tion with the established role of NPH1 in phototropism (*9, 10*), lead us to propose that NPH1 is an autophosphorylating flavoprotein, unrelated to cryptochrome, that serves as a photoreceptor for phototropism in higher plants.

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31. Spectroscopic analysis was performed with a Beckman DU-70 spectrophotometer and a Photon Technology International Alphascan spectrofluorometer. Fluorescence excitation spectra were obtained by monitoring the emission at 535 nm. Fluorescence emission spectra were measured by using an excitation wavelength of 390 nm. For fluorescence measurements, insoluble fractions (1 mg) were treated with 6 M guanidine-HCl, 0.1 M sodium phosphate, and 0.01 M tris-HCl (pH 8.0). For absorption studies, insoluble fractions were treated with 10% trichloroacetic acid. In each case, samples were centrifuged at 16,000*g* for 10 min and the supernatants were analyzed for the presence of chromophore.
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Purification and Cloning of a Protein Kinase That Phosphorylates and Activates the Polo-Like Kinase Plx1

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The *Xenopus* polo-like kinase 1 (Plx1) is essential during mitosis for the activation of Cdc25C, for spindle assembly, and for cyclin B degradation. Polo-like kinases from various organisms are activated by phosphorylation by an unidentified protein kinase. A protein kinase, polo-like kinase kinase 1 or xPlkk1, that phosphorylates and activates Plx1 in vitro was purified to near homogeneity and cloned. Phosphopeptide mapping of Plx1 phosphorylated in vitro by recombinant xPlkk1 or in progesterone-treated oocytes indicates that xPlkk1 may activate Plx1 in vivo. The xPlkk1 protein itself was also activated by phosphorylation on serine and threonine residues, and the kinetics of activation of xPlkk1 in vivo closely paralleled the activation of Plx1. Moreover, microinjection of xPlkk1 into *Xenopus* oocytes accelerated the timing of activation of Plx1 and the transition from G_2 to M phase of the cell cycle. These results define a protein kinase cascade that regulates several events of mitosis.

Progression through the eukaryotic cell cycle relies on the periodic activation or inactivation of various cyclin-dependent protein kinases (Cdks) (*1*). Cell cycle checkpoints monitor the fidelity of events in a given cell cycle phase and control a signaling system that can delay cell cycle progression and changes in Cdk activity. One checkpoint blocks activation of the Cdc2–cyclin B complex in G_2 phase if DNA replication is incomplete. This block to activation is mainly accomplished by maintenance of the phosphorylated state of Thr^{14} and Tyr^{15} in Cdc2 (*2*). These considerations have focused attention on the pathway of activation of the phosphatase Cdc25C, which initiates mitotic entry by dephosphorylating Thr^{14} and Tyr^{15} in Cdc2 (*3*). Overexpression of Cdc25C either in vivo (*4*) or in vitro (*5, 6*) overcomes the replication checkpoint. Cdc25C is activated at the G_2-M transition by serine-threonine phosphorylation (*6, 7*). The Cdc2–cyclin B complex can phosphorylate and activate Cdc25C (*8*), forming a positive feedback loop, but in *Xenopus* initial phosphorylation and activation of Cdc25C results from activation of the polo-like kinase Plx1 (*9, 10*). Plx1 can phosphorylate Cdc25C in vitro at activating sites (*9*), and in vivo activation of Plx1 coincides with the activation of Cdc25C (*10*). Moreover, inhibition of Plx1 delays the activation of Cdc25C, and microinjection of

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wild-type Plx1 into oocytes accelerates activation of Cdc25C and entry into M phase (*10*). Polo-like kinases have also been implicated in the control of spindle assembly and cytokinesis in various systems including *Xenopus* (*10, 11*) and in the activation of the proteolytic machinery required for exit from mitosis (*12, 13*).

Plx1 and other polo-like kinases are activated by phosphorylation (*10, 13–15*). To detect and purify the upstream kinase or kinases responsible for activation of Plx1, termed *Xenopus* polo-like kinase kinases (xPlkks), we devised an assay to detect activation of Plx1 (*16*). The activity of xPlkk in M-phase extracts from unfertilized eggs was 15 times that in extracts from resting (G_2 phase) oocytes (*17*), consistent with increased Plx1 activity in M phase (*10*). Several chromatographic steps were used to purify the xPlkk activity from unfertilized eggs (*18*). After purification about 3200-fold to near homogeneity, a 120-kD polypeptide coeluted precisely with xPlkk activity (Fig. 1, A and B). This polypeptide autophosphorylated after incubation with [γ-^{32}P]adenosine triphosphate (ATP), suggesting that it is an autophosphorylating protein kinase (Fig. 1C). We designated this protein xPlkk1. Purified xPlkk1 activated Plx1, but not catalytically inactive Plx1(N172A), only in the presence of ATP

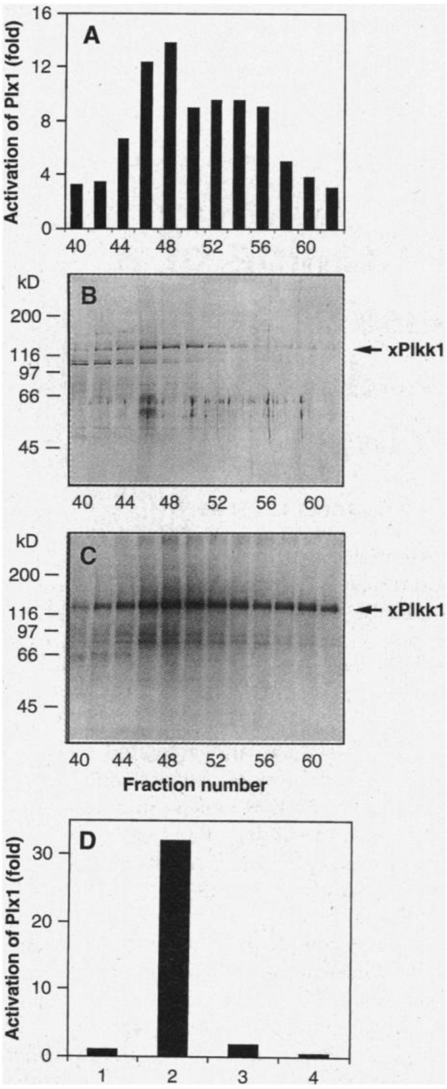


Fig. 1. Purification of a protein kinase that activates Plx1. A partially purified fraction, prepared from unfertilized eggs (*18*), was subjected to chromatography on Mono Q. Fractions were assayed for the activation of Plx1 (**A**) or were incubated in the presence of [γ-^{32}P]ATP and analyzed by SDS gel electrophoresis, silver staining (**B**), and autoradiography (**C**). (**D**) Immune complexes containing Plx1 or Plx1(N172A) were incubated with purified xPlkk1 and subsequently assayed for casein phosphorylation (*16*). Plx1 was incubated with buffer plus ATP (lane 1); xPlkk1 plus ATP (lane 2); xPlkk1 without ATP (lane 3); Plx1(N172A) with xPlkk1 plus ATP (lane 4).

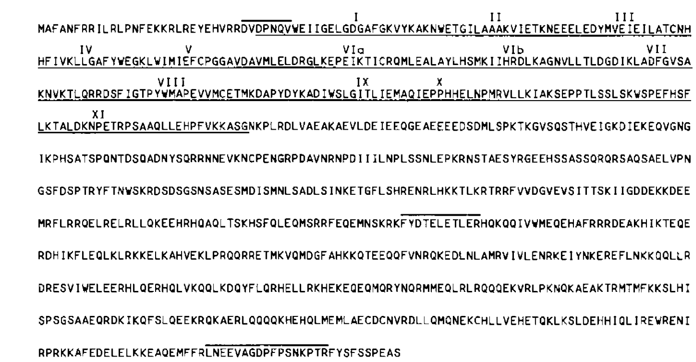
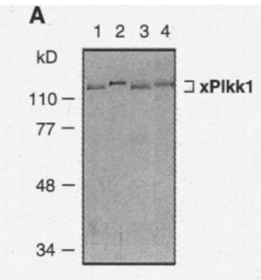


Fig. 2. Predicted amino acid sequence of xPlkk1. The nucleotide sequence of xPlkk1 cDNA (GenBank accession number AF100165) contains an open reading frame predicting 950 amino acids with the Kozak consensus (*27*) at the initiation codon and the initiating ATG preceded by one in-frame stop codon. Sequences corresponding to those obtained from tryptic peptides of xPlkk1 are indicated by lines above the sequence. The kinase domain is underlined, and the 11 conserved subdomains are indicated in Roman numbers above the sequence.



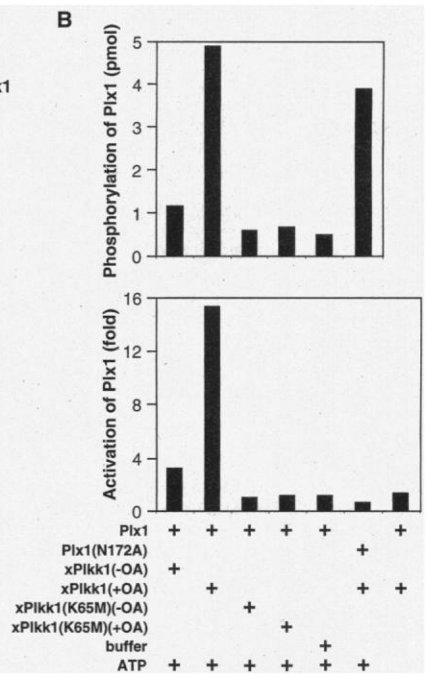


Fig. 3. Phosphorylation and activation of Plx1 by recombinant xPlkk1. (**A**) Samples of recombinant xPlkk1 or catalytically inactive xPlkk1(K65M) purified from Sf9 cells that were left untreated or treated with OA were subjected to SDS gel electrophoresis and Coomassie blue staining (*28*). Lane 1, xPlkk1(−OA); lane 2, xPlkk1(+OA); lane 3, xPlkk1(K65M)(−OA); lane 4, xPlkk1(K65M)(+OA). (**B**) Recombinant Plx1 (*29*) (0.75 μg) was incubated with the various xPlkk1 preparations (20 ng), as indicated, in the presence of [γ-^{32}P]ATP and subjected to SDS gel electrophoresis. The Plx1 bands were excised, and incorporated radioactivity was quantified by liquid scintillation spectrometry (top). Plx1 immunoprecipitated with antibody to Myc (*16*) was incubated with the various preparations of xPlkk1 as indicated, washed, and assayed for casein phosphorylation (bottom).

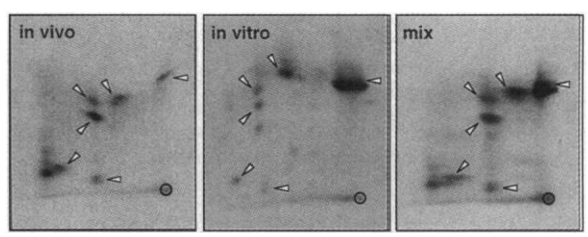


Fig. 4. Phosphopeptide mapping of Plx1. Chymotryptic digests of Plx1 radiolabeled in progesterone-treated oocytes (in vivo) or in vitro by xPlkk1 (in vitro) were separated by electrophoresis (right to left) and chromatography (bottom to top) (*25*). In the right panel (mix) an equal amount of radioactivity from each preparation was analyzed. Arrowheads indicate phosphopeptides common to both preparations, and circles indicate the origin.

(Fig. 1D), indicating that xPlkk1 directly phosphorylates Plx1.

From about 1.2 kg of dejellied eggs, 20 μg of xPlkk1 was isolated, and three of its tryptic peptides were sequenced (*19*). On the basis of the sequences of two of these peptides, polymerase chain reaction (PCR) primers were designed and used to generate a 1-kb fragment of the xPlkk1 gene (*20*). This PCR product was then used to isolate a full-length 3.3-kb cDNA encoding an open reading frame of 950 amino acids with a predicted relative molecular mass (M_r) of 110,000. All three tryptic peptide sequences were present within the open reading frame (Fig. 2). The complete amino acid sequence indicates that xPlkk1 has the typical motifs of a serine-threonine protein kinase, with a catalytic domain in the NH_2-terminal half of the polypeptide. xPlkk1 displays 36% identity to STE20 in the kinase domain (*21*) and 65% overall identity to a murine lymphocyte-specific kinase of unknown function (*22*). However, xPlkk1 is found in embryos (which contain many cell types) and in *Xenopus* tissue culture cells (*17*) and therefore appears not to be functionally related to lymphocyte-specific kinases. The noncatalytic domain of xPlkk1 contains a predicted long coiled-coil structure, which has been found in many proteins that participate in centrosome and spindle function (*23*).

xPlkk1 was expressed as a Flag- and His_6-fusion protein in baculovirus-infected Sf9 cells. Because xPlkk1 activity is high in mitotic extracts (*17*), the cells expressing xPlkk1 were treated with okadaic acid (OA) to drive them into a state resembling mitosis (*24*). Such treatment has been used to isolate active Plx1 from insect cells (*9*). As a control, catalytically inactive xPlkk1(K65M), in which the lysine residue at the ATP-binding site of the catalytic domain was changed to methionine, was also expressed. Both xPlkk1 and xPlkk1(K65M) were purified from Sf9 cells, untreated (−OA) or treated with OA (+OA), by chromatography on TALON metal affinity resin and Mono S, and samples were subjected to SDS gel electrophoresis (Fig. 3A). The xPlkk1(+OA) preparation phosphorylated recombinant Plx1 efficiently, whereas xPlkk1(−OA) did so poorly, and the K65M mutant preparations did not support phosphorylation (Fig. 3B). Treatment of Plx1 by xPlkk1(+OA) increased the activity of Plx1 15-fold, whereas the activity of Plx1 treated with xPlkk1(−OA) increased only 3-fold, and Plx1 treated with the K65M mutant preparations was not activated at all. Moreover, the activation of Plx1 by xPlkk1(+OA) was dependent on ATP, indicating that the observed phosphorylation is required for the activation. Although xPlkk1(+OA) did phosphorylate Plx1(N172A), it did not elicit any casein kinase activity (Fig. 3B). Similar experiments confirmed that Plx1 was activated by xPlkk1 for phosphorylation of Cdc25C to a similar extent as for casein (*17*).

Because recombinant xPlkk1(+OA) phosphorylated and activated Plx1, we tested whether this phosphorylation occurred at physiologically relevant sites. Chymotryptic phosphopeptide maps of Plx1 that had been phosphorylated with xPlkk1(+OA) in vitro or in vivo during progesterone-induced oocyte maturation were prepared (*25*). The map of Plx1 phosphorylated in vivo revealed the presence of six discrete phosphopeptides that comigrated with those from Plx1 phosphorylated in vitro by xPlkk1(+OA) (Fig. 4). These findings indicate that xPlkk1(+OA) phosphorylates Plx1 at physiologically relevant sites and that xPlkk1 may activate Plx1 during oocyte maturation.

The xPlkk1 protein from cells treated with OA migrated more slowly upon SDS gel electrophoresis than xPlkk1(−OA) and was also much more active (Fig. 3). To determine whether phosphorylation accounted for these differences, we treated the xPlkk1 proteins with

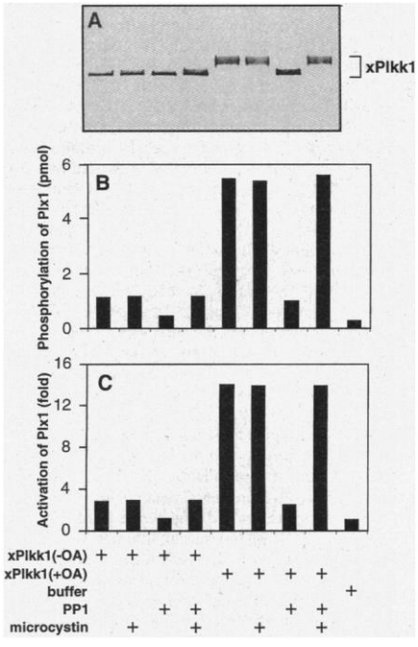


Fig. 5. Activation of xPlkk1 by phosphorylation. Samples of xPlkk1 prepared from Sf9 cells that had been left untreated or treated with OA were incubated with PP1 in the presence or absence of microcystin as indicated (*30*). Samples were subjected to SDS gel electrophoresis and immunoblotting (**A**) and assayed for phosphorylation (**B**) and activation of Plx1 (**C**) as described (Fig. 3B).

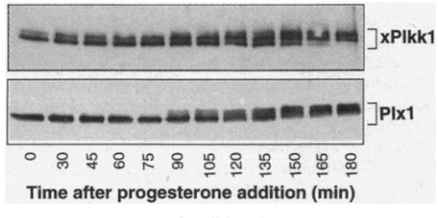


Fig. 6. Activation of xPlkk1 during oocyte maturation. Oocytes were incubated in the presence of progesterone (3.2 μM), and groups of 20 oocytes were frozen at the indicated times. Samples were subjected to immunoblotting with antibodies to xPlkk1 (*31*) and Plx1.

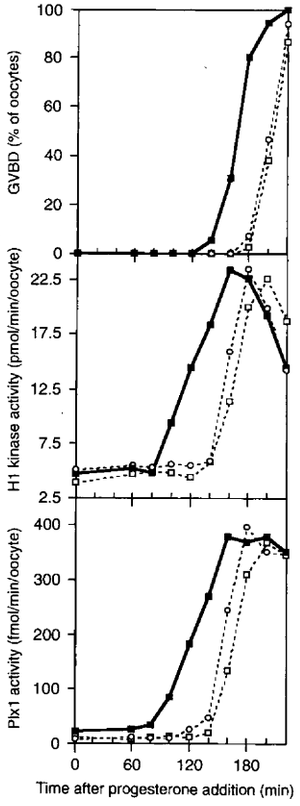


Fig. 7. Accelerated activation of Plx1 in vivo after microinjection of xPlkk1. Oocytes were microinjected with either buffer, wild-type xPlkk1, or catalytically inactive xPlkk1(K65M) (12 ng per oocyte) and then incubated in the presence of progesterone (64 nM). Groups of six oocytes were frozen at the indicated times. The time of germinal vesicle breakdown was established by examining oocytes for white spot formation with a dissecting microscope. Extracts were prepared, and histone H1 kinase and Plx1 activities were determined, all as indicated. Symbols: filled squares, wild-type xPlkk1; open squares, catalytically inactive xPlkk1(K65M); open circles, buffer.

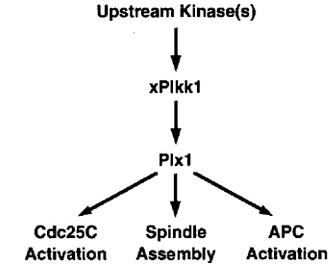


Fig. 8. Schematic diagram of the Plx1 protein kinase cascade.

protein phosphatase 1 (PP1) and again assessed their electrophoretic mobility and activity. Treatment of xPlkk1(+OA) with PP1 increased its electrophoretic mobility and reduced its activity by 85% (Fig. 5). xPlkk1(−OA) treated with PP1 also showed increased electrophoretic mobility and had no detectable activity. These results suggest that xPlkk1 is itself activated by phosphorylation by one or more as yet unidentified protein kinases.

If xPlkk1 activates Plx1 in vivo, then both kinases should be activated in concert during cell cycle transitions such as oocyte maturation. Activation of xPlkk1 did parallel exactly the kinetics of activation of Plx1 in vivo (Fig. 6). As a more direct test of participation in the Plx1 activation pathway, recombinant xPlkk1 was microinjected into oocytes that were subsequently treated with progesterone. Microinjection of xPlkk1 caused acceleration of both the activation of Plx1 and the G_2-M transition (Fig. 7) and also accelerated activation of Cdc25C (*17*), consistent with the activation of Plx1. These results provide further evidence for a role of xPlkk1 in the activation of Plx1 during oocyte maturation.

Our results define a protein kinase cascade that regulates the activity of polo-like kinases (Fig. 8). This cascade is likely to be crucial for mitotic control because of the key role polo-like kinases play in activation of Cdc25C, leading to Cdc2–cyclin B activation (*9, 10*), in organization of bipolar spindle assembly (*10, 11*), and in activation of the anaphase-promoting complex (*11, 13*). It is significant that each of these functions is the target of one or more cell cycle checkpoints.

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16. Oocytes injected with mRNA encoding Plx1 or catalytically inactive Plx1(N172A) (containing an Asn^{172}→Ala mutation), tagged at the COOH-terminus with either Flag or Myc, were lysed, and proteins were immunoprecipitated with antibody to Flag (M2) or Myc (9E10) (*10*). The immune complexes were incubated with samples to be assayed in kinase buffer containing 1 mM ATP at 30°C for 30 min. The immune complexes were then washed and assayed for phosphorylation of α-casein (*10*). Data are expressed as fold activation relative to samples to which control buffer was added.
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18. Dejellied unfertilized *Xenopus* eggs (200 ml) were homogenized in three volumes of extraction buffer [55 mM β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4)] and centrifuged at 27,000*g* for 15 min. All fractions were assayed for the activation of Plx1 (*16*), and all procedures were done at 4°C. The supernatant was subjected to ammonium sulfate fractionation, and the precipitate at 20 to 35% saturation with ammonium sulfate was dissolved in 160 ml buffer A [15 mM β-glycerophosphate, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM NaF, 1 mM DTT (pH 7.5)] containing 0.1 mM Na_3VO_4 and 1 mM PMSF, diluted with one-half volume of H_2O, applied to a 200-ml DEAE Sepharose column equilibrated with buffer A, and eluted with a linear gradient up to 0.5 M NaCl in buffer A. Fractions that contained xPlkk activity (65 to 155 mM NaCl) were pooled, diluted with an equal volume of H_2O, adjusted to pH 7.0, applied to an 8-ml SP Sepharose column equilibrated with buffer B [27 mM β-glycerophosphate, 5 mM $MgCl_2$, 10 mM NaF, 1 mM DTT, (pH 7.0)], and eluted with a linear gradient up to 1 M NaCl in buffer B. Fractions that contained xPlkk activity eluting at 220 to 330 mM NaCl were pooled. The proteins were precipitated with ammonium sulfate, resuspended in buffer C [55 mM β-glycerophosphate, 5 mM $MgCl_2$, 2 mM DTT, 150 mM NaCl, 0.01% Brij-35, 10% ethylene glycol (pH 7.0)], and stored at −80°C. The pooled material from three SP Sepharose column runs was applied to a Superdex 200 column equilibrated with buffer C and eluted with the same buffer. Fractions that contained xPlkk activity eluting with an approximate M_r = 145,000 were diluted with an equal volume of buffer D (2 mM DTT, 0.01% Brij-35, 10% ethylene glycol), applied to a 2-ml hydroxyapatite column equilibrated with buffer D containing 10 mM potassium phosphate (pH 7.0) and eluted with a gradient up to 500 mM potassium phosphate (pH 7.0) in buffer D. Fractions that contained xPlkk activity eluting at 290 to 370 mM potassium phosphate were pooled, diluted with six volumes of buffer E [20 mM tris, 2 mM DTT, 0.01% Brij-35, 10% ethylene glycol (pH 7.0)], applied to a 1-ml Mono Q column equilibrated with buffer E containing 5 mM $MgCl_2$, 2 mM EGTA, and 20 mM NaCl, and eluted with a gradient up to 1 M NaCl. Fractions that contained xPlkk activity were stored at −80°C.
19. Digestion, separation, and peptide sequencing were performed at the Harvard Microchemistry Facility.
20. Two degenerate primers corresponding to the peptide sequences FYDTELETLER and LNEEVAGDPFPSNKPTR (*26*) were designed: ACIGA(A/G)(C/T)TIGA(A/G)ACI(C/T)TIGA(A/G)(A/C)G and GG(A/G)TCICCIGCIAC(C/T)TC(C/T)TC. PCR was done with 0.5 μg of each primer, 20 ng of *Xenopus* oocyte cDNA, and 1 U of Taq polymerase (GIBCO) for five cycles at 93°C for 2 min, 54°C for 3 min, and 72°C for 3 min; and 35 cycles at 93°C for 1 min, 54°C for 1 min, and 72°C for 2 min. The reaction generated a 1-kb DNA, which was subcloned, sequenced, and shown to encode the previously determined peptide sequences. A *Xenopus* oocyte λZAP Express cDNA library was screened with this 1-kb PCR product, and the largest insert was sequenced on both strands. Sequencing was done with an ABI Prism 377 automated DNA fluorescent sequencer (Applied Biosystems) in the DNA Sequencing/Analysis Core Facility at the University of Colorado Cancer Center.
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25. Oocytes that had each been injected with 50 ng of recombinant Plx1 and 8 μCi of [γ-^{32}P]ATP were treated with progesterone and harvested when about 50% of the oocytes had undergone germinal vesicle breakdown. Plx1 was isolated by immunoprecipitation with antibody to Plx1 (*10*). Plx1 was phosphorylated in vitro as described (Fig. 3B). Digests were prepared as described [B. G. Gabrielli, M. S. Lee, D. H. Walker, H. Piwnica-Worms, J. L. Maller, *J. Biol. Chem.* **267**, 18040 (1992)] except that chymotrypsin was used and then separated by electrophoresis (pH 8.9, 1000 V, 60 min) in the first dimension and chromatography [pyridine/butanol/acetic acid/water(3.3:5:1:4)] in the second dimension.
26. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. Full-length xPlkk1 with COOH-terminal Flag and His_6 tags was subcloned into the baculovirus transfer vector pVL1393 (Invitrogen). Recombinant baculoviruses were isolated, and baculovirus-infected Sf9 cells were produced in the Tissue Culture/Monoclonal Antibody Core Facility at the University of Colorado Cancer Center. The catalytically inactive K65M mutant of xPlkk1 was created by PCR with oligonucleotides having the sequences AGCTGCCATGGTAATTGAGACCAAGAATGAG and CAATTACCATGGCAGCTGCTAAAATGCC. Recombinant xPlkk1 proteins were prepared from Sf9 cells untreated or treated with 0.1 μM OA for 3 hours, with the use of TALON affinity resin (Clontech) as described (*10*) except that the resin was washed twice with buffer that also contained 0.5 M NaCl. To further purify the proteins, we diluted the eluate from the resin with three volumes of buffer F (5 mM $MgCl_2$, 1 mM DTT, 0.01% Brij-35, 10% ethylene glycol), applied it to a Mono S column equilibrated with buffer F containing 20 mM Hepes (pH 7.0), and 2 mM EGTA, and eluted it with a linear gradient up to 1 M NaCl. Fractions containing the xPlkk1 proteins (540 to 600 mM NaCl) were stored in small portions at −80°C. The concentration of protein was determined by SDS gel electrophoresis, Coomassie blue staining, and densitometry, with bovine serum albumin as a standard.
29. Recombinant Plx1 proteins prepared from Sf9 cells (*10*) were further purified. The eluate from the TALON resin was diluted with four volumes of buffer D and fractionated by hydroxyapatite chromatography (*18*). Fractions containing the Plx1 proteins (235 to 265 mM potassium phosphate) were pooled, diluted with four volumes of buffer F, and fractionated by Mono S chromatography. Fractions containing the Plx1 proteins were stored in small portions at −80°C, and the concentration of protein was quantified as described above (*28*).
30. Portions of the xPlkk1 preparations (60 ng) were treated with 0.15 U of *Xenopus* PP1 in the presence or absence of 33 μM microcystin at 30°C for 15 min, after which all samples were adjusted to a final concentration of 33 μM microcystin. One unit of PP1 is the amount of enzyme that will release 1.0 nmol of phosphate per minute from phosphorylase *a* at 30°C. The samples were divided into three portions for immunoblotting xPlkk1 with antibody to Flag (M2) and for assay of phosphorylation and activation of Plx1.
31. Flag- and His_6-tagged xPlkk1 bound to TALON resin was used directly to immunize rabbits, and xPlkk1 antibodies were affinity purified on a column of recombinant xPlkk1 coupled to Affi-gel 10 resin.

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