Human Prk Is a Conserved Protein Serine/Threonine Kinase Involved in Regulating M Phase Functions*

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Human prk encodes a novel protein serine/threonine kinase capable of strongly phosphorylating casein but not histone H1 in vitro. prk expression is tightly regulated at various levels during different stages of the cell cycle in lung fibroblasts. The Prk kinase activity is relatively low during mitosis, G1, and G2/S, and peaks during late S and G2 stages of the cell cycle. Recombinant human Prk expressed through the baculoviral vector system is capable of phosphorylating Cdc25C, a positive regulator for the G2/M transition. Human prk shares significant sequence homology with Saccharomyces cerevisiae CDC5 and Drosophila melanogaster polo, both of which are essential for mitosis and meiosis. Full-length prk transcripts greatly potentiate progesterone-induced meiotic maturation of Xenopus laevis oocytes. On the other hand, antisense prk transcripts significantly delay and reduce the rate of oocyte maturation. Taken together, prk may represent a new protein kinase, playing an important role in regulating the onset and/or progression of mitosis in mammalian cells.

Cyclin-dependent kinases (CDKs) control many phosphorylation events during the cell cycle, and are indispensable for eukaryotic cell division (1, 2). Because of their importance in the cell cycle, CDKs are structurally and functionally conserved across a wide spectrum of evolution. For example, human p34cdc2 is capable of functional complementation of temperature sensitive (ts) Cdc2 mutant strains of the fission yeast, Schizosaccharomyces pombe (3). During the past decade, significant progress has been made in structural and functional characterization of individual components such as cyclins and CDKs regulating the transit of cells through various checkpoints of the animal cell cycle (1–5). For example, p34cdc2 plays a rate-limiting role in the transition from G2 into M (6), and Cdc25C gene product, a dual specific protein phosphatase, dephosphorylates p34cdc2 on threonine 14 and tyrosine 15 residues and thereby activates p34cdc2 kinase activity (7). The Cdc25C phosphatase activity is also under regulation by reversible phosphorylation. It has been demonstrated that extensive phosphorylation of Cdc25C amino-terminal domain occurs at the onset of mitosis by a novel enzyme (8), and this phosphorylation strongly activates Cdc25C's phosphatase activity toward p34cdc2 (8, 9).

Recently, an emerging family of protein kinases (termed the polo kinase family) has been described in yeast, Drosophila, and higher animals. In Drosophila, the polo gene encodes a protein serine/threonine (Ser/Thr) kinase that is required for M phase functions (10, 11). Mutants of polo induce hyper-condensed chromosomes, abnormal spindle formation, and polyploidy (10, 11). A polo homolog encoded by CDC5 in Saccharomyces cerevisiae is required for nuclear division in the late mitotic stage (12). Deletion of this gene is lethal, resulting in a dumbbell-shaped terminal morphology with incomplete nuclear division (12). We have recently cloned a cDNA encoding a putative protein Ser/Thr kinase (13) named prk (proliferation-related kinase) which has a predicted molecular mass of 67.8 kDa. Prk shares an extensive homology with the polo family kinases, and the homology is not confined solely to the kinase domain (13). Expression of prk mRNA is inducible by growth factors or cytokines, and appears to be down-regulated in many lung carcinomas (13).

EXPERIMENTAL PROCEDURES

Cell Culture and Synchronization—Human A549 fibroblast cells originally derived from a lung carcinoma were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT) and antibiotics (100 units/ml penicillin and 50 μg/ml streptomycin sulfate, Life Technologies, Inc.). G1 cells were obtained by culturing in methionine-free DMEM containing 10% FBS for 48 h. Cells arrested at G1/S (some refer this stage as early S or S) (14, 15) were achieved by the treatment with aphidicolin (5 μg/ml) and hydroxyurea (0.5 mM) for 48 h. Later S (S/G2) cells were obtained through washing the G1/S-arrested cells as above with phosphate-buffered saline and reculturing them in DMEM with 10% FBS for 6 h. To obtain mitotic metaphase cells, A549 cells were treated with nocodazole (0.4 μg/ml) for 16 h. Aphidicolin, hydroxyurea, and nocodazole were purchased from Sigma.

RNA Isolation and Northern Blotting—Total RNA was isolated from A549 cells or Xenopus oocytes using the guanidine isothiocyanate method (16). Equal amounts of total RNA (15 μg) were fractionated on 1% formaldehyde agarose gels. The fractionated RNA was transferred to Nytran Plus membranes. The RNA blots were baked for 2 h, prehybridized for 2 h, and hybridized with a 32P-labeled prk or β-actin cDNA fragment overnight. After hybridization, the blots were washed and autoradiographed. High stringency hybridization and washing conditions were as described previously (17).

Antibody Production and Western Blotting—Both the N-terminal half (Prk-N, amino acids 20–341) (13) and the C-terminal half (Prk-C, amino acids 334–607) of Prk were expressed as a glutathione S-trans-
ferase (GST) fusion protein using the plasmid pGEX-2T (Pharmacia
Biotech Inc.). Fusion proteins were induced by isopropyl-β-D-thiogalac-
topyranoside and purified through affinity chromatography ac-
cording to the protocol provided by the supplier. Polyclonal anti-Prk
antiserum was produced in rabbits using the purified Prk-C through a
commercial source (Research Genetics, Inc., Atlanta, GA). For Western
topography and purified through affinity column chromatography
for 4 °C for at least 8 h. Immunoprecipitates were collected, washed four
times, and the incubation was continued at
histone H1: 25 mM HEPES, pH 7.4, 25 mM MgCl2; buffer for
immunoprecipitation mixture, and the incubation was continued at
histone H1: 25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 10 mM
MgCl2, 5 mM EDTA, 10 mM NaF, 1 mM dithiothreitol, and resuspended
in 30 μl of the respective kinase buffer. The kinase activity of immu-
noprecipitated Prk was assayed by the addition of a substrate (casein
(20 μg/reaction) or histone H1 (20 μg/reaction), Upstate Biotechology,
Inc., Lake Place, NY) and [γ-32P]ATP (2 μCi) to each kinase mixture,
and the reaction lasted for 30 min at 37 °C. The kinase reaction mix-
tures were analyzed by SDS-PAGE followed by autoradiography. Pur-
fied recombinant His-Prk expressed through the baculoviral expres-
sion system was also assayed for its in vitro kinase activity using
substrates such as casein (15 μg/reaction) and recombinant GST-
Cdc25C (2.5 μg/reaction). GST-Cdc25C was kindly provided by K. Ga-
lakinov. The kinase reaction conditions were the same as above. The
kinase reactions were terminated by the addition of 2 X SDS-polycryl-
amide gel sample buffer and analyzed on a 10 % SDS-polyacrylamide
gel followed by autoradiography. For phosphoamino acid analysis,
the phosphorylated GST-Cdc25C was excised and eluted from the gel.
The eluted GST-Cdc25C was then hydrolyzed in 6 N HCl for 60 min at
110 °C. The hydrolyzed amino acids were analyzed by two-dimensional
thin layer chromatography and autoradiography.
Expression and Purification of Recombinant Human Prk from Bacu-
lovirus-Induced Cell-Letters Recombinant Prk was expressed using the bacu-
viral expression system (PharMingen, San Diego, CA) following the
manufacturer's protocol. Briefly, a cDNA fragment containing the en-
tire open reading frame of human prk was cloned into PVL-1393 trans-
fer vector. To facilitate purification of recombinant Prk protein, a
short nucleotide sequence coding for six histidine residues was in-
serted in-frame immediately after the ATG codon of prk cDNA. Bacu-
viral expression vector BaculoGold™ DNA and the transfer plasmid
PVL-1393-Prk were then co-transfected into insect s/f9 cells. Insect s/f9 cells
insect recombinant Prk were harvested and lysed for purifi-
cation of recombinant Prk using Ni-nitrilotriacetic acid resin (Qiagen,
Chatworth, CA).
Oocyte Maturation Studies—The prk cDNA insert was cloned into
pCDNA3 expression vector (Invitrogen, Inc., San Diego, CA). In vitro
transcription was performed using T7 DNA-dependent RNA polymer-
ase in the presence of a Cap analog (m7G(5′)ppp(5′)) according to the
protocol provided by the supplier (Promega, Madison, WI). Antisense
transcripts were synthesized with SP6 DNA-dependent RNA polymer-
ase in the absence of the Cap analog. In vitro transcribed RNAs were
directly transfected into diethylpyrocarbonate-treated water at a con-
centration of 1 ng/μl. Stage 5–6 oocytes of Xenopus tropicalis (Zymed
Laboratories Inc., South San Francisco, CA) antibody raised in a rabbit and then with
goat-anti-rabbit antibodies conjugated with horseradish peroxidase.
The signals were detected by enhanced chemiluminescence (Amersham
Corp.).

RESULTS
Since prk mRNA expression is rapidly activated by serum or
selected cytokines (13), we first examined the steady state level of
prk mRNA during various stages of the cell cycle. Human A549 cells (21) were arrested at different stages of the cell cycle as
described under "Experimental Procedures." Fig. 1A shows that a low level of prk transcript was present in G1 cells (lane 3). The
prk transcript level was significantly elevated at the G2/S junction (lane 4), peaking at late S and G2 stages of the cell cycle (lane 5). Interestingly, prk mRNA level was markedly reduced during M phase (lane 6), indicating that prk mRNA expression was tightly regulated during the cell cycle. The cell cycle status in all cells was monitored by flow cytometric anal-
ysis of propidium iodide-stained cells (data not shown).

The Prk protein has two apparent domains with the approxi-
mate N-terminal half bearing the typical protein Ser/Thr ki-
nase structure (13). We generated recombinant GST fusion proteins containing either the N-terminal half (Prk-N) or the
C-terminal half (Prk-C) of Prk (data not shown). Since the C-terminal half of Prk shares no structural homology to any
other known proteins except for polo family kinases, purified
Prk-C was used to raise polyclonal anti-human Prk antibodies
in rabbits. An anti-Prk antiserum was first characterized for its
specificity. Western blot analyses showed that the antiserum
reacted with the molecular mass of 68 and 64 kDa, respectively, and
was immunoreactive to an anti-Prk antisera (Fig. 1B, lanes 1 and 2) but not to the preimmune serum (lanes 3 and 4). The 68-kDa
band, but not the 64-kDa band, was competed by an excess amount of purified Prk-C protein (Fig. 1B, lanes 5 and 6), indicating that the band of 68 kDa represents Prk protein. Prk is another known member of the polo family kinases in the
human, and it shares about 50% amino acid sequence identity with human Prk. Western blot analysis showed that Plk is also expressed in A549 cells, and it has a faster mobility of about 66 kDa than the 68-kDa Prk on denaturing gels (Fig. 1B, lanes 7 and 8). To determine Prk antigen levels, unsynchronized A549 cells as well as the cells synchronized at various stages of the cell cycle were analyzed for Prk expression by Western blotting using the anti-Prk anti-serum. Lane 6 represents cell lysates from unsynchronized murine erythroleukemia (MEL) cells. D, the same blot as shown in C was stripped and reprobed with the anti-Plk antibody.

to determine whether the Prk kinase activity is regulated during the cell cycle, cell lysates collected from defined stages of the cell cycle were immunoprecipitated with the anti-Prk antisera (lanes 3–6). As a control, the protein lysates from unsynchronized cells (unsyn) were immunoprecipitated with the preimmune serum (unsyn/pre, lane 1) or the anti-Prk antisera (unsyn, lane 2). The immunoprecipitates were analyzed for in vitro kinase activity using casein as a substrate.

CDK's kinase activity absolutely depends on the association with a specific cyclin. To determine whether Prk's kinase activity requires the association with other protein(s), we expressed full-length Prk using the baculoviral expression system. Western blot analyses revealed that His6-Prk was strongly expressed (Fig. 3A, lanes 2 and 3) in sf9 cells infected with recombinant baculovirus. The purified His6-Prk (Fig. 3A, lane 4) was analyzed for its kinase activity using casein as a substrate. Fig. 3B shows that His6-Prk strongly phosphorylates casein (lane 2, arrowhead casein), and the addition of staurosporine, a protein serine/threonine kinase inhibitor, drastically inhibited phosphorylation of casein by Prk (lane 6). Prk is capable of autophosphorylation (Fig. 3B, arrowhead Prk). The small arrowhead (Fig. 3B) denotes a degradation product of Prk, since it is immunoreactive to anti-Prk antibody (arrowhead in Fig. 3A). Since Cdc25C is extensively phosphorylated by a kinase other than Cdc2 and Cdk2 before the onset of mitosis (8), we tested whether Prk is capable of phosphorylating Cdc25C. Fig. 3B shows that His6-Prk (lane 3), but not its deletion mutant (lane 5), phosphorylates GST-Cdc25C. No phosphorylation was detected when GST alone was used as a substrate (data not shown), indicating the phosphorylation oc-
Role of Prk in Mitosis/Meiosis

FIG. 3. Purified recombinant Prk phosphorylates casein and Cdc25C in vitro. A, recombinant baculovirus-infected sf9 cell lysates (lane 1), partially purified His_{6}-Prk (lane 2), and purified His_{6}-Prk (lane 4) were analyzed by SDS-PAGE and Western blotting using an anti-Prk antibody. Mock purification was also performed with the uninfected sf9 cell lysates using Ni-nitrotriacetic acid resin and the purification product(s), if any, was analyzed in the same manner (lane 3). B, purified His_{6}-Prk (lanes 2 and 3) as well as a truncated version of Prk with deleted kinase domain (lanes 4 and 5) were analyzed for their kinase activities using casein (lanes 2 and 4) or GST-Cdc25C (lanes 3 and 5) as a substrate. Lane 1 denotes the His_{6}-Prk kinase reaction with no substrate addition. Lane 6 (CS+ink) represents the His_{6}-Prk kinase reaction using casein as a substrate in the presence of staurosporine (100 nM). Lane 7 is a negative control for lane 1 (the kinase reaction supplemented with no His_{6}-Prk). C, GST-Cdc25C phosphorylated by His_{6}-Prk was subjected to phosphoamino acid analysis as described under "Experimental Procedures."

curs on Cdc25C moiety. We quantitated substrate phosphorylation by titrating the amount of Cdc25C used for the kinase reaction. His_{6}-Prk phosphorylates Cdc25C to a stoichiometry of 4 mol of phosphate per mol of Cdc25C. To confirm the predicted Ssr/Thr phosphorylation by Prk, GST-Cdc25C protein eluted from the gel was hydrolyzed as described under "Experimental Procedures" and analyzed for its phosphoamino acid content. As shown in Fig. 3C, both phosphoserine and phosphothreonine are present with a ratio of about 3 to 1.

The X. laevis oocyte is a useful bioassay system for assessing complex nuclear events (22–24). Immature oocytes are arrested at the G_{2}/M border of meiosis I. When such oocytes are stimulated with progesterone, they undergo meiotic maturation, which is visibly monitored by GVBD. Microinjection of Cdc2 activators such as cyclin B and Cdc25 also induce a rapid GVBD (23, 24). Since prk homologs in yeast and Drosophila are involved in regulating mitotic and meiotic progression (10–12), we asked whether prk is required for Xenopus oocyte maturation. Fig. 4A shows that injection of either vehicle or prk transcripts alone induced GVBD of about 40% oocytes. However, when treated with progesterone, all oocytes injected with the full-length, but not the short form, prk transcripts reached the GVBD stage (Fig. 4A). The short form refers to Prk with an in-frame deletion of amino acids 2–24 in the kinase domain as described elsewhere (13). Thus, injection of full-length prk transcripts greatly potentiated the progesterone-mediated oocyte maturation. To determine whether or not loss of function could affect induced GVBD, we tested the effect of antisense prk transcripts on the maturation of frog oocytes. Fig. 4B shows that injection of the vehicle or antisense prk transcripts alone had no effect on the maturation rate. However, injection of antisense prk transcripts significantly blocked the progesterone-induced GVBD (Fig. 4B). Thus, these oocyte experiments suggest that a progesterone-induced factor(s) is required for the activation of Prk or that activation of Prk downstream component(s) requires both Prk and a progesterone-one-induced factor. To gain insight into the specificity of anti-sense human prk transcripts, Xenopus oocyte RNA was analyzed for prk-specific transcripts using reverse transcription-PCR. Fig. 4C shows that human prk primers were capable of amplifying a Xenopus DNA product (lane 2) that has the same mobility as human prk fragment on an agarose gel (lane 3). High stringency Southern blotting analyses revealed that the amplified Xenopus cDNA fragment, which is outside the kinase domain, hybridized strongly with a human prk cDNA probe (Fig. 4D, lanes 2). Subsequent DNA sequencing analysis showed that Xenopus did contain prk gene and that there existed a very high homology (97%) between the amplified human and Xenopus prk cDNA fragments (data not shown). The slower mobility band in lane 3 (Fig. 4C) was most likely derived from the unspliced prk transcript. To further determine whether suppression of Prk expression would also delay their maturation, Xenopus oocytes injected with antisense prk transcripts or the vehicle were treated with progesterone and examined for GVBD at various time post treatment. It has been observed (Fig. 5) that vehicle-treated oocytes began to mature 5 h post-progesterone treatment (open squares). On the other hand, oocytes injected with antisense prk transcripts did not undergo GVBD until 10 h post-progesterone treatment (open circles).
hand, a small number of oocytes injected with antisense prk transcripts did not show any signs of maturation until 9 h post-progesterone treatment (Fig. 5, filled circles), suggesting that a loss Prk function also significantly delayed meiotic maturation of oocytes induced by progesterone.

Complementation studies with yeast conditional mutants have led to the cloning of many human genes conserved in cell cycle control and elucidation of their respective functions (3, 25). The polo family kinases share structural homology, and the biological functions of the budding yeast CDC5 and Drosophila polo appear to be similar; ablation of either gene function arrests the cell at the G2/M phase (10–12). The human Prk and yeast CDC5 share 38% amino acid residue identities which extend beyond the kinase domain (Fig. 6A). This structural conservation led us to ask whether human prk can complement a yeast CDC5 ts mutant strain. The wild-type CDC5 gene, used as a positive control, was cloned from the wild-type yeast genome via PCR. Fig. 6B shows that CDC5 ts mutant cells transformed with the vector alone remained temperature-sensitive. In contrast, the full-length human prk gene restored the capacity of mutants to grow at 33 °C, and the growth rate was indistinguishable from CDC5 cells transformed with the wild-type CDC5 gene. In addition, a prk expression construct containing a short in-frame deletion in the conserved kinase domain (Prk-st) was unable to grow at 33 °C (Fig. 6B), indicating that a functional prk is important in rescuing the mutant phenotype.

**DISCUSSION**

All proliferating cells undergo an orderly cyclic process that entails genome duplication and mitosis. Major checkpoints in the animal cell cycle control entry into DNA replication and completion of DNA replication before mitosis. Central to this regulation are families of Ser/Thr kinases (1–6). CDKs were the first family of well documented protein kinases shown to play a major role in the regulation of G$_1$, G$_{1}$/S, and G$_2$/M phase checkpoints (1–6). Recently, the polo family of protein kinases has been implicated in the regulation of cell cycle progression at various stages (10, 13, 25–29). There are at least three genes (fnk/prk, snk, and plk) in the mouse that show significant structural homology to Drosophila polo and S. cerevisiae CDC5 (25–29). In human, plk and prk represent the two known polo family kinases (13, 26, 28). Structurally, polo family kinases differ significantly from CDKs in that the former appears to
contain both kinase and regulatory domains (13), and their in vitro kinase activities do not require the association with other protein components (Fig. 3). Human Plk protein exhibits about 50% amino acid identity with human Prk (13). Plk has been shown to undergo dramatic redistribution as cells progress from metaphase to anaphase (29). Recently, Kumagai and Dunphy (30) have shown that Plx, a Xenopus counterpart of mammalian Plk, associates with phosphorylated, and activates the Xcdc25 (Xenopus Cdc25C) gene product. The activated Xcdc25 in turn dephosphorylates and activates p34\(^\text{cdc2}\) (29). We have demonstrated that human prk is capable of rescuing a yeast CDC5 mitotic mutant, indicating that human prk has a conserved function in regulating mitotic/meiotic progression and is a functional homolog of the yeast CDC5 gene.

Western blot analyses reveal that there are two major proteins immunoreactive with the anti-Prk antiserum (Fig. 1B). The band with a slower mobility on the denaturing gel appears to be Prk, since it has a molecular mass of about 68 kDa, which is consistent with what is predicted from its deduced amino acid sequence (13). In addition, the 68-kDa band, is competed out with excess purified recombinant Prk-C (Fig. 1B). The 64-kDa band is not Plk since it is smaller than Plk and since it is not immunoreactive to the anti-Plk antibody (Fig. 2). Human prk encodes an active protein kinase capable of phosphorylating casein and Cdc25C in vitro (Figs. 2 and 3), and it phosphorylates histone H1 weakly (Fig. 2). The latter observation is consistent with early reports that histone H1 is a poor substrate for polo family kinases (29). We have shown that Prk kinase activity peaks at the late S and G2 stages of the cell cycle (Fig. 2B). Considering that Prk antigen levels showed no significant increase in S/G2 compared with that in G1/S and M (Fig. 1C), Prk kinase activity must be regulated by a post-translational mechanism.

In this report we also demonstrate that antisense human prk transcripts significantly block as well as delay the maturation of Xenopus oocytes induced by progesterone. These results suggest that prk is required for the oocyte meiotic maturation and that frog prk has functional as well as sequence homology to human prk. We have observed that there is about 40% overall homology between human Prk and Drosophila polo at the nucleotide level, and that the level of homology increases to 65% when only the kinase domains are aligned. Thus, it is reasonable to predict that human Prk should show stronger overall homology with the frog counterpart than with polo. Our PCR and Southern blotting analyses (Fig. 4, C and D) suggest that Xenopus has at least one prk gene. Our subsequent DNA sequencing analysis has confirmed the identity of the PCR product as a Xenopus prk fragment. Recently, it has been shown that Plx shares 80% amino acid sequence identity with human Prk (30), also indicating structural conservation of polo family kinases across species. We noted that the short form of human prk transcripts (Prk-st) stimulated to some degree GVBD in the presence of progesterone (Fig. 4A), whereas the same prk structure failed to rescue yeast CDC5 ts mutants (Fig. 6B). One interpretation is that Prk-st may possess a low level of biological activity that is capable of weakly regulating cellular targets in Xenopus. On the other hand, this level of activity is insufficient for complementing the deficiency in yeast because of the evolutionary distance.

p34\(^\text{cdc2}\) kinase, a component of mitosis-promoting factor (6), plays an important role in the transition from G2 into M. However, it remains unclear what molecular component(s) senses the completion of genome replication and initiates mitosis. Although Plx1 was recently shown to interact with and phosphorylate Xcdc25 (30), it remains to be determined whether Plx1 kinase activity in vivo correlates with the activation of Xcdc25 and therefore p34\(^\text{cdc2}\) kinase, since early studies show that Plk kinase activity peaks during mitosis but not at the onset of mitosis and that Plk associates with mitotic spindle (29). In fact, we have also observed that the Plk antigen level is rather low during late S and G2 stages of the cell cycle, and it reaches the highest level at the mitotic metaphase (Fig. 1D). On the other hand, we have demonstrated that prk mRNA expression and its kinase activity reach the peak level during late S and G2 stages (Figs. 1 and 2), correlating with the completion of DNA synthesis and the activation of cyclin-dependent kinase p34\(^\text{cdc2}\). In addition, we have shown that Prk is capable of phosphorylating Cdc25C in vitro, suggesting that Prk may truly represent an important protein Ser/Thr kinase regulating the onset of mitosis/meiosis in animal cells.

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