ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
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GRADUATE PROGRAM IN
THE MOLECULAR BASIS OF HUMAN DISEASE

The role of Extracellular-signal- Regulated Kinase (ERK) activation during Herpes Simplex Virus type-1 (HSV-1) lytic infection

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Ο ρόλος της ενεργοποίησης της ERK κινάσης κατά τη διάρκεια λυτικής λοίμωξης από τον Απλό Ερπητοιό τύπου 1 (HSV-1)

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Summary

Viruses, alike other intracellular organisms, are ultimately dependent upon the host cell for their replication. To establish a successful infection, they need to overcome several host cell obstacles, such as virus binding-triggered apoptosis, induction of innate and inflammatory immune responses, cellular RNA interference, autophagy and restriction on virus gene transcription. Besides using their gene products, viruses have evolved better strategies to eliminate host cell defense. Manipulation of cellular preexisting signaling pathways is probably one of the best strategies used early during infection. Signaling pathways determine the cell's ability to respond to external stimuli. Transduced signals can be interpreted as proliferative , mitogenic, differentiating or apoptotic, depending on the cell type and the quality and duration of the stimulus.

Several DNA and RNA viruses have been documented to use various strategies to activate the Extracellular-signal- Regulated Kinase (ERK) signaling pathway. Conversely, Herpes Simplex Virus type 1(HSV-1), member of the Herpesviridae family of DNA viruses, is well-known to activate several other MAP kinase cascades, such as JNK and p38. To date, no data exist about a possible effect of HSV-1 on ERK pathway. This was the aim of the present study: to demonstrate the impact of HSV-1 infection on the cellular ERK signaling cascade, as well as to investigate the role of a functional ERK pathway on viral replication.

For this purpose, briefly, we used cells receptive to infection from HSV-1 strain 17+ (wild-type). BHK cells were used for viral propagation and titration, while microscopy and biochemistry assays were performed in Vero cells. The latter were fixed or collected at different time points in the course of infection, in the presence or absence of a MEK-specific inhibitor, and immunostained or immunoblotted, respectively, for p-ERK and viral gene products.

Our data demonstrate for the first time that ERK pathway is activated during HSV-1 infection, while this activation favors HSV-1 efficient replication.

Περίληψη

Η αναπαραγωγή των ιών, όπως και άλλων ενδοκυττάριων οργανισμών, εξαρτάται από τα κύτταρα-ξενιστές τους. Για να εξασφαλίσουν μια επιτυχή λοίμωξη χρειάζεται να ξεπεράσουν ορισμένα εμπόδια των κυττάρων-ξενιστών, όπως τη διαμεσολαβούμενη από την πρόσδεση του ιού απόπτωση, την επαγωγή εγγενών και φλεγμονωδών ανοσολογικών αποκρίσεων, την παρεμβολή κυτταρικών RNA, την αυτοφαγία και τον περιορισμό της ιικής μεταγραφής. Οι ιοί, πέρα από το ότι μπορούν να χρησιμοποιήσουν τα γονιδιακά προιόντα τους, έχουν αναπτύξει καλύτερες στρατηγικές για να κάμψουν την κυτταρική άμυνα. Η χειραγώγηση προυπάρχοντων κυτταρικών σηματοδοτικών μονοπατιών αποτελεί πιθανότατα μια από τις καλύτερες στρατηγικές που μπορεί να χρησιμοποιήσει πρώιμα κατά τη διάρκεια λοίμωξης. Τα σηματοδοτικά μονοπάτια καθορίζουν την ικανότητα του κυττάρου να ανταποκρίνεται σε εξωγενή ερεθίσματα. Τα μεταβιβαζόμενα σήματα μπορούν να ερμηνευτούν είτε ως μιτογόνα, πολλαπλασιασμού και διαφοροποίησης, είτε ως αποπτωτικά, ανάλογως του κυτταρικού τύπου, καθώς και του είδους και της διάρκειας του σήματος.

Έχει περιγραφεί για αρκετούς DNA και RNA ιούς η ενεργοποίηση του σηματοδοτικού μονοπατιού της ERK με τη χρήση διάφορων στρατηγικών. Από την άλλη, ο Απλός Ερπητοιός τύπου 1 (HSV-1), DNA ιός και μέλος της οικογένειας των Ερπητοιών , ενεργοποιεί ορισμένα άλλα μονοπάτια MAP κινασών, όπως το JNK και το p38. Δεν έχει περιγραφεί έως τώρα πιθανή επίδραση του HSV-1 στο μονοπάτι της ERK. Αυτός είναι και ο σκοπός της παρούσας μελέτης: η πιθανή επίδραση της λοίμωξης από HSV-1 στο κυτταρικό σηματοδοτικό μονοπάτι της ERK , καθώς και η διερεύνηση του ρόλου ενός τέτοιου λειτουργικού μονοπατιού στον ιικό πολλαπλασιασμό.

Εν συντομία, γι’ αυτό το σκοπό χρησιμοποιήσαμε κύτταρα δεκτικά σε λοίμωξη από το στέλεχος 17+ του HSV-1. Για τον πολλαπλασιασμό και την τιτλοποίηση του ιού χρησιμοποιήθηκαν κύτταρα BHK, ενώ τα πειράματα μικροσκοπίας και βιοχημείας πραγματοποιήθηκαν με τη χρήση κυττάρων Vero. Τα τελευταία μονιμοποιήθηκαν ή συλλέχθηκαν σε διαφορετικά χρονικά σημεία κατά τη διάρκεια της λοίμωξης, παρουσία και απουσία του ειδικού αναστολέα της MEK, και ελέγχθηκαν για τη p-ERK καθώς και για ιικά γονιδιακά προιόντα με ανοσοφθορισμό και ανοσοαποτύπωση, αντίστοιχα.

Τα δεδομένα μας καταδεικνύουν για πρώτη φορά ότι το μονοπάτι της ERK ενεργοποιείται κατά τη διάρκεια λοίμωξης από τον HSV-1 και ταυτόχρονα αυτή η ενεργοποίηση ευνοεί τον αποτελεσματικό πολλαπλασιασμό του ιού. .

Acknowledgements

I wish to thank, first and foremost, my Supervisor Dr George Sourvinos, for trusting me for my thesis, for his continuous guidance and most of all, for his kind patience, while giving me room to work in my own way.

I am also thankful to all members of the Virology lab, and especially Evi Vlachava and Harry Filippakis, who made my work much easier with helpful suggestions and instructions.

Without the encouragement, patience and assistance of John, this thesis would not have been written.

I dedicate my Master's degree to my parents who have supported me throughout my life and without whom I would never have enjoyed so many opportunities.

Contents

1.INTRODUCTION

1.1Herpesviruses

The *Herpesviridae* family is a large group of DNA viruses, containing more than 200 members that infect organisms from fungi to humans. To date, only eight of them have been identified to infect humans, and these are Herpes Simplex Virus 1 and 2 (HSV-1, HSV-2), Varicella-Zoster Virus (VZV), Ebstein-Barr Virus (EBV), Human Cytomegalovirus (HCMV), Human Herpesviruses 6 and 7 (HHV-6, HHV-7), and Kaposi Sarcoma Herpesvirus (KSHV) (Table 1).

All human herpesviruses display similar biological characteristics, which account also for their similar pattern of infection. Apart from their similar structure, they encode for a series of enzymes involved in nucleic acid metabolism (e.g., thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase), DNA synthesis (e.g. DNA polymerase, helicase, primase) and processing of proteins (e.g., protein kinases), although the exact array of enzymes may vary from one herpesvirus to another. DNA synthesis and capsid assembly occurs in the host cell nucleus. Final processing of the virion takes place in the cytoplasm followed by release of viral progeny to adjacent host cells, while the infected cell is lysed *(Baron S. , Medical Microbiology)*. Finally, their most significant biological property is their ability to achieve life-time latent infections in humans. All herpesviruses examined to date are able to remain latent in their natural hosts. In cells harboring latent virus, viral genomes take the form of closed circular molecules, and only a small subset of viral genes is expressed. Latent genomes retain the capacity to replicate and cause disease on reactivation. Their ability to establish lifelong infection is possibly attributed to immune evasion. Herpesviruses have found many different ways to evade the immune system. One such way is by encoding a protein mimicking human interleukin 10 (hIL-10) *(Spencer J., et al., 2002)* and another is by downregulation of the Major Histocompatibility Complex II (MHC II) in infected cells *(Lin A., et al., 2007)*. A virus can be simultaneously latent in some cells and actively proliferating in others. Thus, there are circumstances when (a) virus is latent in essentially all infected cells (the classical example of VZV); (b) virus is lytically active in a subset of infected cells, but there are no associated symptoms (asymptomatic shedding of HSV—the most common state of affairs for all herpesviruses and may be for VZV); and (c) lytic virus activity results in illness, while a subset of cells remains in latency

Herpesviruses display differences concerning their site of latency and target cells for lytic replication. Some have a wide host cell range, multiply efficiently, and rapidly destroy the cells they infect (e.g., HSV-1, HSV-2). Others have a narrow host cell range (EBV, HHV-6) or a long replicative cycle (HCMV). Although all herpesviruses remain latent in a specific set of cells, the exact cell in which they remain latent varies from one virus to another.

These differences allow further classification to α-, β- and γ-herpesviruses. α-herpesviruses (HSV-1, HSV-2 and VZV) have a relatively short reproductive cycle(24h), cause rapid lysis in monolayer cell cultures, have a broad host range and establish latent infections in sensory ganglia; β- herpesviruses (HCMV, HHV-6 and HHV-7) have longer reproducive cycles and restricted host range; and γ-herpesviruses (EBV and KSHV) have a very restricted host range in T and B cells *(Whitley RJ. Fields Virology)*. Based on their tropism regarding latent infections they are further classified into neurotropic (HSV-1, HSV-2 and VZV) and lymphotropic (EBV, HCMV, HHV-6, HHV-7 and HHV-8) *(Britt,W.J. Fields in virology).*

Herpesvirus	Subfamily	Genome size(kbp)	Latency/ targets	Commonest manifestations
HSV-1	α	152	neurons/ epithelial cells	Gingivostomatitis(primary) Cold sores, orofacial herpes, encephalitis, genital lesions(reactivation)
HSV-2	α	152	neurons/ epithelial cells	Genital lesions, cold sores Neonatal herpetic infection(primary)
VZV (HHV-3)	α	125	neurons/ epithelial cells	chickenpox(primary) Herpes zoster(reactivation)
EBV (HHV-4)	γ	172	B cells/ Bcells-epithelial	Infectious mononucleosis Burkitt's lymphoma, nasopharyngeal carcinoma, HIV-associated hairy leukoplakia
HCMV (HHV-5)	β	229	Monocytes-lymphocytes/ monocytes-lymphocytes-epithelial	Mononucleosis-like syndrome, congenital infections Retinitis, hepatitis, colitis, pneumonitis in immunocompromised
HHV-6	β	162	T cells and?	Roseola infantum
HHV-7	β	145	T cells and?	?
KSHV (HHV-8)	γ	170	B cells/ lymphocytes	Kaposi's sarcoma

Table 1: The Herpesviridae family

1.2Herpes Simplex Virus Type 1

1.2.1HSV-1 structure:

The HSV-1 virion often has a pleomorphic appearance when seen by electron microscopy (Fig 1). It has a 150–300nm diameter and is composed of an icosahedral protein capsid containing a double-stranded DNA genome, that is enveloped by a lipid membrane with numerous glycoprotein spikes. Between the envelope and capsid is an amorphous layer, the tegument, which contains multiple virus-encoded proteins (Fig 3).

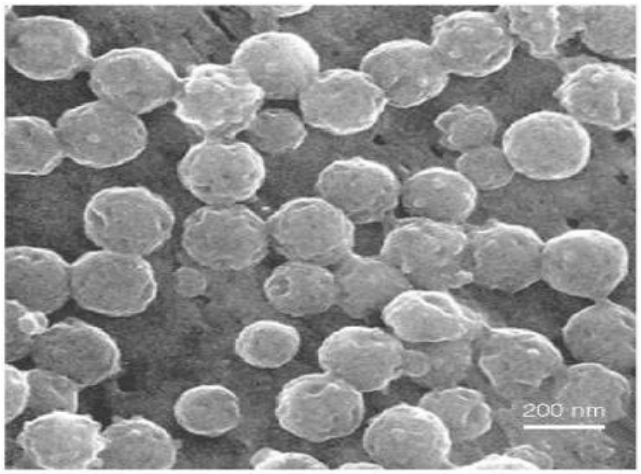
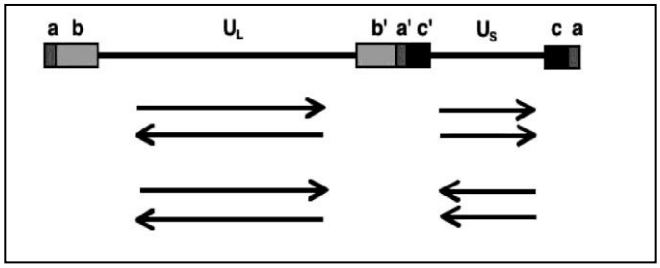


Fig 1: Scanning electron micrograph of HSV-1. Image was taken by SEM after infection of cultured cells.
(Picture courtesy of Dr Charles Grose)

DNA core: HSV-1 DNA is a linear double-stranded molecule. Its size varies for different herpesviruses, from approximately 80,000 to 150,000kDa (125–245kbp, 152 kbp for HSV-1). Within the virion the DNA is a linear molecule with its ends appearing to attach to the inner surface of the nucleocapsid, thus preventing DNA circularization until it is released during infection. It circularizes within the host cells during latency. Because replication takes place inside the nucleus, herpesviruses can use both the host's transcription machinery and DNA repair enzymes to support a large genome with complex arrays of genes.HSV-1 DNA is arranged as long and short unique segments (U_L and U_S) flanked by inverted repeats (ab, b'a' and c', c respectively, fig.2). Inversion of the two segments during DNA replication produces 4 genome isomers at equal frequencies in wild-type strains.

Fig 2: HSV-1 DNA arrangement in long and short unique segments. Schematic representation of inversions during replication resulting in four genome isomers



Nucleocapsid: The icosahedral nucleocapsid is 100–110nm in diameter comprising 162 individual capsomers (i.e. 12 pentavalent and 150 hexavalent capsomers). In HSV-1, individual capsomers are believed to be constructed from the four major capsid proteins VP5, VP26, VP23 and VP19. The interior and exterior of the capsid appear to be linked by transcapsomeric channels. *(Haarr L., et al. 1994).*

Tegument: The historically described as amorphous electron-dense tegument lies between the nucleocapsid and the envelope of the virus. Increasing evidence suggests that there is an ordered addition of tegument during assembly. It contains 20 virus-encoded proteins, which serve important functions after the virus has penetrated the host cell.*(Kelly BJ, et al.. 2009)*

Envelope: The viral envelope is formed from a budding of the original host cell nuclear membrane, in which more than ten viral glycoproteins are embedded. These glycoproteins have functional roles in the process of attachment and penetration to the host cell membrane *(Heldwein EE., et al., 2009)* . In HSV-1 there are at least ten glycoproteins on the viral envelope.

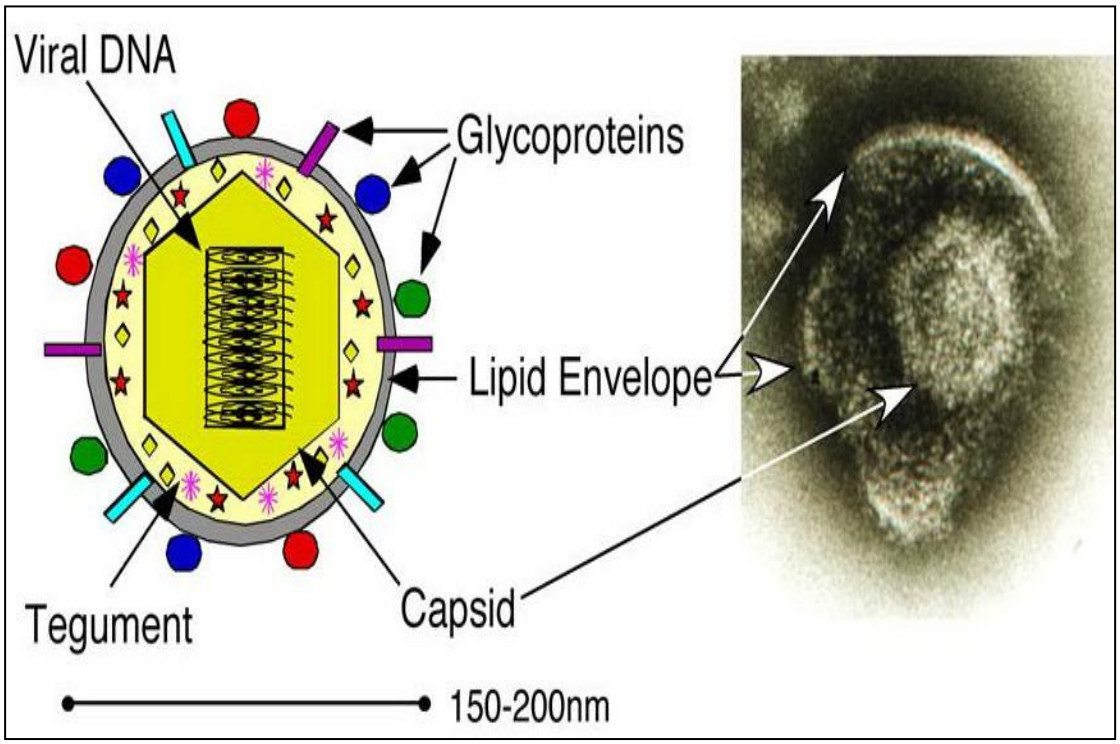


Fig 3: HSV virion structure. The HSV virion is comprised of four major features: (1) the viral double-stranded DNA; (2) an icosahedral capsid shell; (3) a tegument layer containing numerous viral proteins; and (4) a lipid envelope where several viral glycoproteins are embedded. The major structural components of the virion can be seen in an EM photograph (right) where the viral envelope is ruptured and folded back. (Picture courtesy of Travis J Taylor**,** Boston, MA)

1.2.2.HSV-1 replication cycle

1.2.2.1.HSV-1 gene expression during lytic infection

The life cycle of HSV-1 is characterized by an initial phase of lytic infection in epithelial cells, followed by a latent phase in the neurons of the trigeminal ganglion. During the lytic phase, attachment of the virus to the host cell membrane by interaction of viral glycoproteins with cellular receptors leads to membrane fusion and the release of nucleocapsid and tegument components to the cytoplasm. The viral capsid is then transported to the nuclear pores, through which the viral genome is released into the nucleus.

The major viral transcriptional activator protein, virion protein 16 (VP16), which is one of the tegument proteins, is also transported to the nucleus by mechanisms yet undefined. VP16 forms a complex with two cellular proteins : octamer DNA-binding protein (Oct-1) and host cell factor (HCF-1), and binds to specific *cis*-acting sequences in the promoters of immediate early (IE) or α genes to stimulate their transcription. Viral mRNAs are transcribed by cellular RNA polymerase II. IE genes encode for regulatory proteins (infected cell proteins ICP0, ICP4, ICP22, ICP27, ICP47), whose expression peaks at 2-4h postinfection. IE genes are required for the subsequent synthesis of all viral proteins. They transactivate the expression of early (E) or β and late (L) or γ genes. Early genes encode for proteins that regulate DNA replication, such as a DNA polymerase, an ssDNA-binding protein, a helicase, an origin-binding protein and DNA repair enzymes. Their synthesis peaks at 5-7 hours postinfection. Viral DNA replication starts shortly after β protein synthesis and continues up to 15 h p.i. After DNA synthesis, HSV-1 gene expression ends with the synthesis of late proteins, which are structural and assembly proteins *(Lehman IR, et al., 1999)*.

In order to facilitate viral transcription, HSV-1 inhibits rapidly the host cell gene expression. Several mechanisms have been implicated in this inhibition, such as host mRNA degradation by virion-associated host shut-off (vhs) protein, inhibition of host gene splicing by ICP27 and alteration of cellular RNA pol II localization and/or phosphorylation *(Smith R, et al., 2008).*

The nucleus is reorganized to form replication compartments in which viral DNA is replicated and transcribed and progeny nucleocapsids are assembled. The nucleocapsids acquire tegument proteins and an envelope during budding through the inner nuclear membrane. Extracellular virions are produced by de-envelopment of the nucleocapsids at the outer nuclear membrane, which is followed by budding into the Golgi apparatus and secretion to the outside of the cell (fig 4). Progeny viruses can infect surrounding cells and cause either primary herpetic disease or an asymptomatic infection *(Britt,W.J. Fields in virology).*

ICP0, one of the five IE proteins plays a central role in regulating whether HSV-1 progresses to lytic or latent infection. ICP0 is a member of the family of E3 ubiquitin ligase enzymes that have a RING finger zinc-binding domain. This domain confers on ICP0 the ability to induce the proteasome-dependent degradation of a number of cellular proteins. This process results in multiple consequences, including the disruption of cellular nuclear sub-structures known as ND10 or PML nuclear bodies. ND10 structures form around the incoming viral DNA and are implicated in transcriptional repression of viral genomes. This could be a possible way that ICP0 activates transcription. Interestingly, prior to the disruption of ND10, parental HSV-1 genomes become closely associated with these structures during the early stages of HSV-1 infection. The E3 ubiquitin ligase activity of ICP0 correlates very well with its role in the stimulation of lytic virus infection and induction of reactivation of latent or quiescent viral genomes. However, despite intensive investigation, the mechanism of the connection between the E3 ubiquitin ligase activities of ICP0 and its roles during HSV-1 infection remain poorly understood *(Everett RD et al., 2000, 2008, 2009, 2010).*

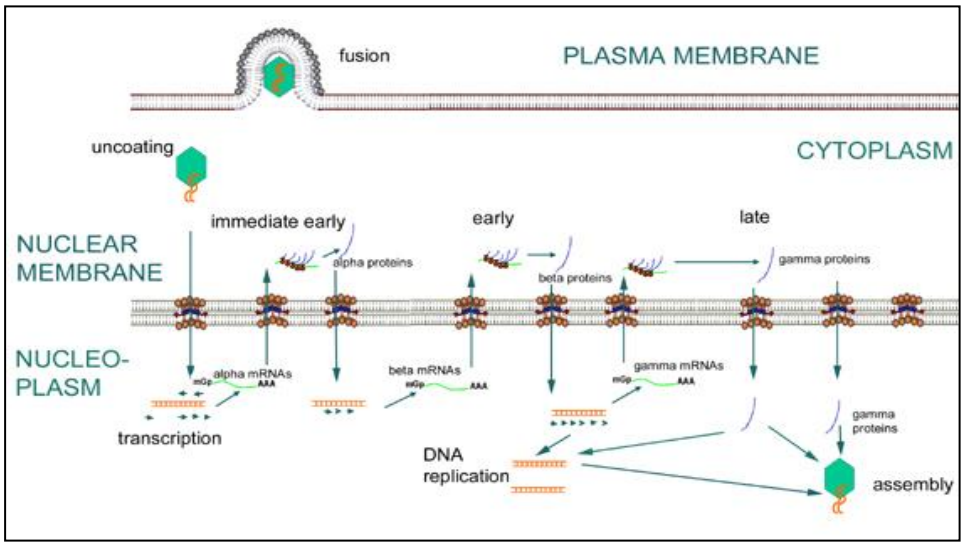


Fig. 4: Schematic representation of productive lytic HSV-1 infection.

1.2.2.2.HSV-1 gene expression during latency

During HSV-1 spread in epithelial cells at the primary site of infection, axonal termini of sensory neurons are also infected. After fusion of the envelope with the axonoplasmatic membrane, the nucleocapsid is carried via retrograde axonal transport to the nucleus in the cell body of the neuron in the respective sensory ganglion. Since HSV-1 infection typically occurs via the oral, ocular or nasal route, the trigeminal ganglia are a primary site for latency. During latency, two changes occur in the organization of the viral genome that may influence viral gene expression. First, the viral DNA is present in a non-replicating state as a circular episome. Second, the viral genome is associated with cellular histones and thus exists as chromatin in latency infected neurons. *(Britt,W.J. Fields in virology).*

Several host and viral factors are implicated in the establishment of latency. Abundant expression of the latency-associated transcript (LAT), repression of α-gene expression, different mechanisms of VP16 reduction in neurons are some of the viral factors reviewed by *Miller CS, et al., 1998* (fig.5). LATs are a highly conserved family of four RNA species derived from sequences within the long, inverted repeat segments of the HSV-1 genome. In sharp contrast to other HSV-1 promoters, the promoter that directs the expression of LAT is activated in sensory neurons only, and therefore LATs are abundantly transcribed in latently infected neurons. LATs do not encode for a protein. They function as primary microRNA (miRNA) precursors that encode four distinct miRNAs in HSV-1 infected cells, which silence immediate-early gene expression *(Umbach JL, et al., 2008)*.

Although the mechanisms for the establishment of latency are yet poorly understood, reactivation requires the immediate early protein ICP0, which is also required for efficient initiation of lytic infection.ICP0 regulates circle formation, and that circle formation may lead to latency, whereas DNA replication during productive infection prefers a linear template *(Jackson SA, et al., 2003)*.

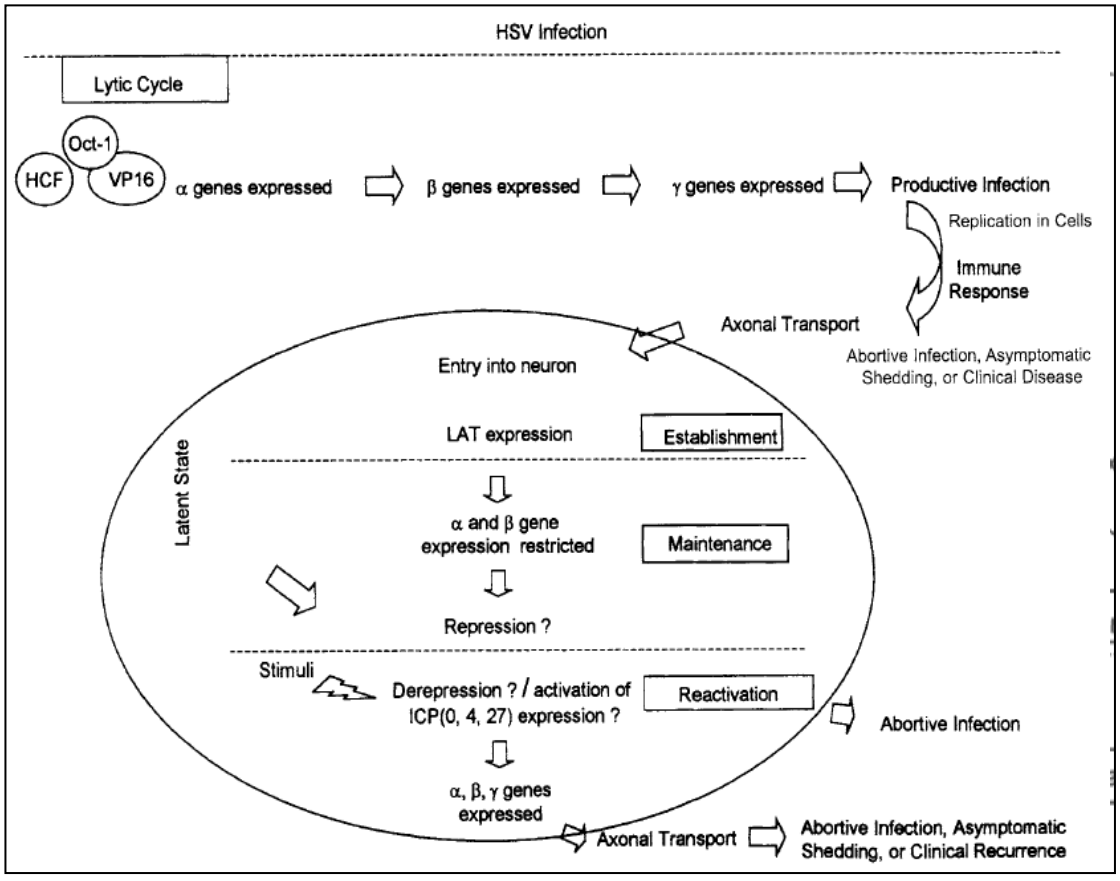


Fig. 5: Gene expression during lytic and latent HSV-1 infection (Picture courtesy of C.S.Miller, Kentucky)

1.3. Viruses versus the host cell

Studies on viral gene function revealed that infected cells mount an unsuccessful multi-layered defense that includes attempts to silence the viral genome at early stages of infection, activation of interferon pathways, turnover of cellular proteins required by the virus, and finally attempts to commit suicide through programmed cell death to salvage the infected host. The surprising finding is that multiple viral functions expressed by different viral proteins or domains thereof target the same cellular pathways to render them dysfunctional. The grand design of viral conquest of the host cell is to sequester and redirect cellular proteins to (i) preclude the infected cells from signaling it status to the environment or be the recipient of signals from the environment, (ii) disrupt the interferon defense mechanisms, and (iii) perform novel functions required by the virus.

Viruses have evolved to hijack host cellular signaling pathways in order to promote their replication. Cell signal modulation during virus infection is mainly a consequence of the binding of virus to its cellular receptor, cross talk between viral and cellular proteins, and stress caused by the infection. Cellular signaling cascade manipulation has been extensively studied for a series of viruses. MAPK pathways, such as the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), and the p38 MAPK cascade, TGF-β pathway, JAK/STAT cascade, IFN and IL-1 related pathways, IGF, apoptotic and survival pathways etc., all have been documented to be employed from viruses. Several viruses are known to activate the ERK pathway early during infection. It is possible that the activation of this pathway may be a strategy used by these viruses to drive cells into hosting virions in a more permissive environment.

1.4.The ERK pathway

ERKs belong to a larger family of mitogen-activated protein kinases (MAPKs). Mitogen-activated protein kinase (MAPK) cascades are important signaling pathways that convert extracellular signals into cellular responses. They regulate proliferation, differentiation, cell activation and immune responses. Four different members organized in separate cascades have been identified so far: ERK (extracellular signal-regulated kinase), JNK (Jun-N-terminal kinase), p38 and ERK5. For each MAPK, different isoforms are known. All these enzymes are activated by phosphorylation mediated by an upstream MAPK kinase (MAPKK, MEKs or MKKs). The MAPKs ERK1/2 are activated by the MAPKKs MEK- 1/2 that are controlled by the MAPKKK Raf. Raf, MEK and ERK form the prototype module of an MAPK pathway (fig. 6).

The ERK cascade has been implicated in the regulation of several cellular processes, including cell growth and survival, proliferation, differentiation, oncogenic transformation, cytokinesis, cell adhesion, migration and apoptosis. Deregulation of the pathway has been associated historically with cancers *(Malumbres M, et al., 2003)*, but also, more recently, with other pathologies, such as polycystic kidney disease, severe developmental disorders and diabetes *(Lawrence MC, et al., 2008)*.

The signaling through the cascade is initiated in the cell membrane in response to many different signals, such as growth factor receptors, G-protein coupled receptors, integrins, src and fyn tyrosine kinases. The canonical ERK pathway is stimulated upon the binding of extracellular growth factors, such as EGF, to their respective receptor tyrosine kinases (RTKs). RTKs dimerize and the autophosphorylation of the cytoplasmic tails of the receptor on tyrosine residues leads to the recruitment of Grb-2, which binds the guanine nucleotide exchange factor SOS. Recruitment of SOS to the cell membrane promotes its interaction with the membrane-localized small GTP-ase Ras protein. SOS promotes the release of GDP from Ras, which subsequently binds GTP. GTP-Ras recruits to the membrane and activates Raf by phosphorylation. Raf in turn phosphorylate MEK1/2 to their active forms, which subsequently activate ERK1/2. *(Torii S, et al., 2004, May LT, et al., 2008)*.

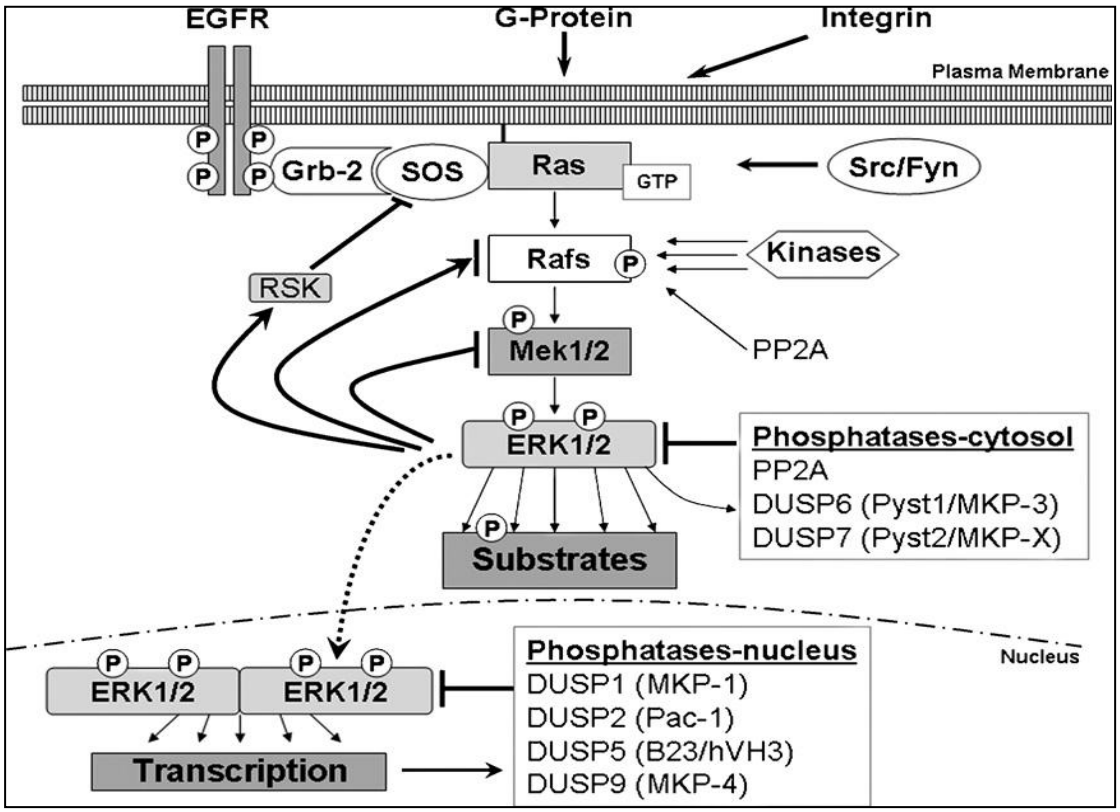


Fig 6: ERK cascade and regulation.(Picture courtesy of Joe W Ramos,USA)

Upon stimulation, ERKs have been demonstrated to phosphorylate a large number of substrates. Some of these substrates are localized in the cytoplasm, while others are

phosphorylated in the nucleus by ERK molecules that are translocated upon activation. Notably, ERKs phosphorylate and activate a series of transcription factors such as Elk1, c-Fos, c-myc, p53, Ets1/2, TFIIIB, and even c-Jun, which are important for the initiation and regulation of proliferation and oncogenic transformation. Other nuclear targets of ERKs are nuclear kinases. They can also phosphorylate various kinases (ribosomal protein S6 kinases-RSKs) and structural proteins in the cytosol, while at the plasma membrane they target proteins that regulate cell adhesion, cell-cell communication and cell survival. The outcome of ERK pathway activation in a given cell will therefore be determined in part by where active ERKs are targeted in the cell and which substrates they have access to at those locations *(Mebratu Y et al., 2009)*.

ERK1 and ERK2 (also known as p44 and p442 MAPK, respectively), are proteins of 44 and 42 kDa that have nearly 85% amino acid identity. Both are ubiquitously expressed in almost all tissues, although their relative abundance in tissues is variable. These quantitative differences could account for functional discrepancies, derived from a competition between ERK1 and ERK2 in binding to the upstream kinase MEK. *(Vantaggiato C, et al., 2006)*. ERK1 KO mice have apparently improved functions (increased rate of learning, better long-term memory) attributable to enhanced ERK2 activity. Moreover, mouse embryo fibroblasts from ERK1 KO mice proliferate faster and have increased transforming activity than control cells, due to ERK2 activity. Those findings suggest an inhibitory effect of ERK1 on ERK2 *(Lloyd AC, et al., 2006)*.

ERK is able to transmit different, and even opposing, signals in the same cells. Several mechanisms determine signal specificity for ERK cascade. Scaffolding proteins enhance or target ERK activation, offer protection from phosphatases, or even recruit different substrates, leading to different functions. Subcellular localization of ERKs may be another mechanism for the final diverse outcomes in the fate of a cell. Nuclear translocation of ERK results in activation of transcription factors mainly implicated in mitogenic responses, while cytoplasmic retention prevents such responses or even potentiates the activation of proapoptotic cytoplasmic proteins *(May LT, et al., 2008; Mebratu Y, et al., 2009)*. Interplay with other cellular signaling pathways may have impacts on the strength of signals transmitted or may modulate the activity of downstream targets of the ERK cascade. Furthermore, the strength, duration and quality of triggers may influence the final signal transmitted. A fifth specificity-determining mechanism might be the existence of various isoforms with distinct regulations or functions in each tier of the ERK pathway. Further details for the complexity of this multi-level regulation of cellular responses by ERK cascade are reviewed in *O'Neill E, et al., 2004; Ramos JW, et al., 2008; Shaul YD et al., 2007*.

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1.5.HSV-1 oncolytic mutant viruses employing the ERK pathway

The use of viruses to destroy tumors, also known as viral oncolysis, dates back to the early 1900's. The earliest case reports of viral oncolysis document spontaneous regression of cervical carcinoma following rabies vaccination *(Dock G., 1904)*, and remission of Burkitt's and Hodgkin's lymphomas after natural infection with measles *(Bluming AZ., et al., 1971; Taqi AM., et al., 1981)*. Since then, advances in tumor biology, molecular biology and virology contributed in the development of oncolytic viruses, which to date have been taken to clinical trials *(Fukuhara H., et al., 2009)*. One of the greatest challenges in viral oncolysis is the development of successful strategies to maximize viral replication in tumor cells and minimize their replication in normal cells. One strategy to target viruses to tumors involves deletion of a viral gene that is necessary for viral replication but whose function can be substituted preferentially by cancer cells. The HSV-1 mutant hrR3 is defective in expression of the ICP6 gene encoding the large subunit of viral ribonucleotide reductase (RR), which is inactivated by insertion of the Escherichia coli b-galactosidase gene.*(Goldstein DJ., et al., 1988)*. HSV-1 is an attractive candidate for viral oncolytic applications as it is highly infectious, can be genetically engineered with ease, has a large capacity for transgenes, and can be grown to high titers. Furthermore, it does not integrate in the cellular genome as retroviruses do, and rarely causes severe disease (*Kuruppu D, et al., 2007)*. Killing of tumor cells is achieved via several different mechanisms, mainly by direct cell lysis due to the completion of viral cycle, and apoptosis due to the heavy biochemical burden of the viral replication *(Everts B., et al., 2005)*. Other mechanisms involve direct cytotoxicity of viral proteins, induction of antitumor immunity or expression of therapeutic transgenes *(Kuruppu .,et al., 2005)*. HSV-1 kills tumor cells via cell lysis upon completion of its replication cycle.

Several oncolytic mutant HSV-1s have been developed , many of which are involved in the ERK signaling pathway of the host cells as a means to preferentially target the malignant ones. The most widely used mutants for assessment of clinical efficacy in the treatment of tumors lack both copies of the $\gamma_1$34.5 gene and are exemplified by the recombinant R3616 *(Veerapong et al., 2007)*. Activation of double-stranded RNA-dependent protein kinase (PKR) constitutes a major host defense to infection by viruses. The key consequence of activation of PKR is phosphorylation of eIF-2α and the ensuing shutoff of protein synthesis. Viruses have evolved numerous mechanisms to block either the activation of PKR or the consequences of the phosphorylation of eIF-2α. In the case of HSV-1, two genes suppress the effects of PKR. One viral protein, US11, if expressed early, blocks the activation of PKR *(Cassady KA, et al., 1998)*. The more important viral protein that has evolved to subvert the consequences of activation of PKR is ICP34.5 *(Chou J, et al., 1990)*. The $\gamma_1$34.5 gene product interacts with cellular protein phosphatase-1 to dephosphorylate eIF-2 and initiate protein translation necessary for viral replication (fig 7). Transduction of a cell line

with a constitutively active MEK gene confers susceptibility to the R3616 mutant virus, whereas cells transduced with a dominant-negative MEK gene become more resistant to the recombinant virus. In cells transduced with the constitutively active MEK, PKR is not activated in contrast to cells transduced with the dominant negative MEK. The cell lines with activated MEK supported increased viral replication, as shown by higher viral yields and more viral proteins *(Smith KD et al., 2006)*. It seems that activated MEK suppresses PKR autophosphorylation and effectively blocks PKR-mediated eIF-2 phosphorylation, and that the status of MEK predicts the ability of these mutant viruses to replicate preferentially in tumor cells. Moreover, it has been reported that systemic delivery of R3616 can selectively target and destroy human xenograft tumors in mice that overexpress MEK activity compared with tumors that express lower MEK activity *(Veerapong J, et al., 2007*). Phase I clinical trials in patients with malignant glioma revealed that HSV-1 mutants lacking the $\gamma_1$34.5 gene (G207 and HSV1716 respectively) are safe following escalating doses *(Markert JM, et al., 2000; Rampling R., et al., 2000).*

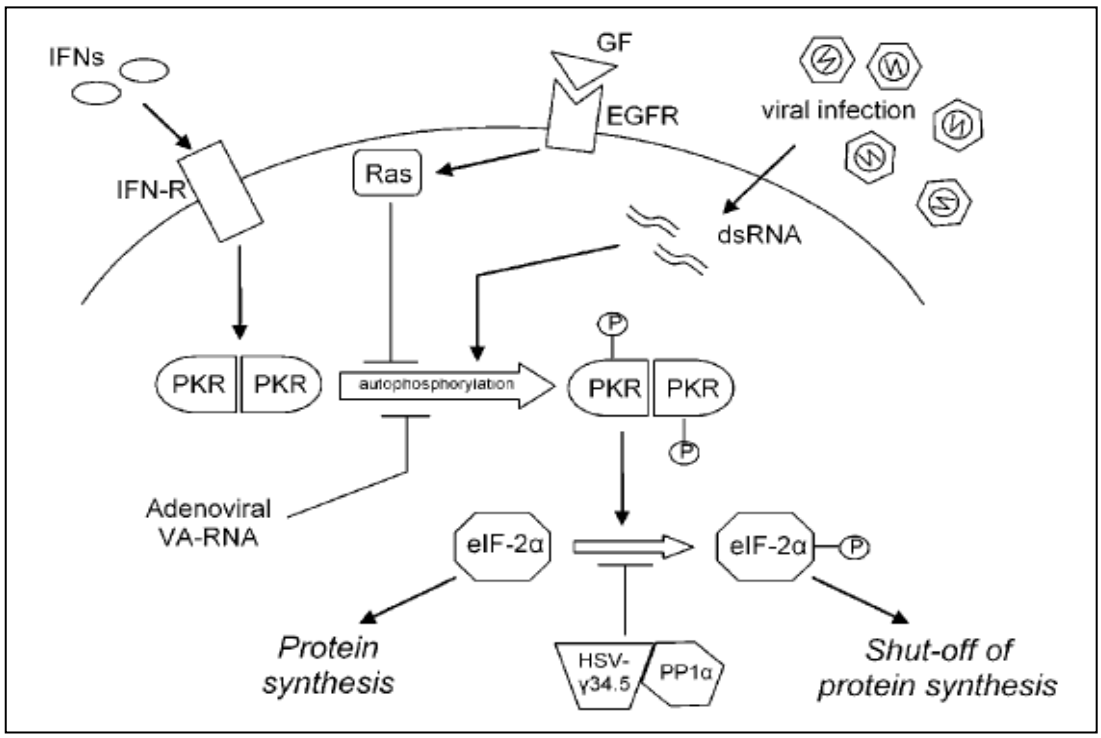


Fig 7: Role of PKR in viral infection.

A few years earlier *(Farassati F, et al., 2001)*, a model of HSV-1 infection has been proposed, in which the outcome of infection is determined by two main parameters: the host cell's Ras signalling activity, which can downregulate PKR function; and the virus's own anti-PKR mechanism (for example, ICP34.5 in HSV-1). Untransformed NIH-3T3 cells have very low anti-PKR activity (owing to low Ras activity) and are therefore not permissive to mutant HSV-1 (R3616) or even wild-type HSV-1 whose anti-PKR mechanism is intact. Cells with intermediate Ras signalling activity (and hence moderate anti-PKR activity, such as MEFs) would be permissive to wild-type

HSV-1 but not to R3616, unless the PKR gene is knocked out in these cells. Cells with high Ras, and therefore high anti-PKR activity (such as Ras-transformed cells), would be permissive to both wild-type HSV-1 and R3616 even though the latter's anti-PKR mechanism has been compromised. In the basis of this model, a mutant virus with its anti-PKR gene mutated or deleted would be better suited as an anti-cancer agent, as it would probably spare untransformed cells but would infect those cells with a high anti-PKR activity. Cancer cells with a highly activated Ras pathway would therefore be susceptible targets. It is interesting to note that another oncolytic virus in its naturally isolated state, reovirus, which is a double-stranded RNA virus unrelated to HSV, also exploits the host-cell Ras pathway to induce a lytic infection in transformed cells *(Marcato P., et al., 2005; Shmulevitz M, et al., 2005).*

G207 is another HSV-1 double mutant in which both copies of $\gamma_1$34.5 are deleted. UL39 (which encodes ICP6 RR) is inactivated by insertion of lacZ *(Kuruppu et al., 2005)*. It has been shown that among five different mouse malignant peripheral nerve sheath tumors (MPNST) cell lines, only the ones with elevated levels of Ras signaling are highly permissive to infection with oncolytic herpes G207. Specific inhibitors of the Ras, ERK, and JNK pathways all reduced the synthesis of viral proteins in MPNST cells. The cell lines that contained lower levels of Ras and decreased activation of downstream signaling components underwent an enhancement in apoptosis upon exposure to G207 *(Farassati F, et al., 2008).*Again, permissiveness to infection with the oncolytic strain is dictated by the ras-mediated PKR inhibition of phosphorylation, in addition to the cellular expression of nibonucleotide reductase *(Mahller Y, et al., 2006).*

A novel oncolytic HSV-1 mutant, named as Signal-Smart 1 (SS1), was recently developed, in which the expression of ICP4 (infected cell protein-4, a viral protein necessary for replication) is controlled by activation of ELK, a transcription factor down-stream of the Ras pathway and mainly activated by ERK *(Pan W, et al., 2009).* This mutant has an ELK binding site in the promoter region of its recombinant alpha-4 gene. Prostate cells with increased ELK activation were preferentially infected by the SS1 virus, as demonstrated by increased levels of viral progeny, gC and overall viral protein production. Exposure of cancer cells to the mutant virus in turn resulted in alteration of cell properties such as decreased proliferation and invasiveness and increased apoptosis. Additionally, high Ras signaling cells infected with SS1 showed a prominent arrest in the G1 phase of the cell cycle as compared to cells exposed to parental HSV-1. ELK in fact could be a suitable factor for predicting tumor susceptibility to oncolytic mutant HSV-1 strains *(Esfandyari T, et al., 2009).*

2. AIMS

We aspired to check for possible activation of ERK pathway upon HSV-1 infection, to define ERK dynamics in the course of lytic cycle of HSV-1 and to investigate the role of a functional ERK pathway on HSV-1 reproducive cycle.

Specific aims:

- To produce adequate HSV-1 WT (17+) stock and titrate the virus
- To define the spatiotemporal localization of p-ERK in the context of viral infection
- To demonstrate the changes in the amount of p-ERK during the infection
- To identify the impact of ERK pathway inhibition on p-ERK localization and amount during the infection
- To investigate the importance of a functional ERK pathway for the replication of HSV-1

3. MATERIALS AND METHODS

3.1.Cell culture and reagents:

HSV grows well in a wide variety of cell types to yield high-titer stocks. Cell lines routinely used to grow HSV include BHK , Vero, RK13 (rabbit kidney) and CVl (monkey kidney). BHK (Baby Hamster Kidney) cells were used for viral propagation and titration, as they are known to be easily infected with HSV-1 and proliferate quite rapidly. They were cultured in Glaskow MEM BHK21 (Biosera, Cat. N^o :L0221-500) enriched with 10% new born calf serum (Biosera, Cat. N^o :NB-112/500), 10% Tryptose phosphate broth (Sigma) and 1ml penicillin/streptomycin from p/s solution 100x (Biosera, Cat. N^o :XC-A4122/100). Microscopy and biochemistry experiments were performed in Vero (African green monkey kidney) cells, commonly used to study HSV-1 replication. They are an ideal ex-vivo model for HSV-1 replication due to the tropism of the virus to epithelial cells and neurons. Vero cells were grown in high glucose DMEM (Dulbecco's modification of Eagle medium- Biosera, Cat. N^o : LM-D1110/500), supplemented with 10% fetal bovine serum (FBS-Biosera) and 1ml P/S as above, for their proliferation, whereas the experiments were performed with overnight serum starvation (1% FBS-DMEM) because ERK can be activated by serum *(Lidke DS, et al., 2009).* Cell lines were grown as monolayers in 25cm^2, 75cm^2 and 175cm^2 cell culture flasks or 12/24-well plates (Nunc-Thermo Fisher Scientific), at 37 °C, in an atmosphere of 5% CO_2 and 80% humidity, to ensure a normal development of the cells in a contamination-free environment.

In some experiments, cells were pretreated (1 h, 37°C) or co-treated(at 4 h p.i., 37°C) with the MEK-specific inhibitor U0126 (Upstate- Lot. N^{o} : 0611046576), at the concentrations given in Results.

3.2.Viruses, viral propagation and purification:

All experiments were performed with the wild-type strain of HSV-1 (HSV-1 17+) provided by Everett RD, Glaskow,UK. , in order to simulate natural viral infection. Viral stocks were obtained and titered as previously described *(Killington RA, et al., 1985)*. More precisely, in order to produce an adequate HSV-1 WT stock, BHK cells were cultured consequently in 25cm^2, 75cm^2 and 175cm^2 flasks and when confluent in the latter one, they were infected with HSV-1 WT. Infected cells were incubated until the production of cytopathic effect, detected as detachment of cells from the flask (48-72h). Cells and supernatant were collected in order to isolate the intracellular virus (Cell Associated Virus – CAV) and the virus produced and released during the lytic infection of cells (Cell Released Virus – CRV), respectively. Cells and supernatant were separated by spinning at 2.000 rpm for 10' at 4 ^{o}C. The cell pellet was resuspended in 1ml fresh GMEM and the suspension was sonicated in order to release CAV. The sonicated suspension was then filtered in sterile conditions (Acradisc Syringe filters 25mm -Life sciences) to withhold cell debris and contaminants and stored at -80^{o}C as cell-associated virus. The supernatant isolated from the first spin was centrifuged at 13.000 rpm for 2h at 4^{o}C and the pellet virions were then resuspended in 1ml fresh GMEM and stored at -80^{o}C as cell-released virus.

3.3.Plaque (viral progeny) titration assay:

The basis of the technique is to measure the ability of a single infectious virus to form a "plaque" on a confluent monolayer culture of cells. When HSV infects a single cell, the surrounding cells will also become infected by spread of progeny virus from cell to cell. This focus of infection normally causes cell necrosis, resulting in a hole in the monolayer with rounded cells at the periphery. Alternatively, certain virus strains can pass from cell to cell and cause fusion of the infected cells, resulting in a syncitium. For either type, these foci of infection are called plaques and are a measure of the number of infectious particles within a virus stock. Plaque assay calculates the viral cytopathic effect in vitro using a series of different dilutions of the viral stock. Briefly, BHK cells were cultured in 35 mm dishes until 90% confluence (4 x 10^{5}cells). The medium was then aspirated leaving approximately 100μl for maintaining the cell monolayer wet. 10μl from the viral stock(CAV or CRV) were diluted in a series of dilutions from 10^{-2} to 10^{-6} or less . 100μl from 10^{-3} – 10^{-6}(or 10^{-8}) dilutions were used to infect 4-6 wells. The last well was mock infected with 100μl PBS(phosphate buffered saline).(fig 8).

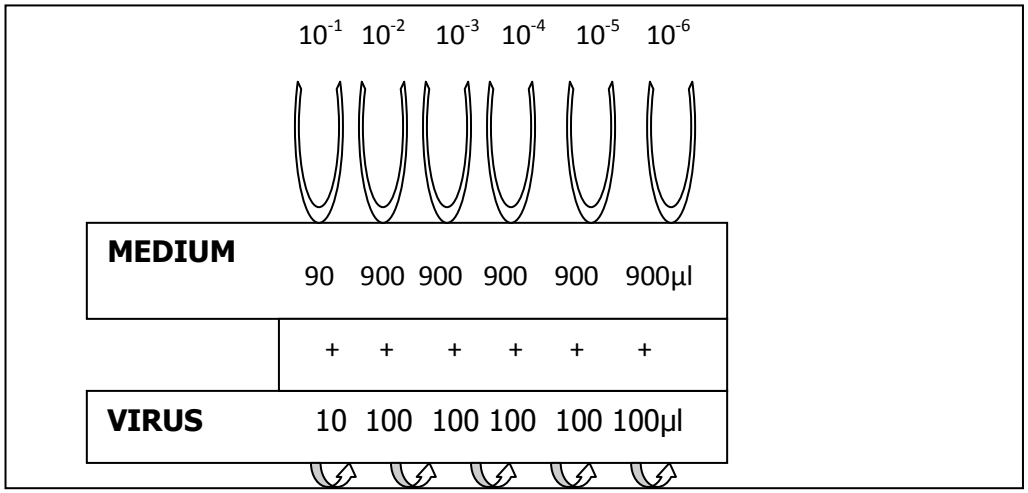


Fig 8: Schematic representation of serial dilutions for plaque assay.

Cells were then incubated for 2 hours at 37°C, 5%CO_2 and gently moved with circular movements every 15 minutes to achieve sufficient viral cell entry throughout the whole monolayer. Approximately 10 minutes before the incubation period, the dilution of anti-HSV-1 human serum (obtained from patients of the University Hospital of Heraklion) was prepared (1:100). At the end of the incubation period the infective inocculum was completely aspirated and replaced by 3ml of BHK fresh medium containing 30µl of anti HSV-1 IgG serum for each well, until the development of cytopathic effects in the form of plaques which usually occurs within 48h. At this point, a monolayer was fixed with methanol and stained with Giemsa solution for 10 minutes. The number of clear plaques was then determined under a stereoscope by using the plate with the maximal dilution of the virus which still had well-defined plaques and counting the corresponding number of viral plaques. The viral titre was expressed in plaque forming units (p.f.u.)/ml as: (number of plaques) x 1/(serial dilution used) x 10 (conversion of 100 µl to 1ml).

3.4.Protein extraction:

Cells from different time points were detached with PBS-1mM EDTA and collected separately at 4°C. Cell lysis and protein extraction was performed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Cat. No: 78503) according to the protocol provided. Cells were centrifuged at 2.500 g for 10min at 4°C and the cellular pellets were resuspended with 40 µl M-PER each and then centrifuged at 14.000 g for 15min. The supernatants containing the protein extracts were recovered and stored at -20°C until use.

3.5.Western Blot Analysis:

Each condition-time point had similar numbers of Vero cells, thus it was assumed that protein concentrations were similar too. Twenty µg of stored protein extracts

were boiled with the appropriate amount of loading dye (10 μl if stock: 3x) for 10min at 95 ^{o}C. The boiled loading mixes were loaded to 0,75 mm 12% polyacrylamide gels together with 8-9 μl of a prestained protein ladder (Fermentas, Lot No: 00032231). The gels were prepared as follows:

Running gel 12%:

Concentrated solution acrylamide 30% (29:1)	4ml
Resolving gel buffer (RGB)	2,5 ml
Water for injection (WFI)	3,3 ml
SDS 10% (BDH)	100 μl
TEMED (Sigma-Aldrich)	4 μl
APS 10%	100 μl

Stacking gel 5%

Concentrated solution acrylamide 30% (29:1)	850 μl
Stacking gel buffer (RGB)	625 μl
Water for injection (WFI)	3,4 ml
SDS 10%	50 μl
TEMED	5 μl
APS 10%	50 μl

After the loading, the gels were placed in the apparatus, where more than 500 ml of running buffer 1X were added. The samples were separated at 80-120 V and transferred to a nitrocellulose membrane (Thermo Scientific, Lot No: JF1143451) in a bath containing cold transfer buffer at 350 mA for 60min. (chromatography paper: Whatman, Cat. No.:3030 861). The blots were incubated (1 h at RT) in Tris-NaCl-Tween buffer (TBS-T) containing 5% milk to block non-specific binding and then exposed overnight at 4 ^{o}C to appropriate primary antibodies diluted in TBST-1% milk. The antibodies used were anti-mouse HSV-1 ICP0 1:200 (Santa Cruz Biotechnology, 11060:sc-53070), anti-rabbit p-ERK1/2 1:1.000 (Millipore, Cat. No: 05-797R), anti-mouse HSV-1 ICP8 1:200 (Santa Cruz Biotechnology, 10A3:sc-53329), and anti-rabbit ERK2 1:200 (Santa Cruz Biotechnology, C-14:sc-154). After extensive washing, the membranes were exposed to anti-mouse (1:2.000 dilution, Chemicon International, Cat No:AP124P) and anti-rabbit (1:10.000 dilution, Millipore, Cat No: AP132P) IgG conjugated to horseradish peroxidase (HRP). Again after extensive washing, bands were detected using enhanced chemiluminescence substrates for the detection of HRP (Thermo Scientific, Lot No: JI123201)) and
exposure to film manually.

3.6.Immunofluorescence:

Vero cells were plated on glass coverslips in 24-well plates and when confluent, they were washed with phosphate buffered saline (PBS), fixed with 1x fixation solution (Chemico, Cat No:3247) for 10min, washed with PBS-1%FBS and then permeabilized with 1x permeabilization solution (Chemico, Cat. No:3247) for 10min. The coverslips were incubated for 1 h at room temperature in dark humid conditions with primary antibodies diluted in PBS-1% FBS (20 μl of antibody per coverslip diluted 1:100) and then washed several times before treatment with secondary antibodies (20μl per coverslip diluted 1:500) in the same manner. The primary antibodies used were anti-rabbit p-ERK1/2 and anti-mouse HSV1-ICP0. The secondary antibodies used were Alexa 488 conjugated anti-mouse or anti-rabbit IgG (Invitrogen, Cat. No: A11008) or Cy3 conjugated anti-rabbit (Invitrogen, Cat. No: A10520) respectively. Cells were counterstained with DAPI (2μl DAPI working solution in a slight amount of PBS-1%FBS) for less than 5min.The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA. Then the coverslips were mounted on slides. Dried slides were stored at 4^0C and examined with a microscope. Fluorescence activity was tested at UV light with LEICA inverted microscope.

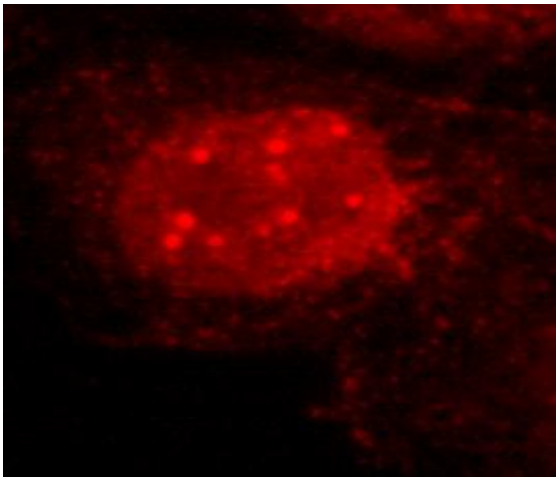
4. RESULTS

4.1.HSV-1 infection induces the activation of ERK and promotes the translocation of p-ERK in the cell nucleus

In order to define p-ERK spatiotemporal localization, Vero cells, plated on coverslips in 24-well plates, were infected with 4 μl HSV-1 WT after overnight serum starvation and fixed at 15min, 30min, 1h, 2h, 4h, 8h, 12h post-infection. A coverslip not infected served as mock. Immunostaining for mouse ICP0 (1:50)/Alexa488(1/500) and rabbit p-ERK2 (1:50)/Cy3(1:500) was performed. ICP0 (green) staining served as a marker of successful progressive viral replication, while DAPI counterstaining was used to trace the nuclei.

p-ERK (red) is initially located in the cytoplasm and translocates to the cell nucleus later in the course of infection, with progressively increasing fluorescence. Cells not triggered by HSV-1 (mock, fig 11) exhibit a baseline weak diffuse expression of p-ERK, mainly in the cytoplasm. At the beginning of the infection (15 min-1h), p-ERK's signal gradually increases in a perinuclear pattern, while 2 hours after the infection, activated ERK massively translocates in the cell nuclei. This indicates that ERK activation occurs first in the cytoplasm, and accumulates markedly in the nucleus afterwards. At 2 h p.i., HSV-1 immediate-early (IE) gene expression can be detected by the intense nuclear fluorescence of ICP0, which coincides with the peak of ERK phosphorylation and nuclear translocation.

Fig 9: Immunofluorescence for p-ERK. Intranuclear aggregates of p-ERK formed in the course of HSV-1 infection. They become more prominent and defined at 4h p.i.



Notably, p-ERK starts forming several punctate foci mainly at the nucleus periphery, which become larger circular well-defined nuclear aggregates at 4 h p.i.(fig. 9). As the infection progresses, a slight attenuation of p-ERK fluorescence is observed, with a second peak of massive nuclear translocation occurring at 8h p.i. This is the only time point that p-ERK locates exclusively within the nuclei (fig. 11). A similar biphasic ERK activation has been previously documented for other herpesviruses, such as *VZV (Rahaus M., et al., 2006).* Progeny nucleocapsids start to leave the nucleus at 12 h p..i. at half the time of the completion of HSV-1 replication cycle. At the same time point, p-ERK starts to relocate from the nuclei back to the cytoplasm (fig 10).

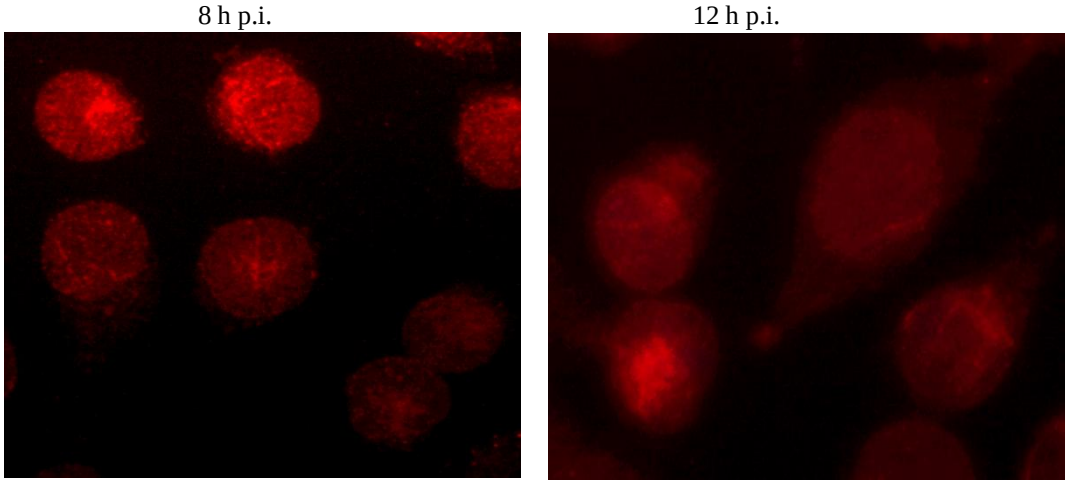


Fig . 10:Immunofluorescence for p-ERK at 8 and 12h after infection with HSV-1. Note that activated ERK exhibits an exclusively nuclear pattern of fluorescence at 8 h p.i. Four hours later, although still mostly intranuclear, p-ERK starts to diffuse back to the cytoplasm.

Although ERK is weakly activated in untriggered cells, HSV-1 infection induces a robust phosphorylation of ERK and its transient massive nuclear translocation in two waves.

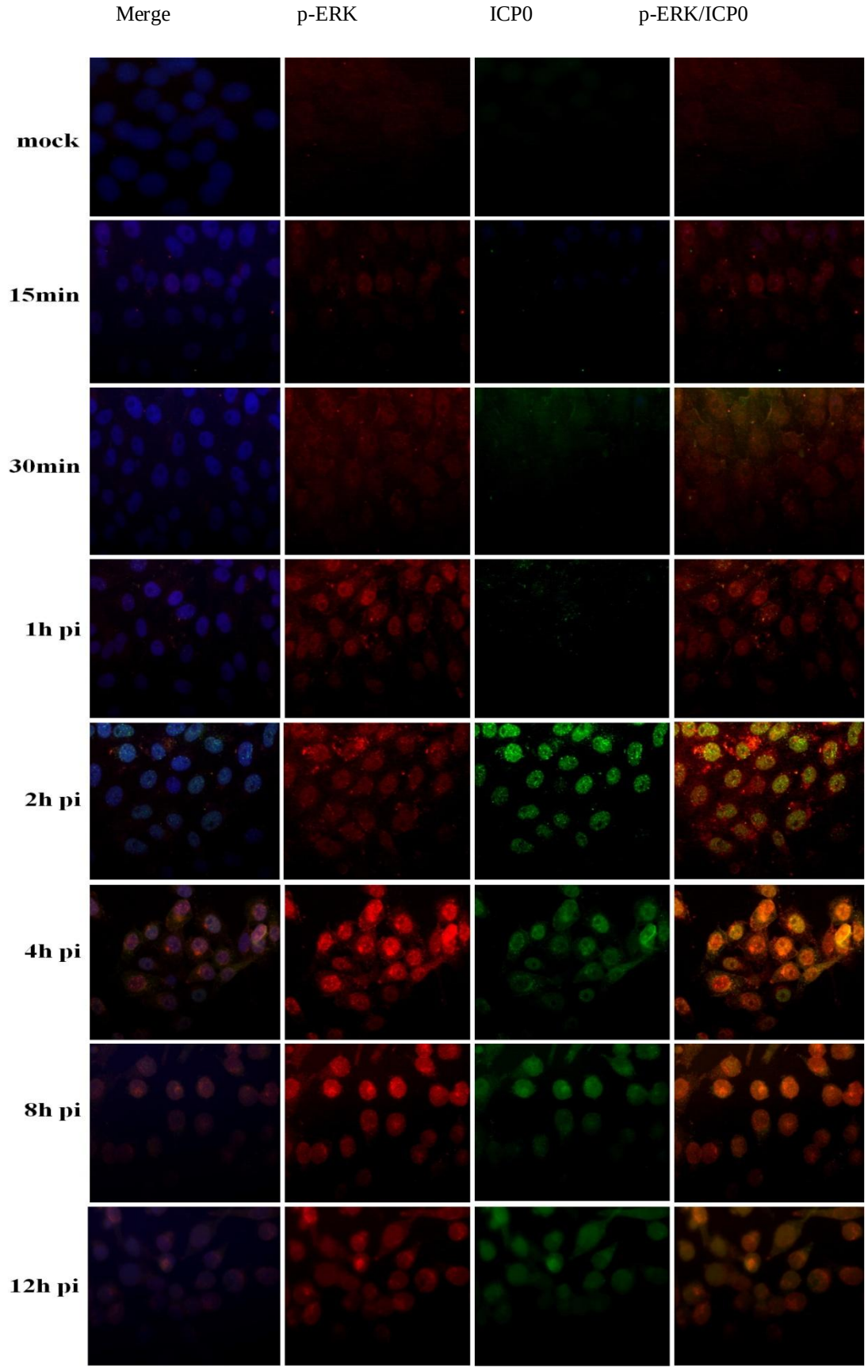


Fig. 11: Immunofluorescence for p-ERK (red) and ICPO (green) after HSV-1 WT infection at 15min, 30min, 1h, 2h, 4h, 8h, 12h. DAPI counterstaining.

4.2. p-ERK expression increases during HSV-1 infection

In order to demonstrate in another way the changes in the amount of p-ERK in the course of HSV-1 infection, Vero cells, cultured in 12-well plates, were infected with HSV-1 WT after overnight serum starvation for 15min, 30min, 1h, 2h, 4h, 8h and 12h. Proteins from cells of each time point were extracted and Western Blot for ICP0, ICP8, gG, p-ERK1/2 and ERK2 was performed. ERK2, as an abundant intracellular protein, served as the loading control and ICP0, ICP8, gG viral antibodies confirmed the progress of the infection. The expression of ICP0 and gG from 4 and 8 h p.i., respectively, demonstrates a successful infection (fig. 12). The amount of p-ERK, assuming that band intensity represents a semi-quantitative measure, seems to increase concomitantly to the time points of its increase in fluorescence intensity, as previously described. Uninfected (mock) cells have weakly detectable p-ERK.

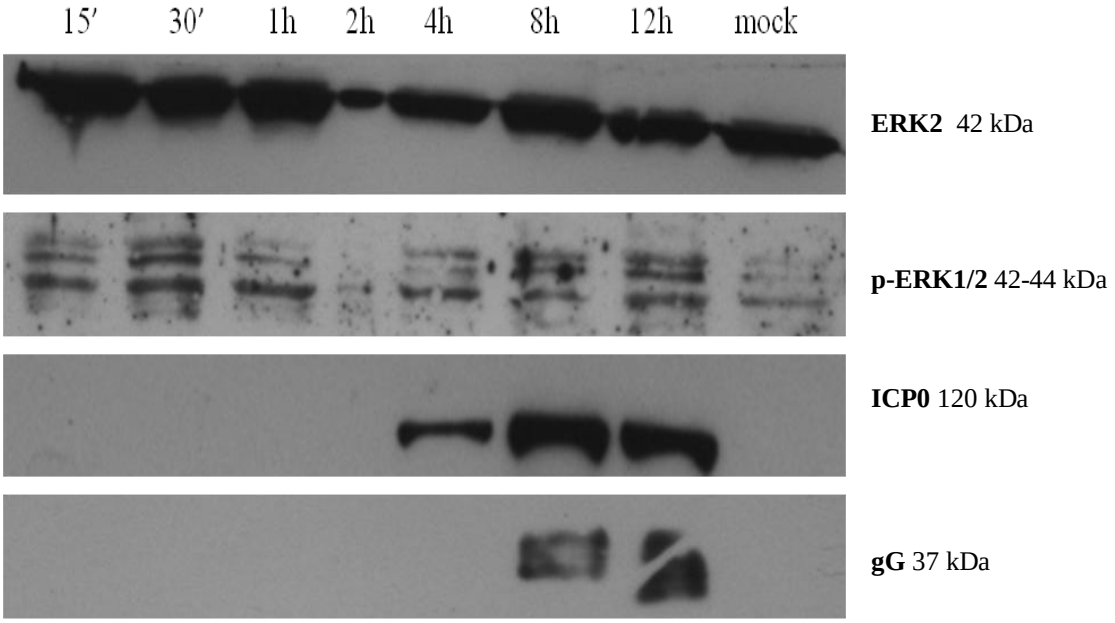


Fig.12: Western Blotting for ERK2, p-ERK1/2, ICP0 and gG at 15min, 30min, 1h, 2h, 4h, 8h, 12h after infection with HSV-1 WT. ERK2 represents the loading control. ICP0 and gG signify the presence of inection. P-ERK1/2 double bands were detected after overnight incubation of primary antibody at room temperature. Note the biphasic increase of band intensity at 30min-2h and 8-12h.

4.3. ERK pathway inhibition during HSV-1 infection results in a significant abrogation of the first wave of ERK nuclear translocation

In order to define the impact of ERK pathway inhibition on ERK dynamics, Vero cells were plated on coverslips in 24-well plates and exposed to overnight serum

starvation with DMEM-1%FBS. Then, after an 1 hour-course treatment with 1 μl of the MEK-specific inhibitor UO126 , they were infected with 4 μl HSV-1 WT and fixed at 15min, 30min, 1h, 2h, 4h, 8h, 12h post-infection. A coverslip not infected served as mock. Moreover another mock coverslip, subjected to 1 hour-course UO126 pretreatment, was used. Immunostaining for mouse ICP0 (1:50)/Alexa488(1/500) and rabbit p-ERK2 (1:50)/Cy3(1:500) was performed. ICP0 (green) staining served as a marker of successful progressive viral replication, while DAPI counterstaining was used to trace the nuclei.

Uninfected cells, both pretreated and not pretreated with UO126 inhibitor, exhibit a similar baseline weak diffuse expression of p-ERK, mainly in the cytoplasm. As the infection progresses, p-ERK fluorescence increases, but the same diffuse cytoplasmic pattern remains at 1-2 h postinfection. Indeed, at 2h p.i, p-ERK fluorescence does not outline clearly the nuclear periphery, as opposed to its well-defined and almost exclusive nuclear location in cells with no MEK inhibition (fig. 13). The first peak of massive nuclear translocation observed in uninhibited cells is clearly abrogated.

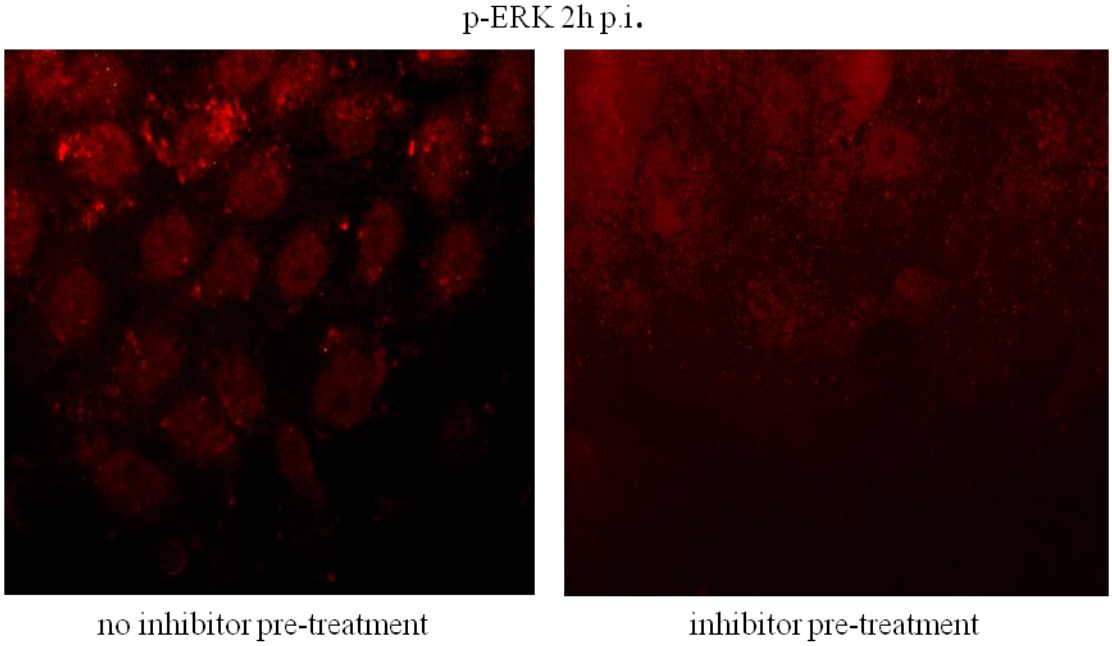


Fig 13: Immunofluorescence for p-ERK after 2 h of HSV-1 WT infection in cells either mock-treated or pretreated with the MEK inhibitor UO126. The massive nuclear translocation wave in the absence of inhibitor is abrogated in its presence.

While the first peak of activated ERK's nuclear translocation is inhibited in the case of UO126 pretreatment, the second peak does not seem to be influenced (fig. 14).

At 8 and 12 hours in the course of HSV-1 infection, p-ERK fluorescence derives mainly from within the nuclei. The fact that the later peak remains unaffected may be attributed to pharmacokinetic parameters of UO126, such as its relatively short biological half-life.

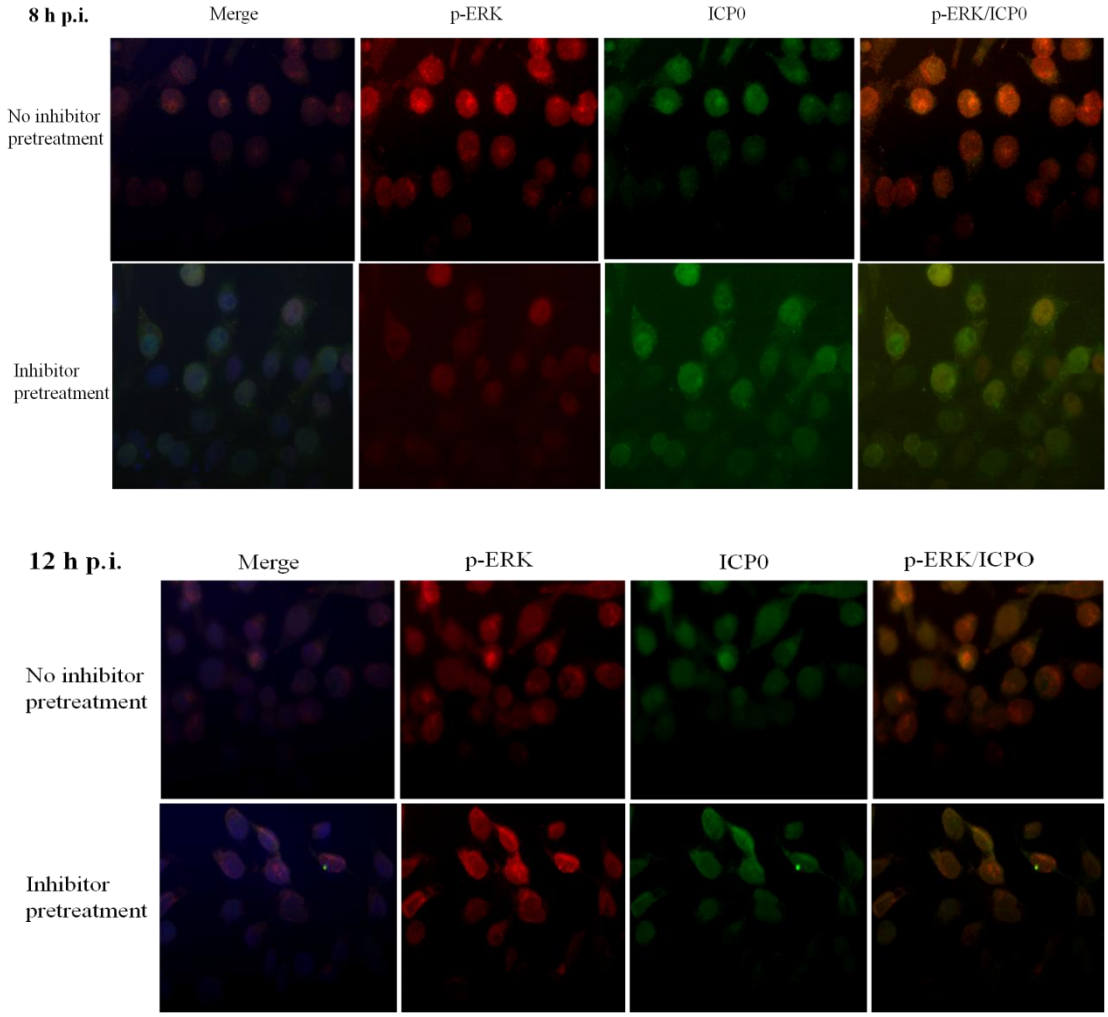


Fig 14:Immunofluorescence for p-ERK (red) and ICPO (green) after HSV-1 WT infection at 8h (upper panel) and 12 h (lower panel) in cells either mock-treated or pretreated with the MEK inhibitor UO126. DAPI counterstaining.

4.4.HSV-1-induced ERK pathway activation is important for the maintenance of its own replication

In order to investigate the effect of a functional ERK pathway for the replication of HSV-1, supernatants of infected cells were titrated when either pretreated or intermediately treated with the MEK inhibitor UO126, and mock-treated. For this purpose, Vero cells were cultured with DMEM-10% FBS in a 12-well plate and when they reached a 100% confluence, the medium was replaced with serum-free medium for overnight starvation. Then the infection and treatment were performed as follows:

Well No.1: infected with 8 μl of HSV-1 WT for 24h

Well No.2: pretreated with 2 µl of UO126 for 1h and then infected with 8 µl of HSV-1 WT for 24h
Well No.3: infected with 8 µl of HSV-1 WT for 24h and treated at 4h p.i. with 2 µl of UO126

Supernatants were collected at 24h p.i. and titrated on BHK cells. Ten µl of each three supernatants were serially diluted from 10^{-2} to 10^{-8} and 100µl from 10^{-3} – 10^{-8} dilutions were used to infect 6 wells. Plaque assay revealed the presence of well-defined plaques (see fig. 15) in the following maximal viral dilutions:

	serial dilution used	**No. of plaques**	**p.f.u/ml**
No inhibitor	10^{-6}	1	10^{7}
Inhibitor pre-treatment	10^{-5}	1	10^{6}
Inhibitor at 4h p.i.	10^{-4}	6	$6x10^{5}$

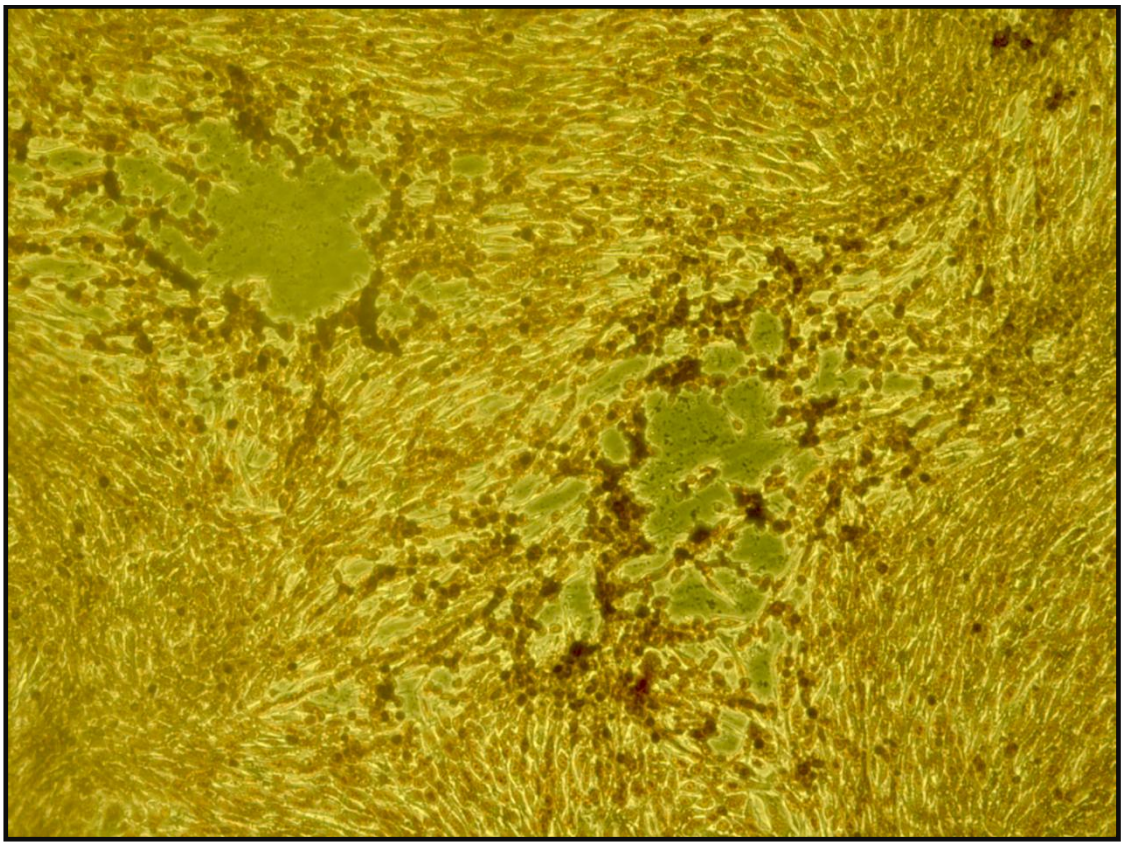


Fig 15:HSV-1 plaques under a microscope. In the center of the picture, two typical plaques are shown. They have well-defined margins, rounding and clumping of cells in the periphery, while their center is void of cells.

Cells not subjected to ERK pathway inhibition could maintain the production of significantly more viral progeny (10^7 p.f.u/ml) compared to those treated with the MEK inhibitor (10^6 and $6x10^5$ p.f.u/ml, respectively) (fig. 16).

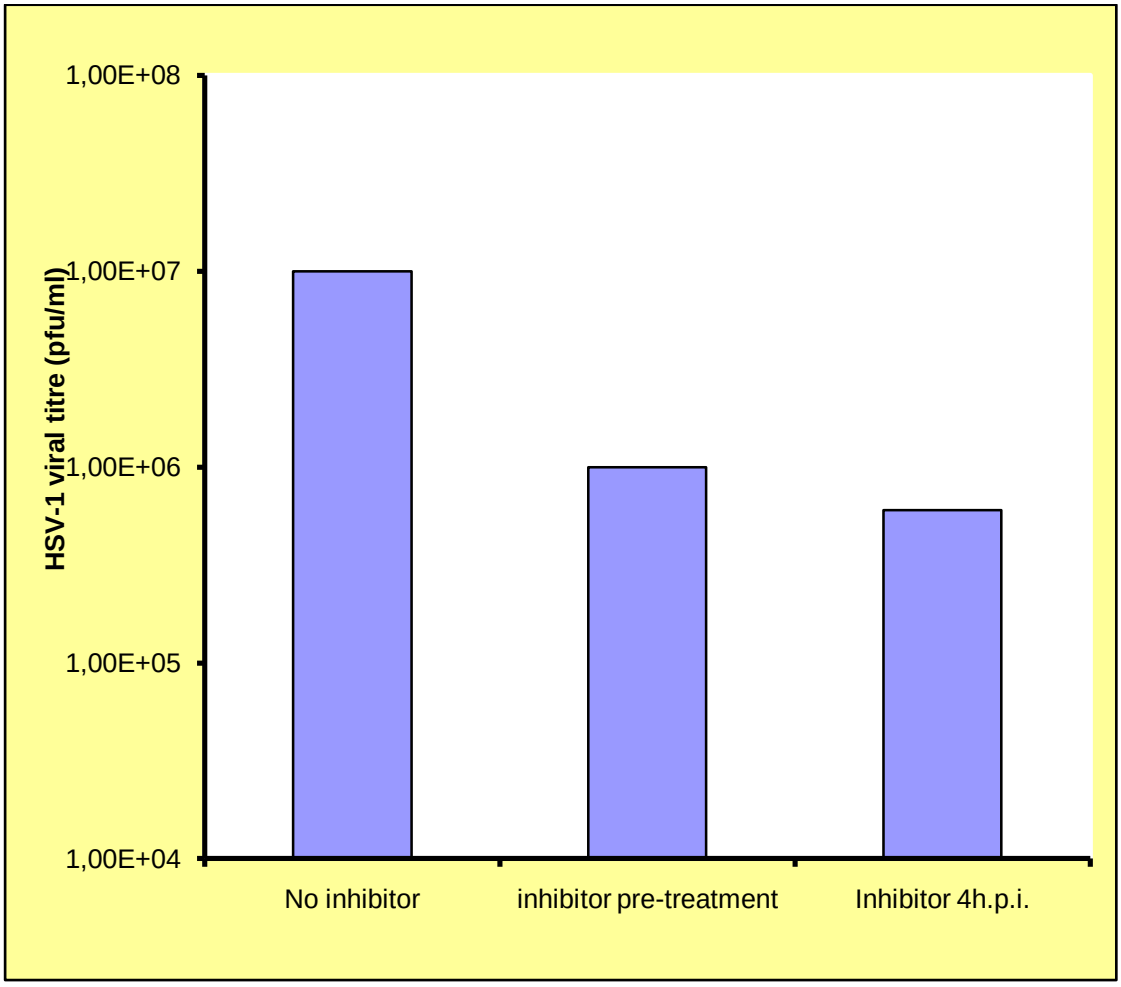


Fig. 16:Titration of the HSV-1 progeny virus in cells either mock-treated or pre-treated with the MEK inhibitor or treated at 4h p.i.

HSV-1 infection induces ERK activation, which in turn supports viral effective replication. Host cell ERK pathway inhibition, either by MEK inhibitor pretreatment or by intermediate inhibition, accounts for a significant decline in viral yields, compared to those where ERK cascade was left intact.

5.DISCUSSION

Viruses depend on host cells for their replication. DNA viruses partly use the DNA synthesis machine of host cells for their replication. Moreover, they hijack preexisting signaling pathways or induce them through various strategies. To date, a series of DNA and RNA viruses have been studied, concerning their effect on host cell cascades, such as the MEK/ERK pathway. *(Liu Q et al., 2003; Silva PN et al., 2006; Pleschka S et al.,2008;Ndjomou J et al., 2009; Wang Q et al.; 2009).* Whether ERK activation upon viral infection represents a cell-directed defense against the virus or a specific tactic orchestrated by the virus for its own means is not entirely clear, but it likely depends on the type of virus and the host cell infected. If ERK activation represents a virus-directed strategy for its efficient replication, a possible mechanism involved is phosphorylation of downstream TFs that could enhance the expression of IE/E genes. Another scenario is that activated ERK targets cellular activities that would lead to a more permissive environment for viral replication.

As far as the Herpesviridae family is concerned, viral infection has been linked to ERK activation and the exact mechanisms, interactions and molecules involved in this process have been identified for several members of the family. HSV-2 has been extensively studied and identified as an activator of the ras/ERK pathway through phosphorylation and inactivation of Ras-GAP mediated by ICP10 (RR1 PK) *(Aurelian L, 1998; Smith CC, et al., 2000; Perkins D, et al., 2002; Smith CC 2005; Wales SQ, et al., 2007).* Other herpesviruses , like VZV *(Rahaus M, et al., 2006),* HHV-8 *(Sharma-Walia N, et al., 2005),* EBV *(Lee YH, et al., 2008),* PRV *(Lyman MG, et al., 2006) and* HCMV *(Rodems SM et al., 1998, Chen J et al. 2002, Filippakis C., et al., unpublished data)* are reported to depend upon ERK cascade signaling for replication, and in some cases, viral proteins that induce activation of ERKs have been identified.

HSV-1 has been studied concerning its effect in other MAP kinase signaling pathways, notably SAPK/JNK and p38MAPK, and is reported to induce host cell apoptosis and to facilitate the lytic cycle via the activation of these pathways *(McLean TI, et al., 1999; Zachos G, et al., 1999; Galdiero S, et al., 2004; Diao L, et al., 2005; Gillis PA, et al., 2009).* However, the ERK signaling pathway in the course of HSV-1 infection has not been investigated yet. This series of experiments demonstrates for the first time that HSV-1 activates ERK pathway of host cells in the course of an infection and that the availability of a functional ERK cascade is essential for HSV-1 replication.

We have demonstrated here that ERK pathway status is altered during HSV-1 infection. Normally, in response to extracellular signals, ERK is stimulated by sequential activation of a series of protein kinases and then rapidly attenuated by phosphatases. All experiments were performed on serum-starved cells to reduce the

ERK activation background so as to more easily detect any increases in ERK activation that may have been induced by infection. While basal preexisting activation of ERK occurs in untriggered cells, HSV-1 trigger induces a sustained increase in p-ERK expression. The fact that ERK phosphorylation starts even at 15-30 minutes after infection , leads us to the hypothesis that this activation is most probably mediated by components acting early in the course of infection. Virion binding to cell surface receptors could initiate a receptor-mediated signal cascade, virion glycoproteins or tegument proteins could induce p-ERK expression or ERK cascade could be activated even by immediate-early gene expression. Mutant viruses defective for selective virion proteins and UV-irradiated viruses should be used in future experiments to clarify which putative molecule(s) is/are mediating this activation.

It is also important to determine where exactly the responsible molecule(s) is/are integrated into the ERK cascade. HSV-1 infection is inducing p-ERK expression rather than simply activating preexisting ERK. If preexisting ERK was activated, it should be expected to trace only a translocation and not a gradual increase in fluorescence, as it has been shown indeed. It is certain that this activation is mediated through a ras/raf/MEK-dependent mechanism triggered by the virus, and not through a crosstalk from other HSV-1-activated intracellular signaling pathways, otherwise the UO126 inhibitor would not affect the outcome of HSV-1 infection. This does not imply that the participation of other cascades is excluded, but that MEK/ERK activation has a primordial role in this process. Yet, the precise molecular events of interaction between ERK cascade and HSV-1 remain to be explored.

p-ERK dynamics within the cell during the first hours of HSV-1 infection has been clearly traced. An initially weak perinuclear and nuclear fluorescence gives place to two massive(1-2h p.i. and 8-12h p.i.) nuclear translocations of activated ERK. The nucleus has been shown to be a critical site for p-ERK localization. Indeed, many of the activated ERK substrates are nuclear transcription factors *(Volmat V, et al., 2001)*. During these time courses, intense active transcription is anticipated. At 2-4 h p.i., several well-defined dense p-ERK nuclear aggregates are visible but their composition, function and role in active transcription of the host cells are tempting to specify. Moreover, the biphasic ERK phosphorylation has been recently documented for VZV, member of the Herpesvirus family *(Rahaus M., et al., 2006)*. Given that both HSV-1 and VZV share similar biological properties and duration of replicative cycle (24 hours), it is not surprising that HSV-1 also exhibits a 2-wave induction of ERK pathway. The second wave of induction (8-12h) could implicate mechanisms others than those concerning the first one (e.g. early or late gene expression induction of ERK activation), but further experiments are needed to address this issue.

Activation of ERK provides a beneficial environment for viral replication. An indirect evidence comes from immunofluorescence experiments, when comparing ICP0 fluorescence in the presence and absence of UO126 inhibitor. After 12 hours of infection, viral tracing through ICP0 seems weaker when the ERK pathway is

inhibited. This finding suggests that viral progeny production is dependent on an intact and activated ERK pathway. Furthermore, true evidence derives from plaque assays, where the virus was titrated in the presence and absence of an activated ERK pathway. Cells not subjected to ERK pathway inhibition were capable to maintain the production of significantly more HSV-1 progeny. The effect of virus-induced ERK activation on the virus has been clarified, although the mechanism by which this is achieved remains to be elucidated. It could be possible that once activated, ERK would phosphorylate cellular transcription factors that could enhance the transcription and expression of immediate-early and early genes. Another possible scenario is that ERK activation could be targeting cellular activities and upregulating cellular genes that would lead to a more permissive and viable intracellular environment for viral replication. Yet, the impact of this activation on the cell fate is unknown. This could be complex and cell-type specific, since activation of ERK under different conditions in different cell types correlates with cell growth, proliferation, differentiation, oncogenic transformation and apoptosis *(Shaul YD, et al., 2007; Ramos JW, et al., 2008, Mebratu Y, et al., 2009).* Downstream ERK substrates that are activated should be identified, in order to define the molecular events that lead finally to the increased replication potential of HSV-1.

Whatever its precise significance in the viral life cycle, it is clear that activation of ERK by HSV-1 is one of the important stimulatory effects of HSV-1 infection on cellular biology. This effect is likely to play a significant role in the interaction between HSV-1 and the cells it infects.

All above findings would have significant implications in the better understanding of the physiology of HSV-1 anti-host mechanisms. Recently, much attention has focused on the potential use of HSV-1 as an oncolytic agent *(Veerapong J, et al., 2007; Farassati F, et al., 2001; Pan W, et al., 2009; Smith KD, et al., 2006; Farassati F, et al., 2008).* Production of effective oncolytic mutants requires a full understanding of the biological mechanisms activated by wild type viral infection and a detailed analysis of the host cell response during the immediate-early and early phases of viral invasion.

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