autoradiographs (average of two experiments). Values are expressed in arbitrary units relative to the activity of WT Plx1 (defined as 1).

Fig. 8. Plx1 phosphorylation sites. A, schematic view of a tryptic phosphopeptide map of Plx1 isolated from a Xenopus M phase extract. All phosphorylation sites identified in this study are annotated. B, diagram of Plx1 domain structure, with phosphorylation sites marked. The large box represents the catalytic domain, and the small box represents a highly conserved motif known as the Polo-box. C, alignment of Plx1 phosphorylation sites with corresponding regions in human Plk1, Drosophila Polo, as well as the Xenopus Plk family members Plx2 and Plx3. Residues corresponding to Plx1 phosphorylation sites are boxed in gray.

FIG. 8.

FIG. 9. Activating T-loop mutation mimics OA-induced Plx1 activation. Recombinant WT and mutant His<sub>6</sub>-Plx1 proteins were expressed in Sf9 cells. Following purification from cells that had been treated with (+) or without (−) OA, Plx1 proteins were assayed for in vitro kinase activity, using casein as a substrate. The recovery of recombinant proteins was assessed by immunoblotting with an anti-Plx1 antibody (upper panel, arrow points to Plx1), and <sup>32</sup>P incorporation into casein was monitored by autoradiography (middle panel, arrowhead points to casein). The histogram shows a quantification of kinase activity, as determined by densitometric scanning of autoradiographs (average of two experiments). Values are expressed in arbitrary units relative to the activity of WT Plx1 (defined as 1).

Analysis of Kinases Potentially Acting upon Plx1—Three recombinant kinases, Cdk1/cyclin B, xPlkk1, and PKA, were tested for their ability to phosphorylate and activate Plx1 in vitro. The proline-directed Cdk1/cyclin B was a likely candidate for phosphorylating Ser-340, and xPlkk1 has previously been shown to phosphorylate Plx1 (40). PKA was analyzed because of the sequence similarity between the Thr-201 site and a PKA consensus site. As shown in Fig. 11A, all three kinases were able to phosphorylate a catalytically inactive Plx1. However, under the assay conditions used here, only PKA was able to activate wild-type Plx1 in a time-dependent manner (Fig. 11, B–D). Tryptic phosphopeptide mapping (Fig. 12) demonstrated that Cdk1/cyclin B phosphorylated Plx1 exclusively on Ser-340 (phosphopeptide B), whereas PKA phosphorylated both Thr-201 (peptides c and f) and a peptide comigrating with spot A. Surprisingly, no detectable phosphorylation of Thr-201 could be seen following incubation of recombinant Plx1 with xPlkk1. Instead, xPlkk1 phosphorylated a major phosphothreonine site that was identified by mass spectrometry as Thr-10 (data not shown).

DISCUSSION

Using a combination of biochemical approaches centered on tryptic phosphopeptide mapping, we have performed a detailed analysis of the phosphorylation state of the Polo-like kinase Plx1 in both Xenopus egg extracts and OA-treated Sf9 insect cells. These studies have led to the identification of threonine 201 and serines 260, 326, and 340 as four major phosphorylation sites in Plx1. As described in detail elsewhere, serines 260 and 340, as well as a fifth site, serine 25/26, were also identified by a novel mass spectrometric approach based on the sequential use of ICP-MS, LC-ESI, and ESI-MS/MS (56). These studies were facilitated by the observation that Plx1 is phosphorylated upon expression from a recombinant baculovirus in Sf9...
insect cells. In particular, treatment of Sf9 cells with the phos-
phatase inhibitor OA results in both hyperphosphorylation and
activation of Plx1 (Refs. 9, 18, and this study). As shown here
by comparative tryptic phosphopeptide mapping, the phospho-
rylation events occurring in insect cells largely mimic those
occurring in Xenopus egg extracts. In fact, virtually identical
phosphopeptide patterns were observed upon analysis of either
recombinant Plx1 isolated from OA-treated Sf9 cells or endog-

FIG. 11. In vitro phosphorylation of Plx1 by Cdk1/cyclin B, xPlkk1, and PKA. A, catalytically inactive His$_6$-Plx1 (N172A) was incubated in the presence of [$\gamma$-$^{32}$P]ATP with purified Cdk1/cyclin B, xPlkk1, or PKA, as indicated. Reaction products were then resolved by SDS-PAGE and stained with Coomassie Blue (upper panels) or subjected to autoradiography (lower panels). Note that phosphorylation by Cdk1/cyclin B causes a retarded electrophoretic mobility of Plx1 (left panel). B and C, His$_6$-Plx1 (N172A) and wild-type Plx1 were incubated for the times indicated with Cdk1/cyclin B (B) or with xPlkk1 or PKA (C). In the former case, incubations were performed in the presence or absence of the Cdk inhibitor roscovitin. Then, Plx1 was immunopre-

cipitated and analyzed for activity in the presence of [$\gamma$-$^{32}$P]ATP, using a constant incubation time of 15 min and casein as an exogenous substrate. Equal recovery of Plx1 was assessed by SDS-PAGE and Coomassie Blue staining (upper panels), whereas phosphorylation of casein was monitored by autoradiography (lower panels). D, the casein phosphorylation shown in panel C was quantified using phosphorimaging. Data were normalized with the value of 1 assigned to the activity of untreated Plx1. Note that only Plx1, preincubated with PKA, showed an increasing casein kinase activity in response to increasing time of pretreatment.

FIG. 12. Phosphopeptide analysis of in vitro phosphorylated Plx1. Catalytically inactive His$_6$-Plx1 (N172A) was incubated in the presence of [$\gamma$-$^{32}$P]ATP with purified Cdk1/cyclin B, xPlkk1, or PKA, as indicated. Reaction products were then resolved by SDS-PAGE, and Plx1 was subjected to tryptic phosphopeptide mapping. A map derived from wild-type Plx1 phosphorylated in the Xenopus M phase extract is shown for comparison (upper left panel).
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Px1 activity by serine phosphorylation. However, these results are difficult to interpret, because we cannot rule out combinatorial interactions between the sites. Thus, to reveal regulation it may be necessary to analyze appropriate double, triple, or quadruple mutants. Alternatively, it is equally possible that phosphorylation on serine sites may regulate aspects of Plk function other than activity, such as localization, stability, or the interaction with other proteins. In any event, we have clearly shown that phosphorylation of Ser-340 is responsible for the characteristically retarded electrophoretic mobility of Px1 that is seen in both M phase extracts and OA-treated Sf9 insect cells.

With regard to kinases acting upon serine residues, it appears that Px1 itself phosphorylates both Ser-260 and Ser-326, most likely by an intramolecular mechanism. This is inferred from the observation that neither of these residues is phosphorylated upon incubation of a catalytically inactive recombinant Px1 mutant in M phase Xenopus extracts, although endogenous Px1 is expected to be active in these extracts (Fig. 2A, left panel). On the other hand, phosphorylation on Ser-340 does not require Px1 activity, clearly demonstrating that this residue is phosphorylated by an upstream kinase. This kinase could be Cdk1/cyclin B, as demonstrated here by in vitro phosphorylation experiments. However, we cannot exclude that other proline-directed kinases, particularly members of the MAP kinase family, might also act on this site.

Threonine 201 Is a Major Site of Plx1 Activation—On both theoretical grounds and on the basis of previous studies (10), Thr-201 appeared as a likely phosphorylation site. This threonine is located within the T-loop of Plx1 (kinase subdomain VIII (51)) in a position that is occupied by a phosphorylatable residue in many serine-threonine kinases, including all members of the Plk family. Furthermore, the activity of many kinases depends on phosphorylation at this position (50). In some cases (e.g. in the case of PKA), T-loop phosphorylation is virtually constitutive, whereas in other cases it clearly represents a regulatory switch (e.g. in the case of MAPKs). Although it had not previously been shown that the T-loop is phosphorylated in any of the Plks, mutation of both Thr-201 in Px1 (39) and T210 in Plk1 (25, 38) to aspartic acid was found to activate the corresponding kinase. From this it was inferred that the incorporation of an acidic residue into the T-loop of Plks mimics an activating phosphorylation event. As shown here by tryptic phosphopeptide mapping, this supposition was correct. Our data further indicate that phosphorylation at Thr-201 activates Px1 and that most of the Plx1 activation seen in response to OA treatment of S19 insect cells may be attributable to phosphorylation of Thr-201. These results thus identify T-loop phosphorylation as a major regulatory switch underlying the activation of Plks.

At present, we have no definitive information on the identity of the kinase(s) acting upon Thr-201. Autophosphorylation appears unlikely, because a catalytically inactive N172A Px1 mutant was still phosphorylated on the T-loop-derived peptides (f and c) when incubated in Xenopus egg extracts (see Fig. 2A, left panel). One attractive upstream regulatory kinase that might act on this site is xPlkk1, because this enzyme was previously shown to phosphorylate a synthetic peptide spanning Thr-201 (39). However, under the assay conditions used here, recombinant xPlkk1 did not activate purified Px1, nor did it phosphorylate the T-loop within the intact protein. Instead, the major site phosphorylated by xPlkk1 was identified as T10. We emphasize that this negative result does not exclude an important regulatory role for xPlkk1, but it suggests that in vivo activation of Px1 by this kinase may require as yet unidentified accessory proteins. Alternatively, it would seem premature to exclude that other kinases might also activate Plks by T-loop phosphorylation. In particular, the basic residues N-terminal to Thr-201 create a consensus motif for PKA, and, indeed, we show here that PKA can readily phosphorylate the T-loop within Px1 and thereby activate the enzyme. Whether PKA plays a physiological role in Px1 regulation remains to be determined. Finally, we note that the sequence at the Thr-201 site matches the consensus (pT-XCGT) for phosphorylation by PDK1 (52), although Plks lack the FXXF-negative charge-F/Y docking motif seen in several physiological PDK1 substrates (e.g. Refs. 53–55).

In conclusion, the studies described here provide direct information on physiological phosphorylation sites within a member of the Plk family. We show that Xenopus Plx1 is phosphorylated on multiple sites and that phosphorylation at all sites increases substantially during M phase. Most importantly, the T-loop residue Thr-201 in Px1 is identified as a major activating phosphorylation site. In contrast, no evidence could be obtained for phosphorylation at Ser-128, although mutation of this residue to aspartic acid clearly activates recombinant Plks (38, 39). Instead, several novel serine phosphorylation sites were identified. These results set the stage for future exploration of the functional consequences of Plk multisite phosphorylation and for the identification of kinases and phosphatases involved in Plk regulation.

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