Increased Levels of Wee-1 Kinase in G₂ Are Necessary for Vpr- and Gamma Irradiation-Induced G₂ Arrest

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Human immunodeficiency virus type 1 (HIV-1) Vpr induces cell cycle arrest at the G₂/M transition and subsequently apoptosis. Here we examined the potential involvement of Wee-1 in Vpr-induced G₂ arrest. Wee-1 is a cellular protein kinase that inhibits Cdc2 activity, thereby preventing cells from proceeding through mitosis. We previously showed that the levels of Wee-1 correlate with Vpr-mediated apoptosis. Here, we demonstrate that Vpr-induced G₂ arrest correlated with delayed degradation of Wee-1 at G₂/M. Experimental depletion of Wee-1 by a small interfering RNA directed to wee-1 mRNA alleviated Vpr-induced G₂ arrest and allowed apparently normal progression through M into G₁. Similar results were observed when cells were arrested at G₂ following gamma irradiation. Thus, Wee-1 is integrally involved as a key cellular regulatory protein in the signal transduction pathway for HIV-1 Vpr-induced cell cycle arrest.

MATERIALS AND METHODS

Cell culture, synchronization, and gamma irradiation. HeLa cells and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine calf serum and penicillin-streptomycin. Aliquots of 1.5 × 10⁶ HeLa cells per well in a six-well plate or 1.0 × 10⁶ cells per 60-mm-diameter plate (Becton Dickinson, Mountain View, Calif.) were seeded 1 day before synchronization. HeLa cells were synchronized at G₁/S by culturing them in the presence of 2 mM thymidine (Sigma-Aldrich, St. Louis, Mo.) in DMEM-10% calf serum for 18 h. They were then allowed to recover in complete growth medium that lacked thymidine for 8 h and then propagated again in 2 mM thymidine for an...
95% of HeLa cells were arrested in G2 phase at 24 h postinfection. The equivalents described previously (26). Virus stock was titrated to ensure that more than \( \text{H9262} \) produced in 293T cells by cotransfection of 293T cells with 12.5 \( \text{vector, 12.5} \text{H9262} \) wee-1 HA was from Covance Inc., Princeton, N.J. Wee-1, Cdc2, and anti-phospho-cdc2 were purchased from Santa Cruz Biotech, Santa Cruz, Calif. Anti-phospho-cdc2 was performed as described previously (33). Wee-1, Cdc2, and western blots were obtained in three independent experiments. Western blotting results obtained in three independent experiments.

FACScan II cell sorter (Becton Dickinson) and analyzed with the Cell Quest software package. A total of 10,000 events were collected and analyzed in each sample. (B) Whole-cell lysate (10 μg) was separated by SDS–7.5% (Wee-1) or 15% (others) PAGE and probed with antibodies to Wee-1, phosphorlated Cdc2, total Cdc2, HA-tagged Vpr, and actin (as a loading control). Data shown are representative of Western blotting results obtained in three independent experiments.

Vpr viruses were from Thermo, and HR

\[ \text{H11032} \text{EGFP} \] were used as negative controls. Infections and flow cytometry. Cells were infected and analyzed for cell cycle status as previously described (27). Cell cycles were measured by DNA staining with propidium iodide (Sigma-Aldrich). All stained cells were acquired on a FACScan II cell sorter (Becton Dickinson) and analyzed with the Cell Quest software package. A total of 10,000 events were collected and analyzed in each sample.

Western blot and Cdc2 kinase assay. The Western blot and Cdc2 kinase assay was performed as described previously (33). Wee-1, Cdc2, and β-actin antibodies were purchased from Santa Cruz Biotech, Santa Cruz, Calif. Anti-phospho-cdc2 (Tyr15) was from Cell Signaling, Beverly, Mass., and antihemagglutinin (anti-HA) was from Covance Inc., Princeton, N.J.

Real-time quantitative RT-PCR assay. Real-time quantitative reverse transcription-PCR (RT-PCR) was performed to measure the level of wee-1 mRNA. Total RNA was prepared using the total RNA isolation kit (RNaseasy; Qiagen, Valencia, Calif.) and treated with DNase I (RNase-free DNase set; Qiagen) according to the manufacturer’s instructions. Quantification of the mRNA with wee-1 and glyceraldehyde-3-phosphate dehydrogenase (gpdh) was performed using ABI PRISM7700 (Applied Biosystems, Foster City, Calif.) with the QuantiTect SYBR Green RT-PCR kit (Qiagen). The reaction mixture contained QuantiTect SYBR Green RT-PCR Master Mix, QuantiTect RT Mix, each primer at 100 nM, and 25 ng (for wee-1) or 5 ng (for gpdh) of total RNA. After incubation for 30 min at 50°C (for the RT reaction) and then for 15 min at 95°C (for activation of Hot Start Taq polymerase), we carried out 45 cycles, with each cycle consisting of denaturation for 1 s at 95°C, annealing for 30 s at 59°C, and extension for 30 s at 72°C. The following primers were used in RT-PCR for these specific mRNAs: (i) human wee-1, 5′ primer, 5′-ATTCTCTGGCGTGCCCGAG AAG-3′, and 3′ primer, 5′-CAAAGGAGATCCTTCACCTGC-3′; (ii) human gpdh, 5′ primer, 5′-TGCAACCACCCGTAGC-3′, and 3′ primer, 5′-GGCATGGACTGTGTCATGAG-3′.

Pulse-chase analysis of endogenous Wee-1. Synchronized HeLa cells were cultured for 7 h after infection with HR Vpr or HR EGFP virus and were washed once with methionine- and cysteine-free DMEM, followed by starvation in the same medium supplemented with 10% dialyzed fetal calf serum (FCS, Gibco BRL, Grand Island, N.Y.) for 30 min. The cells were pulse-labeled at 37°C by adding 100 μCi of \( ^{35} \text{S} \)-labeled L-methionine and L-cysteine (Redivue Pro-Mix \( ^{35} \text{S} \) in vitro cell labeling mix; Amersham Biosciences, Uppsala, Sweden) per 10\(^6\) cells in methionine- and cysteine-free medium supplemented with 10% dialyzed FCS and glutamine for 10 min. It has been reported that \( ^{35} \text{S} \)-methionine in order to avoid this effect (data not shown). The pulse-labeling was stopped by washing the cells twice with DMEM, and DMEM–10% FCS containing unlabeled methionine and cysteine at a concentration of 200 μM was added to cultures for the chase periods indicated. After a chase, the cells were washed with ice-cold phosphate-buffered saline and were lysed in 1 ml of ice-cold radioimmunoassay buffer (1% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.2% NP-40, 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl [pH 7.4], 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na\(_2\)VO\(_4\)) containing protease inhibitor cocktail (PI860; Sigma-Aldrich) and phosphatase inhibitor cock-
HIV-1 Vpr was used to infect a population of HeLa cells that was synchronized at the G1/S border by a double thymidine block and release protocol (23). Flow cytometry was used to monitor the ability of cells to traverse the cell cycle after release from the block (Fig. 1A). Cells infected with control virus HR’EGFP proceeded normally through the S, G2, and M phases of the cell cycle. Mitosis occurred 11 h after release at G1/S. In agreement with our previous observations the majority of Vpr-infected cells (>95%) were arrested in G2 (Fig. 1A).

Western blot analysis was used to monitor the levels of Wee1 and Cdc2 after G1/S release (Fig. 1B). As expected, the level of Wee1 in cells infected with control virus decreased at 11 and 12 h after release as a result of protein degradation during M phase. We observed an elevated level of Wee1 in Vpr-infected cells at the same time points.

The electrophoretic mobility of Cdc2 can be used as an indicator of cell cycle position and to assess the phosphorylation status of Cdc2. The higher-molecular-weight species represents the phosphorylated form of Cdc2 (Tyr15) that is inactive. The smaller species is the active unphosphorylated form of the kinase. We monitored the mobility of Cdc2 using antibodies that allowed us to distinguish the phosphorylation status of Cdc2 as cells cycled from G1/S through M. As previously reported, in the control cells we observed the disappearance of the upper band (phosphorylated form of Cdc2) at 10 h and then a decrease in the overall level of Cdc2 at 12 h as cells went through mitosis (Fig. 1B). In contrast equivalent amounts of the phosphorylated and unphosphorylated forms of Cdc2 were observed in the Vpr-infected cells throughout the time course. The transient increase in the level of Wee1 and the presence of the phosphorylated form of Cdc2 in Vpr-arrested cells are consistent with the cell cycle-inhibitory role of Wee1 at the G2/M checkpoint.

**Vpr causes a delay in reduction of the levels of Wee1 at G2/M.** The results presented above demonstrate that an increase in the levels of Wee1 following infection with HR Vpr is paralleled by increased levels of the phosphorylated (inactive) form of Cdc2. The relationship between induction of G2 arrest and increased levels of Wee1 at the G2/M transition...
over time suggested the hypothesis that a delay in reduction of Wee-1 may be responsible for the failure of Vpr-arrested cells to progress through mitosis. To further test this hypothesis, we determined the level of 35S-labeled Wee-1 in Vpr-infected cells by pulse-chase analysis (Fig. 2A). Cells were released from G1/S at the time of infection and 35S-pulse-labeled 7 h after infection/release. In HR Thy-infected cells the level of labeled Wee-1 decreased following release at G1/S. Wee-1 had a half-life of approximately 2.5 h. In contrast a sustained level of labeled Wee-1 is observed in the presence of Vpr. Over the 5-h time course there is a delay in degradation of Wee-1 relative to cells infected with HR Thy. In this case the half-life of Wee-1 is about twice as long as it is in the absence of Vpr (Fig. 2B).

Quantitative RT-PCR analysis of wee-1 mRNA. Vpr has been shown to weakly transactivate transcription from heterologous promoters (4). In order to determine if the increased level of Wee-1 was due to an increase in the level of wee-1 mRNA, we used quantitative real-time RT-PCR to monitor levels of wee-1 mRNA as cells transitioned from G1/S to M (Fig. 3). Total RNA was isolated from cells infected with HR’EGFP or HR’Vpr. The level of mRNA was determined relative to gapdh mRNA. Our results indicate that the level of wee-1 mRNA in Vpr-infected cells over time was not significantly different from that in control cells. Thus, the delayed loss of Wee-1 protein appears to be the major cause of increased levels of Wee-1 in the presence of Vpr.

Cdc2 activity is reduced at G2/M in HR’Vpr-infected cells. We investigated whether Vpr had an effect on Cdc2 kinase activity. Wee-1 regulates Cdc2 kinase activity through phosphorylation of Tyr15, resulting in inactivation of Cdc2 and cell cycle arrest at the G2/M checkpoint. Our previous finding that the level of Wee-1 was elevated in Vpr-infected cells lent support to the idea that we should expect to see a decrease in Cdc2 kinase activity in the presence of Vpr. We immunoprecipitated Cdc2 from HeLa cells infected with HR Thy or HR’Vpr at various time points after release at G1/S. Utilizing a histone H1 phosphorylation assay, we observed three- to fourfold less Cdc2 kinase activity specifically in Vpr-infected cells 14 h after release (Fig. 4). This result is consistent with the elevated level of Tyr15 phosphorylation of Cdc2 that we observed in Vpr-infected cells.

Depletion of Wee-1 by siRNA Wee-1 alleviates Vpr-induced cell cycle arrest. Our results indicate that increased levels of Wee-1 prior to M at the G2/M transition correlate with cell cycle arrest induced by Vpr, leading to the hypothesis that the higher levels of Wee-1 are directly responsible for the failure of Vpr-infected cells to progress into mitosis. We tested this hypothesis further by using an siRNA directed to wee-1 mRNA that specifically eliminates Wee-1 expression. Since the timing of Wee-1 depletion within the cell cycle is critical for this experiment, we utilized cells synchronized at G1/S by a double thymidine block. Wee-1 siRNA was transfected into the cells immediately following release from the double thymidine block.
block. Four hours later, cells were infected with HR/Vpr and the effect of Wee-1 depletion upon Vpr-mediated cell cycle arrest was monitored over time as the cells progressed through the G2/M transition. Control cells were transfected with an irrelevant siRNA directed against luciferase and infected with control virus HR/Thy. We confirmed that the siRNA knocks down Wee-1 levels by analysis of Wee-1 levels at 14 h post release from the double thymidine block by Western blot analysis (Fig. 5B). Cell cycle progression was analyzed about the G2/M transition (9 to 14 h following release). As expected, in control cells with siRNA to luciferase mRNA (siLuc.), the virion-associated Vpr in HR/Vpr induces cell cycle arrest within 11 to 14 h, whereas normal cells (siLuc. and HR/Thy) progressed through mitosis into G1. In contrast, transfection of siRNA to wee-1 mRNA (siWee-1) prior to introduction of Vpr resulted in a nearly normal cell cycle progression through the G2/M checkpoint (compare HR/Thy/siLuc. with HR/Vpr/siWee-1). We also counted the viable cell number by trypan blue dye exclusion and confirmed that the number of cells with HR/Vpr/siWee-1 was similar to that of control cells (HR/Thy/ siLuc.) or cells with siWee-1 alone (HR/Thy/siWee-1) at 14 h after infection, indicating progression through mitosis and cell division (data not shown). Cells depleted of Wee-1 without Vpr showed a slightly accelerated progression into G1 (Fig. 5A, compare lanes 1 and 2 at 14 h), consistent with the depletion of Wee-1. Thus, the presence of Wee-1 during G2 is essential for the induction of cell cycle arrest by Vpr. As previously reported, at later time points (24 to 48 h) in the presence of Vpr, siWee-1, or both, cell death via apoptosis is evident (data not shown) (25, 33).

Depletion of Wee-1 by siWee-1 partially alleviates gamma irradiation-induced G2/M arrest. Gamma irradiation induces cell cycle arrest at different stages of the cell cycle. The mechanisms of arrest are complex and involve multiple pathways (28). In some cells, including HeLa cells, DNA damage induced by gamma irradiation induces a cell cycle arrest at G2/M that phenotypically resembles Vpr-induced arrest (Fig. 6A). We observed that the level of Wee-1 is increased in gamma-irradiated cells (Fig. 6B). As expected, the level of Wee-1 increases and remains constant throughout the 24-h time

FIG. 5. Wee-1 depletion by siRNA Wee-1 alleviates Vpr-induced arrest. HeLa cells (1.5 × 10⁵) were synchronized at G1/S by double thymidine block and transfected with siWee-1 or small interfering luciferase (siLuc.). Cells were then infected with HR/Vpr or HR/Thy virus (0.3 μg of viral p24). (A) Cell cycle profiles were determined at various time points following infection. P.R., postrelease. (B) Fourteen hours after infection whole-cell lysates were prepared and analyzed by Western blotting.
point. In control cells a decrease in Wee-1 is seen at 11 h postinfection (at the G2/M transition) followed by an increased level of Wee-1 at 24 h when the majority of cells proceeded to S phase. Thus, Vpr and gamma irradiation-mediated cell cycle arrest give a similar phenotype in regard to Wee-1 levels. These results indicate that, like Vpr, increased levels of Wee-1 prior to M at the G2/M transition correlate with cell cycle arrest induced by gamma irradiation. We tested the role of Wee-1 directly in a fashion similar to that above by depleting Wee-1 with an siRNA to wee-1 mRNA. Cells were synchro-

FIG. 6. Gamma irradiation-induced G2 arrest results in stabilization of Wee-1. HeLa cells (1.5 × 10^6) were synchronized by double thymidine block at G1/S and gamma irradiated with 4,000 rads following release. (A) Cells were collected at various times after gamma irradiation, stained with propidium iodide, and analyzed with a FACScan cell sorter. (B) Cell lysates were analyzed at the indicated time points with an antibody specific for Wee-1. (C) HeLa cells were transfected with siRNA to wee-1 (siWee-1) or luciferase (siLuc.) for 4 h following release and irradiation. Cells were then collected for cell cycle analysis at the indicated time points. (D) Fourteen hours after release siWee-1-transfected cell lysates were analyzed for Wee-1 expression. IR, irradiation; P.IR, postirradiation.
nized at G1/S by a double thymidine block and exposed to gamma irradiation following release. Cells were immediately transfected with siLuc or siWee-1. The effect of Wee-1 depletion upon gamma irradiation-mediated cell cycle arrest was monitored as the cells progressed through the G2/M transition. Western blotting analysis of Wee-1 levels at 14 h post-release from the double thymidine block confirmed the effect of siWee-1 (Fig. 6D). Cell cycle progression was analyzed by the G2/M transition (9 to 14 h following release). As expected, in control cells with siRNA Luc, the gamma irradiation-induced cell cycle arrest within 11 to 14 h (Fig. 6C). In contrast, transfection of siRNA to wee-1 mRNA following gamma irradiation significantly alleviated the radiation-induced G2 arrest.

**DISCUSSION**

Previous studies established the role of Wee-1 kinase in negative regulation of Cdc2 activity. Our results demonstrate that, in cells arrested in G2 by Vpr, Wee-1 levels are elevated. Most importantly, depletion of Wee-1 by an siRNA prior to infection with Vpr virus demonstrated that elevated levels of Wee-1 are essential for the induction of cell cycle arrest by Vpr. These results support our hypothesis that a delay in reducing the levels of Wee-1 may be one determinant responsible for the failure of Vpr-arrested cells to progress through mitosis.

The greater levels of Wee-1 in the presence of Vpr correlated with an increase in the level of the phosphorylated form of Cdc2 and a decrease in Cdc2 kinase activity. The observed reduction in Cdc2 kinase activity is consistent with the inhibitory role of Wee-1 via Tyr15 phosphorylation of Cdc2 (24). Ultimately, insufficient levels of Cdc2 kinase activity are responsible for the failure of cells to progress into mitosis.

The regulation of Wee-1 activity is complex. Here, we find that at least one reason for increased levels of Wee-1 is due to enhanced protein stability. Wee-1 is a target for ubiquitination in *Xenopus* oocytes (17) and caspase-3 cleavage in human Jurkat cells (34). Vpr expression has also been reported to result in increased nuclear herniations resulting in transient cytoplasmic release of Wee-1 green fluorescent protein fusion proteins (8). It is possible that a change in subcellular compartmentalization reduces the stability and or activity of Wee-1. It has also been reported that Wee-1 kinase activity is regulated by changes in phosphorylation; however, we did not examine that directly here.

We previously demonstrated that a decline in Wee-1 levels subsequent to Vpr-mediated cell cycle arrest correlated with the apoptosis that occurs 1 to 3 days following prolonged cell cycle arrest by Vpr. Ectopic overexpression of Wee-1 during the Vpr-mediated apoptotic phase results in an attenuation of the apoptosis (33). Taking that together with the results presented here, we propose that Wee-1 has two roles in the deregulation of cell cycle progression by Vpr. First, elevated levels of Wee-1, mediated principally through enhanced protein stability, are critical for the initiation of G2/M cell cycle arrest induced by Vpr. Second, once cell cycle arrest is initiated by Vpr, subsequent decreases in Wee-1 levels appear to be important for the induction of Vpr-mediated apoptosis. During normal cell cycle progression, Wee-1 is established as a negative regulator of Cdc2 kinase. This role of Wee-1 is likely to be the reason why elevated levels of Wee-1 in the presence of Vpr lead to cell cycle arrest at G2/M. The role of Wee-1 in apoptosis is less well understood. We previously reported an enhanced Cdc2 kinase activity concomitant with the declines in Wee-1 levels and proposed that this inappropriate activation of Cdc2 may lead to cell death via an abnormal mitotic process known as mitotic catastrophe (33). Thus, the dual and opposing effect of Vpr on Wee-1 at different points in the cell cycle may indicate the role of Wee-1 in linking cell cycle arrest to induction of apoptosis.

**REFERENCES**