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Minireview

G2/M cell cycle arrest in the life cycle of viruses

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Abstract

There is increasing evidence that viral infection, expression of viral protein or the presence of viral DNA causes the host cell cycle to arrest during G2/M. The mechanisms used by viruses to cause arrest vary widely; some involve the activation of the cellular pathways that induce arrest in response to DNA damage, while others use completely novel means. The analysis of virus-mediated arrest has not been proven easy, and in most cases the consequences of arrest for the virus life cycle are not well defined. However, a number of effects of arrest are being investigated and it will be interesting to see to what extent perturbation of the G2/M transition is involved in viral infections.

Keywords: G2/M arrest; HPV; HIV; Life cycle; Replication

Introduction

An important feature of many viral infections is subversion of the host cell cycle. Often this is apparent as a stimulation of S phase entry in cells that would otherwise be in G1 or G0. However it is becoming increasingly evident that the G1/S boundary is not the only transition that is targeted by viruses, and that many viruses are able to induce cell cycle arrest at G2/M. In some cases the molecular basis for G2/M arrest is known, while for others the specific pathways involved remain unclear. In most cases it is not yet well understood what function this modulation of the cell cycle may play in the virus life cycle. This review summarizes the different mechanisms used by viruses to affect the G2/M boundary, and aims to collate the limited knowledge that is currently available on the consequences of arrest. By considering the strategies that have so far been used to study G2/M arrest mechanisms among viruses, it may be possible to develop a general approach to establish the significance of arrest in the life cycles of the different viruses.

The normal cell cycle and G2/M arrest

The life cycle of a dividing cell can be split into four stages: G1, S, G2 and mitosis, with cells that are no longer cycling being said to be quiescent or in G0. The two gap phases, G1 and G2, separate S phase, during which the DNA is replicated, and mitosis, in which it is divided between two new nuclei. After mitosis, the cell itself divides and each daughter cell begins the cycle again from G1, or exits the cell cycle into G0. Progression from one stage to the next is controlled by the activities of kinase complexes made up of cyclins bound to cyclin-dependent kinases (Cdk). These complexes are in turn regulated by a multitude of pathways that allow response to external stimuli, e.g., via mitogen-activated protein kinase (MAPK) signaling, as well as to the internal conditions of the cell, e.g., via the checkpoint pathways.

Control of mitotic entry

The control of the G2/M boundary during the normal cell cycle, and in response to DNA damage or incomplete replication, is complex. Here we will focus only on those pathways that will be encountered in the subsequent discussions of virally induced G2/M arrest that are summarized in Fig. 1. A more complete overview is provided by Stark and Taylor (2006).

During G2, cyclin B1 accumulates and forms a kinase complex with Cdk1. The complex is kept inactive by inhibitory

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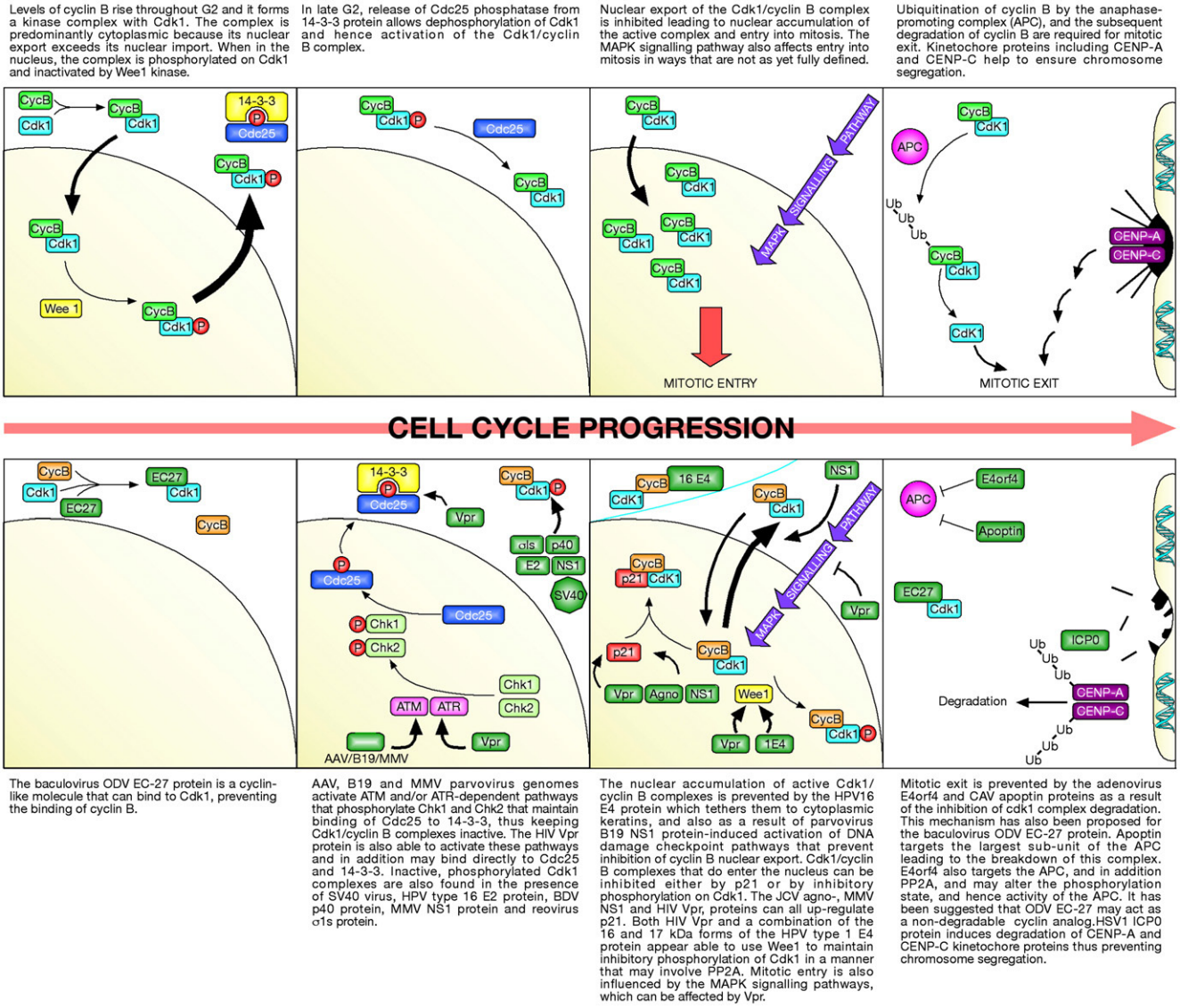

Fig. 1. G2/M events targeted by viruses. The upper panels illustrate aspects of normal progression through G2/M. The lower panels show the sites where viruses and viral proteins are observed to cause changes to events of the G2/M transition (but do not necessarily imply direct action at this place; the virus/viral protein may in fact be acting somewhere upstream). Abbreviations: AAV—adeno-associated virus; APC—anaphase promoting complex; BDV—Borna disease virus; CAV—chicken anemia virus; HPV—human papillomavirus; HSV1—herpes simplex virus type 1; JCV—JC polyomavirus; MAPK—mitogen-activated protein kinase; MMV—mice minute virus; ODV—occlusion-derived virus; PP2A—protein phosphatase 2A. Reovirus in this instance refers to serotype 3.

phosphorylation of Cdk1, catalyzed by nuclear Wee1. Although the Cdk1/cyclin B1 complex can enter the nucleus in G2, due to its fast rate of nuclear export it is predominantly cytoplasmic. In late G2, Cdc25C activates the kinase complex by dephosphorylating Cdk1. Inhibition of cyclin B1 nuclear export promotes the accumulation of the complex in the nucleus, where it stimulates entry into mitosis. Eventually the Cdk1/cyclin B1 complex is inactivated by the anaphase-promoting complex (APC), a ubiquitin ligase that targets cyclin B1 for degradation. Without the activity of the APC, the cells are unable to exit mitosis. Passage through mitosis also requires that the mitotic spindle fibers attach to the chromosome via a complex of proteins called the kinetochore, and pull the sister chromatids apart. Successful G2/M transition and exit from mitosis are regulated by a multitude of other kinases and phosphatases, including protein phosphatase 2A, PP2A, which has multiple cell cycle targets and therefore pleiotropic effects on the cell.

In the presence of DNA damage or incomplete replication, it is important that the cell prevents mitotic entry. The pathways that monitor and induce G2 arrest under these conditions are examples of “checkpoints” and result in the activation of the ATM and ATR kinases. Both ATM and ATR phosphorylate Chk1 and Chk2 leading to the activation of these kinases and subsequent phosphorylation of Cdc25C. The binding of 14-3-3 to phosphorylated Cdc25C sequesters this phosphatase in the cytoplasm, preventing it from activating the Cdk1/cyclin B1 complex. In addition to maintaining inactive Cdk1/cyclin B1 complexes, the checkpoint pathways can also prevent their nuclear accumulation. After the initial induction of G2 arrest, the arrest may be sustained via activation of other pathways that are

typically regulated by p53. One regulator whose expression can be controlled by p53 is the p21 protein. By binding directly to Cdk/cyclin complexes, p21 is able to inhibit their kinase activity.

The diversity of G2/M arrest strategies used by viruses

A variety of viruses have been associated with G2/M arrest, including DNA viruses, RNA viruses and retroviruses but the methods by which arrest is achieved appear very diverse (see Fig. 1). Although in many cases the mechanisms are not fully characterized, observations have been made that implicate particular pathways. What follows is an attempt to broadly group the strategies used by the different viruses.

Inhibition of Cdk1/cyclin B1 kinase activity

In a number of instances, G2 arrest has been linked to an inhibition or delay in the activation of Cdk1/cyclin B1 kinase activity (Fournier et al., 1999; De Beeck et al., 2001; Planz et al., 2003). This can result either from the induction of Cdk inhibitors such as p21, for example as occurs with JC polyomavirus agnoprotein (Darbinyan et al., 2002), or via maintenance of Cdk1 phosphorylation (Poggioli et al., 2001; Scarano et al., 1994), as proposed for the human papillomavirus (HPV) type 1 E4 protein (Knight et al., 2006). The mechanisms used by these viruses to mediate arrest appear to vary widely, and are not always fully understood, but it appears that in several cases, components of the DNA damage checkpoint pathways may be activated. Our current understanding of how these viruses achieve arrest is outlined diagrammatically in Fig. 1.

Inhibition of nuclear accumulation of Cdk1/cyclin B1 complexes

In some instances, mitosis can be inhibited even in the presence of active Cdk1/cyclin B1 complexes, provided they are prevented from accumulating in the nucleus (see Fig. 1). The parvovirus B19 NS1 protein mediates G2 arrest despite the presence of active Cdk1/cyclin B1 in the cytoplasm (Morita et al., 2001). The NS1 protein has nicking and helicase activities that may damage cellular DNA (Momoeda et al., 1994; Poole et al., 2006), and although the precise mechanism of arrest is unclear, it may involve checkpoint pathways that regulate cyclin B nuclear export. An alternative strategy is used by the E4 protein of HPV16, which prevents the nuclear entry of the Cdk1/cyclin B complex by sequestering the Cdk1/cyclin B kinase complex in the cytoplasm (Davy et al., 2005). The association of HPV16 E4 with the cytokeratin network is thought to contribute to the ability of E4 to achieve this effect *in vivo* (Wang et al., 2004).

Inhibition of mitotic exit

In some cases of virally induced G2/M arrest, the cells are able to enter but not exit mitosis. Again a variety of strategies have been proposed, and are described more fully in Fig. 1. Among these are the inhibition of the APC (Kornitzer et al., 2001) by the chicken anemic virus (CAV) apoptin protein (Teodoro et al., 2004), the interference with kinetochores by herpes simplex type 1 (HSV-1), ICP0 protein (Everett et al., 1999; Lomonte et al., 2001), and the expression of the baculovirus EC27 protein (*Autographa californica* nucleopolyhedrovirus), which may act as a non-degradable Cdk1/cyclinB analog (Belyavskyi et al., 1998). The mechanism underlying EC27-mediated arrest is however only poorly understood (Ikeda and Kobayashi, 1999).

The HIV Vpr protein—an illustration of multiple potential arrest mechanisms

By far the most studied viral G2 arrest is that induced by the HIV Vpr protein. Although in the last 10 years, well over 20 papers, from a variety of laboratories have been published on the subject, the exact mechanism(s) remains unclear. The Cdk1/cyclin B1 complex is clearly inactive in Vpr-arrested cells (He et al., 1995; Re et al., 1995), but more contentious are the upstream pathways that lead to this inhibition. The mechanisms that have been proposed to explain how Vpr expression may contribute to G2/M arrest include (1) Vpr binding to chromatin and/or splicing factors, which causes activation of ATR, and leads eventually to changes in the activity of the Wee1 kinase and/or the Cdc25 phosphatase (Lai et al., 2005; Terada and Yasuda, 2006); and the less well characterized phenomena of (2) direct binding to Cdc25C and inhibition of its phosphatase activity (Goh et al., 2004); (3) alteration of the level of Wee1 protein (Yuan et al., 2004); (4) effects on PP2A (Elder et al., 2001); (5) activation of the p21 promoter and p21-mediated Cdk1/cyclin B inhibition (Chowdhury et al., 2003); and (6) downregulation of expression of genes in the MAPK pathway (Yoshizuka et al., 2005) (see Fig. 1).

It may be that the range of potential mechanisms identified for Vpr is a unique feature of this viral protein, and that the G2 arrest proteins encoded by other viruses do not target so many pathways. Alternatively, it may be that the proteins encoded by other viruses do possess redundant or synergistic arrest mechanisms, but that a greater effort that has been put into understanding the biology of HIV.

G2/M arrest is not necessarily dependent on viral proteins

It is not only viral proteins that are able to elicit G2/M arrest; adeno-associated virus (AAV)-induced G2 arrest can result from the presence of the viral genome, a single-stranded DNA molecule with terminal hairpin loops (Cotmore and Tattersall, 1994; Raj et al., 2001). It appears that these unusual DNA structures, and similar ones found in other parvoviruses (Op De Beeck and Caillet-Fauquet, 1997), are sufficient to activate a DNA damage response. Whether these viruses have evolved to exploit this natural cellular defence mechanism to their advantage remains to be seen.

The consequences of G2/M arrest during the virus life cycle

In contrast to the extensive information available on the mechanisms of G2/M arrest, few publications have examined its role in the life cycles of the different viruses. One obvious effect may be to prevent new cell production, which might be of benefit to the virus if (for example) it disrupts the anti-viral

immune response by preventing the clonal expansion of infected lymphocytes. Interestingly, HIV-infected T lymphocytes isolated from patients are arrested in G2 which may limit the immune response to the virus (Zimmerman et al., 2006). However for the majority of viral infections where the target cells are not those of the immune system, other aspects of the G2/M arrest may be more significant.

Effect of G2/M arrest on very early virus life cycle events

Experiments with small molecule inhibitors have shown that arrest at G2/M can benefit the early stages of the HIV life cycle by increasing the number of integrated proviruses (Groschel and Bushman, 2005), and it is hypothesized that Vpr-induced arrest might act in a similar way. Significantly, the infectious HIV virion contains sufficient Vpr to induce G2 arrest following infection, and thus Vpr may be able to affect aspects of the virus life cycle that occur prior to viral protein expression (Poon et al., 1998).

G2/M arrest and the cell cycle control of transcription and translation

Using a variety of mechanisms, the expression of many cellular proteins has been found to fluctuate during the cell cycle. Virus-induced G2/M arrest may exploit these mechanisms of regulating protein levels within the cell, in order to control the expression of viral or cellular genes that are important for the completion of the virus life cycle. A specific example of this occurs during infection with the avian coronavirus, infectious bronchitis virus, where the levels of viral protein expression appear increased in G2/M (Dove et al., 2006). Although this correlates with an increase in virus progeny, the mechanism for the upregulated expression is not yet understood. In contrast, the expression of HSV1 late proteins appears dependent on Cdk1 activity, although evidence for increased protein synthesis in G2 remains to be tested (Advani et al., 2000). Enhanced expression of papillomavirus capsid proteins in the G2 phase of the cell cycle has recently been proposed for HPV6 and BPV1 (Kong-Nan Zhao, University of Queensland, Australia, personal communication). Mechanisms for increased protein expression may involve transcription and/or translation, and for viruses with RNA genomes, higher levels of transcription during G2/M might lead to the production of more viral genomes. HIV has been reported to be more transcriptionally active in G2 (Goh et al., 1998) and Vpr may also modulate translation via Cdk1/cyclin B1-induced changes in the activity of poly (A) polymerase (Mouland et al., 2002). Another way in which viruses make use of different cell cycle phases to regulate the extent of protein expression is via internal ribosome entry site (IRES)-mediated translation. The IRES sequences from hepatitis C and encephalomyocarditis viruses appear to be downregulated in G2/M (Venkatesan et al., 2003), while that of HIV appears upregulated (Brasey et al., 2003).

Induction of a pseudo-S phase state to allow viral replication

For a number of DNA viruses, there is evidence that one consequence of arrest is to establish a pseudo-S phase state, in which normal cellular DNA replication is complete, but where the cell still remains competent for replication. Why might this be beneficial to the virus? By maintaining an S phase environment, complete with substrates and the machinery for DNA replication, DNA viruses will extend the time available to them for the replication of their genomes. The continued replicative state can lead to an increase in viral genome copy number and to the replication of cellular DNA beyond 4n. This occurs during infections with murine and SV40 polyomaviruses (Lehman et al., 1994, 2000), and following expression of the HPV type 31 E2 protein (Frattini et al., 1997). Alternatively, it may be solely viral DNA levels that increase, as is the case with Aleutian mink disease parvovirus (ADV) infection (Oleksiewicz and Alexandersen, 1997).

To create a pseudo-S phase state, inhibition of the G2 transition is not sufficient on its own, and it is necessary for the viruses to ensure that the proteins required for DNA replication are also present. The strategies that viruses use to achieve this vary. Baculovirus virions contain both the viral EC27 protein and the cellular PCNA protein, which may contribute to S phase entry following infection (Belyavskyi et al., 1998). DNA tumor viruses express gene products that are specifically able to stimulate cell cycle entry, as is the case with the HPV E6 and E7 proteins (Munger et al., 2004).

The HPV life cycle—an illustration of the potential significance of pseudo-S phases

The E4 proteins (full-length proteins also called E1^E4), of several HPV types have been shown to stimulate cell cycle arrest in G2 (Davy et al., 2002; Knight et al., 2004; Nakahara et al., 2002) and it has been shown *in vivo* that viral genome amplification coincides with E4 expression (Doorbar et al., 1997; Peh et al., 2002). It has also been observed in an organotypic raft culture system that recreates the HPV18 life cycle, that viral genome amplification occurs in cells that are in G2 in the absence of cellular DNA replication (Louise Chow, University of Alabama, Birmingham, USA, personal communication). Although the exact details of the life cycle and the mechanism of E4-mediated G2 arrest appear to vary somewhat between different papillomavirus types (see Fig. 2), knock-out experiments have demonstrated an important role for E4 in the amplification of viral genomes in the upper layers of infected epithelium (Nakahara et al., 2005; Peh et al., 2004; Wilson et al., 2005). In this instance, it is thought that E4 contributes to the elevated level of replication by stimulating a G2 arrest environment, which in the presence of E7 expression (and the stimulation of replication proteins) leads to the formation of a replication competent pseudo-S phase state. The mechanism by which E4, which acts at the G2/M boundary, and E7, which acts at the G1/S boundary are thought to work together during natural infection is shown in Fig. 2.

Effect of G2/M arrest on late virus life cycle events

Late events such as virion assembly and release may also be affected by cell cycle stage. It has been hypothesized that

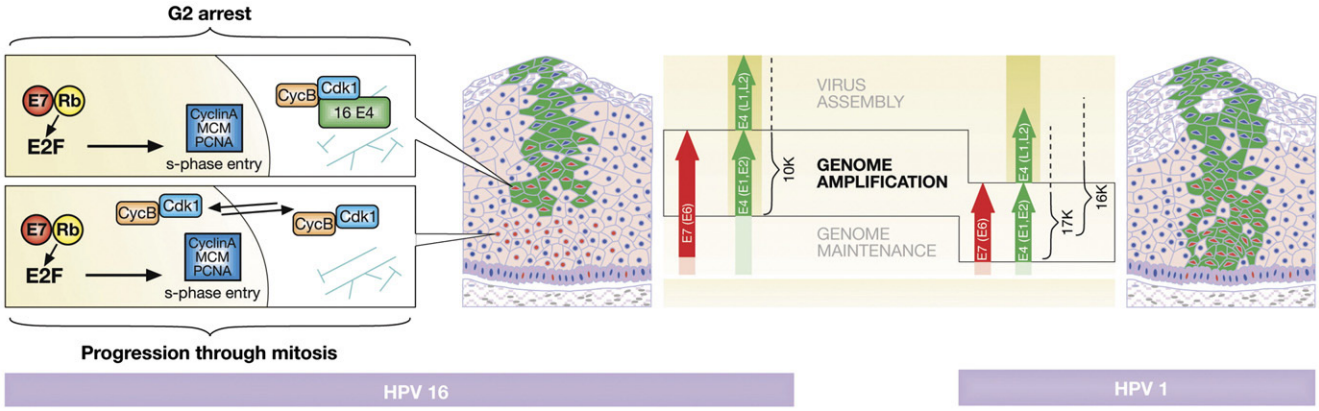

Fig. 2. G2 Arrest during the human papillomavirus (HPV) life cycle. The expression patterns of key HPV proteins including E7, which drives cells into S phase, and E4, which arrests cells in G2/M, are shown as vertical arrows adjacent to diagrammatic representations of infected epithelium. The images represent a model of the HPV life cycle, and are based on data presented in Middleton et al. (2003) and Peh et al. (2002). Initial infection occurs in the basal cells (purple) and expression of the viral E1 and E2 proteins facilitates replication of the viral episome. As the cells divide and leave the basal layer, they would normally become quiescent and differentiate but following HPV infection they can re-enter the cell cycle as a result of E7 expression. As the cells are pushed towards the epithelial surface the levels of E1, E2 and E4 expression increase, and the high levels of E4 protein cause the cells to arrest in G2. The continued expression of E7 in cells arrested in G2/M is thought to create a pseudo-S phase state, which facilitates viral genome amplification rather than cell proliferation. Active genome amplification ceases following the downregulation of E7, E1 and E2, and upregulation of the viral capsid proteins (L1 and L2). The timing of viral gene expression varies between HPV16 and HPV1 lesions but G2 arrest and amplification of the viral genomes occurs where the expression of E4 coincides with the expression of E7. The 1E4 protein is proteolytically cleaved during differentiation with the N-terminally truncated 16-kDa form, in addition to the full-length 17-kDa form, appearing to play a role in causing G2 arrest. Although proteolytic cleavage of HPV16 E4 occurs during differentiation, it is the full-length gene product that is responsible for growth arrest in G2. The HPV16 E4 protein prevents the nuclear accumulation of Cdk1/cyclin B1 that is required for mitosis by binding to the complex and tethering it to cytoplasmic keratins. This is illustrated to the left of the figure.

changes to nuclear microvesicles as a result of baculovirus EC27 protein-induced G2/M arrest are involved in aspects of virus particle maturation that are required for inter-insect transmission of the occlusion-derived (ODV) form of the virus (Braunagel et al., 1998). The spread of some viruses might also be aided by the fact that cells arrested in mitosis tend to adhere less well to neighboring cells than cells in other stages of the cell cycle.

The relationship between mechanism and outcome of virus-induced G2/M arrest

Although viruses from widely diverse evolutionary groups may have evolved to target G2/M, and may even have converged on similar mechanisms, there are likely to be fundamental differences in the outcome of arrest for the different viruses. The cell cycle requirements for optimal replication of RNA viruses are presumably quite different from those of DNA viruses. Among more closely related virus groups we might expect to see greater similarities in the consequence of arrest. The potential benefit of maintaining a replicative state for viral DNA may explain why G2/M arrest in some form or another has been observed in the small DNA tumor viruses, adenovirus, HPV and polyomaviruses. Indeed all of these viruses have well-characterized proteins that can push cells out of G1 and into S phase; E1A (adenovirus), E7 (HPV), and large T (polyomaviruses). It will be interesting to see whether they all simultaneously express G2 arrest proteins during genome amplification in order to create a pseudo-S phase state.

The differing requirements of viruses may in part explain the diverse mechanisms used to induce G2/M arrest, and their cellular targets may reflect the need to activate other important cellular pathways. For example, HIV requires activation of a DNA damage response in order to efficiently integrate into the host cell chromosome (Daniel et al., 2005). By targeting early components of the checkpoint pathway, it may be that Vpr can simultaneously promote G2/M arrest, which increases levels of viral transcription, and activate the DNA repair mechanisms that are required for integration. By contrast, HPV type 16 does not require activation of the DNA damage response since its DNA is maintained as an extrachromosomal episome and therefore it can target cyclin B1 directly, which is a more downstream component of the G2/M pathway (see Fig. 2).

Difficulties associated with the analysis of viral G2/M arrest

For most viruses it has been proven very difficult to precisely define the mechanisms and outcomes of G2/M arrest. Such problems are evidenced by the abundance of papers that have examined the mechanism of Vpr-induced arrest in HIV. For example, Vpr may target mitotic entry in at least five different ways, and it remains unknown how many of the identified pathways are relevant. The challenge is to identify those that are of biological significance. To do this successfully, a number of issues must be considered. Some of these are common to other areas of biology, while others relate to difficulties in working with viruses and/or the cell cycle.

The complexities of cell cycle control

The cell cycle is controlled not by a series of discrete, linear pathways but by a complex web of interactions that as yet are not fully understood. Viral proteins may appear therefore to be

acting through a number of regulatory pathways, when in fact their target is a common upstream event. In some cases of course, viral proteins may indeed have multiple targets, but there is still a need to distinguish events that lead to G2/M arrest from changes that ensue in the arrested cell. Cause and effect can be a particular problem in cell cycle experiments, where changes that might be expected to cause arrest (such as abnormal levels of Cdk/cyclin activity) may instead be the result of arrest by other mechanisms. The use of siRNA and/or cellular knock-outs has been proven a powerful technique for addressing this problem. Unfortunately, for the analysis of many cell cycle proteins this can be problematic, either because the results would be too detrimental to normal cellular proliferation or because of degeneracy among the activities of cell cycle control proteins. Cells may also react differently to viral proteins depending on when in the cell cycle these proteins are expressed, and this may cloud interpretation when cells are synchronized using drugs or other methods. The use of drugs to modulate specific cell cycle pathways can also be problematic, as many such drugs are known to exert pleiotropic effects on the cell (e.g., the use of okadaic acid to inhibit PP2A).

Choice of model system

Rarely can viral G2/M arrest be studied during a natural infection. For ease, experiments are often carried out in transformed cell lines but as these often exhibit abnormal cell cycle pathways, e.g., as a result of p53 deletions, the real effect of the viral protein may be masked. Ideally a number of cell types should be assayed, and the choice should reflect as far as possible the natural host of the virus, because different cell types respond differently to arrest stimuli. During our analysis of the mechanism of HPV16 E4-induced G2 arrest we have observed discrepancies between results obtained from *Schizosaccharomyces pombe* (a commonly used model system for cell cycle experiments) and those from mammalian cells (C. Davy and D. Jackson, unpublished observations).

Reliance on mutants, knock-outs or over-expression

The multifunctional nature of viral proteins makes it difficult to attribute one activity to a particular role in the life cycle. For example, expression of the HPV type 31 E2 protein can increase viral copy number but it is not straightforward to say what contribution E2-induced G2 arrest makes to this because another function of E2 is to recruit replication proteins to the viral *ori*. Such difficulties are often compounded in experimental systems by the problem of over-expression, which can facilitate non-physiological interactions as a result of alterations in protein abundance or subcellular localization.

Experiments that use mutant viral proteins also need to be interpreted cautiously as several binding partners and activities may be affected, some of which may not be known. Such experiments are useful in disproving causal relationships. For example, the significance of the Vpr binding protein HHR23A which was initially thought to be critical for G2 arrest was shown to be less important after a mutant Vpr was identified that was no longer able to bind to HHR23A, but which was still able to induce G2 arrest (Mansky et al., 2001). The need to carefully interpret mutant data is further emphasized by the R80A G2 arrest mutation in Vpr. R80A does not bind to PP2A, but neither does it activate the p21 promoter, associate with Cdc25C, nor downregulate gene expression in the MAPK proliferation pathway, which makes functional interpretation of its significance particularly difficult.

Summary

The ability to induce G2/M arrest is a feature of viruses from a range of different families. Interestingly, the arrest mechanisms vary widely, and do not correlate with their taxonomic classifications. Although the outcomes of arrest are in general less well characterized than the mechanisms, a number of potential consequences have been identified. In some cases these do seem to be common to virus groups, such as the increase in viral genome copy number that is seen among some DNA viruses, but there is still much work to be done before the role of G2/M arrest in the life cycle of different viruses is properly understood. The most well-established mechanisms and outcomes are those that have been determined using a variety of approaches, and which make use of model systems that closely mimic natural infection. Hopefully by using such multi-faceted approaches, the mechanism of action, and the consequence of G2/M arrest can be established in the life cycle of the different viruses.

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