Viral infections and cell cycle G2/M regulation

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ABSTRACT

Progression of cells from G2 phase of the cell cycle to mitosis is a tightly regulated cellular process that requires activation of the Cdc2 kinase, which determines onset of mitosis in all eukaryotic cells. In both human and fission yeast (*Schizosaccharomyces pombe*) cells, the activity of Cdc2 is regulated in part by the phosphorylation status of tyrosine 15 (Tyr15) on Cdc2, which is phosphorylated by Wee1 kinase during late G2 and is rapidly dephosphorylated by the Cdc25 tyrosine phosphatase to trigger entry into mitosis. These Cdc2 regulators are the downstream targets of two well-characterized G2/M checkpoint pathways which prevent cells from entering mitosis when cellular DNA is damaged or when DNA replication is inhibited. Increasing evidence suggests that Cdc2 is also commonly targeted by viral proteins, which modulate host cell cycle machinery to benefit viral survival or replication. In this review, we describe the effect of viral protein R (Vpr) encoded by human immunodeficiency virus type 1 (HIV-1) on cell cycle G2/M regulation. Based on our current knowledge about this viral effect, we hypothesize that Vpr induces cell cycle G2 arrest through a mechanism that is to some extent different from the classic G2/M checkpoints. One the unique features distinguishing Vpr-induced G2 arrest from the classic checkpoints is the role of phosphatase 2A (PP2A) in Vpr-induced G2 arrest. Interestingly, PP2A is targeted by a number of other viral proteins including SV40 small T antigen, polyomavirus T antigen, HTLV Tax and adenovirus E4orf4. Thus an in-depth understanding of the molecular mechanisms underlying Vpr-induced G2 arrest will provide additional insights into the basic biology of cell cycle G2/M regulation and into the biological significance of this effect during host-pathogen interactions.

Keywords: HIV-1, Vpr, cell cycle G2/M regulation, DNA damage, DNA replication, checkpoints, viral infections, fission yeast, *Schizosaccharomyces pombe.*

CELL CYCLE G2/M CONTROLS AND CHECKPOINTS

To insure the accurate transmission of genetic information, eukaryotic cells have developed an elaborate network of checkpoints to monitor the successful completion of every cell cycle step and to respond to cellular abnormalities such as DNA damage and replication inhibition as they arise during cell proliferation. The two best characterized checkpoints are those for DNA damage and DNA replication (Fig.1A, top. For reviews, see ref [1-4]). Activation of either of these two checkpoints causes a cell cycle arrest, and they were first characterized in detail by genetic analysis in fission yeast (*Schizosaccharomyces pombe*). The G2 to M transition is controlled in fission yeast by the phosphorylation status of Tyr15 on Cdc2, the cyclin-dependent kinase which regulates the cell cycle in all eukaryotic cells [5]. In fission yeast, Tyr15 is phosphorylated by the Wee1 and Mik1 kinases to hold the cell in G2, and rapid dephosphorylation by the Cdc25 phosphatase triggers the G2 to M transition [5-8].

The DNA damage checkpoint (Fig. 1A, top) is activated by ionizing radiation or ultraviolet light, and activation of this checkpoint leads to inhibitory Tyr15 phosphorylation of Cdc2 by a multistep pathway [9, 10]. The early genes in the pathway, which include Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1, are thought to sense the DNA damage and lead to phosphorylation of the Chk1 protein [11]. In response to double strand DNA breaks (DSBs) induced by ionizing radiation, for example, Rad17 acts as a checkpoint-specific loading factor, which responds to the DNA damage by loading a 9-1-1 protein complex onto the sites where DNA is damaged [12, 13]. The 9-1-1 protein com-

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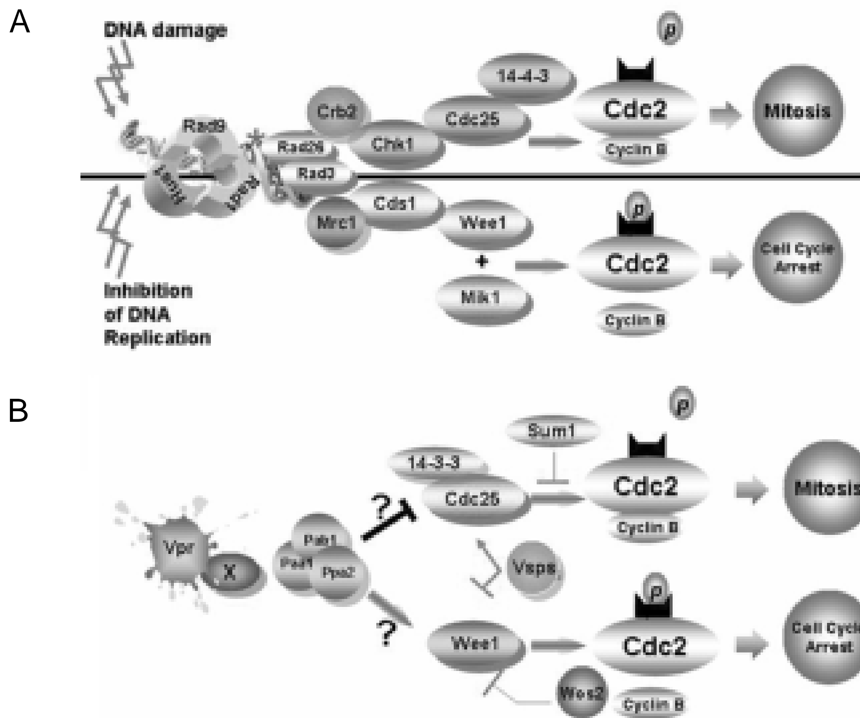

Fig. 1 Models for the classic cell cycle G2/M checkpoints and HIV-1 Vpr-induced cell cycle G2/M regulation. (**A**) Pathway for DNA damage and replication checkpoints in fission yeast. (**B**) Current model for Vpr-induced G2 arrest. The X indicates that Vpr may bind to cellular protein(s) and regulates the PP2A holozyme. →, activation; ⊣, inhibition; ?, unknown.

plex is also known as the checkpoint clamp complex (CCC), which is composed of Rad1, Rad9 and Hus1 [13]. In addition, the Rad3-Rad26 protein complex also binds to sites of DNA damage independently of the 9-1-1 protein complex. The independent binding of two protein complexes to DNA damage to initiate the DNA structure checkpoint is believed to protect the cell against inappropriate checkpoint activation [2, 13, 14]. Activation of Chk1 is mediated by Crb2, which may bridge Rad3 and Chk1 [15-17]. The activated Chk1 kinase then directly phosphorylates the Cdc25 phosphatase [18]. The phosphorylated Cdc25 binds Rad24/25 (14-3-3) protein, and this complex is transported out of the nucleus to render Cdc25 inactive [19]. The activated Chk1 also regulates the Mik1 kinase to inhibit Cdc2 [20]. DNA damage thus initiates a Chk1-mediated protein phosphorylation cascade ending in the inactivation of Cdc25 phosphorylase and activation of Mik1 kinase to increase inhibitory phosphorylation of Tyr15 on Cdc2.

The DNA replication checkpoint (Fig. 1A, bottom) is activated by treatment with hydroxyurea, which inhibits DNA replication, and this checkpoint also controls the G2 to M transition through inhibitory phosphorylation of Cdc2 [10]. Parts of this DNA replication checkpoint are shared with the DNA damage checkpoint as Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1 are required for both checkpoints in fission yeast [21]. The same 9-1-1 and Rad3-Rad26 checkpoint protein complexes may associate with the DNA replication complex [13]. However, the DNA replication checkpoint acts primarily through phosphorylation of Cds1 kinase, which is mediated by another protein Mrc1 [22, 23], with minor participation of Chk1 kinase, and either kinase is sufficient by itself to give cell cycle arrest when DNA synthesis is inhibited [24]. Activated Cds1 kinase inactivates Cdc25 through a similar mechanism as Chk1 [1, 24]. Cds1 also activates the Mik1 kinase, which phosphorylate Tyr15 of Cdc2 [25].

Cell cycle G2/M controls, which were often initially defined in fission yeast, are highly conserved, and most of the genes required for the checkpoints have human homologues (Tab. 1). In general, these homologues have similar, although not always identical, roles in the control of the human cell cycle. There is a tendency for multiple, partially redundant checkpoints in human cells compared to

Tab. 1 Functional conservation of cell cycle G2/M machinery between human and fission yeast

Fission Yeast	Human	Putative Activity
Mitotic regulators		
Cdc2	CDK1	Cyclin B-dependent kinase
Cdc13	Cyclin B	B-type cyclin
Wee1	WEE1	Tyrosine kinase
Mik1	——	Tyrosine kinase
Cdc25	CDC25A/B/C	Tyrosine phosphatase
DNA damage and replication checkpoints		
Rad1	hRAD1	Nuclease
Rad3	ATM/ATR	Protein kinase
Rad9	hRAD9	3’-5’ exonuclease
Rad17	hRAD17	Unknown
Rad24/25	14-3-3	Binds to phosphorylated ser
Rad26	ATRIP	Rad3 regulatory protein
Hus1	hHUS1	A PCNA-related protein
Chk1	CHK1	Serine/Threonine Kinase
Cds1	CHK2	Serine/Threonine Kinase
Crb2	BRCA1	Unknown
Mrc1	CLSPN	Unknown
Cellular proteins involved in Vpr-induced G2 arrest		
PP2A	PP2A	Protein phosphatase 2A
Paa1	A	A regulatory subunit
Pab1	B	B regulatory subunit
Ppa2	C	C catalytic subunit
Ppa1	C	C catalytic subunit
Wos2	P23	Inhibitor of Wee1

Note: “—”, not found.

simpler checkpoints in yeast probably reflecting the more complex requirements for cell cycle control in multicellular eukaryotes. For example, the single *rad3* gene in fission yeast is required for both the DNA damage and replication checkpoints and activation of the *chk1* and *cds1* checkpoint kinases [2, 13, 14]. In human cells there are two homologues of *rad3*, *ATM* and *ATR*. The primary role of *ATM* is in the DNA damage checkpoint initiated by double strand breaks and activation of *CHK2*, the human homologue of *cds1*, whereas the primary role of the essential *ATR* gene is in the DNA replication checkpoint or responses to many forms of DNA damage and activation of *CHK1* [26, 27]. Similarly, there is only one tyrosine phosphatase Cdc25 that dephosphorylates Cdc2 in fission yeast. In human cells, there are three CDC25 homologues, CDC25A, CDC25B and CDC25C, and each of them can be phosphorylated by CHK1 [28]. All three of these phosphatases have been shown to be involved in the control of the G2/M transition, even though their specific roles in this process have not yet been well characterized [29-31]. The *p53* gene is an example of an additional level of cell cycle control in human cells.The p53 transcription factor, which has no homologues in yeast, has multiple roles including regulating the cell cycle and apoptosis with an essential role in the G1 DNA damage checkpoint [32]. It also has important roles in the G2 damage checkpoint. It inhibits Cdc2 through activation of Gadd45, p21, and 14-3-3σ. In addition, it is involved in regulatory feedback loops with ATM/ATR and CHK1 [33]. The conservation of checkpoints even extends to the regulatory mechanisms as illustrated by the negative regulation of CDC25 by relocation to the cytoplasm from the nucleus in both fission yeast and human cells. This relocation in both organisms is dependent on 14-3-3 proteins [19, 34].

CELL CYCLE G2/M ARREST INDUCED BY HIV-1 VPR

The HIV-1 Vpr protein induces cell cycle G2 arrest

through inhibitory phosphorylation of Cdc2 both in fission yeast and human cells, suggesting that Vpr affects a conserved cellular process. Specifically Vpr induces hyper-phosphorylation of fission yeast Cdc2 or human CDK1, the human homologue of Cdc2 [35-37]. It exerts its inhibitory effect through T14A and Y15F of CDK1 and Y15F of Cdc2, as expression of nonphosphorylatable Cdc2 mutants, T14A Y15F of CDK1 and Y15F of Cdc2, prevents Vpr-induced G2 arrest [36, 38]. Furthermore, Vpr inhibits Cdc25 phosphatase [39, 40] and activates Wee1 kinase [39, 41] to promote phosphorylation of Cdc2/CDK1 during induction of G2 arrest. Consistent with the roles of Wee1 and Cdc25 in Vpr-induced G2 arrest, proteins which regulate Cdc25 or Wee1 have been shown to either augment or alleviate Vpr-induced G2 arrest. For example, fission yeast Wos2, which is a homologue of human p23 and a Wee1 inhibitor [42], is a Vpr suppressor when overproduced [39]. The Cdc25 inhibitor *rad25* [19], which is a homologue of human 14-3-3 proteins, enhances Vpr-induced G2 arrest when overproduced in fission yeast [39]. Recent studies further show that Vpr binds to Cdc25C and to 14-3-3 in human cells [43-45].

Given that the DNA checkpoints and Vpr both induce G2 arrest through inhibitory phosphorylation of Cdc2 which is regulated by Wee1 and Cdc25, Vpr might induce G2 arrest through a checkpoint pathway. This possibility has been evaluated in fission yeast by expressing *vpr* in mutant fission yeast strains defective in early and late steps of the checkpoint pathways. None of the early checkpoint-specific mutants (*rad1*, *rad3*, *rad9* and *rad17*) showed a significant effect on the induction of G2 arrest by Vpr [38, 39, 46]. Furthermore, mutations in both *chk1* and *cds1*, which are thought to be the last steps specific for the checkpoint [1, 18, 24], also do not block Vpr-induced G2 arrest [39, 46]. Therefore, Vpr does not appear to use the DNA-damage or DNA-replication checkpoint pathways to induce G2 arrest in fission yeast.

Early data in human cells tended to support the conclusion that Vpr does not induce G2 arrest through the DNA damage checkpoint pathways. Vpr still induced G2 arrest in cells from patients with ataxia telangiectasia (AT) [40]. These AT cells are mutant for the ATM gene, which is a human homologue of fission yeast Rad3, and they do not arrest in G2 in response to DNA damage caused by ionizing radiation [47-49]. However, recent reports show that Vpr activates ATR, the second human homologue of fission yeast Rad3, and other steps in this checkpoint pathway such as RAD17, HUS1, BRCA1 and γ-H2AX [50, 51]. Down-regulation of ATR or CHK1 by RNAi partially attenuated Vpr-induced G2 arrest [50, 51]. These studies suggest that Vpr may induce G2 arrest through a cellular response to DNA replication stress or to a signal that "mimics" DNA damage. However, expression of *vpr* does not increase gene mutation frequency [52], which argues against the possibility that Vpr actually causes DNA damage. It is thus reasonable to think that other signals other than actual DNA damage triggers DNA damage-like cellular responses. These cellular responses could include the nuclear herniation caused by Vpr [53] or cellular stress responses to *vpr* gene expression [54-56]. Since ATR and CHK1 have primary roles in the responses to changes in DNA replication, an alternative possibility is that Vpr may interfere with DNA replication. This possibility is certainly supported by a number of reports showing that Vpr induces genomic instability, formation of micronuclei and aneuploidy [57, 58]. All of these changes in DNA structures could be perceived as replication stresses, which would trigger cell cycle arrest.

Considering that G2/M DNA checkpoints are highly conserved between mammalian and fission yeast cells (Tab. 1), it is unclear at the moment why human ATR and CHK1 are activated by Vpr but *rad3* (the fission yeast homologue of ATR/ATM) or *chk1*/*cds1* (CHK1/CHK2) double deletion in fission yeast does not block Vpr-induced G2 arrest [38, 39]. One factor possibly contributing to the observed activation of ATR in mammalian cells is that retroviral integration appears to activate ATR [59], and the experiments showing activation of ATR by Vpr were done with lentiviral vectors which might therefore activate ATR to some extent independently of Vpr. In addition, it was noticed that activation of ATR and CHK1 only accounts for part of the G2 arrest induced by Vpr [50]. Other as yet unidentified molecular mechanism(s) may explain at least half of the G2 cell population induced by Vpr. Interestingly, Roshal et al [60] showed that treatment of Vpr-producing mammalian cells with caffeine completely blocks Vpr-induced G2 arrest. Caffeine is part of the methylxanthine family, and similar to the caffeine effect, another methylxanthine pentoxifylline (PTX) also inhibits Vpr-induced G2 arrest in mammalian cells [61]. Similarly, both PTX and caffeine suppress Vpr-induced G2 arrest in fission yeast [39, 58] [our unpublished data]. Since PTX or caffeine inhibits Vpr-induced G2 arrest in fission yeast where the classic DNA checkpoints apparently play no role, these observations suggest molecular mechanisms other than the classic DNA checkpoints may be involved in activation of ATR and regulation of Cdc25 and Wee1.

The additional molecular mechanism might involve protein phosphatase 2A (PP2A). Although this protein phosphatase has no known role in the DNA checkpoints, it has an important role in Vpr-induced G2 arrest. Okadaic acid is a specific inhibitor of PP2A, and okadaic acid was shown to inhibit Vpr-induced G2 arrest both in human [35] and fission yeast cells [37]. Further evidence for an important

role of PP2A comes from PP2A mutant strains. PP2A is composed of three subunits, one catalytic (C) and two regulatory (A and B) subunits. When *vpr* was expressed in a strain with a deletion for a catalytic subunit (*ppa2*) or a regulatory subunit (*pab1)* of PP2A, Vpr-induced G2 arrest was reduced [39, 46]. Taken together, it is possible that a concerted cellular mechanism interlinks PP2A and possibly ATR/CHK1 in the cellular response to *vpr* gene expression during the induction of G2 arrest.

POTENTIAL ROLE OF PP2A IN CELL CYCLE G2/M REGULATION DURING VIRAL INFECTIONS

Other evidence supporting involvement of PP2A in Vpr-induced G2 arrest comes from other viral proteins with effects on cell cycle G2/M controls. PP2A appears to be a common viral target since other viruses such as simian virus 40 (SV40), polyoma virus, human T lymphotrophic retrovirus and adenovirus affect the enzymatic activity of at least a subset of PP2A proteins (see review [62]). Even though these viruses are not otherwise related, they all seem to have adapted a similar strategy to affect cellular processes by direct interaction with PP2A. Similar to the Vpr effects, both adenoviral E4orf4 [63-65] and HTLV Tax protein induce cell cycle G2 arrest [59]. These two viral proteins both bind to PP2A and affect its enzymatic activity [63, 66]. Interestingly, similar to Vpr, Tax-induced G2 arrest is reversed by caffeine [59]. Further examinations indicated that Tax binds to CHK2 in Jurkat T-cells [59] but it complexes with CHK1 in other T-cells [67]. In contrast, the T-antigens of both SV40 and polyoma virus promote cell proliferation by down-regulation of PP2A through direct competition with the B regulatory subunit (Reviewed in [68]). Further studies on the interactions of Vpr and these other viral products with PP2A should provide additional insight into the role of PP2A in cell cycle G2/M regulation.

A NEW MODEL FOR CELL CYCLE G2/M TRANSITION

Based on our current knowledge about the effect of Vpr on cell cycle G2/M regulation, a new model for the cell cycle G2/M transition is proposed for HIV-1 Vpr as shown in Fig. 1B. Although this new model may not be mutually exclusive from the classic G2/M checkpoint pathways (Fig. 1A), it nevertheless has the unique feature of a PP2A-mediated mechanism or mechanisms for G2/M control by Vpr. We hypothesize that Vpr induces G2 arrest at least in part by going through an alternative cellular pathway(s) in which expression of *vpr* activates PP2A activity either by direct association with the PP2A enzyme complex or by association with an intermediate protein(s) X. In addition, there may also be a concerted cellular mechanism which interlinks PP2A and ATR/CHK1 in the cellular response to *vpr* during the induction of G2 arrest. A protein phosphorylation cascade (depicted as "?") including PP2A is probably in part responsible for activation of the mitotic inducer Wee1 by altering its phosphorylation levels, which in turn inhibits Cdc2 by Tyr15 phosphorylation. While Wee1 plays the major role in the induction of G2 arrest by Vpr, Cdc25 appears to play a minor role and to be partially inhibited by this proposed regulatory pathway or by direct interaction with Vpr. Since overexpression of Sum1, a putative Cdc25 inhibitor, shows a synergistic effect with Vpr, Sum1 may be required by Vpr to inhibit Cdc25 either from upstream or downstream of Cdc25. Wos2 is another possible step in the pathway, and Vpr most likely inhibits Wos2 during the induction of G2 arrest. Since Wos2 physically interacts with Cdc2, Wos2 may be a downstream inhibitor of Wee1 (Fig. 1B). One possible mechanism for the inhibition of Wee1 activity by Wos2 is that Wos2 physical blocks the Tyr15 phosphorylation site on Cdc2. Vpr might then inhibit Wos2 by preventing Wos2 from binding to Cdc2. Based on the demonstrated similarities for cell cycle controls between fission yeast and human cells, it seems likely that pathway for Vpr-induced G2 arrest in fission yeast will also apply in most respects to that part of Vpr-induced G2 arrest in human cells which is not solely dependent on ATR.

SUMMARY

Besides the two most well-characterized cell cycle G2/M regulation pathways, i.e., DNA damage and replication checkpoints, some viral infections appear to modulate host cell cycle machinery in a fashion that is characteristically different from the classic checkpoints. This unique host-pathogen interaction is exemplified here in the induction of cell cycle G2 arrest by HIV-1 Vpr. PP2A seems to be a common cellular target for unrelated viruses. Therefore, further studies on the interactions of these viral proteins with PP2A and other related cellular factors should provide insight into the basic biology of cell cycle G2/M regulation and the biological significance of viral-induced G2 arrest during host-pathogen interactions.

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