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HERPES SIMPLEX VIRUS TYPE 2: UNIQUE BIOLOGICAL PROPERTIES INCLUDE NEOPLASTIC POTENTIAL MEDIATED BY THE PK DOMAIN OF THE LARGE SUBUNIT OF RIBONUCLEOTIDE REDUCTASE

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1. ABSTRACT

The prevalence of herpes simplex virus type 2 (HSV-2) infections in the US has increased approximately 30%. Like HSV-1, which causes facial lesions, HSV-2 causes symptomatic lesions (at genital sites) and establishes latent infections of the sensory ganglia. However, the two viruses are biologically distinct, suggesting that they possess unique functions which are mediated by different viral genes. Unlike HSV-1, HSV-2 is a tumor virus. It causes neoplastic transformation of cultured human cells and tumors in animals. The oncogene is at the 5'-terminal of a chimeric gene that also codes for the large subunit of viral ribonucleotide reductase (RR1). It was captured from the cell and it codes for a novel growth factor receptor serine-threonine protein kinase (PK) the minimal genetic information of which can adapt to a relatively wide functional diversity due to the flexibile use of additional and alternate catalytic sites and protein interaction motifs which are organized in an efficient, almost superimposed fashion. By contrast to other growth factor receptor serine-threonine kinases studied so far, the HSV-2 oncoprotein (RR1 PK) activates the RAS signaling pathway, thereby providing a biological bridge to the tyrosine growth factor receptor kinases. Expression of the oncogene is required for neoplastic transformation and tumor growth *in vivo* is inhibited by antisense inhibition of oncogene expression. The virus conserved the captured oncogene because it provides a biological advantage for its survival. In cultured cells, RR1 PK is required for viral IE gene transcription. *In vivo*, RR1 PK is likely to be involved in latency reactivation.

2. INTRODUCTION

The spread of sexually transmitted diseases continues unabated, despite educational efforts made in response to the epidemic of human immunodeficiency virus (HIV). Recent studies indicate that the age-adjusted prevalence of herpes simplex virus type 2 (HSV-2) in the US is now 20.8%, an increase of approximately 30% over the past 13 years (1). In addition to its ability to cause symptomatic recurrent genital lesions, HSV-2 is a tumor virus. It is mutagenic, it causes chromosome breakage, gene amplification and neoplastic transformation (reviewed in 2). The increasing rate of HSV-2 acquisition among young adults increases the likelihood that infants will be exposed to HSV-2 at delivery, resulting in an infection that, despite antiviral therapy, is still life-threatening (3). New concerns about HSV-2 infection is that it causes previously undescribed hyperproliferative lesions and it facilitates the spread of HIV as well as increasing the severity of the disease (4).

While it is often assumed that HSV-2 is molecularly indistinguishable from the type 1 virus (HSV-1) which causes facial lesions, the overall homology of HSV-2 and HSV-1 DNA is only 50% and it is significantly lower in some genome regions. The two viruses are biologically distinct. They have a predilection for different body sites, a different propensity to cause recurrent disease (60% and 30% for HSV-2 and HSV-1 respectively), they are associated with different neurological diseases (primarily meningitis for HSV-2 and encephalitis for HSV-1), and only

HSV-2 has neoplastic potential. These differences presumably reflect the distinct function and regulation of some viral genes. Here, we briefly review available data on virus pathogenesis with particular emphasis on aspects unique to HSV-2, notably neoplastic potential.

3. THE HSV VIRION AND ITS GENOME

The HSV-1 and HSV-2 virions are morphologically indistinguishable. They consist of an electron dense core containing the genome which is surrounded by an icosahedral capsid. In turn, the capsid is surrounded by an amorphous mass (called tegument) and a lipid bilayer envelope on the surface. Present understanding of genome organization comes from studies of HSV-1, the entire genome of which has been sequenced (5). The genome consists of approximately 150kb of double stranded linear DNA and has a unique organization. There are two unique stretches, a long stretch (UL) and a short stretch (US) which are flanked by inverted repeats. 15kb of DNA sequences represent inverted repeats of terminal regions inserted between the UL and US domains. Genes are contained both within the unique and repeat sequences. HSV-1 is known to express at least 84 different proteins. Of these, 5 open reading frames (ORFs), which map in the inverted repeats, are present in 2 copies/viral genome. In addition, infected cells contain transcripts from genome domains not known to specify proteins. These include the latency associated transcipts (LATs) that have been implicated in latency regulation and a RNA (Oris RNA) derived by transcription of two of the three origins of viral DNA synthesis which map in the inverted repeats. Thirthy eight HSV-1 ORFs cannot be deleted without ablating the capacity of the virus to replicate in cell culture (reviewed in 6). By contrast, the HSV-2 genome has not been entirely sequenced and ORFs that are essential for virus replication are not yet identified. Genome organization is similar to that of HSV-1, but overall homology is only 50%. Certain genome regions, such as the 5'-terminal one-third of the gene which codes for the large subunit of ribonucleotide reductase (RR1), have relatively little (38%) homology. Others, including the 3'-terminal two-thirds of RR1 have very high (80-90%) homology (7,8).

Approximately one half of the HSV-1 structural proteins are contained within the tegument structure. Many of them are functional proteins which may play critical roles in the initiation of the replicative cycle. They include: (i) immediate early (IE) proteins ICP4 and ICP0, which regulate the expression of the other viral genes (9,10), (ii) UL36 (VP1/2) which is associated with the release of viral DNA from incoming capsids and the cleavage and packaging of newly synthesized viral DNA (11), (iii) UL41, (*vhs*) which is responsible for decreased mRNA stability and inhibition of host cell translation (12), (iv) UL48 (also known as Vmw65, VP16 or alpha-TIF) which is responsible for the *trans*-activation of IE genes (13), and (v) UL13 which has protein kinase (PK) activity (14). At least VP16, *vhs*, UL13 and RR1 (also known as ICP10) are also present in HSV-2 virions.

4. HSV GENES AND THE REPLICATIVE CYCLE

The most common site of primary HSV infection is at mucosal membranes, facial for HSV-1 and genital for HSV-2. The virus replicates in cells at the site of infection and is transported to sensory neurons that inervate the site. Some neurons support productive infection and are destroyed. Others retain the virus DNA in a latent state, generally throughout the lifetime of the host. Periodic reactivation of the latent viral genome results in virus replication often causing recurrent disease.

The HSV-1 and HSV-2 reproductive cycle has been studied using cultured cells. During acute infection, gene expression is coordinately regulated and sequentially ordered in a cascade fashion. To accomplish this, the virus brings into the newly infected cells proteins that, as mentioned above, are packaged into the tegument and function to create the environment required for initiation of viral replication. The IE genes, also known as alpha, are expressed first in the infected cells [at approximately 2-4 hrs post infection (p.i.)], and continue to accumulate until late in infection at non-uniform rates. They were initially defined as those genes which are expressed in the absence of other viral protein synthesis. They have an octamer *(oct-1)*/TAATGAARAT sequence which is recognized by a complex consisting of the virion protein VP16 and *oct-1*, within several hundred base pairs (bps) upstream of the cap site. There are five IE proteins, ICP0, ICP4, ICP22, ICP27 and ICP47. ICP4 is a phosphoprotein required for the induction of the next kinetic classes of viral proteins. It also acts as a repressor of its own expression, and that of other HSV genes. ICP27 is primarily required for the regulation of late gene expression and it appears to play a role in the shut-off of host protein synthesis. ICP27 also affects early gene expression in the absence of ICP4, and it can affect the ability of ICP4 to bind to DNA. In transient expression assays, ICP0 is a promiscuous transactivator. Virus deleted in ICP0 is growth impaired, but only at low multiplicity of infection (moi). ICP4 and ICP0 can act synergistically to activate early genes. Early (E) proteins (also known as beta) are involved in viral DNA replication and reach peak synthesis rates at 5-7 hrs p.i. Examples include ICP8 (the major DNA binding protein), RR2 (the small subunit of ribonucleotide reductase) and TK (thymidine kinase). Their synthesis requires a functional ICP4 protein. The late (L) genes (also known as gamma) encode virion structural components. They form a continuum differing in their timing and dependence on viral DNA synthesis for expression. Their expression is dependent on the continued presence of ICP4 and, also requires the action of ICP27 (reviewed in 6).

Lytic infection results in rapid and profound repression of RNA polymerase II (polII) transcription on

host cell promoters. Pol II is the central component of the RNA transcription machinery, responsible for the regulated transcription of eukaryotic genes. It requires ATP and general transcription factors, the assembly of which on the promoter of protein-coding genes is a multistep process. In addition to sites of catalysis and RNA and DNA binding, the large subunit of polII also contains a carboxy-terminal domain (CTD) that consists of 52 repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. It is phosphorylated by the cdk-activating (Cak) complex which is a component of transcription factor (TF) IIH. *In vivo*, polII occurs in two major forms that differ in the extent of CTD phosphorylation. It is generally accepted that the non-phosphorylated form enters into the preinitiation complex when CTD interacts with members of the activated transcription machinery, thereby recruiting polII in the vicinity of the promoter. Once a competent preinitiation complex is assembled Cak phosphorylates CTD, releasing it from its contacts at the promoter, and elongation ensues (26-28).

An important question is the mechanism whereby polII is re-directed from cellular to viral genes after HSV infection. In HSV-1 infected cells, the IE protein ICP22 was implicated in the alteration of polII nuclear localization and CTD phosphorylation by an, as yet, unknown kinase (29). Complexation of viral DNA with ICP4, ICP22, a cellular nucleolar protein, and polII appears to be important for late gene expression (30), confirming our previous finding that HSV-1 growth is inhibited by antisense oligonucleotides which inhibit ICP22 synthesis (31,32). The virion transactivating protein VP16 binds the p62 subunit of TFIIH and a CTD kinase (other than Cak) which may be involved in elongation (33), and it stimulates transcriptional elongation and its initiation (34). However, inasmuch as CTD kinases(s) are activated by phosphorylation (35), an alternative (but not mutually exclusive) mechanism for re-directing polII to viral IE promoters may reside in altered phosphorylation of CTD kinases by virion-associated PKs.

As first shown in our laboratory for HSV-2, the RR1 protein differs from its counterparts in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique amino-terminal domain that has serine-threonine specific PK activity (16-24). The HSV-1 virion-associated RR1 appears to lack PK activity, although the failure to expose the tegument, prior to enzymatic assay complicates data interpretation (15). By contrast, the HSV-2, virion-associated RR1 has PK activity (16). In HSV-1 infected cells, the IE major transactivating protein ICP0 is phosphorylated by UL13 in a cell type-dependent manner (25). However, UL13 is not involved in altered phosphorylation of CTD kinases (36). By contrast to HSV-1 virions, HSV-2 virions contain two kinase activities (UL13 and RR1 PK) and we recently found that RR1 PK is required for IE gene expression (Smith *et al.*, submitted), suggesting that it may be involved in altering the phosphorylation of CTD kinases.

5. HSV GENE EXPRESSION AND LATENCY

In vivo, HSV-1 and HSV-2 infections can be divided into four stages: (i) acute infection, (ii) establishment of latency, (iii) maintenance of the latent state, and (iv) reactivation of latent virus. During acute infection, virus replicates at the site of inoculation on epithelial surfaces resulting in primary lesions. Virus then enters into nerve terminals where it travels by axonal transport to associated sensory ganglia. At this stage, in some animal models there is a short period of virus replication in the ganglia, which can be detected by virus isolation from cell-free homogenates of the ganglia. It is unknown whether this also occurs in humans and it may be an artifact due to the animal species, route of inoculation or the large virus inoculum used in experimental models. In the second stage, occurring at approximately 2-4 weeks p.i., a latent infection is established in which viral DNA is maintained in the neurons as an episome and there is limited HSV gene expression. Infectious virus is no longer detected in cell-free homogenates, but it can be detected by explantation and organ culture of the ganglia. In the last stage, certain stimuli may result in activation of virus replication with concomitant axonal transport of virus progeny to a peripheral site, at or near the portal of entry. Such stimuli include physical or emotional stress or peripheral tissue damage. In some animal models (guinea pigs), latent virus reactivates spontaneously. In others it can be experimentally reactivated by neurectomy, trauma to the ganglia, electrical stimulation, epinephrine iontophoresis or cadmium treatment. Depending on the host immune response, the outcome of virus reactivation may vary from severe debilitating lesions (in immunosuppressed individuals) to no lesions, or barely visible ones. In humans, HSV-1 genital recurrences are less frequent than HSV-2 and the converse relationship holds for orolabial recurrences (37), suggesting that there is an evolutionary divergence between HSV-1 and HSV-2 which allows different routes of colonization/reactivation. Viral functions responsible for the different rates of reactivation are unknown.

A still unanswered question is why latency ensues after infection of sensory neurons. The decision for or against latency appears to be made before IE gene expression. However, a HSV function that is directly required for the establishment of latency, has not yet been identified. In mouse neuronal cells, a repressor activity (possibly, *oct-2*) was shown to bind the *oct-1*/TAATGARAT motifs in the ICP4 promoter and inhibit the expression of a transfected pICP4-cat, suggesting that neuronal factors may inhibit expression of viral genes. Yet after the repressor factor was titrated out by binding with the TAATGARAT element, expression did not match that seen in nonneuronal cells, suggesting that neuronal cells also lack, or have insufficient levels, of positive-acting factors needed for high level IE gene expression (38). In animal models, peripheral (or neuronal) virus replication is not absolutely required for latency establishment. This is evidenced by the

observation that viruses lacking the essential genes ICP4 and ICP27, which do not replicate in any cell *in vivo*, establish and maintain latency, albeit at greatly reduced levels. These viruses reactivate poorly and this may be due to a low number of latently infected neurons (39).

In latently infected ganglia the virus genome persists as an episome and its expression is restricted to a small region termed LAT. Originally identified as an abundant nuclear RNA, LAT is now believed to be a 2kb intron that can itself be spliced and is derived from a 8.3kb primary transcript the domain of which encompasses all, but approximately 1kb each of the UL flanking inverted repeats (40). The function of LAT remains elusive. It appears that it is not essential for productive infection or establishment and maintenance of latency (41), but may be necessary for HSV-1 reactivation, involving the first 1.5kb of the LAT gene (42). At least 16 ORFs were identified in the LAT transcript. Two of these (ORF-O and ORF-P), which are read antisense to LAT on the HSV genome, were recently identified in cells latently infected with HSV-1. ORF-P has been classified as a pre-alpha, the definition of which includes induction by VP16 and firm shut-off by onset of IE gene expression (43). The role of these transcripts in latency is unknown, and similar studies were not done for HSV-2.

What determines the switch which initiates virus reactivation? ICP0, LAT and TK were implicated in HSV-1 latency reactivation (44-46). According to a recent proposal, in addition to IE genes, E genes involved in viral DNA synthesis must be turned-on for limited DNA replication. This stimulates a viral function that upregulates IE gene expression leading to the lytic cascade and the production of infectious virus (47). However, this proposal does not address the mechanism whereby IE and E genes are turned on by distinct reactivating stimuli. Using a highly sensitive PCR-based RNA assay, mouse neurons latently infected with HSV-1 were shown to express low, but correlated, levels of ICP4 and TK mRNAs, and it was suggested that ICP4 expression leads to E gene transcription in some of the latently infected ganglia (48). This is unlikely, however, because the levels of detected ICP4 RNA were too low to generate the amounts of ICP4 protein which are required in order to initiate the ensuing lytic cascade. As will become evident later, we propose that RR1 PK, the expression of which is independent of viral IE genes, plays a central role in HSV-2 latency reactivation.

6. HSV-2 RR1 IS A CHIMERA THAT CONTAINS CELLULAR SEQUENCES

As first shown in our laboratory for HSV-2, the RR1 proteins of both HSV types are unique among known HSV proteins in that their expression is regulated with IE kinetics and it does not require a functional ICP4 (51,53). RR1 is synthesized as early as 2hrs p.i., and it continues to accumulate throughout most of the replicative cycle. Like the IE genes, it is expressed in the absence of other viral proteins and it has the octamer/TAATGAARAT sequence within several hundred bps of the cap site. RR1 responds to activation by the VP16/oct-1 complex, also like IE genes, and its expression is also increased by the promiscuous trans-activator ICP0 (49-53). The RR1 promoter is unique in that it has response elements for AP-1 (51,52), a broad class of transcription factors that are frequent components of complex regulatory elements in IE cellular genes (54), but are not present in other viral genes. Significantly, AP-1 factors are required for basal expression of HSV-2 RR1, and even for its activation by ICP0 (52). Inasmuch as the small subunit of the viral ribonucleotide reductase (RR2) is an E gene, the synthesis of which requires a functional ICP4, it is evident that the IE regulation of RR1 is unrelated to ribonucleotide reductase activity and is required for its PK activity.

Important questions are the origin and role of RR1 PK in virus growth. By analogy to oncogenic retroviruses that contain captured cellular genes originally discovered by homology to transforming viral genes (55), various members of the Herpesviridae including Epstein Barr virus (56), the recently isolated human herpesvirus 8 (57) and HSV contain DNA sequences with cellular homology. The HSV genes with cellular homology are g134.5, a region of which is homologous to GADD34 and MyD116 that are involved in growth arrest (58), and RR1 PK (8).

Philogenetic analyses indicate that the cellular homologue of RR1 PK is a novel member of the family of serine-threonine growth factor receptor PKs (59) which are involved in the regulation of cell proliferation, growth inhibition, cellular differentiation and apoptosis (60). The only other known member of the family branch is FAST, a recently identified protein that is activated during Fas-mediated apoptosis (61). Implicit in the interpretation that RR1 PK was captured from the cell, is the conclusion that the upstream (5') recombination site is within the promoter of the ancestral HSV-2 RR1 gene and the C-terminus (3') recombination site, at the junction of the PK and RR domains of the chimeric protein, presumably occurred within the promoter region of the ancestral HSV-2 RR1. The presence of enhancer core and functional promoter elements at the PK and RR junction is consistent with this interpretation (62). However, since the HSV-1 and HSV-2 RR1 PKs share relatively little homology (7), it is unclear whether they originated from the same cellular progenitor, diverging under evolutionary pressure, or whether different members of the growth factor receptor family were independently captured by HSV-1 and HSV-2 because they were destined to fulfill different functions in virus pathogenesis.

The conservation of RR1 PK over many evolutionary cycles suggests that the captured cellular gene provided the virus with a growth advantage. HSV-1 mutants

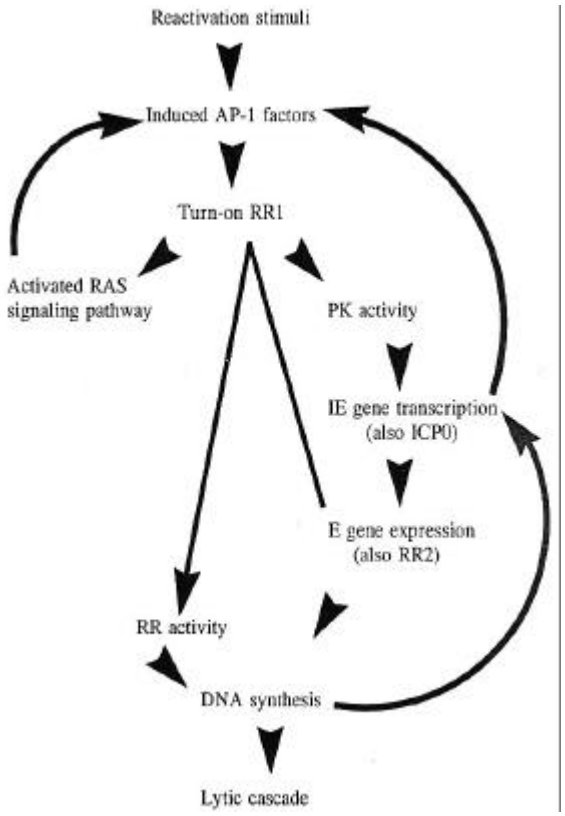

Figure 1. HSV-2 RR1 PK plays a crucial role in latency reactivation. Because RR1 is the only viral gene the basal expression of which is AP-1 dependent, we propose that it is the earliest viral response to latency reactivation stimuli which are known to induce AP-1 factors. The turned-on RR1 supplies the PK activity which is required for IE gene transcription, thereby initiating expression of other viral genes and DNA synthesis. The latter is amplified by the RR enzymatic activity resulting from complexation of RR1 and RR2. IE gene ICP0 interacts with AP-1 to further amplify RR1 expression. RR1 activates the RAS signaling pathway further amplifying AP-1 levels. The outcome is increased DNA synthesis and initiation of the lytic cascade.

deleted in RR1 fail to replicate in neurons, and are significantly less neurovirulent, and less able to reactivate from latency than the wild type virus. However, these defects are due to the failure of the growth restricted neurons to supply the ribonucleotide reductase activity that is required for DNA synthesis (63-66). A HSV-2 temperature sensitive mutant which is defective in RR1 expression at the non-permissive temperature failed to grow under these conditions, but the data did not differentiate between the contribution of the PK vs RR domains (67). We recently constructed a HSV-2 mutant which is deleted in the PK domain of RR1 but retains an intact RR domain and ribonucleotide reductase activity and showed that it is defective in IE gene expression, particularly ICP4, ICP27 and ICP22 which are essential for the expression of the other viral proteins and for DNA synthesis (Smith et al., submitted). These findings indicate that HSV-2 RR1 PK is required for HSV-2 growth in cultured cells.

We propose that RR1 PK is also required for latency reactivation. Indeed, latency reactivating stimuli induce AP-1 transcription factors (68). Because RR1 is the only viral gene that can respond to AP-1 with basal expression, it seems reasonable to conclude that they turn-on RR1. AP-1 levels are further amplified by RR1 PK which functions to activate the RAS signaling pathway (69,70). Furthermore, by virtue of its role in IE gene expression, RR1 PK initiates the cascade of viral protein synthesis, including IE proteins ICP4 and ICP0 and E proteins, such as RR2. This results in limited levels of DNA synthesis which, in turn, upregulates gene expression (47). In neurons, ICP4 activity appears to be determined by serine phosphorylation, which is also supplied by RR1 PK. ICP0 co-operates with AP-1 to further increase RR1 synthesis (52). RR1 complexes with RR2 to generate the ribonucleotide reductase activity that is required for DNA synthesis in neurons (66), resulting in increased DNA levels. The outcome is initiation of the lytic cascade and production of infectious virus (figure 1).

7. RR1 PK CODES FOR A NOVEL KINASE ACTIVITY WHICH IS REQUIRED FOR NEOPLASTIC TRANSFORMATION

Philogenetic studies indicate that RR1 PK originated from a growth factor receptor serine-theronine kinase (59). Indeed, the PK is a serine-threonine kinase located within amino acids 1-411 of the HSV-2 chimeric RR1 protein. Its catalytic domain contains 8 conserved catalytic motifs [as compared to 12 motifs in many eukaryotic PKs (71)]. It is preceded by a single transmembrane (TM) helical segment followed by a basic amino acid which is responsible for TM anchorage within the cell membrane. There is a short (88 amino acids) extracellular domain that initiates with a signal peptide (17,18). The RR1 PK protein is localized on the cell surface and is internalized by the endocytic pathway, as evidenced by immunogold electron microscopy (70). Known substrates for HSV-2 RR1 PK include calmodulin, some but not all histones, ras-GAP and the viral protein RR2 (17-22,59,69). With its 8 catalytic motifs RR1 PK bridges the gap between the previously described eukaryotic PKs which have twelve catalytic motifs and the newly identified classes which only conserve one catalytic motif (72).

How does HSV-2 RR1 PK function with only 8 catalytic motifs? Catalytic motif I is a Gly-rich loop localized at position 106-110. Mutational analyses indicated that it is not essential for kinase activity (20), as also reported for several other PKs (73). The two conserved charged residues that respectively constitute PK catalytic motifs II (Lys^{176}) and III (Glu^{209}) presumably form an ion pair that, in the ternary complex, provides a docking site for MgATP. In tyrosine kinases, which are typically expressed

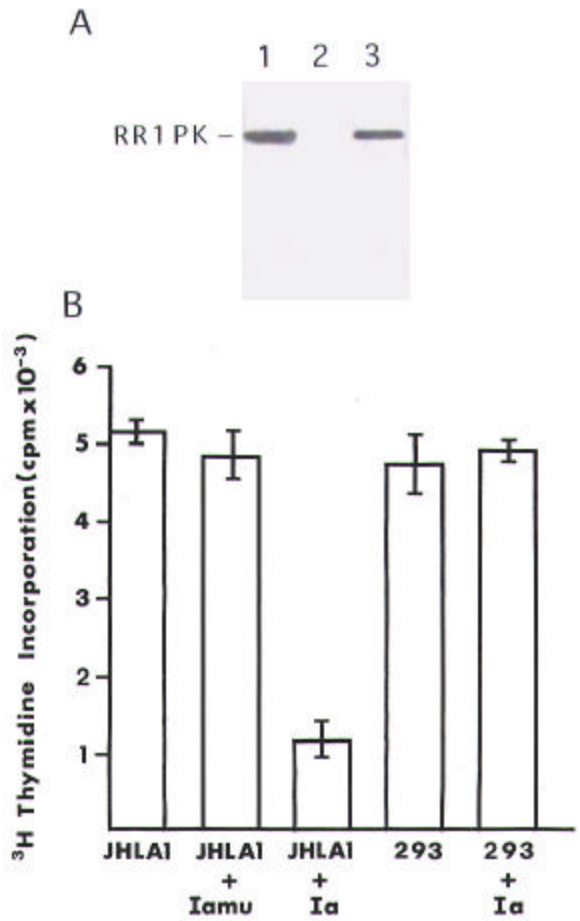

Figure 2. Inhibition of HSV-2 RR1 PK and cell growth by antisense oligonucleotides (ODNs). **A**. Human cells transformed by HSV-2 RR1 PK were treated with PBS (lane 1), antisense ODN Ia which is complementary to the HSV-2 RR1 PK translation initiation site (lane 2), or a mutant of ODN Ia (I*amu)* in which the two central bases were inverted (lane 3). Treatment was with 100 μM for 44 hrs at 37°C. The cells were then pulse-labeled with [^{35}S]-methionine for 4 hrs and precipitated with a monoclonal antibody (MAb) specific for HSV-2 RR1 PK. **B**. Human cells (5x10^3) untransformed (293) or transformed with HSV-2 RR1 PK (JHLa1) were untreated or treated for 24 hrs with 100 μM of the antisense ODN complementary to HSV-2 RR1 PK translation initiation site (Ia) or its mutant (I*amu)*. They were assayed for [^{3}H]-TdR incorporation after a 6 hrs pulse. Results are average counts ._SEM.

at low levels, replacement of the Lys residue in catalytic motif II is sufficient to show a null phenotype (71). However, in the yeast cAMP kinase catalytic subunit and in isocitrate dehydrogenase PK, both of which are expressed at high levels, replacement of this Lys residue reduces PK activity but is insufficient to cause a null phenotype. This is achieved by replacement of two adjacent non-conserved Lys residues (74). A similar situation holds for HSV-2 RR1 PK. Replacement of the Lys residue in catalytic motif II (Lys 176) reduces its kinase activity (Km = 1.2 and 6.6 μM for RR1 PK and Lys176 mutant respectively) and a similar reduction (Km = 9.4 μM) is achieved by replacement of Lys259. A null phenotype is only achieved by mutation of both residues (22). Both Lys residues bind ATP, since: (i) the Lys176 and Lys259 mutants evidenced a similar decrease in FSBA binding relative to the wild type protein, (ii) FSBA binding was specifically competed with another ATP analogue (AMP-PNP), and (iii) FSBA binding was not achieved when both Lys residues were mutated (22). Replacement of Glu209 (catalytic motif III) severely compromised both MnATP-dependent and MgATP-dependent PK activity, suggesting that the ion pair which is presumably formed between the two charged residues (Lys and Glu) provides a docking site for either MnATP or MgATP (22). Another basic shared function common to all PKs, including RR1 PK, is catalysis which involves the Asp and Asn residues in catalytic motif VI. Asp is believed to accept the proton from the attacking substrate hydroxyl group during phosphotransfer and Asn chelates the secondary Mg2+ ion and may serve to stabilize the loop (71,74). In RR1 PK, the Asp residue is replaced by Glu (Glu324) which has a similar charge and is likely to fulfill the same function. Catalysis can also be accomplished by an alternate motif (265DSPGN269) which contains Asp and Asn residues in a functionally appropriate configuration. Indeed, a bacterially expressed truncated RR1 protein (pp29la1) which lacks catalytic motif VI, retains PK activity which is lost by replacement of Asp265 (19,22).

Both HSV-1 and HSV-2 cause mutagenesis and gene amplification, activate DNA synthesis, induce cellular genes, including endogenous retroviruses, and cause morphologic transformation. However, only HSV-2 causes neoplastic transformation of rodent and human cells and induces tumors when injected in animals (75). Transformation and tumor formation in vivo, are achieved with inactivated virus, temperature sensitive mutants, sub- or supraoptimal infection temperatures, sheared viral DNA and subgenomic fragments. Depending on conditions, the transformed cells retain and express the viral DNA or not, suggesting that viral genes cause increased cell proliferation or they initiate the tranformation process which subsequently can progress independently. For example, HSV-2 DNA fragment BglII N (mu 0.58-0.63) which does not have protein coding functions and is not retained, causes morphologic transformation of rodent cells through the activity of sequence motifs functioning as transcriptional promoter elements which may alter the expression of cellular genes. By contrast, HSV-2 DNA fragment BglII C (mu 0.42-0.58) which contains both an immortalizing function and the transforming function mediated by the RR1 PK oncogene, is retained by the tumor cells (reviewed in 2,76).

HSV-2 RR1 PK expression is required for human cell tranformation, indicating that the oncoprotein plays an active role in increased cell proliferation. Indeed, HSV-2 mutants rendered PK negative by site-directed modification

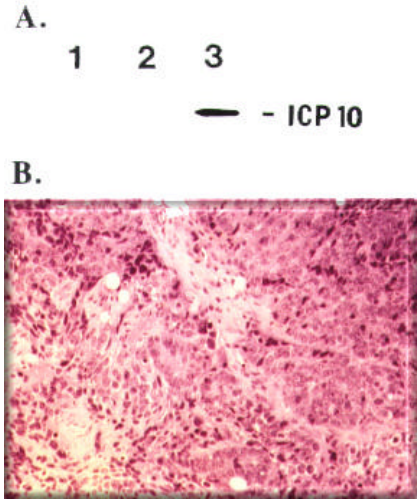

Figure 3. Tumors caused by cells transformed with HSV-2 RR1 PK express the oncoprotein. **A**. Proteins from a tumor induced by HSV-2 RR1 PK transformed cells and treated (5 days, 50 μM daily) with antisense ODN Ia (complementary to the translation initiation site of HSV-2 RR1 PK) (lane 1), adjacent normal tissue (lane 2) and an untreated tumor (lane 3) were immunoblotted with MAb to the HSV-2 RR1 PK oncoprotein. RR1 PK (ICP10) is expressed only in the untreated tumor cells. **B**. H&E stained section of an adenocarcinoma induced in nude mice by injection of human cells transformed with HSV-2 RR1 PK.

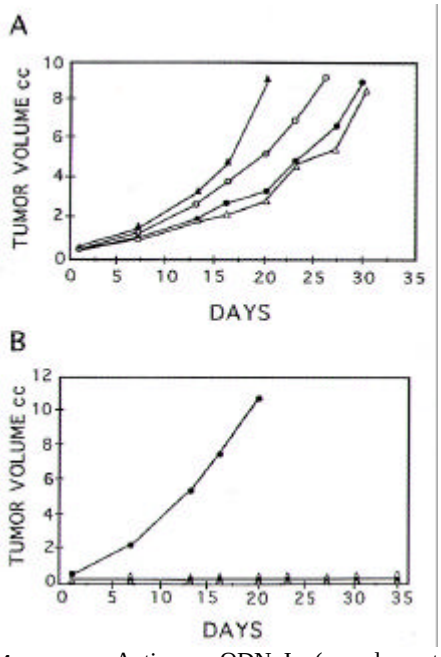

Figure 4. Antisense ODN Ia (complementary to the translation initiation site of HSV-2 RR1 PK) inhibits tumor growth. Groups of 4 mice were injected subcutaneously (sc) with human cells transformed by HSV-2 RR1 PK ($1x10^6$/mouse). Beginning 24 hrs later and daily for 9 days they were given sc injections of medium (Panel A) or antisense ODN Ia (50 μM at the site) (Panel B) and tumor volume was measured as described (78).

of amino acids within the ATP binding sites or by TM deletion, do not cause neoplastic transformation (69,70). Furthermore, cellular DNA synthesis and cell proliferation are inhibited (figure 2B) by exposure of the transformed cells to an antisense oligonucleotide (ODN) that is complementary to the RR1 PK translation initiation site and inhibits RR1 PK synthesis (figure 2A). Human cells transformed by HSV-2 RR1 PK cause poorly differentiated adenocarcinomas when injected into nude mice (figure 3B). The tumors express RR1 PK (figure 3A) and are amenable to gene therapy by antisense expression of RR1 PK. In studies done in collaboration with Dr. M. Colvin, groups of 4 mice each were given sc injections at the site of cell inoculation with medium or with the antisense oligonucleotide to RR1 PK, beginning 24 hrs after cell injection and daily for 9 days. Tumor growth was measured every 2 days and tumor volumes calculated using width (a) and length (b) measurements [a2b/2 where a<b (78)]. At 35 days after exposure, tumors were seen in 1/4 mice given the antisense oligonucleotide as compared to 4/4 mice given medium alone (figure 4), indicating that expression of the RR1 PK oncoprotein is required for tumor growth.

The RR1 PK activity and the neoplastic potential of a chimeric protein in which the RR1 extracellular domain (upstream of the TM) was replaced with that of the epidermal growth factor receptor are ligand-inducible (77), suggesting that the RR1 PK oncoprotein is constitutively activated. Presumably, this occured during capture of the cellular homologue by HSV-2. However, final conclusions depend on the results of ongoing studies designed to clone and characterize the cellular progenitor. Taken in toto, these findings indicate that HSV-2 is a DNA tumor virus. However, it is unique among the viruses in this group (SV40, polyoma and adenovirus), in that its oncogene was captured from the host cell, like those of the transforming retroviruses.

Consistent with its relatively low homology to the HSV-2 oncoprotein (7), the HSV-1 RR1 PK is structurally and functionally distinct. It lacks the conserved Lys of PK catalytic motif II (corresponds to Lys176 in HSV-2 RR1 PK) and it does not bind ATP at a Lys residue which corresponds to Lys259. The ATP binding site is downstream of amino acid 350, distant from the ion-binding Glu residue (23). This gives rise to a secondary structure which is incompatible with good levels of enzymatic activity. Indeed, the Km of the native HSV-1 RR1 PK is significantly (10-fold) lower than that of the HSV-2 RR1 PK (Lee and Aurelian, in preparation) and may explain the failure of the HSV-1 RR1 PK to evidence transphosphorylating activity (23,24). HSV-1 RR1 PK also differs from its HSV-2 counterpart in that its TM is not followed by a negatively charged amino acid which is required for TM anchorage, and consequently it does not localize to the plasma membrane (79). It does not have transforming activity (2,76) and its biologic function is still unknown.

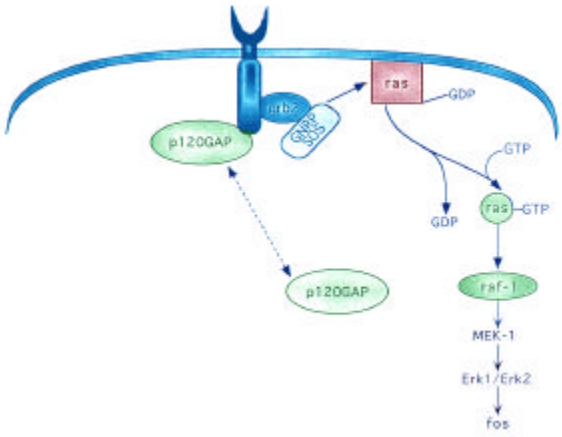

Figure 5. HSV-2 RR1 PK activates the RAS signaling pathway. The constitutively activated oncoprotein binds the Grb_2-hSOS complex thereby bringing hSOS in the vicinity of RAS. This results in the conversion of RAS-GDP to RAS-GTP (RAS activaton). HSV-2 RR1 PK also binds the downregulatory factor RAS-GAP (p120GAP) which it inactivates through phosphorylation. The activated RAS initiates the kinase cascade that leads to the nucleus and results in AP-1 (fos) activation

8. TRANSFORMATION BY HSV-2 RR1 PK INVOLVES ACTIVATION OF THE RAS SIGNALING PATHWAY

What is the mechanism of human cell transformation by RR1 PK? As shown in figure 5, it involves signaling through the RAS pathway. The activated (autophosphorylated) oncoprotein binds the Grb2-hSOS complex, thereby bringing the guanine transfer factor hSOS in the vicinity of RAS. The latter converts the inactive RAS.GDP to the active form RAS.GTP. To overcome the downregulatory activity of RAS-GAP, the activated RR1 PK also binds RAS-GAP and transphosphorylates it, thereby decreasing its GTPase activity. The combined effect of RAS activation by hSOS and RAS-GAP inactivation through phosphorylation, results in significantly increased levels of activated RAS. This in turn stimulates the downstream kinase cascade, which includes activation (through phosphorylation) of raf and Erk, and culminates in increased expression of nuclear AP-1 transcription factors and cell transformation. PK negative mutants of RR1 PK do not activate RAS and do not transform cells (69,70).

How does HSV-2 RR1 PK bind signaling proteins? Proline-rich motifs in the oncoprotein are consensus binding sites for SH3 domains involved in protein-protein interaction (22). Thus, SH3 binding domains share a common PXXP motif and have residues that contribute to specificity. Class I motifs have an amino to carboxy terminal binding orientation and include sites specific for the Src oncoprotein. Class II motifs are more promiscuous in their binding. They have a carboxy to amino terminal binding orientation, PXXP can be in either of two positions and they have basic residues at the C-terminus (80,81). The first proline-rich domain in the HSV-2 RR1 PK oncoprotein is located in the insert between PK catalytic motifs I and II. It consists of a class I, Src-specific SH3 binding motif (^{140}RTPEPQGP^{147}) followed by a promiscuous class II motif (^{149}AVPPPPPPPFPWGH^{159}) similar to that recently shown to bind Abl, Src, Fyn and Crk. The second HSV-2 RR1 PK proline-rich domain located between PK catalytic motifs VII and VIII (^{396}LPPVPPNAYT^{405}) is a class II SH3 binding site with basic residues (His^{408} and/or Arg^{410}) at the carboxy-terminus. HSV-2 RR1 PK oncoprotein binds the Grb_2/hSOS complex primarily at this latter site, as evidenced by a 20-fold binding reduction after mutation (22). Binding is competed by a peptide which represents the carboxy-terminal SH3 motif of Grb_2 at concentrations within the range of those which compete the high affinity hSOS binding (22), suggesting that Grb_2 binds RR1 PK at its carboxy-terminus and hSOS at its amino-terminal SH3 (82) with similar affinities (22). RAS-GAP binding by the RR1 PK oncoprotein involves the RAS-GAP amino-terminal SH2 domain and phosphothreonine residues at position 106-178 of the oncoprotein. An oncoprotein mutant deleted in amino acids 106-178 retains PK activity and its ability to bind Grb_2-hSOS, but it does not bind RAS-GAP, such that its transforming activity is 10-20-fold higher than that of the wild type oncoprotein (Nelson *et al.*, in preparation). The transforming activity of this mutant is as high as that of the strongest oncogenes (83).

9. HSV-2 CAUSES HYPERPROLIFERATIVE LESIONS IN HUMAN PATIENTS

A newly described complication of HSV-2 infections is the causation of hyperproliferative lesions which are often difficult to treat. We reported the case of a 27 year old woman with large recurrent hyperproliferating lesions caused by acyclovir resistant HSV-2. On histopathology the lesions revealed pseudo-epitheliomatous hyperplasia, multinucleated keratinocytes containing molded nuclei and intranuclear inclusions typical of HSV. Laboratory results included a somewhat decreased level of IgG [376 mg/dl (normal range 694-1618 mg/dl)] which is indicative of common variable immunodeficiency. However, the patient was not immunosuppressed. She had normal levels of HSV-2 antibody and there was positive immunity to the anergy panel (mumps, trichophyton, candida). She was negative for human papillomavirus (HPV) 6/11, 16/18, 31/33/35 as well as HIV, VDRL, chlamydia and gonorrhea. She was successfully treated with a 6 week course of intravenous foscarnet, but the lesions recurred, this time when the patient was pregnant, greatly complicating therapy and leading to the termination of her pregnancy (84).

10. CONCLUSIONS

During the last 10 years, significant information

has been accumulated on the molecular biology of HSV-1. While less is known about the molecular aspects of disease pathogenesis, we have witnessed major advances in our understanding of the viral genes involved in HSV-1 disease causation and their regulation. Much less is known about HSV-2, which is biologically distinct. In this review we have underscored the relative limitations of our present understanding, emphasizing those properties which are unique to HSV-2. This is of particular importance since there has been a major increase in the rate of HSV-2 acquisition resulting, among others, in an increased likelihood of life-threatening neonatal infections, possible facilitation of HIV spread and increased disease severity, and the emergence of novel disorders, such as hyperproliferative lesions which are often difficult to treat and are associated with unknown risks.

A major difference between HSV-2 and HSV-1 is the ability of HSV-2 to cause neoplastic transformation of human cells. Transformation is mediated by the RR1 PK oncogene which was captured from the cell, presumably through an ancestral recombinational event. It belongs to an as yet poorly described subfamily of serine-threonine growth factor receptors in which minimal genetic information can adapt to a relatively wide functional diversity and has the necessary flexibility to use additional and alternate catalytic sites, as required. The oncoprotein contains various sites for protein-protein interaction which are organized in an efficient, almost superimposed fashion. By contrast to other growth factor receptor serine-threonine kinases studied so far, the HSV-2 oncoprotein binds secondary messenger proteins that interact with growth factor receptor tyrosine kinases and it activates the RAS signaling pathway, thereby providing a biological bridge between these two groups of growth factor receptors. Activation of the RAS signaling pathway by the HSV-2 RR1 PK oncoprotein is essential for transforming activity. The transformed cells cause tumors in animals and their growth depends on RR1 PK expression. Antisense inhibition of the oncoprotein inhibits tumor growth.

Inasmuch as transforming activity is of no evolutionary benefit for the virus, it seems logical to assume that the captured oncogene has been conserved because its PK activity provides a biological advantage for virus survival. In cultured cells, RR1 PK is required for IE gene transcription. *In vivo*, RR1 PK might be required for virus replication at the site of infection, efficient latency establishment and/or reactivation from latency. The HSV-1 RR1 PK protein is structurally and enzymatically different, it does not have signaling potential and does not cause neoplastic transformation. Its role in virus growth is still unclear.

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