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Human Raf-1 Proteins Associate With Rad24 and Cdc25 in Cell-Cycle Checkpoint Pathway of Fission Yeast, *Schizosaccharomyces pombe*

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Abstract Raf-1 is a serine/threonine protein kinase that connects cell surface receptor signals to nuclear transcription factors. By screening *Schizosaccharomyces pombe* (*S. pombe*) cDNA library, we isolated Rad24, which is a 14-3-3 homolog that is important in the DNA damage checkpoint in *S. pombe*, as a Raf-1 interacting protein. The interaction found in yeast was confirmed by co-immunoprecipitation. Furthermore, Cdc25, which has been known to bind to Rad24, also associated with Raf-1 and was phosphorylated in vitro by catalytically active Raf-1. However, in the presence of Raf-1, an interaction between Rad24 and Cdc25 was inhibited in triple hybrid assay, indicating that Raf-1 inhibits the interaction between Rad24 and Cdc25. An in vitro competition assay showed that the binding of Cdc25 and of Rad24 to Raf-1 is mutually exclusive. Western blots of whole cell lysates probed with polyclonal antibodies specific for tyrosine-15-phosphorylated Cdc2 showed that overproduction of Rad24 led to the dephosphorylation of tyrosine residue on Cdc2, which is known to be activated through dephosphorylation by Cdc25 phosphatase. Unexpectedly, overexpression of catalytically inactive mutant protein of Raf-1, S624A, also caused tyrosine dephosphorylation of Cdc2. Thus, these data suggest that Raf-1 may interfere with the role of Rad24 by competing with Rad24 for binding to Cdc25 or a direct phosphorylation of Cdc25, bypassing the checkpoint pathway in DNA repair through Cdc25 activation. J. Cell. Biochem. 101: 488–497, 2007.

Key words: Raf-1; Rad24; Cdc25; cell cycle; two-hybrid

In eukaryotic cells, the onset of mitosis is triggered by simultaneous activation of Cdc25 phosphatase and inactivation of Wee1 kinase [Russell and Nurse, 1986]. In Cdc25, the presence of a Cdc2/Cyclin B-mediated positive feedback activation loop has been suggested to be critical for promoting entry into mitosis [Margolis et al., 2006]. In addition, Plx1 (polo-kinase; *plo1* in *S. pombe*, *Plxl* in *Xenopus* and *Plks* in mouse and human) was reported as the Cdc25-activating kinase [Nigg, 1998; Myer et al., 2005]. Karaiskou et al. [1999] suggested that the initiation of the Cdc2/Cdc25 feedback loop requires two steps of phosphorylation reactions on Cdc25; one is Cdc2 kinase-dependent and the second requires Plx1 activity and protein phosphatase 2A inhibition. The other laboratories also suggested the implication of Raf-1 kinase as a Cdc25 activating kinase [Galaktionov et al., 1995; De Smedt et al., 1999]. In addition, a significant colocalization of Ras, Raf-1, and Cdc25 was observed at the cell membrane using double-immunofluorescence [Jessus and Ozon, 1995]. Originally, Raf-1 is known to be a cytoplasmic serine/threonine protein kinase, and couples Ras to the mitogen-activated protein (MAP) kinase cascade [Williams et al., 1993; Avruch et al., 1994]. However, it has been reported that the mechanisms responsible for activating Raf-1 during mitosis, and subsequent downstream effects, are distinct from those involved in

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Ras-dependent signaling [Laird et al., 1999; Harding et al., 2003].

Genetic analyses in the yeasts *Schizosaccharomyces pombe* (*S. pombe*) and *Saccharomyces cerevisiae* (*S. cerevisiae*) have identified a number of genes whose functions are essential for the correct cell-cycle response to both DNA damage and the completion of DNA synthesis. Many laboratories used complementation of yeast mutants to detect human homolog genes with similar functions [Freire et al., 1998; Novoa et al., 1999]. Thus, it is of great significance that Raf-1-interacting proteins are firstly identified in *S. pombe*, which is a relatively simple and easy-handling eukaryote compared to human, and then their homolog in mammalian cells are screened using *S. pombe* cDNA. In this study, we used the yeast two-hybrid system to search for Raf-1-interacting proteins. By screening a fission yeast *S. pombe* cDNA library with Raf-1 bait, we isolated a cDNA encoding Rad24, 14-3-3 protein homolog, which is essential for DNA damage checkpoint and cell proliferation in *S. pombe* [Ford et al., 1994]. In mammalian cells, dimeric 14-3-3 is required to maintain Raf-1 in an inactive state in the absence of GTP-bound Ras and to stabilize an active conformation of Raf-1 produced during activation in vivo [Irie et al., 1994; Aitken, 1996]. However, in *S. pombe*, Rad24 acts downstream of Chk1 in the DNA damage checkpoint and *rad24* null cells enter mitosis prematurely resulting in a small cell size at division [Ford et al., 1994; Peng et al., 1997]. Here we show that mammalian Raf-1 proteins can associate with both Rad24 and Cdc25, connecting mitogenic signaling to cell-cycle machinery in *S. pombe*.

MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions

S. pombe haploid strain ED668 (h^+, *ade6-M216*, *ura4-D18*, *leu1-32*) was transformed using lithium acetate transformation procedure [Jang et al., 1997]. The cells were grown in Edinburgh minimal medium (EMM) with the appropriate supplements in the presence or absence of 25 μM thiamine, or in rich yeast extract (YEPD) medium at 30°C.

Yeast Two-Hybrid Screens

S. cerevisiae strain HF7c was used as host for yeast two-hybrid vectors. *S. pombe* two-hybrid cDNA libraries in GAL4 activation domain of pGADGH were obtained from Clontech (Mountain View, CA). Raf-1, which was used as the bait protein, was fused to the sequences encoding the GAL4 DNA-binding domain of pGBT9 (Stratagene, La Jolla, CA). Cotransformation of HF7c with pGBT-Raf-1 and *S. pombe* cDNA library in the pGADGH vector gave 91 colonies that could be grown in the absence of histidine. These $Leu^+Trp^+His^+$ transformants were further assayed for the production of β-galactosidase on filter. After retesting, 22 positive clones were identified. Of these, seven clones encode 14-3-3 homolog of *S. pombe*, Rad24 and Rad25. The coding region DNA of Rad24 was amplified by PCR from *S. pombe* cDNA and cloned into the pGAD424 vector. The pGBT-Raf-1 and pGAD-Rad24 plasmids were introduced into *S. cerevisiae* strain SFY526 and interaction between Raf-1 and Rad24 proteins was confirmed by examining the β-galactosidase activity. As positive controls for specific protein interaction, plasmids pGBT-p53 (pVA3) and pGAD-SV40 large T-antigen (pTD1) were co-transformed. For triple hybrid assay, the *Eco*RI/*Pst*I fragments of *cdc25* were cloned into the MCS I site of the vector pBridge (Clontech), while the *Not*I fragments of *raf-1* were cloned into the MCS II site of the vector pBridge.

Expression of Recombinant Proteins in *E. coli*

For expression of Rad24 and Cdc25 proteins in *E. coli*, *rad24*, and *cdc25* cDNAs were amplified from *S. pombe* cDNA. For *rad24*, the *Eco*RI and *Sal*I sites were introduced during amplification by PCR using the oligonucleotides 5′-gaattcatgtctactacttctcg-3′ and 5′-gtcgacctatgcgtccgccttgg-3′. The PCR products were cloned into the vectors pGEX-4T-1 (Amersham Bio-Sciences Corp., Piscataway, NJ) and pMAL-c1 (New England Biolabs, Beverly, MA). For *cdc25*, the primers for the 5′- and 3′-end were 5′-gaattcatggattctccgctttc-3′ and 5′-ctcgagttaacgtctggggaagc-3′, respectively. The *Eco*RI/*Xho*I fragment of *cdc25* was cloned into the vector pGEX-4T-1. Human full-length Raf-1, BxB (N-terminally truncated, oncogenically active Raf-1 mutant; the C-terminal fragment starting with the 302nd amino acid of Raf-1) and kinase inactive mutant of Raf-1, S624A (mutation of serine 624 to alanine) (Fig. 1A), were generated by using the pεMTH-Raf-1, pεMTH-BxB, and pεMTH-RafS624A plasmid [(a generous gift

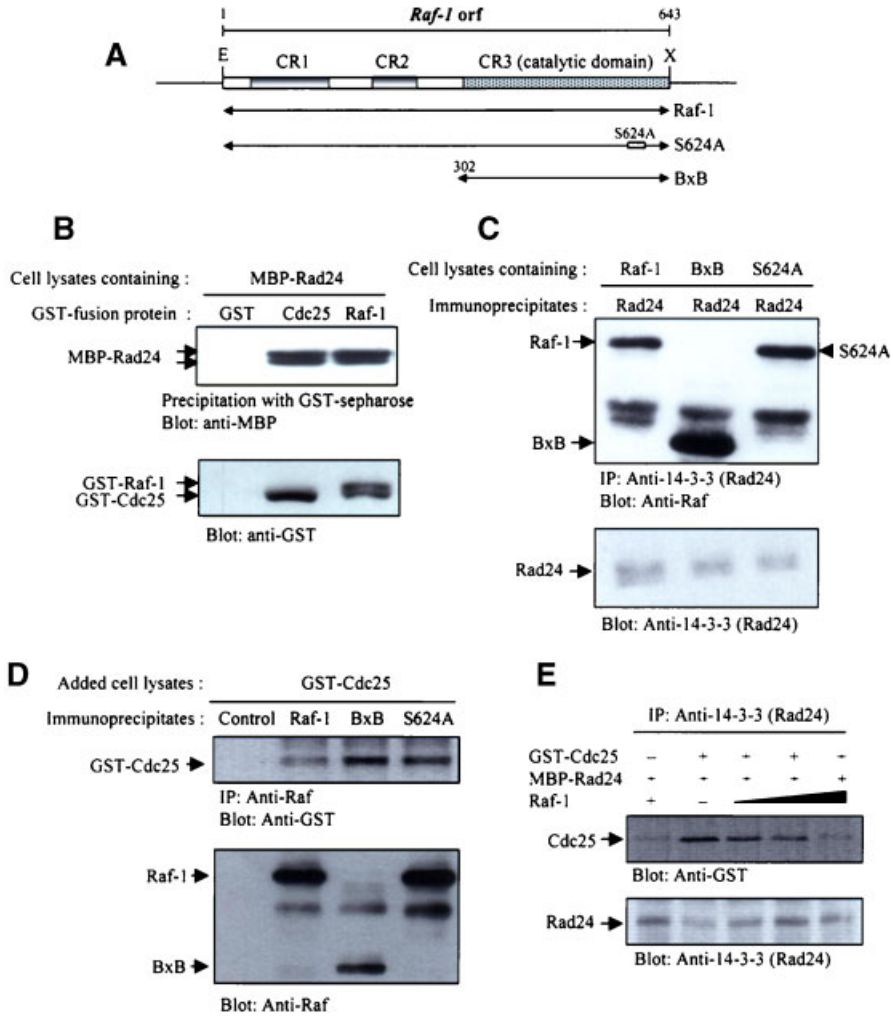

Fig. 1. In vitro binding of mammalian c-Raf-1 with *S. pombe* Rad24 and Cdc25. **A**: Schematic representation of recombinant Raf-1 mutants tested for interaction with Rad24 or Cdc25. Raf-1 kinase has three highly conversed regions called CR1, CR2, and CR3. The CR1 and CR2 regions negatively regulate the Raf-1 kinase domain (CR3) that is located in the carboxy terminal of Raf-1. S624A: kinase inactive mutant of Raf-1 (mutation of serine 624 to alanine); BxB: N-terminally truncated, oncogenically active Raf-1 mutant (the C-terminal fragment starting with the 302nd amino acid of Raf-1). **B**: **Upper panel**: GST-pull down assay was performed using a fusion protein consisting of Cdc25 or Raf-1 fused in frame to GST. Cell lysates containing GST-Cdc25 or GST-Raf-1 fusion proteins were incubated with glutathione (GSH)-sepharose to precipitate the GST-fusion proteins. These precipitates or control GST beads were then incubated with the crude *E. coli* cell extracts containing MBP-Rad24 fusion proteins for 2 h at 4°C. The proteins on the beads were subjected to electrophoresis on SDS–PAGE, and then blotted onto a nitrocellulose membrane. Immunoblot analysis was performed using anti-MBP antibody. **Lower panel**: The same blot was stripped and then reprobed with anti-GST antibody to confirm the expression of GST-Raf-1 or GST-Cdc25 fusion proteins in *E. coli*. **C**: **Upper panel**: Rad24 was immunoprecipitated from the cell lysates containing Rad24 using anti-14-3-3 antibody with protein A-agarose beads. Immunoprecipitates containing Rad24 were then incubated with the crude *E. coli* cell extracts containing each recombinant Raf-1 protein. Immunoblot analysis was performed using anti-Raf-1 antibody. **Lower panel**: The same blot was stripped and then reprobed with anti-14-3-3 antibody to confirm the expression of Rad24 proteins in *E. coli*. **D**: **Upper panel**: Each recombinant Raf-1 protein was immunoprecipitated from the cell lysates containing Raf-1, BxB or S624A using anti-Raf antibody with protein A-agarose beads. Immunoprecipitates containing each recombinant Raf-1 protein or control protein A-agarose were then incubated with the crude *E. coli* cell extracts containing GST-Cdc25 fusion proteins. Immunoblot analysis was performed using anti-GST antibody. **Lower panel**: The same blot was stripped and then reprobed with anti-Raf-1 antibody to confirm the expression of each recombinant Raf-1 protein in *E. coli*. **E**: In vitro competition assay using purified Raf-1 proteins (1, 2, or 5 μg) to compete out the binding of Cdc25 to Rad24. GST-Cdc25- or MBP-Rad25-containing cell lysates were mixed with the purified Raf-1 proteins. The polyclonal anti-14-3-3 was used to immunoprecipitate Rad24. Immunoblot analysis was performed using anti-Raf antibody. The same blot was stripped and then reprobed with anti-14-3-3 antibody to show that equal amounts of cell lysates containing MBP-Rad24 were added. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

from Wayne B. Anderson (NIH, Bethesda, MD)] as templates, and the following primers, 5′-gaattcatggagcacatacag-3′ (5′ end), 5′-ctcgagctagaagacaggcagcctcggggacgtgg-3′ (3′ end) containing *Eco*RI and *Xho*I sites. Amplified fragments were cloned into the vector pET-21a(+) (Novagen Inc., Madison, WI). Plasmids were transformed into *E. coli* strain either DH5α or BL21. The cells containing these plasmids were grown at 37°C and expression of proteins was induced by addition of 1 μM IPTG for 2 h at 37°C.

Expression of Raf-1 Proteins in *S. pombe*

For expression of recombinant Raf-1 mutants in *S. pombe*, the *Eco*RI/*Xho*I fragments of Raf-1, BxB, and S624A from pET-21a(+) were first cloned into pGEX-4T-1. The fragments of each recombinant Raf-1 cDNA were moved from pGEX-4T-1 into pREP2, which contained thiamine-regulated *nmt1* promoter of *S. pombe*, using the *Bam*HI site and blunt-ended *Xho*I site in the polylinker of pGEX-4T-1. *S. pombe* ED668 cells transformed with pREP2 containing Raf-1 were grown in the presence of 25 μM thiamine to repress the *nmt1* promoter until expression was desired. To induce expression, cells were grown to early log phase, washed with EMM lacking thiamine, and resuspended in EMM. After 20 h of induction in the absence of thiamine in the medium, the cells were harvested and resuspended in PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% Triton X-100) containing protease inhibitor. The cells were lysed with acid-washed glass beads and vortexing. The cell lysates were centrifuged at 10,000*g* for 10 min. Aliquots of lysates were stored at −80°C.

In Vitro Binding Assay

To precipitate glutathione S-transferase (GST) fusion proteins, 1 ml of cell lysate was incubated with 50 μl of glutathione (GSH)-sepharose (Amersham Bio-Sciences Corp.,) at 4°C for 2 h. The beads were washed four times with PBST. For in vitro binding, the beads containing GST-Cdc25 or MBP-Rad24 were incubated with the crude cell extract containing each recombinant Raf-1 protein for 2 h at 4°C. For in vitro competition assays, 1–5 μg of purified Raf-1 (Upstate, Charlottesville, VA) was incubated with the crude cell lysates containing MBP-Rad24 or GST-Cdc25. The anti-14-3-3 (Rad24) antibody immobilized on protein A-agarose beads was added to the mixtures and incubated with mixing at 4°C for 2 h. After washing the beads with PBST buffer, the beads were denatured in Laemmli sample buffer, resolved by SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and immunoblot analysis was performed using the antibodies described in figure legends. Recombinant Raf-1 mutants and GST fusion proteins were detected using a 1:750 dilution of anti-Raf-1 antibody and a 1:4,000 dilution of anti-GST antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Tyrosine phosphorylated Cdc2 was detected using a 1:1,000 dilution of phospho-Cdc2 (Tyr 15) antibody (Cell Signaling Technology, Inc., Danvers, MA). Immune complexes on nitrocelluose were detected by enzyme-linked chemiluminescence (Amersham Bio-Sciences Corp.).

Raf-1 Protein Kinase Assay

Raf-1, BxB (constitutively active C-terminal Raf-1 fragments), and S624A (catalytically inactive Raf-1 mutants) proteins were specifically immunoprecipitated from the lysates of either *S. pombe* cells or bacterial cells overexpressing each recombinant Raf-1 protein. Immunoprecipitation was performed on the cell lysates using anti-Raf-1 (Santa Cruz Biotechnology) and protein A-agarose beads (Roche Diagnostics Corp., Indianapolis, IN). After incubation for 2 h at 4°C, immunoprecipitates were washed twice with ice-cold lysis buffer. The washed immunoprecipitates of each recombinant Raf-1 protein were incubated in 40 μl of kinase buffer containing 10 μM ATP, GST-Cdc25, and 10 μCi of [γ-^{32}P]ATP at 30°C for 30 min as described previously [Lee et al., 2004].

RESULTS

Isolation of Raf-1 Interacting Proteins

To identify Raf-1-interacting proteins, we screened *S. pombe* yeast two-hybrid cDNA library using human Raf-1 as bait. Among the 91 clones that were grown in the absence of histidine, 22 clones showed lacZ expression on X-gal induction plates. Three of these clones encode the full-length *S. pombe* Rad24 protein. The four clones encode the *S. pombe* Rad25. Both Rad24 and Rad25 were found to be a 14-3-3 homolog in *S. pombe*, and have an important role in determining the timing of mitosis [Ford et al., 1994].

Interaction of Mammalian Raf-1 With *S. pombe* Rad24 and Cdc25

We performed additional two-hybrid tests to verify positive interaction. Especially, we focused on Rad24 proteins because it has been known that *S. pombe rad24* null cells had a cytokinesis defect much greater than *rad25* null cells. Results presented in Table I confirmed that Raf-1 protein interacted with Rad24 after 3-day incubation. The interaction of Raf-1 and Cdc25 was also detectable after 5-day incubation in two-hybrid assay. Additionally, the strong interaction of Rad24 with Cdc25 was observed as previously reported [Peng et al., 1997]. However, we did not observe an interaction between Rad24 and Cdc25 in the presence of Raf-1 in triple hybrid assay (Table II), indicating that Raf-1 inhibits the interaction between Rad24 and Cdc25.

To investigate further the direct interaction between Raf-1 and Rad24 proteins, in vitro binding between Raf-1 and Rad24 was tested using GST-Raf-1 fusion protein and maltose binding protein (MBP)-Rad24 fusion protein produced in *E. coli*. The amount of each protein expressed was verified by SDS–PAGE and by Western blotting. Purified bacterially produced MBP-Rad24 migrated as a doublet. Interaction between the GST and MBP moieties was not observed. As found in yeast two-hybrid assay, Raf-1 showed direct binding to Rad24 (Fig. 1B). The strongest interaction was found with the oncogenically active, amino-terminal truncated Raf-1 mutant, BxB (amino acids 304–648) and Rad24 (Fig. 1C). The binding between Rad24 and the kinase inactive Raf-1 mutant (S624A) was similar to that with wild-type Raf-1. Further, to test whether the interaction of Cdc25 and Raf-1 is direct, we expressed the recombinant Raf-1 mutants in bacterial cells and assessed binding of these proteins to bacterially expressed, purified GST-Cdc25 fusion proteins. We observed strong binding of Cdc25 to BxB proteins (Fig. 1D). Table II suggested the possibility that Raf-1 inhibits the interaction between Rad24 and Cdc25. Thus, an in vitro competition assay was performed to test if Raf-1 could prevent Cdc25 from binding to Rad24. As shown in Figure 1E, immunoprecipitation of Rad24 pulled down Cdc25. Addition of increasing concentrations of purified Raf-1 proteins reduced the amount of Cdc25 immunoprecipitated with Rad24, suggesting that the binding of Raf-1 and of Cdc25 to Rad24 are mutually exclusive.

TABLE I. Interaction of Mammalian Raf-1 With *S. pombe* Rad24 and Cdc25 in the Two Hybrid System

	BD-Raf-1			BD-Cdc25		
Days	3	5	8	3	5	8
AD-Cdc25	–	+	–			
AD-Rad24	++	+	–	++++	++++	++++

The two-hybrid system was used to determine interactions between the indicated constructs. Proteins were expressed as fusion proteins with either the GAL4 DNA- binding (BD) or the activation domains (AD). For LacZ expression (the qualitative β-galactosidase activity), ++++ indicates a strong interaction, + indicates an intermediate interaction, – means no blue color development.

TABLE II. The Effect of Mammalian Raf-1 on Interaction of *S. pombe* Rad24 With Cdc25 in the Triple Hybrid System

	BD-Cdc25					
	Raf-1		BxB		S624A	
Bridge protein	w/o	w/	w/o	w/	w/o	w/
AD-Rad24	++++	–	++++	+	++++	–

The triple-hybrid system was used to determine interactions between the indicated constructs. Cdc25 protein was expressed as fusion protein with DNA-binding domain of the yeast transcriptional factor GAL4 (BD), while the recombinant Raf-1 mutants were conditionally expressed from the P_{Met25} promoter. Rad24 protein was fused with activation domain of yeast transcriptional factor GAL4 (AD). For LacZ expression (the qualitative β-galactosidase activity), ++++ indicates a strong interaction, + indicates an intermediate interaction, – means no blue color development.

Phosphorylation of Cdc25 by Raf-1 Protein

Since serine/threonine-proline sequences of Cdc25, which are the phosphorylation sites for mitotically active kinases, are found to be conserved among Cdc25 homologs from evolutionary distant species [Jessus and Ozon, 1995], we examined whether mammalian Raf-1 phosphorylates *S. pombe* Cdc25. The phosphorylation of GST-Cdc25 fusion proteins was directly measured by immunoprecipitation with anti-GST antibody, followed by in vitro kinase assay. MEK-1 protein was used as a positive substrate for Raf-1 kinase. As shown in Figure 2, *S. pombe* Cdc25 phosphatase was phosphorylated in vitro by Raf-1 kinase expressed and purified from *S. pombe* cells (lanes 2 and 3, Fig. 2). Especially,

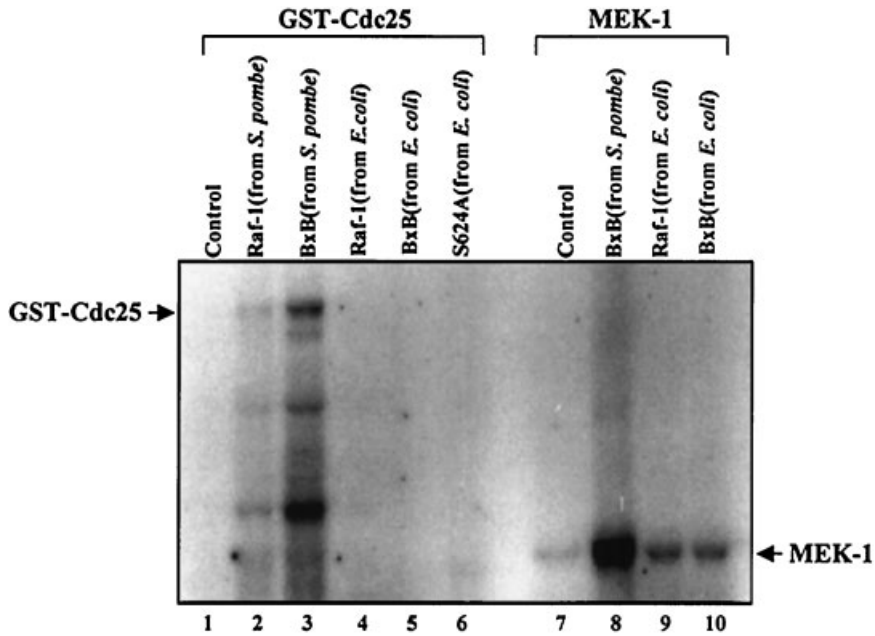

Fig. 2. In vitro phosphorylation of Cdc25 by Raf-1 protein. Immunoprecipitates from *S. pombe* cells overexpressing recombinant Raf-1 proteins were incubated with either *S. pombe* GST-Cdc25 (**lanes 1–6**) or recombinant MEK-1 proteins (**lanes 7–10**) in a kinase buffer with [γ-^{32}P]ATP followed by SDS–PAGE.

the phosphorylation of Cdc25 by a constitutively active BxB fragment of Raf-1 kinase was much more evident. However, bacterially produced Raf-1 had no stimulatory effect both on Cdc25 and MEK1 (lanes 4–6 and 9–10).

The Effect of Either Raf-1 or Rad24 Overexpression on Cdc2 Tyrosine-15 Phosphorylation

Since tyrosine 15 dephosphorylation and activation of Cdc2 is induced by the active Cdc25 phosphatase, we determined the tyrosine phosphorylation state of Cdc2 as a marker for Cdc25 activation (Fig. 3). Western blots of whole cell lysates were probed with polyclonal antibodies specific for tyrosine-15-phosphorylated Cdc2. As a control for sample variation, the blots were reprobed with polyclonal antibodies against the PSTAIR motif of Cdc2. Unexpectedly, analysis of phospho-Cdc2 showed that overproduction of Rad24 led to dephosphorylation of Cdc2 molecules (low phospho-Cdc2/total Cdc2 ratio) (lane 2). More surprisingly, overexpression of catalytically inactive mutant protein of Raf-1, S624A, also caused tyrosine dephosphorylation of Cdc2 (lane 5).

DISCUSSION

The mammalian Raf-1 protein kinase plays a central role in many of the mitogenic and developmental signaling pathways [Avruch et al., 1994; Marshall, 1995]. A functional homolog of Raf-1 signaling cascade, Ras1/Gpa1/Byr2 pathway, also exists in a fission yeast *S. pombe* [Hughes et al., 1993; Masuda et al., 1995] and plays an essential role for both conjugation and sporulation of *S. pombe* [Xu et al., 1994]. In this study, we isolated Rad24 of

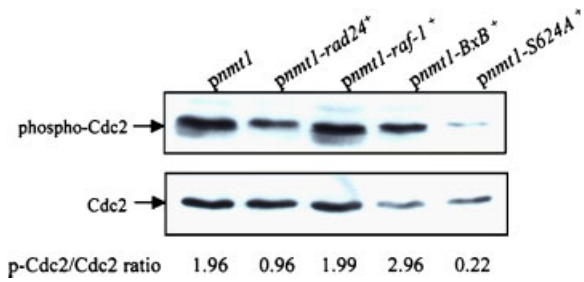

Fig. 3. The effect of either Raf-1 or Rad24 overexpression on Cdc2 tyrosine-15 phosphorylation. *S. pombe* cells overexpressing each Raf-1 recombinant were grown in minimal medium at 25°C for 24 h in the absence of thiamine for induction of the recombinant proteins. The tyrosine phosphorylation of Cdc2 in the samples was analyzed by Western blotting with an antibody specific to Cdc2 phosphorylation on tyrosine-15. The blots were reprobed with an anti-PSTAIR antibody to visualize the total amount of Cdc2 present in the sample. Numbers listed below each band indicate value quantified by Quantity One software (Bio-Rad), expressed as ratios of phospho-Cdc2/total Cdc2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

S. pombe, a 14-3-3 homolog, as a mammalian Raf-1 interacting protein. In *S. pombe*, Rad24 is known to be essential for proper cell-cycle responses to both DNA damage and completion of DNA synthesis [Ford et al., 1994] and is thought to function as an attachable nuclear export signal that enhances the nuclear export of Cdc25 [Lopez-Girona et al., 1999]. It was also demonstrated that Rad24 negatively regulates Byr2, a homolog of Raf-1 kinase, by affecting its localization in *S. pombe* [Ozoe et al., 2002]. Our finding that mammalian Raf-1 can recognize *S. pombe* 14-3-3 homolog, Rad24, and as well as Cdc25 that is sequestered and inactivated by Rad24 [Peng et al., 1997], supports that Raf-1 can function in DNA damage-induced cell-cycle control. Phosphorylation of Cdc25 by catalytically active Raf-1 confirmed this possibility. This result is consistent with the previous finding by others [Jessus and Ozon, 1995] that mammalian Cdc25A can be phosphorylated in vitro by Raf-1 kinase and activated in a Raf1-dependent manner.

When DNA is damaged in *S. pombe*, Chk1 kinase of *S. pombe* is activated and phosphorylates Cdc25, and this in turn promotes its binding to Rad24 protein and preventing it from activating Cdc2 [Peng et al., 1997; Lenormand et al., 1999]. Raf-1 is unlikely to form a triple complex with Rad24 and Cdc25. Rather, triple-hybrid assay (Table II) actually indicated that Raf-1 proteins inhibited the interaction between Rad24 and Cdc25. Also, an in vitro competition assay showed that the binding of Raf-1 and of Cdc25 to Rad24 were mutually exclusive. Thus it is possible that binding of Raf-1 kinase to Cdc25 may interfere with its interaction with Rad24 leading to inhibition of inactivation of Cdc25 by Rad24. Yeast two-hybrid assay revealed the following relative strength of each interaction; Cdc25 · Rad24 > Raf-1 · Rad24 > Cdc25 · Raf-1 (Table I). Therefore, we suggest that Raf-1 may be transported out of the nucleus by binding to Rad24 rather than prevent Cdc25 from interacting with Rad24. That is, when Raf-1 proteins are overexpressed, excess Raf-1 may compete with Rad24 for binding to Cdc25 and induce a sustained activation of Cdc25. However, the observation that Raf-1 protein can regulate the cell-cycle machinery after direct binding with Cdc25 suggested that Raf-1 should be entering the nucleus. Even though Raf-1 is suggested to be mostly in the cytoplasm and is activated at the membrane, it has recently been demonstrated that significant amounts of Raf-1 are detected in the nucleus after 30 min of serum stimulation [Wang et al., 1998]. However, since Raf-1 lacks an identifiable nuclear localization signal, its mechanism of translocation is not clear.

The timing of mitosis in a normal cell cycle is controlled by the phosphorylation of Cdc2 on Tyr15 which maintains Cdc2-cyclin B complexes at an interphase level of activity, high enough to initiate replication and prevent re-replication, but too low to trigger mitosis [Rhind and Russell, 1998a]. At the G_2-M transition, the balance of kinase and phosphatase activity shifts in favor of dephosphorylation, Cdc2 is activated, and mitosis ensues. Thus the inhibitory tyrosine-15 phosphorylation of Cdc2 is used by the DNA damage checkpoint to prevent mitosis [Rhind and Russell, 1998b]. However, we observed that tyrosine-phosphorylation of Cdc2 reduced in the cells overexpressing S624A (catalytically inactive form of Raf-1) or Rad24 whose overexpression arrests cell cycling at G(2)/M phase [Masuda et al., 2000]. Although Cdc25 was found to be exported from the nucleus by associating with Rad24 during a replication or DNA damage checkpoint arrest [Zeng and Piwnica-Worms, 1999], the effect of Rad24 overexpression on tyrosine phosphorylation of Cdc2 have not been known. Wang et al. [2000] showed that, from yeast two-hybrid screening, Rad24 was also able to bind to Wee1 tyrosine kinase, which functions as a mitotic inhibitor by directly phosphorylating Cdc2 on Tyrosine 15 [Parker et al., 1992]. Thus, there is a possibility that overexpression of Rad24 also leads to nuclear export of Wee1 kinase as well as Cdc25, resulting in dephosphorylation of Cdc2. In case of catalytically inactive Raf-1 kinase, S624A, excess amount of S624A to compete for binding toRad24 might result in the release of Cdc25 from Rad24-induced nuclear export. However, catalytically active Raf-1 kinase form appeared to increase the phosphorylation of Cdc2 on Tyrosine 15. We cannot exclude the possibility that, even though the exact phosphorylation sites is need to be identified, Raf-1 stimulates Wee1 kinase activity through phosphorylation, such as the phosphorylation of Wee1 kinase by Chk1, which correlated with the induction of inhibitory tyrosine 15 phosphorylation on Cdc2 [O'Connell et al., 1997]. On the other hand, Knudsen et al. [1996] reported

that *S. pombe* cells arrested in response to hydroxyurea with fully activated Cdc2 kinase activity. It is also reported that when M phase is blocked by activation of the spindle microtubule-assembly checkpoint using nocodazole or paclitaxel, cyclin A/B-Cdc2 complexes are held in an active state [Poon et al., 1997]. Therefore, it seems likely that overexpression of either Rad24 or catalytically inactive form of Raf-1, S624A, causes cell-cycle arrest at G_2/M with active Cdc2.

Studies have established that, in response to DNA damage, Chk1 phosphorylates the nuclear fraction of Cdc25 that is required to activate Cdc2 [Furnari et al., 1997]. This phosphorylation creates a binding site in Cdc25 for 14-3-3 proteins such as Rad24, which escorts the associated Cdc25 from the nucleus by a Crm1-dependent process, resulting in G_2/M arrest [Yang et al., 1999; Zeng and Piwnica-Worms, 1999]. Thus, the mechanism that we suggest was summarized in Figure 4. The transiently active Cdc25 reverts back to inactive form through the negative regulation in which Rad24 may be involved. Although Rad24 also binds to Raf-1 to transport out of the nucleus, excess Raf-1 may compete with Rad24 for binding to Cdc25 and induce a highly activated, sustained form of Cdc25 when Raf-1 proteins are overexpressed.

In view of the striking degree of homology of mitotic control mechanisms in fission yeast and mammals, we expect that Raf-1 proteins function as a regulator of the DNA damage checkpoint mechanism in mammalian cells. In fact, it was proposed by Rittinger et al. [1999] that all mammalian 14-3-3 isotypes contain a C-terminal amphipathic α helix acting as a putative nuclear export signal that is responsible for binding to Crm1. In addition, the 14-3-3$\sigma^{-/-}$ cells were unable to maintain cell-cycle arrest and died ("mitotic catastrophe") as they entered mitosis [Chan et al., 1999], such as *rad24* null cells of *S. pombe*. However, unlike *S. pombe* Rad24 proteins, mammalian 14-3-3 binding may not be essential for cytoplasmic localization of Raf-1 [Muslin and Xing, 2000]. Rather, several other proteins in the cytoplasm, including HSP90 and $p50^{CDC37}$, may in some way facilitate cytoplasmic localization [Perdew et al., 1997; Helmbrecht et al., 2000]. Therefore, further experiments are required to determine

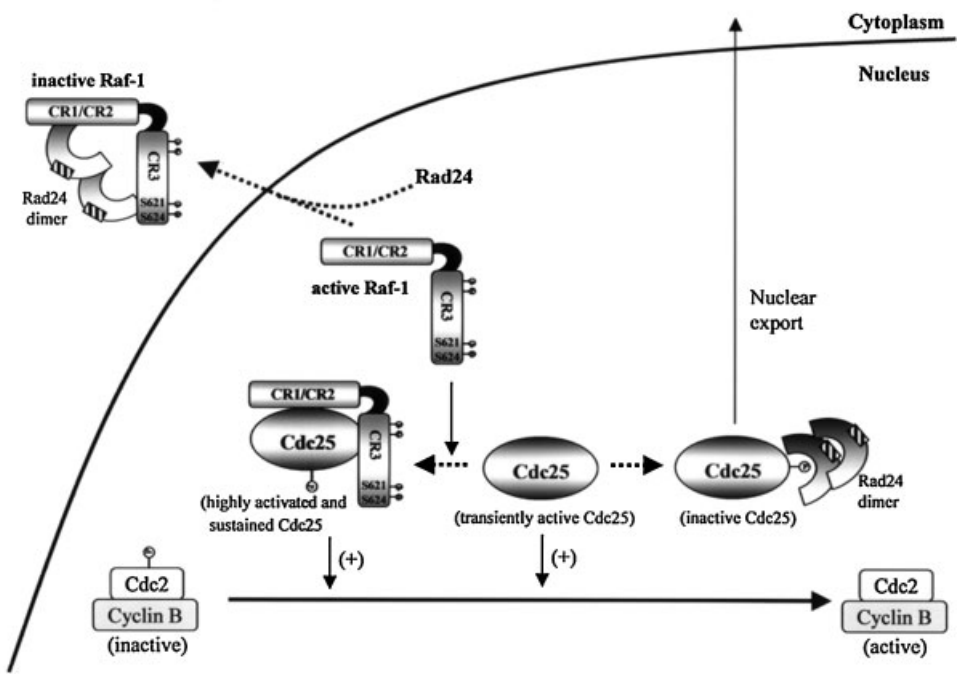

Fig. 4. Model of the inhibition of interaction of Rad24 and Cdc25 by catalytically active Raf-1 kinase. The transiently active Cdc25 is involved in cell proliferation and then goes back to an inactive form through the negative regulation in which Rad24 may be involved. In response to DNA damage, Rad24 escorts the associated Cdc25 from the nucleus by a Crm1-dependent process, resulting in G_2/M arrest. However, the ability to induce cell-cycle arrest in fission yeast can be abolished by activation of Cdc25 by Raf-1. Although Rad24 may also bind to Raf-1 to transport out of the nucleus, excess Raf-1 may compete with Rad24 for binding to Cdc25 and induce a sustained activation of Cdc25 when Raf-1 proteins are overexpressed.

if Raf-1 is involved in DNA replication checkpoint pathway containing 14-3-3 and mammalian Cdc25 following DNA damage, using a catalytically inactive dominant negative mutant of Raf-1 in mammalian cells.

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