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Genetic Studies with the Fission Yeast *Schizosaccharomyces pombe* Suggest Involvement of Wee1, Ppa2, and Rad24 in Induction of Cell Cycle Arrest by Human Immunodeficiency Virus Type 1 Vpr

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Accessory protein Vpr of human immunodeficiency virus type 1 (HIV-1) arrests cell cycling at G_2/M phase in human and simian cells. Recently, it has been shown that Vpr also causes cell cycle arrest in the fission yeast *Schizosaccharomyces pombe*, which shares the cell cycle regulatory mechanisms with higher eukaryotes including humans. In this study, in order to identify host cellular factors involved in Vpr-induced cell cycle arrest, the ability of Vpr to cause elongated cellular morphology (*cdc* phenotype) typical of G_2/M cell cycle arrest in wild-type and various mutant strains of *S. pombe* was examined. Our results indicated that Vpr caused the *cdc* phenotype in wild-type *S. pombe* as well as in strains carrying mutations, such as the *cdc2-3w*, Δ*cdc25*, *rad1-1*, Δ*chk1*, Δ*mik1*, and Δ*ppa1* strains. However, other mutants, such as the *cdc2-1w*, Δ*wee1*, Δ*ppa2*, and Δ*rad24* strains, failed to show a distinct *cdc* phenotype in response to Vpr expression. Results of these genetic studies suggested that Wee1, Ppa2, and Rad24 might be required for induction of cell cycle arrest by HIV-1 Vpr. Cell proliferation was inhibited by Vpr expression in all of the strains examined including the ones that did not show the *cdc* phenotype. The results supported the previously suggested possibility that Vpr affects the cell cycle and cell proliferation through different pathways.

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of AIDS. In addition to the viral genes, such as *gag*, *pro*, *pol*, and *env*, common to all of the replication-competent retroviruses, the HIV-1 genome has genes for accessory proteins that are thought to play important roles in viral replication and pathogenesis. One of the HIV-1 accessory proteins, Vpr, is a virion-associated protein of 14 kDa. Despite its small size, Vpr has been shown to have multiple functions including nuclear translocation of the preintegration complex (20, 53, 63), regulation of apoptosis (3, 61), inhibition of cell proliferation (30, 50), induction of cell differentiation (30), and host cell cycle arrest at G_2/M phase (22, 55). The G_2/M cell cycle arrest by Vpr is conserved among primate lentiviruses including HIV-2 and simian immunodeficiency viruses (14, 24, 51, 62), suggesting that it may play an important role in viral replication. It has been shown that the transcriptional activity of the HIV-1 long terminal repeat is elevated in the cells at G_2 phase, leading to efficient virus production, and that the virus with intact Vpr can be selected for in vivo (17). It has also been suggested from a recent study that Vpr might enhance the fidelity of DNA repair through its ability to arrest the cell cycle at G_2 phase and might protect unintegrated HIV provirus from intracellular defenses against exogenous DNA (21). Therefore, the ability of Vpr to cause G_2/M arrest appears to be instrumental to HIV-1 propagation.

To elucidate the molecular mechanism for Vpr-induced cell cycle arrest, a number of attempts to identify the host proteins which physically interact with Vpr have been made. The results of those studies revealed that Vpr could bind various proteins, including uracil DNA glycosylase (UNG) (7), HHR23A (18, 65), and a human homologue of mov34 (33), which have been implicated in cell cycle control. However, the functional significance of the interaction between Vpr and these cellular proteins is still unclear. In fact, it was shown that the ability of Vpr to bind UNG did not correlate with its ability to induce cell cycle arrest (60). Therefore, a different approach for identifying host factors functionally involved in Vpr-induced cell cycle arrest appeared necessary.

Previous studies have shown that Vpr-induced cell cycle arrest is associated with inactivation of $p34^{cdc2}$ kinase, a key regulator of the G_2/M transition (19, 54). The kinase activity of $p34^{cdc2}$ is mainly regulated by proteins Wee1 and Cdc25 (28, 46). Specifically, $p34^{cdc2}$ is inhibited by Wee1 via phosphorylation of its tyrosine residue at position 15 (Y15) when a cell is not ready for mitosis (37, 58). When a cell is prepared for mitosis, $p34^{cdc2}$ is activated by Cdc25-mediated dephosphorylation of Y15, leading to G_2/M transition (39). When DNA replication or repair of damaged DNA is incomplete, a checkpoint control mechanism is induced, which results in inhibition of $p34^{cdc2}$ probably through activation of Wee1 or inactivation of Cdc25, causing cell cycle arrest at the G_2/M boundary (28, 46). Therefore, it is possible that Vpr causes cell cycle arrest by affecting Wee1, Cdc25, or other checkpoint control molecules.

For elucidation of cell cycle regulatory mechanisms, the fission yeast *Schizosaccharomyces pombe* has been used as a good model system for a number of years, because (i) it shares the cell cycle regulatory mechanisms with higher eukaryotes including humans, (ii) a variety of well-defined mutant strains are available, facilitating genetic studies, and (iii) G_2/M cell cycle arrest is manifested as an easily noticeable elongated morphology called the *cdc* phenotype (28, 40, 44). It has been

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TABLE 1. *S. pombe* strains used in this study

Brief genotype	Genotype[a]	Description	Source
Wild type	Wild type	Normal cell cycle; normal DNA damage and replication checkpoints	Our stock
cdc2-1w	*cdc2-1w*	Constitutively active Cdc2 refractory to Wee1-mediated Y15 phosphorylation	Our stock
cdc2-3w	*cdc2-3w*	Cdc2 activated by Cdc25-independent dephosphorylation of Y15	Our stock
Δ*wee1*	*ura4-D18 wee1*::*ura4*$^+$	No expression of Wee1, a negative regulator of Cdc2	Our stock
wee1-50	*wee1-50*	Wee1 *ts* mutant functional at 23°C, but not at 32.5°C	Our stock
Δ*nim1*	*ura4-D18 nim1*::*ura4*$^+$	No expression of Nim1, a negative regulator of Wee1	Our stock
wee1-50 Δ*mik1*	*ura4-294 wee1-50 mik1*::*ura4*$^+$	No expression of Mik1, the twin kinase of Wee1, with the *wee1-50* mutation	Our stock
cdc2-3w Δ*cdc25*	*cdc2-3w ura4-D18 cdc25*::*ura4*$^+$	No expression of Cdc25, a positive regulator of Cdc2, with the *cdc2-3w* mutation	P. Nurse
rad1-1	*rad1-1*	Nonfunctional Rad1, a transducer for DNA damage and replication checkpoints	Our stock
Δ*chk1*	*ura4-D18 chk1*::*ura4*$^+$	No expression of Chk1, a transducer for DNA damage and replication checkpoints	Our stock
Δ*rad24*	*ura4-D18 rad24*::*ura4*$^+$	No expression of Rad24 involved in cell cycle control and DNA damage checkpoint	Our stock
Δ*cds1*	*ura4-D18 cds1*::*ura4*$^+$	No expression of Cds1, a transducer for DNA replication checkpoint	Our stock
Δ*ppa1*	*ura4-D18 ppa1*::*ura4*$^+$	No expression of Ppa1, a catalytic subunit of PP2A	M. Yanagida
Δ*ppa2*	*ura4-D18 ppa2*::*ura4*$^+$	No expression of Ppa2, a catalytic subunit of another isozyme of PP2A	M. Yanagida

[a] The genetic background common to all of the strains (*h*$^-$ *leu1-32*) is omitted.

demonstrated that Vpr also causes cell cycle arrest in *S. pombe*, suggesting that this organism may be a useful model for studying the molecular mechanism of Vpr-induced G_2/M arrest (67, 68). Indeed, *S. pombe* has successfully been used for studying the antagonism of pentoxifylline against the effects of Vpr (69) and the structure-function relationship of Vpr (10). In this study, to identify host cellular factors involved in Vpr-induced cell cycle arrest, HIV-1_{NL4-3} Vpr was expressed in wild-type and various mutant strains of *S. pombe* and its effects on cellular morphology and proliferation were examined. The results demonstrated that among the genes involved in cell cycle regulation, *wee1*$^+$, *rad24*$^+$, and *ppa2*$^+$ were necessary for induction of the *cdc* phenotype by Vpr, suggesting that the products of these genes, Wee1, Rad24, and Ppa2, may play important roles in Vpr-induced cell cycle arrest.

MATERIALS AND METHODS

Molecular cloning of the HIV-1 *vpr* gene into an *S. pombe* expression vector. The *vpr* gene fragment was prepared by PCR using an infectious DNA clone of HIV-1_{NL4-3} (1) given by Akio Adachi (Tokushima University, Tokushima, Japan) as a template and a pair of oligonucleotide primers (5′-CGGGATCCCG AGGACAGATGGAACAAGCCC-3′ and 5′-CAATAGCAATTGGTACAAG CAGTTTTAGGC-3′). The amplified product was digested with *Bam*HI and *Mfe*I and subcloned between the *Bam*HI and *Eco*RI sites of pBluescript SK II(+) (Stratagene), and its nucleotide sequence was verified. Then, the *Bam*HI-*Eco*RV fragment containing the Vpr-coding region was prepared from the plasmid and inserted between the *Bam*HI and *Sma*I sites of the pREP-1 vector (36) downstream of the thiamine-repressible *nmt1* promoter. The constructed vector was named pREP1-vpr. With pREP1-vpr as a template, a mutant *vpr* gene fragment carrying C-to-T and T-to-C substitutions at the first and second nucleotides, respectively, of codon 67 was generated by primer-directed PCR mutagenesis as described previously (35). Introduction of the mutation was verified by nucleotide sequencing so that the mutant gene encodes Vpr whose Leu^{67} is replaced by Ser. The fragment was cloned in the pREP-1 vector to construct pREP1-L67S.

Culture and transformation of *S. pombe*. *S. pombe* strains used in this study are listed in Table 1. Mutant strains were originally obtained from Paul Nurse (Imperial Cancer Research Fund, London, United Kingdom), Antony M. Carr (University of Sussex, Brighton, United Kingdom), and Mitsuhiro Yanagida (Kyoto University, Kyoto, Japan). Strains designated as "our stock" in Table 1 were generated in our laboratory by modifying their nutrition requirement properties through mating and tetrad analysis. Fission yeast cells were grown at 30°C in minimal medium (MM) supplemented or not supplemented with leucine (250 μg/ml), using standard culture techniques (2). As for temperature-sensitive (*ts*) mutants, different conditions were used as specified below. For repression of the *nmt1* promoter, 10 μM thiamine was added to the medium. To induce transcription from the *nmt1* promoter, cells were washed twice with MM without thiamine and then reinoculated into MM lacking thiamine. Transformation of *S. pombe* with plasmid DNA was carried out by the lithium acetate method as described previously (47).

Examination of proliferation and morphology of yeast cells. Yeast cells were grown in MM supplemented or not supplemented with thiamine, and an aliquot was taken at various time points. The number of cells in the sample was measured by using a particle counter (Z1; Beckman Coulter, Inc.). Morphology of the cells was observed under a phase-contrast microscope (Nikon Corp.) without fixation, and representative pictures were taken by using a charge-coupled device camera (KV-26B; Hitachi Denshi, Ltd.) and printed by a video copy processor (SCT-P67; Mitsubishi Electric Corp.). Staining of the nucleus with 4′,6-diamidino-2-phenylindole (DAPI) was carried out by the standard method (2).

Protein analysis. *S. pombe* cells in the mid-log growth phase were seeded in MM supplemented or not supplemented with thiamine at a density of 3×10^5 per ml and grown at 30°C for 15 h with vigorous shaking. Then, cell extracts were prepared in HB buffer as described previously (41), electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) gel, and blotted to a polyvinylidene difluoride (PVDF) membrane. HIV-1 Vpr was detected by anti-HIV-1_{NL4-3} Vpr rabbit serum (NIH AIDS Research and Reference Reagent Program) and a peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) mouse antibody (Amersham Pharmacia Biotech). The binding of the antibodies was visualized by using a BM chemiluminescence blotting kit (Roche Diagnostics).

Cell cycle analysis. *S. pombe* cells in the mid-log growth phase were seeded in nitrogen-limited MM supplemented or not supplemented with thiamine at a density of 2×10^5 per ml and grown at 30°C for 36 h with vigorous shaking. Then, the cells were fixed with 70% ethanol, treated with RNase A (0.1 mg/ml) in 50 mM sodium citrate (pH 7.0) for 2 h, and stained with propidium iodide (10 μg/ml) overnight. Next day, DNA contents of the cells were measured by a flow cytometer (EPICS XL; Beckman Coulter, Inc.).

RESULTS

Wild-type Vpr, but not the L67S mutant, induced G_2/M cell cycle arrest in *S. pombe* associated with the *cdc* phenotype. It has previously been shown that expression of HIV-1 Vpr in *S. pombe* causes cell cycle arrest (67, 68). To confirm the observation, the *vpr* gene was cloned in thiamine-repressible expression vector pREP-1 and introduced into wild-type *S. pombe* cells. When the cells were grown in the absence of thiamine,

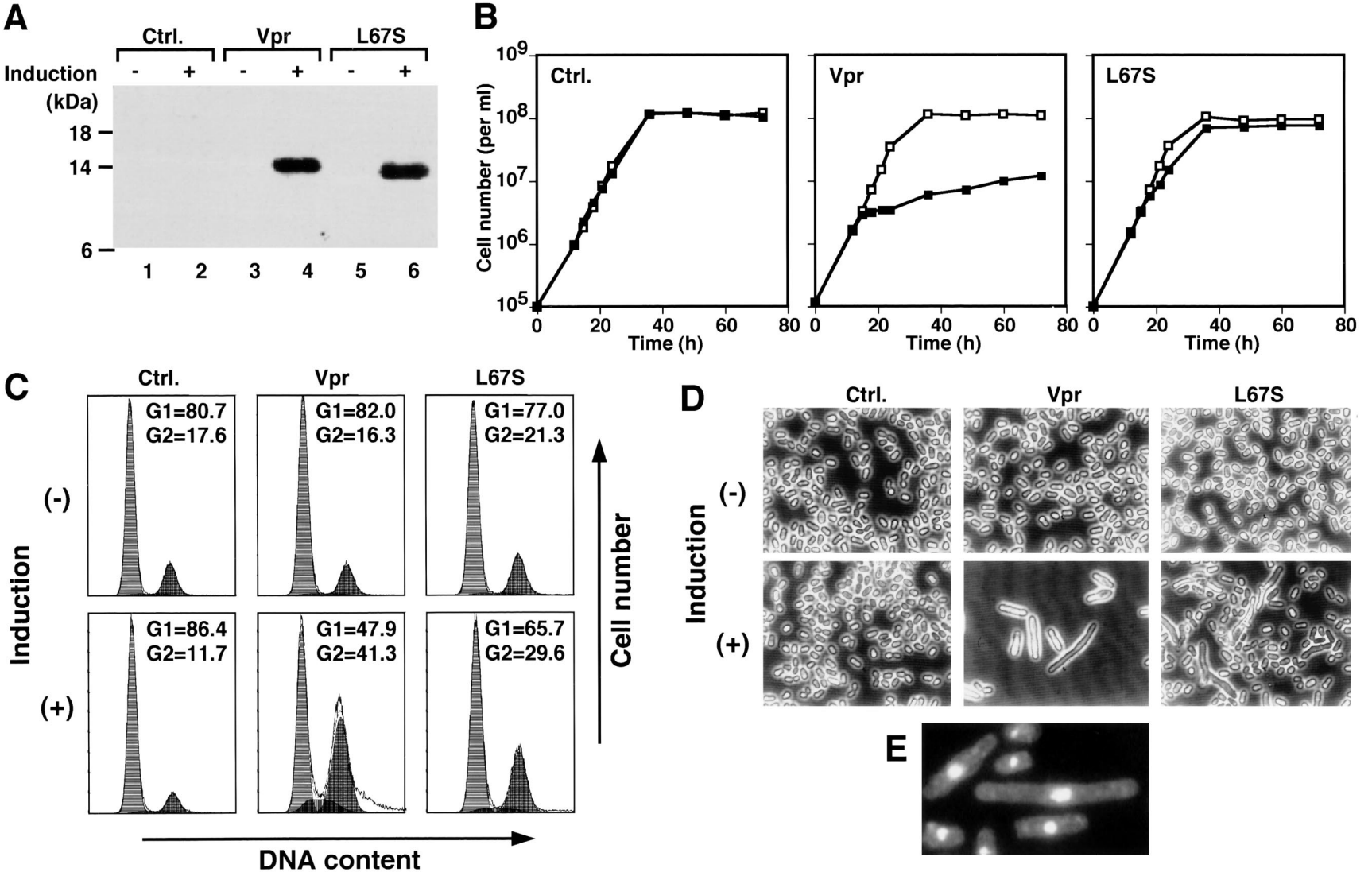

FIG. 1. Effects of wild-type Vpr and Vpr^{L67S} expression on *S. pombe* cell growth properties. Wild-type *S. pombe* cells carrying pREP-1 (Ctrl.), pREP1-vpr (Vpr), and pREP1-L67S (L67S) were compared. (A) Immunoblot analysis of Vpr expression. Cells were grown in the presence (−) or absence (+) of thiamine for 15 h, and cell extracts were prepared, fractionated by SDS–10% PAGE, and transferred to a PVDF membrane. Vpr was detected by a rabbit antiserum to HIV-1_{NL4-3} Vpr (NIH AIDS Research and Reference Reagent Program) and a peroxidase-conjugated mouse anti-rabbit Ig antibody. Binding of the secondary antibody was visualized by using a BM chemiluminescence blotting kit (Roche Diagnostics). (B) Cells were grown under Vpr-inducing (■) or noninducing (□) conditions and were counted at the indicated time points. (C) Cells were grown in the low-nitrogen medium supplemented (−) or not supplemented (+) with thiamine for 36 h, fixed with ethanol, treated with RNase A, and stained with propidium iodide. Then, cellular DNA content was measured by flow cytometry. The numbers in each graph indicate the percentages of the cells in G_1 and G_2 phases. (D) Photomicrographs of cells grown under Vpr-inducing (+) and noninducing (−) conditions for 36 h. Original magnification, ×400. (E) DAPI staining of wild-type *S. pombe* manifesting the Vpr-induced *cdc* phenotype. Original magnification, ×630.

Vpr expression was induced (Fig. 1A, lane 4) and cell proliferation was markedly inhibited (Fig. 1B). Normally, nitrogen-starved *S. pombe* cells are arrested in G_1 phase of the cell cycle, as shown by flow cytometric analysis of the control cells carrying the pREP-1 vector (Fig. 1C). Although *S. pombe* cells carrying pREP1-vpr showed a similar cell cycle profile under Vpr-repressing conditions, a large proportion of Vpr-expressing cells were arrested at G_2/M phase (Fig. 1C). Microscopic observation demonstrated that the Vpr-expressing cells manifested an elongated morphology typical of the *cdc* phenotype representing G_2/M cell cycle arrest (Fig. 1D). In addition, DAPI staining of the Vpr-expressing cells revealed that the elongated cells carried a single nucleus (Fig. 1E), further confirming that the cells were arrested at G_2/M phase. The Leu^{67}-to-Ser (L67S) substitution in Vpr has been shown to decrease the ability of the protein to induce G_2/M cell cycle arrest in human cells (34). When the Vpr with the L67S substitution (Vpr^{L67S}) was expressed in *S. pombe* (Fig. 1A, lane 6), cell proliferation was affected only slightly and the level of G_2/M arrest was reduced compared with the arrest induced by wild-type Vpr (Fig. 1B and C). Most of the Vpr^{L67S}-expressing cells failed to manifest the *cdc* phenotype, consistent with the reduced effects on the cell cycle (Fig. 1D). These results indicated that the effects of wild-type and mutant Vpr on the cell cycle of *S. pombe* cells were similar to those on the human cell cycle, suggesting that a common mechanism is involved in Vpr-induced cell cycle arrest in these different species. It was also shown that microscopic detection of the elongated morphology (*cdc* phenotype) was useful for evaluating the level of Vpr-induced G_2/M arrest.

***wee1*$^+$, but not *cdc25*$^+$, was required for induction of the *cdc* phenotype by Vpr.** In order to examine whether Vpr affects the cell cycle through the Wee1 or the Cdc25 pathway, Vpr expression was induced in wild-type *S. pombe* and the *cdc2* mutant *cdc2-3w* and *cdc2-1w* strains (Table 1). *cdc2-3w* encodes $p34^{cdc2}$, whose Y15 is dephosphorylated in a Cdc25-independent manner. On the other hand, *cdc2-1w* encodes $p34^{cdc2}$, which is refractory to Wee1-mediated Y15 phosphorylation. Although both of them are defined as constitutively active mutants, *cdc2-3w* responds to the negative regulation by overexpression of Wee1, whereas *cdc2-1w* does not (58). When Vpr was expressed in the *cdc2-3w* strain, the *cdc* phenotype was clearly observed and cell proliferation was inhibited (Fig. 2A and B). On the other hand, Vpr expression in the *cdc2-1w* strain failed to show the *cdc* phenotype, and only inhibition of cell proliferation was observed (Fig. 2C and D). These results suggested that Wee1-mediated phosphorylation of $p34^{cdc2}$ might be necessary for Vpr-induced cell cycle arrest. To further examine this possibility, Vpr was expressed in a Δ*wee1* mutant (Table 1). As shown in Fig. 3A and B, the *cdc* phenotype was not manifested by Vpr in the Δ*wee1* strain, whereas cell proliferation was inhibited. The level of Vpr expression in the Δ*wee1* strain in the absence of thiamine was comparable to that in wild-type *S. pombe* (Fig. 4, lanes 2 and 4). When the effects of Vpr expression on the viability of wild-type and Δ*wee1* strains of *S. pombe* were compared, no significant difference was observed (data not shown). Therefore, failure of the Δ*wee1* strain to manifest a Vpr-induced *cdc* phenotype was not due to lack of Vpr expression or a higher susceptibility to Vpr-mediated cell killing. The requirement of Wee1 activity for the Vpr-induced *cdc* phenotype was also confirmed with *ts* mutant *wee1-50* strain (Table 1). The *wee1-50* cells grown at a permissive temperature (23°C) clearly showed the *cdc* phenotype in response to Vpr expression (Fig. 3C). In contrast, manifestation of the distinct *cdc* phenotype was no longer observed when the temperature was shifted to 32.5°C (Fig. 3E), demonstrating that Wee1 activity was required for Vpr-induced cell cycle arrest. Proliferation of the *wee1-50* strain was inhibited by Vpr at both temperatures (Fig. 3D and F). A temperature shift from 23 to 32.5°C did not affect the susceptibility of wild-type *S. pombe* to the effects of Vpr (Fig. 3G and H). As a "twin kinase" of Wee1, Mik1 plays a supplementary role in regulating $p34^{cdc2}$ through phosphorylation of Y15 (29, 31). Vpr expression in a double mutant *wee1-50* Δ*mik1* strain (Table 1) at 23°C caused both the *cdc* phenotype and inhibition of proliferation (Fig. 3I and J), indicating that Mik1 was not required for Vpr-induced cell cycle arrest. At 32.5°C, the *wee1-50* Δ*mik1* strain manifested a lethal phenotype both in the presence and absence of induction of Vpr expression (data not shown). The effects of Vpr in the absence of Cdc25 on a Δ*cdc25 cdc2-3w* double mutant were examined (Table 1) since *S. pombe* carrying the *cdc25* null mutation alone is not viable (57). Induction of Vpr expression in this strain caused the *cdc* phenotype as well as inhibition of proliferation (Fig. 3K and L), indicating that Cdc25 was dispensable for Vpr-induced cell cycle arrest. In a mutant Δ*nim1* strain (Table 1) deficient in the kinase which negatively regulates Wee1 (11, 48, 66), Vpr expression induced the *cdc* phenotype as well as inhibition of proliferation (data not shown).

Requirement of *rad24*$^+$ for the Vpr-induced *cdc* phenotype. It has previously been suggested that Vpr may cause cell cycle arrest through a pathway similar to the DNA damage checkpoint pathway (52). To examine this possibility, the effects of Vpr on various cell cycle checkpoint mutants were examined. Rad1 and Chk1 are signal transducers required for both DNA damage and replication checkpoints (46). Strains carrying both *rad1-1*, which encodes nonfunctional Rad1, and Δ*chk1*, which expresses no Chk1, manifested the *cdc* phenotype in response to Vpr expression (Table 1; Fig. 5A and C). DAPI staining of the Vpr-expressing *rad1-1* and Δ*chk1* cells revealed that most of the elongated cells carried one nucleus (data not shown). Although proliferation of the *rad1-1* and Δ*chk1* strains was clearly inhibited under Vpr-inducing conditions at earlier time points up to 36 h postinduction, their growth curves appeared to catch up with that of the controls at later time points (Fig. 5B and D). These results indicated that both of *rad1*$^+$ and *chk1*$^+$ were dispensable for Vpr-induced cell cycle arrest, while they may play some role in sustaining the arrest. *rad24*$^+$ was identified as a multicopy suppressor of a radiation-sensitive mutation of *S. pombe* and is thought to be involved in regulation of the cell cycle timing and DNA damage checkpoint control through negative effects on Cdc25 (12, 15, 16, 49, 59). Unlike *rad1-1* and Δ*chk1* mutants, the Δ*rad24* strain (Table 1) failed to reveal the *cdc* phenotype in response to Vpr expression, although its proliferation was markedly inhibited (Fig. 5E and F). The level of Vpr expression in the Δ*rad24* strain was comparable to that in wild-type *S. pombe* (Fig. 4, lane 8). Another mutant, the Δ*cds1* strain, deficient in the DNA replication checkpoint (Table 1) was susceptible to both induction of the *cdc* phenotype and inhibition of proliferation by Vpr (Fig. 5G and H).

Vpr failed to induce the *cdc* phenotype in the Δ*ppa2* strain. It has been shown that okadaic acid, a potent inhibitor of protein phosphatase 2A (PP2A), can abrogate Vpr-induced cell cycle arrest in mammalian and fission yeast cells, suggesting that PP2A may be required for manifestation of the effects of Vpr (54, 68). Fission yeast PP2A consists of a catalytic subunit, either Ppa1 or Ppa2, and two regulatory subunits, Paa1 and Pab2 (25, 27). To investigate the possibility that PP2A might be involved in Vpr-induced cell cycle arrest, the effects of Vpr expression on cellular morphology and proliferation in mutant Δ*ppa1* and Δ*ppa2* strains were examined (Ta-

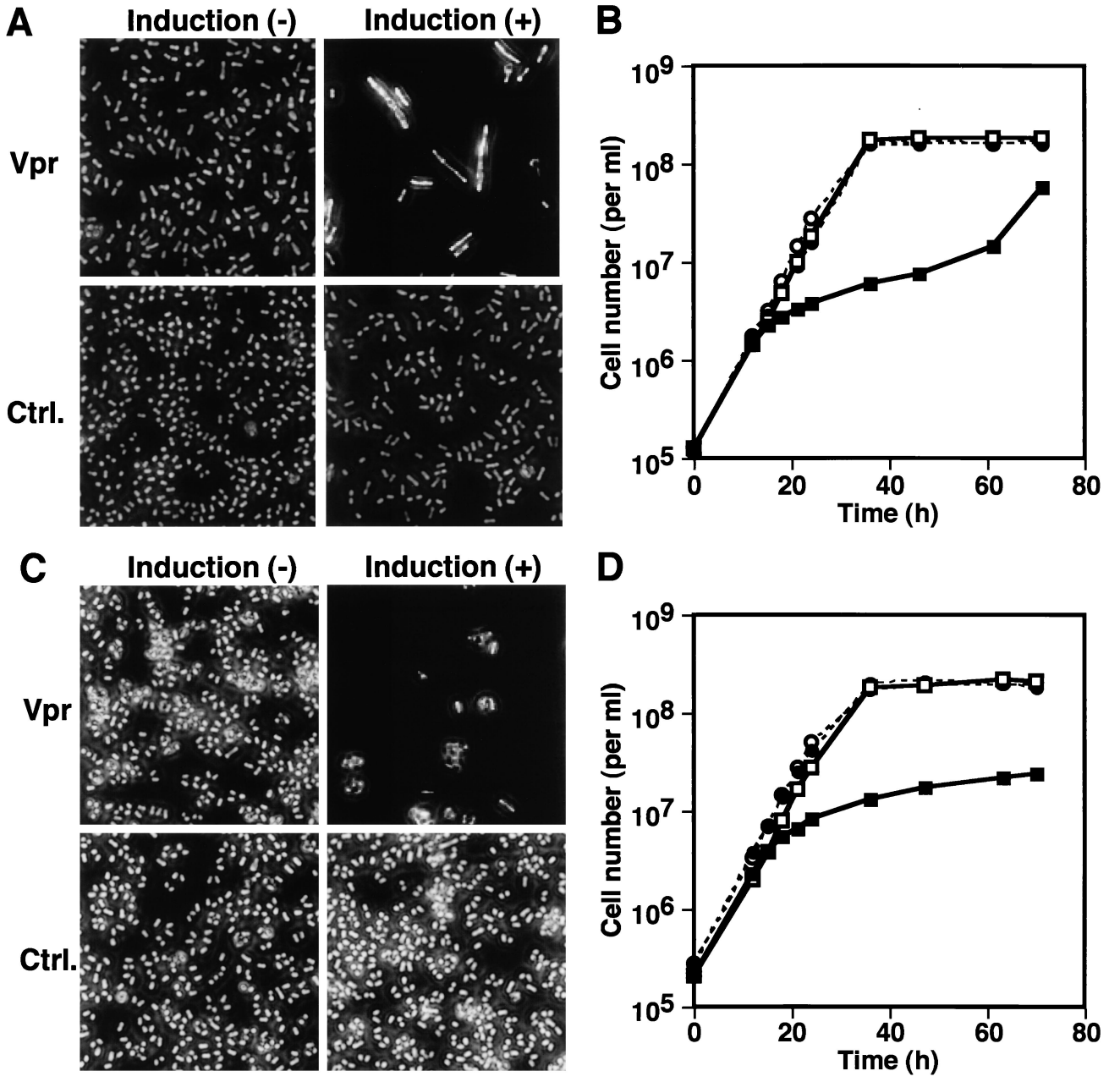

FIG. 2. Effects of Vpr expression on morphology and proliferation of the *cdc2-3w* and *cdc2-1w* strains of *S. pombe*. *S. pombe* cells bearing the *cdc2-3w* (A and B) or *cdc2-1w* (C and D) mutation transformed with pREP1-vpr (Vpr; squares) or pREP-1 (Ctrl.; circles) were grown in the presence (−; open symbols) or absence (+; solid symbols) of thiamine. Photomicrographs (A and C) show representative morphology of the cells at 36 h. Graphs (B and D) indicate the numbers of the cells counted at the indicated time points.

ble 1). Vpr expression in the Δ*ppa1* strain caused clearly elongated morphology and inhibition of cell proliferation (Fig. 6A and B). On the other hand, Vpr affected the morphology of the Δ*ppa2* strain only marginally (Fig. 6C). Vpr was expressed in the Δ*ppa2* strain as efficiently as in wild-type *S. pombe* (Fig. 4, lane 6) and inhibited cell proliferation (Fig. 6D). These results suggested that $ppa2^{+}$, but not $ppa1^{+}$, was necessary for the inhibitory effects of Vpr on the cell cycle.

Susceptibility of several mutants to Vpr-induced cell cycle arrest was discordant with their responsiveness to DNA damage. In order to compare the mechanisms of Vpr-induced cell cycle arrest and the DNA damage checkpoint, several mutant strains of *S. pombe* carrying pREP1-vpr were grown under Vpr-repressing conditions and treated with bleomycin, which induces DNA double-strand breaks. As expected from previous studies (16), wild-type *S. pombe* and the Δ*wee1 wee1-50* Δ*mik1* strains incubated at the permissive temperature responded to bleomycin-induced DNA damage, revealing the *cdc* phenotype, whereas the *cdc2-3w* Δ*cdc25* strain did not (Table 2). The size of *cdc2-3w* cells was somewhat increased by bleomycin treatment (Table 2), probably because the mutant remains responsive to DNA damage-induced Cdc25 inhibition (16). However, the change in the cell size of the *cdc2-3w* strain was marginal compared with the *cdc* phenotype induced by

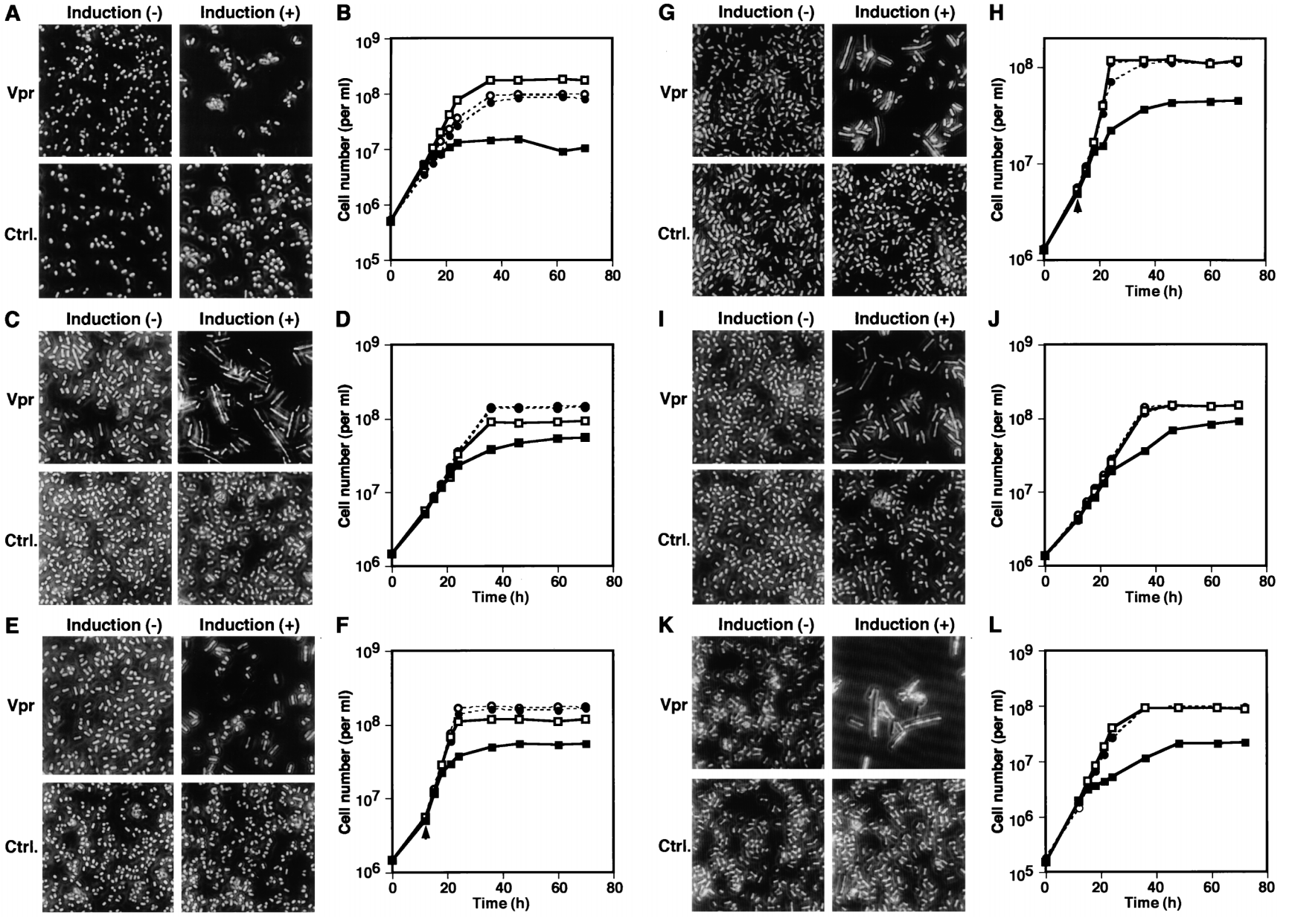

FIG. 3. Requirement of *wee1* for manifestation of the Vpr-induced *cdc* phenotype. Δ*wee1* (A and B), *wee1-50* (C, D, E, and F), wild-type (G and H), *wee1-50* Δ*mik1* (I and J), and Δ*cdc25 cdc2-3w* (K and L) *S. pombe* strains transformed with pREP1-vpr (Vpr; squares) or pREP-1 (Ctrl.; circles) were grown in the presence (−; open symbols) or absence (+; solid symbols) of thiamine at 30 (A, B, K, and L) or 23°C (C, D, I, and J) throughout the experiment or at 23°C until 12 h (arrowhead) and 32.5°C thereafter (E, F, G and H). Photomicrographs (A, C, E, G, I, and K) show representative morphology of the cells at 36 h. Graphs (B, D, F, H, J, and L) indicate the numbers of the cells counted at the indicated time points.

TABLE 2. Effects of genetic background of *S. pombe* on induction of cell cycle arrest in response to Vpr and DNA damage

Brief genotype	*cdc* phenotype induction in response to:	
	Vpr[a]	DNA damage[b]
Wild type	+	+
cdc2-3w	+	+/−[c]
cdc2-3w Δcdc25	+	−
Δwee1	−	+
wee1-50 Δmik1[d]	+	+
rad1-1	+	−
Δrad24	−	−
Δppa1	+	+
Δppa2	−	+

[a] Data shown in Fig. 2, 3, 5, and 6 are summarized.
[b] Cells in the mid-log growth phase under Vpr-repressing conditions were treated with bleomycin (50 mU/ml) for 6 h and were examined for the presence or absence of the *cdc* phenotype with a microscope.
[c] Cell size was marginally increased, but not to the level of an unequivocal *cdc* phenotype.
[d] At 23°C.

Vpr. Other radiation-sensitive mutants, such as the *rad1-1* and *rad24* strains, failed to respond to bleomycin as expected (15, 56). The sensitivity of PP2A mutants to DNA damage has not previously been described, and our data indicated that both *Δppa1* and *Δppa2* strains manifested the *cdc* phenotype in response to bleomycin treatment (Table 2). These results demonstrated that in *S. pombe* susceptibility to Vpr-induced cell cycle arrest was not necessarily correlated with responsiveness to DNA damage.

DISCUSSION

In this study, we exploited the fission yeast *S. pombe* for identifying the cellular factors involved in Vpr-induced cell cycle arrest. The fission yeast *S. pombe*, serving as a useful model organism for elucidating the mechanism for cell cycle regulation, has been shown to be susceptible to Vpr-induced cell cycle arrest (67, 68). Although it has previously been suggested that the effects of Vpr on the cell cycle were species or cell type specific (43, 62), a recent study demonstrated that the effects of the mutations in Vpr on its functions in *S. pombe* and human cells were similar (10). Our data on Vpr^{L67S}, which was not examined in *S. pombe* in the previous study (10), also indicated that the effects of the Vpr mutation in *S. pombe* were similar to those in human cells (34). These observations suggest that Vpr may induce cell cycle arrest in *S. pombe* and human cells through a common mechanism despite the large phylogenetic distance between these species.

It has previously been shown that G_2/M cell cycle arrest by HIV-1 Vpr is associated with inactivation of $p34^{cdc2}$ kinase, a key regulator of the G_2/M transition (19, 54). The kinase activity of $p34^{cdc2}$ is mainly regulated by the relative activities of Wee1 and Cdc25, although other mechanisms also appear to contribute to the regulation (28, 46). Our data demonstrated that Wee1, but not Cdc25, was required for induction of the *cdc* phenotype by Vpr in *S. pombe*, suggesting that Vpr may affect the host cell cycle by increasing the Wee1 activity rather than inhibiting Cdc25. Like Wee1, Mik1 has been shown to negatively regulate $p34^{cdc2}$ through Y15 phosphorylation. However, our data indicated that Mik1 was dispensable for the Vpr-induced *cdc* phenotype.

What might be the mechanism by which Vpr affects the Wee1 activity? Although Vpr may activate Wee1 directly (Fig. 7), Vpr has no known domain or activity that mediates immediate interaction with Wee1. Therefore, it is possible that Vpr affects Wee1 through a mechanism which involves additional cellular factors. In *S. pombe*, kinase Nim1 negatively affects Wee1 activity (11, 48, 66). However, Vpr caused the *cdc* phenotype in a *Δnim1* mutant, making it unlikely that Nim1 or the pathway upstream of Nim1 is involved in Vpr-induced cell cycle arrest. A Nim1-related kinase, Cdr2, which may also negatively regulate Wee1, has recently been identified (8, 23). Additional experiments using a *cdr2* mutant may reveal whether Cdr2 plays any role in the effects of Vpr on the cell cycle. Alternatively, it is possible that Vpr blocks the G_2/M transition by inhibiting Wee1 degradation, since a recent study suggested that degradation of Wee1 might be a prerequisite for entry into mitosis (38). These possibilities are currently being investigated in our laboratories.

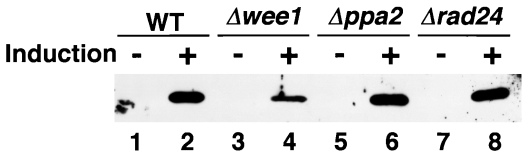

FIG. 4. Immunoblot analysis of Vpr expression. Wild-type (WT), *Δwee1*, *Δppa2*, and *Δrad24 S. pombe* cells carrying the pREP1-vpr vector were grown under noninducing (−) or inducing (+) conditions for 15 h, and cell extracts were prepared, fractionated by SDS–10% PAGE, and transferred to a PVDF membrane. Vpr was detected by an rabbit antiserum to $HIV\text{-}1_{NL4\text{-}3}$ Vpr (NIH AIDS Research and Reference Reagent Program) and peroxidase-conjugated mouse anti-rabbit Ig antibody. Binding of the secondary antibody was visualized by using a BM chemiluminescence blotting kit (Roche Diagnostics).

PP2A is also thought to play a role in cell cycle regulation through its positive or negative effects on Wee1 or Cdc25, respectively (26). *S. pombe* PP2A consists of two regulatory subunits, Paa1 and Pab2 (25), and a catalytic subunit, Ppa1 or Ppa2; Ppa2 contributes to the majority of the phosphatase activity and is a target of the inhibitory effect of okadaic acid (27). Our data demonstrated that Ppa2, but not Ppa1, was required for manifestation of the Vpr-induced *cdc* phenotype. This observation is compatible with the previous studies showing that okadaic acid rescues mammalian and *S. pombe* cells from Vpr-induced cell cycle arrest (54, 68).

It is possible that Vpr mimics cell cycle checkpoint control, which delays the G_2/M transition, in response to DNA damage and incomplete DNA replication. Our data showed that Vpr caused the *cdc* phenotype in the *cdc2-3w* strain, which appears to be specifically defective in the DNA replication checkpoint (13). We also showed that $cds1^+$, which is involved in the DNA replication checkpoint (5, 42), was dispensable for the Vpr-induced *cdc* phenotype. Therefore, Vpr does not seem to utilize the DNA replication checkpoint pathway for inducing G_2/M arrest. Involvement of DNA damage checkpoint control in Vpr-induced cell cycle arrest has previously been suggested (52). However, another study reached the opposite conclusion that Vpr induced cell cycle arrest by a mechanism which differs from DNA damage checkpoint control (4). A currently proposed model for DNA damage checkpoint control (9, 46) speculates that DNA damage is recognized by an as yet unidentified sensor molecule and that the signal is transduced through the *rad* checkpoint pathway, which involves multiple proteins, including Rad1 (56), and effector molecules, such as Wee1 (Fig. 7). There is some evidence that Chk1, which can phosphorylate Wee1, mediates the DNA damage signal from the *rad* pathway (45, 64) (Fig. 7). However, our data indicated that neither Rad1 nor Chk1 was required for induction of the *cdc*

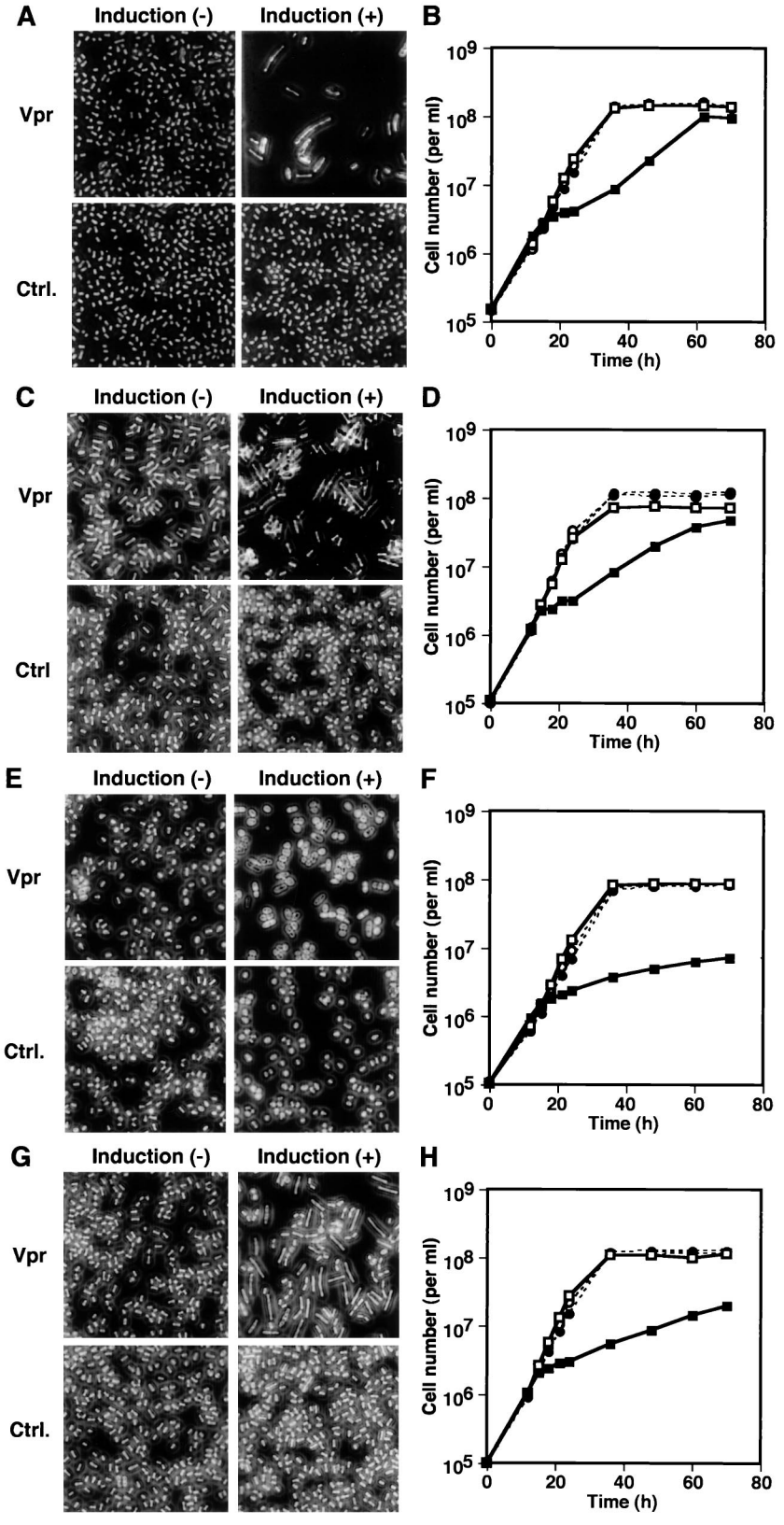

FIG. 5. Influence of cell cycle checkpoint molecules on the susceptibility to the effects of Vpr. *rad1-1* (A and B), Δ*chk1* (C and D), Δ*rad24* (E and F), and Δ*cds1* (G and H) *S. pombe* cells transformed with pREP1-vpr (Vpr; squares) or pREP-1 (Ctrl.; circles) were grown in the presence (−; open symbols) or absence (+; solid symbols) of thiamine. Photomicrographs (A, C, E, and G) show representative morphology of the cells at 36 h. Graphs (B, D, F, and H) indicate the numbers of the cells counted at the indicated time points.

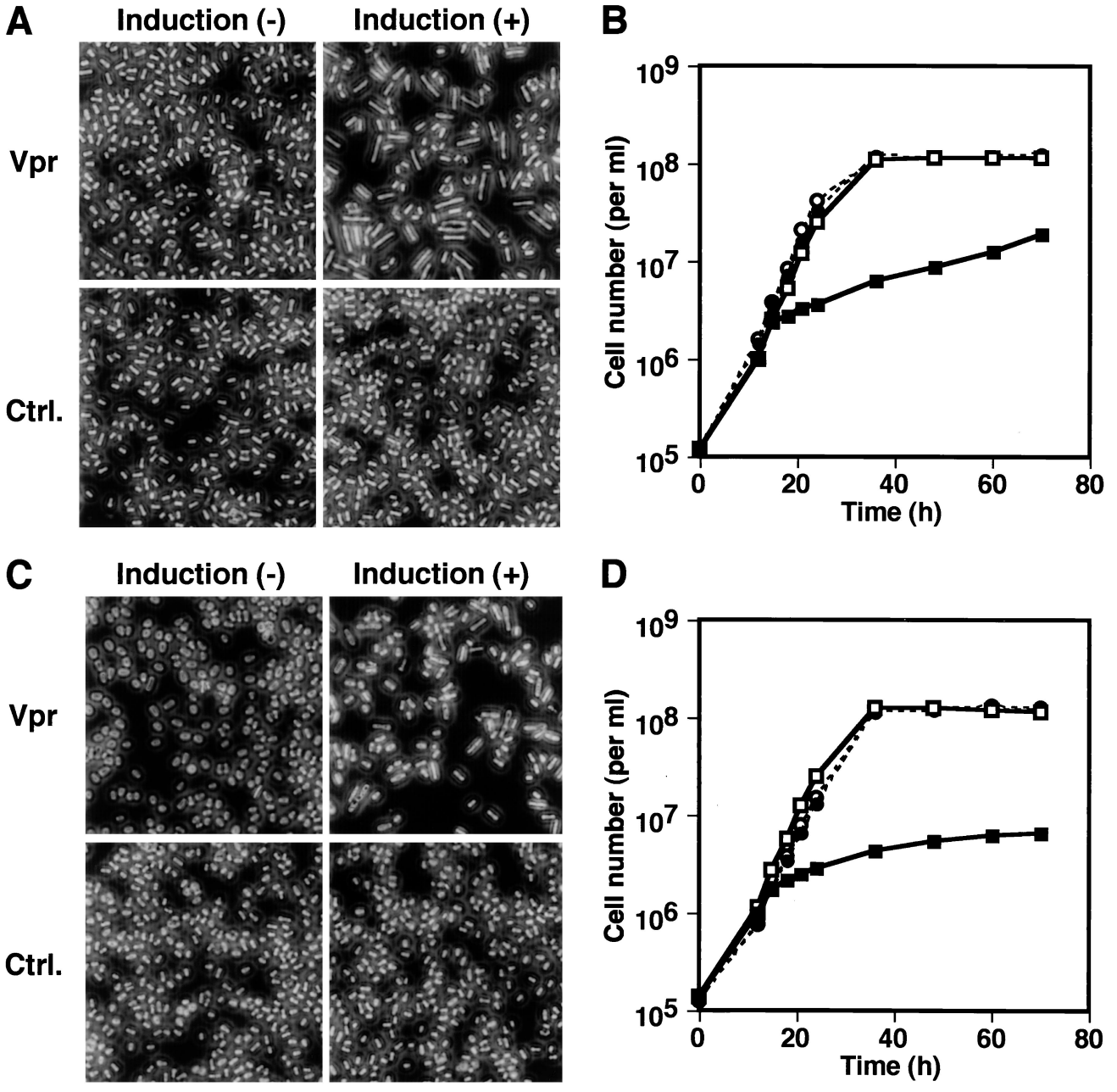

FIG. 6. Requirement of *ppa2*, which encodes a catalytic subunit of PP2A, for the Vpr-induced *cdc* phenotype. Δ*ppa1* (A and B) and Δ*ppa2* (C and D) *S. pombe* cells transformed with pREP1-vpr (Vpr; squares) or pREP-1 (Ctrl.; circles) were grown in the presence (−; open symbols) or absence (+; solid symbols) of thiamine. Photomicrographs (A and C) show representative morphology of the cells at 36 h. Graphs (B and D) indicate the numbers of the cells counted at the indicated time points.

phenotype by Vpr. These results suggest that Vpr does not utilize Rad1, Chk1, or the DNA damage checkpoint control pathway upstream of these molecules for inducing cell cycle arrest. It should be mentioned that the *rad1-1* and Δ*chk1* strains appeared to recover from the effects of Vpr after prolonged induction of Vpr expression (Fig. 5B and D), suggesting that Rad1 and Chk1 might play some role in the maintenance, if not the induction, of cell cycle arrest by Vpr. Unlike *rad1*$^+$ and *chk1*$^+$, *rad24*$^+$ was shown to be necessary for manifestation of the Vpr-induced *cdc* phenotype. Rad24, an *S. pombe* homolog of the mammalian 14-3-3 protein, was identified as a DNA damage checkpoint molecule which determines the timing of mitosis (15). Recent studies have suggested that 14-3-3 binds to Cdc25 phosphorylated by Chk1, inhibits its nuclear translocation, and thereby prevents it from dephosphorylating p34^{cdc2} (12, 16, 49, 59). Our data indicated that Cdc25 does not play a major role in induction of the *cdc* phenotype by Vpr. Therefore, it is possible that Rad24 negatively regulates p34^{cdc2} through an alternative pathway, which may involve Wee1 (Fig. 7). Taken together, these findings suggest that Vpr does not mimic the entire DNA damage checkpoint control, but rather may partially utilize it for inducing G_2/M arrest.

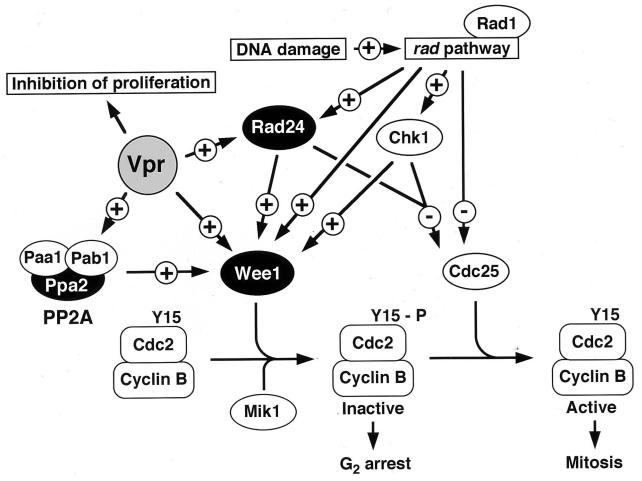

FIG. 7. A putative mechanism of Vpr-induced cell cycle arrest. A model for relevant pathways of cell cycle regulation and DNA damage checkpoint has been summarized from previous studies (9, 46). Positive and negative regulations are indicated by arrows labeled + and −, respectively. P, Y15 phosphorylation.

Also supporting this possibility are the data in this study indicating that the susceptibility of *S. pombe* mutants to Vpr-induced cell cycle arrest is not always correlated with their responsiveness to DNA damage (Table 2).

Vpr inhibited proliferation of all of the strains used in this study including the ones that did not show the *cdc* phenotype in response to Vpr expression. It has been suggested in a previous study (67) that Vpr-induced cell cycle arrest may be Wee1 independent, based on an observation that Vpr inhibited the proliferation of the *cdc2-1w* strain. However, the morphology of *cdc2-1w S. pombe* cells expressing Vpr was not described in the study. Our present study reveals that Vpr could inhibit proliferation of *cdc2-1w* cells without causing the *cdc* phenotype, indicating that Wee1 is required for the Vpr-induced *cdc* phenotype but not inhibition of cell proliferation. Therefore, Vpr appears to affect the cell cycle and proliferation through distinct pathways (Fig. 7) as suggested by previous studies (43, 69). Since the toxicity of Vpr has been documented, not only for mammalian cells but also for other systems including bacteria (6) and the budding yeast *Saccharomyces cerevisiae* (32), Vpr may affect the viability of *S. pombe* by deteriorating a basic biological function common to various species.

Although it is still unknown whether the human homologs of Wee1, Ppa2, and Rad24 are involved in Vpr-induced G_2/M arrest, our preliminary data suggested that expression of human WEE1 in the Δ*wee1* strain of *S. pombe* restored susceptibility to the Vpr-induced *cdc* phenotype (Y. Nagai and M. Masuda, unpublished data). Further studies using *S. pombe* on the mechanism by which Vpr affects human cellular functions may provide useful insights into the molecular basis for AIDS pathogenesis and development of a novel therapeutic intervention.

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