A Feedback Loop in the Polo-like Kinase Activation Pathway*

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The Xenopus polo-like kinase Plx1 plays important roles during entry into and exit from mitosis (M phase). Previous studies revealed that Plx1 is activated by phosphorylation on serine and threonine residues, and purification of an activating enzyme from mitotic Xenopus egg extracts led to cloning and characterization of Xenopus polo-like kinase kinase (xPlkk1), which can phosphorylate and activate Plx1 in vitro. In the present study, a positive feedback loop between Plx1 and xPlkk1 was shown to result in each kinase phosphorylating and activating the other. Sequencing of radiolabeled xPlkk1 after phosphorylation by Plx1 identified three phosphoserine residues that act as an upstream activating enzyme during the G2/M transition.

Studies in many laboratories have shown that entry into mitosis is controlled by the activation of cyclin B/Cdc2 following dephosphorylation of Tyr-15 in Cdc2 by the dual specificity phosphatase Cdc25C. Cdc25C itself is activated at the G2/M transition by phosphorylation on serine and threonine residues (1–3). Although this activating phosphorylation can be performed by cyclin B/Cdc2 itself (forming a positive feedback loop), evidence in Xenopus eggs and extracts and in mammalian cells suggests that initial activation of Cdc25C depends on phosphorylation by a polo-like kinase (4–9). The Xenopus polo-like kinase Plx1 binds Cdc25C through a noncatalytic domain, thought to represent a phosphorylation sequence in Cdc25C (10–12). Recent evidence (13) suggests that initial phosphorylation of Cdc25C by p38 may facilitate binding and activation of Cdc25C by Plx1. However, prior phosphorylation is not obligatory, because Plx1 can efficiently phosphorylate bacterially expressed Cdc25C (see Fig. 2C), which presumably is not phosphorylated. Other studies on the interaction of Plx1 and Cdc25C have shown that overexpression of activated Plx1 accelerates phosphorylation of Cdc25C (14, 15), and depletion of Plx1 from oocyte extracts blocks Cdc25C activation and the G2/M transition (6, 8). In addition to Cdc25C activation, polo-like kinase is also required for bipolar spindle formation, activation of the anaphase-promoting complex/cyclosome, and cytokinesis (14–21). Because Plx1 controls both the activation and deactivation of cyclin B/Cdc2, it is a major player in the control of mitosis. This has focused attention on the pathway of Plx1 activation. Earlier studies showed that polo-like kinases were activated by phosphorylation, implicating an unidentified protein kinase as being responsible for activation (14, 22, 23). Purification and cloning of an activity from M-phase Xenopus egg extracts that could phosphorylate and activate Plx1 in vitro led to characterization of a novel Ste20-related kinase, termed Xenopus polo-like kinase kinase 1 (xPlkk1) (24). Related kinases from mammalian cells have been identified that also phosphorylate (25–27) and activate (26, 27) the polo-like kinase Plk1 in vitro. The activity of these enzymes is not increased in mitosis, and whether these enzymes are relevant for activation of Plk1 in vivo has not yet been clarified (25, 26). However, overexpression of xPlkk1 in oocytes accelerates the activation of Plx1 and the Cdc25C-dependent G2/M transition (24). xPlkk1 itself could be deactivated by treatment with serine/threonine phosphatases, indicating the existence of a polo-like kinase kinase (24). These results suggest that a protein kinase cascade regulates activation of Plx1.

When characterizing any new protein kinase, it is highly desirable to generate gain-of-function mutants to probe specific functions of the kinase itself in the absence of complex upstream signaling pathways. For protein kinases activated by phosphorylation, one approach to generating gain-of-function mutants is to mutate sites of activating phosphorylation to acidic residues, such as aspartic acid or glutamic acid, where the negatively charged amino acid may mimic the effects of phosphorylation. Such an approach has been used previously to generate constitutively active forms of Plx1, Mek1, and p90Rsk (15, 28–31). In this paper, we identified several sites of phosphorylation in xPlkk1 and evaluated their role in the activation process. We also utilized immunodepletion approaches to evaluate whether either kinase is required for activation of the other.

**EXPERIMENTAL PROCEDURES**

Mutagenesis and Preparation of Recombinant Proteins—Cloning and insertion of PLX1 and XPLKK1 cDNAs into the vectors pOTVF and pOTVKe

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1 The abbreviations used are: xPlkk1, Xenopus polo-like kinase kinase 1; PKI, heat-stable inhibitor of the cAMP-dependent protein kinase; MBF, myelin basic protein; GVBD, germin vesicle (nuclear) breakdown.  
2 R. L. Erikson, personal communication.
pVLFH have been described previously (14, 24). pOTVF is the vector pOTV that also encodes a C-terminal FLAG tag, and pVLFH is the baculovirus transfer vector pVL1393 (Invitrogen) that also encodes C-terminal FLAG and hexahistidine tags. The catalytically inactive form of Plx1, Plx1(N172A), (5, 14) and the equivalent inactive form of xPlkk1, xPlkk1(N162A), were also used. These are denoted as Plx1(NA) and xPlkk1(NA), respectively. Site-directed mutagenesis of XPLKK1 cDNA was performed by the QuikChange method (Stratagene, La Jolla, CA) on nucleotides 1315–2229, which had been subcloned from pOTVF-XPLKK1 into pGEM-T Easy (Promega, Madison, WI). The three serine residues, 482, 490, and 486, were mutated to Ala in that order with the following pairs of oligonucleotides: 5′/H11032-GCATGGACATCGCAATGAATT-TGTCTGCGG-3′/H11032 and 5′/H11032-CCGCAGACAAATTCATTGCGATGTCCATG-C-3′/H11032; 5′/H11032-GTCTGCGGACCTGGCCATTAACAAGGAAACTGG-3′/H11032 and 5′/H11032-CCAGTTTCCTTGTTAATGGCCAGGTCCGCAGAC-3′/H11032; 5′/H11032-CATCGCAATGAATTTGGCCGCGGACCTGGCCATTAAC-3′/H11032 and 5′/H11032-GTTAATGGCCAGGTCCGCGGCCAAATTCATTGCGATG-3′/H11032. The relevant Ala codons are underlined. The mutated region was excised with the restriction enzymes AfeI and BbvCI (nucleotides 1323–1924) and ligated into AfeI-BbvCI-cut XPLKK1 or XPLKK1(N162A) in the vector pOTVF to generate pOTVF-XPLKK1(SA3) and pOTVF-XPLKK1(NA;SA3), respectively. mRNAs were transcribed from these pOTVF constructs with the use of the mMessage mMachine kit (Ambion, Austin, TX).

The full-length XPLKK1(SA3) and XPLKK1(NA;SA3) cDNAs were then subcloned into pVLFH. All cDNAs were fully sequenced to confirm the relevant mutations and the absence of incidental mutations. Plx1 and xPlkk1 proteins expressed in baculovirus-infected Sf9 cells were isolated with TALON metal-affinity resin (Clontech, Palo Alto, CA) and eluted. Plx1 proteins were further purified by hydroxyapatite (CHT-1; Bio-Rad) and MonoS (HR5/5; Amersham Biosciences) chromatography, and the xPlkk1 proteins were further purified by MonoS chromatography. The basal specific activity of xPlkk1(SA3) was similar to that of xPlkk1, 2 nmol/min/mg, with myelin basic protein (MBP) as the substrate. Where indicated, the Sf9 cells were treated with 100 nM okadaic acid for 3 h prior to harvest to generate highly active enzymes (5, 24); these preparations, depicted as (OA) in Figs. 2 and 4, have specific activities 10-fold higher than the corresponding preparations from untreated cultures. The enzymes were stored in small aliquots at

FIG. 1. Reciprocal phosphorylation of Plx1 and xPlkk1. A, Plx1 and xPlkk1 proteins (50 and 25 ng, respectively), as indicated, were incubated in kinase buffer for 2.5 min and subjected to SDS-PAGE, and the products of the reaction were visualized by autoradiography. Exposure was for 6 h. Molecular mass markers (in kDa; Amersham Biosciences) are indicated on the right, and the positions of Plx1 and xPlkk1 are indicated on the left. B, samples of each of the Plx1 and xPlkk1 preparations (300–500 ng) used in A and in further experiments were subjected to SDS-PAGE and visualized by Coomassie Blue staining. Upper panel, lane 1, Plx1; lane 2, Plx1(NA); lane 3, Plx1(OA); lane 4, Plx1(NA;OA). Lower panel, lane 5, xPlkk1; lane 6, xPlkk1(NA); lane 7, xPlkk1(OA); lane 8, xPlkk1(SA3); lane 9, xPlkk1(NA;SA3). Molecular mass markers (in kDa) are indicated on the right, and the positions of Plx1 and xPlkk1 are indicated on the left.

FIG. 2. Activation of xPlkk1 by Plx1. A, xPlkk1 (25 ng) was preincubated with or without Plx1(OA) (50 ng), and at the indicated times, 5-μl samples were removed and assayed for phosphorylation of MBP, as described under “Experimental Procedures.” B, procedures were the same as described for A, except enzymatically inactive Plx1(NA;OA) was used. This enzyme was also prepared from okadaic acid (OA)-treated Sf9 cells to control for potential contaminating okadaic acid-activated Sf9 cell enzymes. C, activation of Plx1 by xPlkk1. Procedures were the same as described for A, but Plx1 (100 ng) was preincubated with or without xPlkk1(OA) (50 ng). At the indicated times, 2.5-μl samples were removed and assayed for phosphorylation of Cdc25C (residues 1–264).
---SDGSNSASESMDISMNLSDSINKETGFL---

Fig. 3. Sites in xPlkk1 that are phosphorylated by Plx1. The serine residues identified as being phosphorylated by Plx1 are highlighted and numbered.

−80 °C as described previously (24). The glutathione S-transferase-tagged N-terminal half of Xenopus Cdc25C (residues 1–264) was prepared according to standard procedures.

Preparation of Oocytes and Oocyte Extracts—Xenopus laevis females were obtained from Nasco (Ft. Atkinson, WI). Techniques for dissection, culture, and microinjection of oocytes and for preparation of oocyte extracts have been described previously (6, 14, 32). Preparation of affinity-purified antibodies against Plx1 and xPlkk1 and of control IgGs depleted of antibody against Plx1 or xPlkk1 was as described previously (14, 24). The purified xPlkk1 antibody was suitable for immunoblotting and immunoprecipitation but proved unsuitable for immune-complex kinase assays. G₂/prophase oocyte extracts were depleted of Plx1 or xPlkk1 by incubation with antibody covalently coupled to Affi-Prep protein A support beads (6).

Phosphorylation Reactions—Samples were incubated at 30 °C in kinase buffer (20 mM HEPES, pH 7.2, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 0.01% Brij-35). For phosphorylation of Plx1 and xPlkk1, the kinase buffer was supplemented with 100 μM of ovalbumin/ml and 100 μM [γ-³²P]ATP (2–4 cpm/fmol). Reactions were stopped by the addition of one-fourth volume of 15 mM ATP. At the indicated times, the samples were removed, added to kinase buffer supplemented with 100 μM [γ-³²P]ATP (2–4 cpm/fmol), and incubated at 30 °C for 5 min with MBP (500 μg/ml) for xPlkk1 or Cdc25C (residues 1–264) (16.7 μg/ml) for Plx1. The reactions were analyzed as described above, and incorporation of radiolabel was quantified by liquid scintillation spectrometry of the excised gel bands.

For identification of phosphorylation sites, 1 μg of Plk1 and 2 μg of xPlkk1 were incubated together for 30 min in a total volume of 60 μl in kinase buffer supplemented with 100 μM [γ-³²P]ATP (10–15 cpm/fmol). Under these conditions incorporation of phosphate into xPlkk1 was ~15 mol/mol. It proved necessary to remove radiolabeled Plx1 from the sample because radiolabeled peptides from Plx1 interfered with identification of peptides from xPlkk1. To accomplish this, the reaction was stopped by the addition of one-eighth volume of 5× concentrated sample buffer and incubated at 95 °C for 3 min. The sample was diluted with 15 volumes of buffer (10 mM Tris, pH 7.2, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate), supplemented with 10 μg of anti-xPlkk1 and incubated overnight at 4 °C. The immunocomplexes were adsorbed to Protein A beads and washed several times with kinase buffer. Radiolabeled xPlkk1 on the beads was cleaved with cyanogen bromide, and the peptides were eluted with 0.1% trifluoroacetic acid. The sample was then subjected to Edman degradation, and release of radioactivity was monitored at each cycle. The results were analyzed by SDS-PAGE, Coomassie Blue staining, and autoradiography. For activation experiments, Plx1 and xPlkk1 were preincubated together in 10 μl of kinase buffer supplemented with 200 μM ATP. At the indicated times, the samples were removed, added to kinase buffer supplemented with 100 μM [γ-³²P]ATP (2–4 cpm/fmol), and incubated at 30 °C for 5 min with MBP (500 μg/ml) for xPlkk1 or Cdc25C (residues 1–264) (16.7 μg/ml) for Plx1. The reactions were analyzed as described above, and incorporation of radiolabel was quantified by liquid scintillation spectrometry of the excised gel bands.

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Immunoblot Analysis—Immunoblotting was performed as described previously (14). Phosphospecific antisera (anti-xPlkk1-3P sera) against a peptide phosphorylated on all three phosphorylation sites were prepared by PhosphoSolutions (Aurora, CO). For preliminary characterization of these sera, xPlkk1(NA) was phosphorylated by Plx1(1/2A) as described above for the identification of phosphorylation sites, but the specific activity of the ATP was 650 cpm/pmol. Affinity-purified antibodies were prepared from these sera on a column containing the triply phosphorylated peptide.

RESULTS AND DISCUSSION

A Feedback Loop Between xPlkk1 and Plx1—Initial studies on the characterization of both Plx1 and xPlkk1 showed that both enzymes undergo autophosphorylation (14, 24). Further analysis of reactions containing both Plx1 and xPlkk1 revealed greatly increased phosphorylation of each enzyme in the presence of the other (Fig. 1, lanes 1–3). The effect was much larger for xPlkk1 than for Plx1. The phosphorylation of Plx1 by xPlkk1 has been shown previously to activate Plx1 (24). To assess the effect of phosphorylation of xPlkk1 by Plx1 on its activity, xPlkk1 was preincubated with highly active Plx1, and its phosphotransferase activity was quantified. Time course studies demonstrated that xPlkk1 was activated by Plx1-dependent phosphorylation in a manner additive with activation due to autophosphorylation (Fig. 2). Activation was completely dependent on the presence of ATP during the preincubation (data not shown). The Plx1 used in these experiments was highly active enzyme purified from okadaic acid-treated SF9 cells; identical results were obtained when a constitutively active mutant of Plx1, Plx1(T201D), was used (data not shown). In addition, Fig. 2C confirms that xPlkk1 can activate Plx1 in
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During oocyte maturation xPlkk1 becomes highly phosphorylated, as judged by retarded mobility upon SDS-PAGE (24). To investigate the phosphorylation of these three serine sites of xPlkk1 in a physiologically relevant system, we generated a phosphospecific antibody against a peptide triply phosphorylated by Plx1 (data not shown) and thus were not due to autophosphorylation of xPlkk1. Two of these sites are conserved in the related mammalian kinases, Stk10, SLK, and LOK (25, 26, 34). To determine whether phosphorylation of these sites is necessary for activation, these three amino acids were all changed to Ala, the mutant enzyme (xPlkk1(SA3)) was expressed in baculovirus-infected SF9 cells, and its ability to be phosphorylated and activated by Plx1 was assessed. As shown in Fig. 4, the ability of Plx1 to both phosphorylate and activate xPlkk1(SA3) in vitro was greatly reduced.

xPlkk1 Phosphorylation in Vivo during Oocyte Maturation—During oocyte maturation xPlkk1 becomes highly phosphorylated, as judged by retarded mobility upon SDS-PAGE (24). To investigate the phosphorylation of these three serine sites of xPlkk1 in a physiologically relevant system, we generated a phosphospecific antibody against a peptide triply phosphorylated at Ser-482, -486, and -490. Western blots show that this antibody recognizes xPlkk1 phosphorylated by Plx1 but not unphosphorylated xPlkk1 or autophosphorylated xPlkk1 (Fig. 5A). This situation is analogous to that of phosphorylase kinase, where activating sites phosphorylated by the cAMP-dependent protein kinase are distinct from the sites involved in activation by autophosphorylation (35). Moreover, in oocytes expressing ectopic xPlkk1, this antibody recognized xPlkk1 from M-phase (GVBD) oocytes but not from G2-phase oocytes (Fig. 5B). This antibody could also detect phosphorylation of endogenous xPlkk1 at these sites (Fig. 5C). Interestingly, a second protein that migrated with a slightly lower Mr, than xPlkk1 was also recognized by this antibody in M-phase. This could represent an xPlkk1 degradation product not recognized by our anti-xPlkk1 antibody (Fig. 7C). These results indicate that phosphorylation at these sites occurs in vivo during the G2/M transition and are consistent with previous evidence that both Plx1 and xPlkk1 are phosphorylated/activated in M-phase oocytes (14, 24).

These results identify a feedback loop between Plx1 and xPlkk1 in which each kinase can phosphorylate and activate the other. In any positive feedback loop, it can be difficult to determine upstream/downstream relationships. To further elucidate the relationship between these two enzymes, we examined their activity in extracts from G2-phase oocytes. Upon
treatment with the heat-stable inhibitor of the cAMP-dependent protein kinase, PKI, these extracts initiate the key molecular events that occur at the G2/M transition during oocyte maturation, including activation of Plx1, Cdc25C, and cyclin B/Cdc2 histone H1 kinase activity (6, 32). An advantage of this system is that it makes immunodepletion/reconstitution experiments feasible, and previously this approach was used to establish that the activation of Cdc25C is dependent on Plx1 (6). Thus, we immunodepleted xPlkk1 and assessed the effect on activation of Plx1, and conversely, we immunodepleted Plx1 and assessed the effect on the phosphorylation of xPlkk1. As shown in Fig. 6, B and C, depletion of xPlkk1 did not prevent activation of either Plx1 or histone H1 kinase activity. In contrast, immunodepletion of Plx1 did prevent activation of histone H1 kinase activity (Fig. 7B), as shown previously (6), and moreover, phosphorylation of xPlkk1 was impaired (Fig. 7C). Both of these effects were reversed by the addition of recombinant Plx1 to the depleted extract. Together, these results demonstrated that xPlkk1 is not required for the activation of Plx1 and suggested that it may instead be a downstream target of Plx1, at least during the G2/M transition.

Evidence indicates Plx1 and xPlkk1 can be co-immunoprecipitated from M-phase extracts, and both kinases are localized on the mitotic spindle (data not shown), suggesting this feedback loop is likely to operate in a supramolecular “module” in M phase, analogous to the assembly of multiple mitogen-activated protein kinase cascade components on a Ste5 scaffold (36). The three sites of phosphorylation identified are likely to be necessary for activation because the SA3 mutant of xPlkk1 was unable to be activated in vitro by purified Plx1. However, when these same three phosphorylation sites were mutated to either Asp or Glu, the enzyme expressed in Sf9 cells was not highly active (data not shown). Therefore, in this case, substitution of even a multiple phosphorylated residue(s) with an acidic resi-
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due(s) does not result in a constitutively active enzyme. Given the high stoichiometry of phosphorylation of xPlkk1 by Plx1, it seems likely that additional phosphorylation sites in xPlkk1 contribute to activity.

It is of some interest that the three sites identified are spaced at intervals of four amino acids (Fig. 3). Two of these sites fit the consensus acidic-X-pSer-hydrophobic, which has been determined for several other substrates of polo-like kinases (37–40). However, other phosphorylation sites that do not fit this paradigm have also been documented in substrates of polo-like kinases (41, 42). A similar pattern of phosphorylation at four-amino acid intervals has been reported for the phosphorylation of glycogen synthase by glycogen synthase kinase-3 (43, 44). In that case, the enzyme recognizes phosphoserine as part of its consensus motif. It seems unlikely that Plx1 also recognizes phosphoserine as part of its consensus motif, because there is no serine residue in xPlkk1 downstream of Ser-490 in the appropriate position (Fig. 3). Further work is necessary to determine whether the phosphorylation of xPlkk1 by Plx1 is ordered and to assess the relative contribution of each of the phosphorylation sites to enzyme activity.

In summary, the results in this paper identify Plx1 as a relevant activating kinase for xPlkk1 and identify sites of phosphorylation in xPlkk1 required for activation. The identification of protein kinases that function physiologically as upstream polo-like kinase kinases remains an important avenue for further work.

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