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Activation of c-Jun N-terminal kinase (JNK) pathway by HSV-1 immediate early protein ICP0

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Abstract

The immediate early protein ICP0 encoded by herpes simplex virus 1 (HSV-1) is believed to activate transcription and consequently productive infection. The precise mechanisms of ICP0-mediated transactivation are under intensive study. Here, we demonstrate that ICP0 can strongly activate AP-1 responsive genes specifically. This activation is inhibited by c-Jun (S73A), c-Jun (S63/73A), TAK1 (K63W), but not by p38 (AF), ERK1 (K71R), ERK2 (K52R) and TRAF6 (C85A/H87A). We further investigate the relevancy of ERK, JNK and p38 MAPK pathways using their respective inhibitors PD98059, SP600125 and SB202190. Only SP600125 significantly attenuates the AP-1 responsive gene activation by ICP0. Consistent with these, the JNK is remarkably activated in response to ICP0, and this JNK activation is shown to be significantly attenuated by TAK1 (K63W). It turns out that ICP0 interacts specifically with TAK1 and stimulates its kinase activity. These findings reveal a new molecular mechanism ICP0 explores to regulate gene expression.

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Keywords: HSV-1; ICP0; AP-1; JNK; TAK1

Introduction

Herpes simplex virus (HSV) is one of the most common agents infecting humans of all age ranges [1]. The virus occurs worldwide and produces a variety of illnesses, including infectious corneal blindness, gastrointestinal disorders, esophageal disorders and approximately 25% of the genital herpes infections [2,3]. The best characterized member of this family, HSV type 1 (HSV-1), accounts for 10% to 20% of all cases of viral encephalitis which is associated with high mortality (60% to 70%), and the neurological impairment in the survivors is invariable [4]. Generation of effective vaccines is complicated by the fact that HSV-1 establishes life-long latent infections in sensory ganglionic neurons [5]. While the mechanism which controls the balance between the lytic and latent states is still inadequately understood, current evidence suggests that ICP0 plays a key role in the efficient initiation of lytic cycle and reactivation of quiescent virus in cultured cells [6].

The 775-amino-acid regulatory protein ICP0 was originally defined as a “promiscuous activator” because it can activate its own expression as well as other cellular genes. While infection of HSV-1 mutant deficient in functional ICP0 resulted in repression of viral transcription and establishment of quiescent viral genomes, re-introduction of exogenous ICP0 allowed reactivation of the quiescent genomes and the entry into a normal lytic cycle [7]. Although there have been many attempts to understand the mechanism by which ICP0 achieves these

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effects, the results still remain controversial. Initially, it was shown that ICP0 increased both viral and cellular protein production by stimulating mRNA synthesis [8]. However, it failed to identify specific response elements in the target promoters recognized by ICP0; nor was it able to demonstrate any affinity between ICP0 and DNA [9], which suggested that the mode of ICP0 functioning may be an indirect one [6]. One possibility is that it functions via interactions with other proteins, as have been demonstrated for many other viral proteins that modulate gene expression. Previously, it was suggested that ICP0 interacted with various proteins, including the viral regulatory molecules such as the major HSV-1 transcriptional regulator ICP4 [10], translation elongation factor EF-1δ [11], cyclin D3 [12], the ubiquitin-specific protease USP7 [13] and the transcription factor BMAL1 [14]. Although the exact mechanism by which ICP0 functions has yet to be determined, it is now generally accepted that an intact Zinc-binding Ring finger domain near the N-terminus of the protein is indispensable. In parallel, BICP0, the HSV-1 functional counterpart in bovine, shares a number of biological properties with ICP0 in protein functions [3].

The pleiotropic cellular transcription factor AP-1 (activator protein 1) consists of a broad set of the Jun, Fos and ATF families of proteins [15]. These proteins associate to form a variety of homo- and heterodimers. The Jun–Jun and Jun–Fos dimmers preferentially bind to the phorbol 12-*O*-tetradecanoate-13-acetate (TPA)-responsive element (TRE, which has the consensus sequence TGACTCA), whereas the Jun–ATF dimmer or ATF homodimers prefer the cAMP responsive element (CRE, this element has the consensus sequence TGACGTCA) [16]. The AP-1 is important in the regulation of gene expression, cell growth, differentiation, tumorigenesis and development in immune response to different stimuli [17]. It has been reported previously that infection with the DNA viruses SV40 [18] and the herpes virus cytomegalovirus (CMV) [19] resulted in an increased activity of AP-l or activation of the c-*fos* and c-*jun* genes in the infected cell. HSV-2 ICP10 PK was reported to activate the ERK survival pathway in hippocampal neurons and function as a dominant regulator of apoptosis [20,21]. Although HSV-1 infection has been shown to result in a similar increased AP-1 activity [22], it remains unknown which protein from HSV-1 genome performs this function and how it activates this signaling pathway. Recent research showed that in HEp-2 cells HSV-1 immediate early gene expression was required for the induction of apoptosis related to AP-1 activation, but the exact protein was not clearly demonstrated [23]. In this report, we showed that the HSV-1 immediate early regulatory protein ICP0 could upregulate the activity of AP-1 efficiently, whereas expression of c-Jun (S73A), c-Jun (S63/73A) and TAK1 (transforming growth factor-β-activated protein kinase 1) (K63W) had a remarkably inhibitory effect. SP600125, a JNK (c-Jun NH_2-terminal kinase) inhibitor, could also block the ICP0-induced AP-1 activation significantly. In addition, overexpression of ICP0 resulted in a robust increase in JNK activity, which could be abolished significantly by dominant-negative TAK1, but not by dominant-negative TRAF6 (TNF receptor-associated factor 6). Coimmunoprecipitation assay demonstrated that ICP0 interacted specifically with TAK1, but not with JNK or TAB1. Importantly, ICP0 could stimulate TAK1 kinase activity potently. In summary, our data demonstrated that ICP0 could stimulate AP-1 activity via the c-Jun N-terminal kinase cascade. This study uncovers a new signaling pathway that ICP0 hijacks to activate a myriad of viral and cellular genes.

Materials and methods

Plasmids and reagents

cDNA construct for ICP0 was a gift from Dr. Yange Zhang of University of Nebraska-Lincoln. This cDNA was subcloned into pDNA3.1-N-Flag (a gift from Dr. Hai Wu in UT Southwestern Medical Center). Truncating mutants of ICP0 were made either by PCR or directly by cutting out the corresponding segments and cloned into pDNA3.1-N-Flag. The mutants covered one segment of the full-length proteins: 1–234 aa for ICP0 exon1 + 2, 518–775 aa for ICP0 MC and 555–775 aa for ICP0 AC. To generate the mutant of the C_3HC_4 Zinc Ring finger domain, the gene sequence of ICP0 (bp 311–535) carrying the artificial mutants was synthesized by Shanghai Sangon Biological Engineering and Technology and Service Co. Ltd. Then, the synthetical fragment was digested with *Xho*I and *Pvu*I, purified from a 2% agarose gel and introduced into the same sites of pDNA3.1-N-Flag-ICP0 construct. The resulting mutant had three changes in conserved amino acids of the C_3HC_4 Zinc Ring finger domain (Fig. 3b), and the construct was designated ICP0 (RM). cDNAs encoding TAK1, TAK1 (K63W), TAB1, TRAF6, TRAF6 (C85A/H87A) and MKK6 (K82A) have been previously described [51,55]. pBS-his-c-Jun (S73A) and pBS-his-c-Jun (S63/73A) were kindly provided by Professor Dirk Bohmann in Medical Center of University of Rochester. pcDNA3-Flag-p38 (WT), pcDNA3-Flag-p38 (AF), pCEP4-ERK1 (K71R) and pCEP4-ERK2 (K52R) were kindly provided by Professor Melanie H. Cobb in University of Texas Southwest Medical Center. GST-ATF2 (1–109), pcDNA3-HA-JNK, GG-JNK (K55M) and GG-JNK (T183A/Y185F) were kindly provided by Professor Li Lin. AP-1-Luc and Gal4-Luc reporter plasmids were purchased from Stratagene. Another luciferase reporter plasmid, the LEF-Luc construct, was kindly provided by Professor Grosschedl (University of California at San Francisco). All these constructs were confirmed by automatic DNA sequencing. His-MKK6 (K82A) was purified from *E. coli* by Qiagen Kit. GST-ATF2 (1–109)

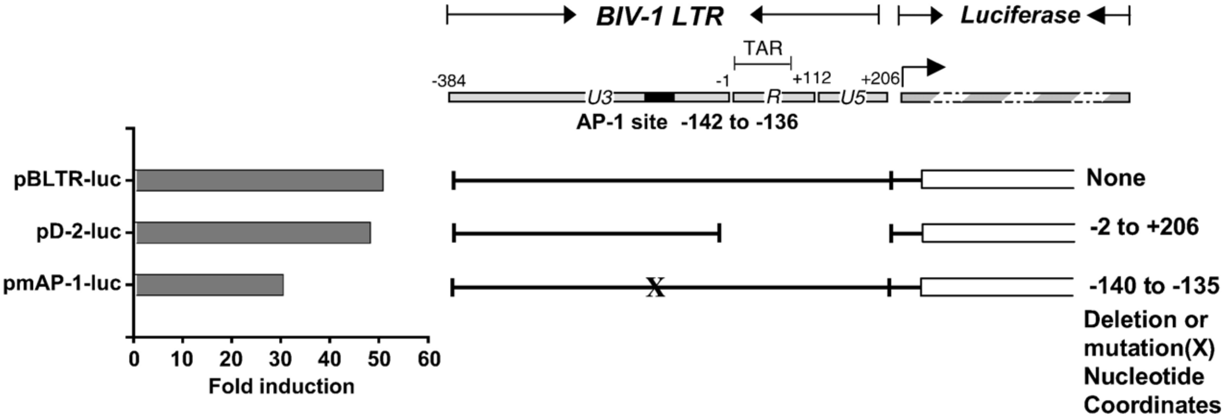


Fig. 1. Activation of BIV-LTR by BICP0 is dependent on an AP-1 site on the enhancer. A map of the BIV-LTR is indicated at top. The solid black bar is used to highlight the existence of the AP-1 site. Diagrams of the BIV-LTR mutants are shown at the right side. Black lines indicate the sequences retained in each mutant construct. The gap indicates the region deleted, and the 'X' indicates the nucleotide at the AP-1 site that was mutated from TGATTCA to TGAGGGC. BICP0 was co-transfected into 293T cells with BIV-LTR or its mutants, respectively. Stimulation of these constructs by BICP0 is represented at the left side as fold increase of luciferase readout. A *Renilla* luciferase reporter vector was included in all transfection experiments as an internal control to correct for transfection efficiency. Results are mean of triplicate determinations.

was purified from *E. coli* by Glutathione Sepharose™ 4B (Pharmacia Biotech Code No. 17-0756-01). The MAPK inhibitor PD98059, SP600125 and SB202190 were purchased from Calbiochem (Merck). All antibodies were from Santa Cruz Biotechnology if not specified otherwise. From Aldrich-Sigma were purchased anti-β-actin antibody, anti-Flag bead and protein-A bead. The mouse polyclonal antibody against ICP0 was made by subcutaneously injecting 6-week Kunming mice with the purified his-ICP0(C) protein expressed from *E. coli* BL21. The antibody recognized only its immunogens specifically.

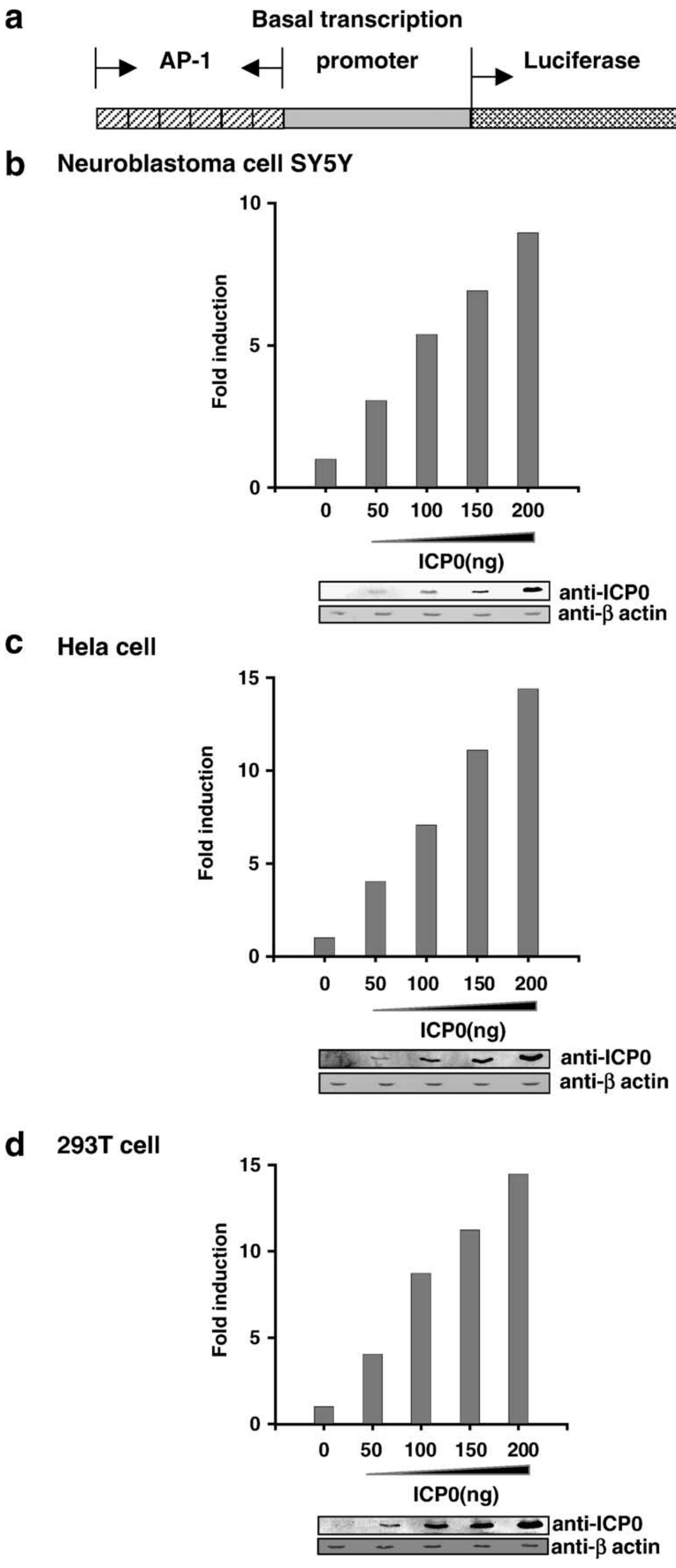
Cell culture, transfection and dual-luciferase reporter assay

293T and SH-SY5Y cells were from ATCC in USA. Hela cell was kindly provided by Professor Lin Li. These cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (50 U/ml) and streptomycin (50 μg/ml). 2.0×10^6 cells and 1.5×10^6cells were seeded in 6-well plates for luciferase assay and 6-cm dishes for other experiments, respectively. Transfection with the indicated plasmids was carried out by either calcium phosphate precipitation method [51] or lipofectamine™ 2000 (Invitrogen Cat. No. 11668-027) according to the manufacturer's instructions. Cells were harvested at different time after transfection as indicated. For reporter gene assay, cells were transiently transfected with indicated plasmids and 20 ng 6 × AP-1 luciferase reporter plasmid (Fig. 2a) plus 0.5 ng *Renilla* luciferase vector (Promega) and harvested at 48 h after transfection. For inhibitor treatment experiments, cells were cultured with the indicated reagents 24 h after transfection and harvested after one more hour of incubation. For dual-luciferase reporter assay, Cells were pelleted, lysed and assayed for relative luciferase activity (firefly: *Renilla*) per manufacturer's protocol (Promega) using Berthold Detection Systems Sirius (Germany) 20/20 luminometer, with settings of a 2 s delay followed by a 8 s measurement period for each assay. Each datum was from a representative experiment reproducibly repeated at least three times. The cell lysates for luciferase assay were all checked with Western blot to ensure protein expression of the various constructs.

Preparation of soluble cell extracts

For reporter gene assay, cells in each well were washed once with PBS, and lysates were prepared by applying 300 μl of passive lysis buffer (Promega) to each culture well and incubating at room temperature with gentle rocking for 15 min. Aliquots of each lysate were immediately assayed for firefly and *Renilla* luciferase activities. For other experiments, infected or mock infected cells were rinsed in PBS twice, harvested, pelleted by centrifugation and solubilized at 4°C in mammalian cell lysis buffer [0.5% Nonidet P-40/20 mM Tris, pH 7.5/20 mM β-glycerol phosphate/10 mM NaF/0.5 mM Na_3VO_4/150 mM NaCl/1 mM DTT/1 mM PMSF/0.2 mM EGTA pH 7.0/5 μg/ml pepstatin, leupeptin and chymostatin]. 15 min later, lysates were clarified by

Fig. 2. ICP0 can activate AP-1 responsive reporter gene in different cells. (a) Schematic representation of AP-1-Luc luciferase reporter gene used in this study. Note that the AP-1 sequence of this construct is an authentic one that is slightly different from the AP-1 site identified in Fig. 1. ICP0 was co-transfected into cells along with this reporter gene and internal control for 48 h. Shown in panel (b) was the mean fold induction of the AP-1-Luc expression by gradient amount of ICP0 in neuroblastoma SH-SY5Y cells. The same luciferase assay experiments were carried out in (c) Hela cells and (d) 293T cells. The gradient expression of ICP0 and the loading control were shown in panels.


a
Basal transcription
AP-1
promoter
Luciferase
b
Neuroblastoma cell SY5Y
Fold induction
0
5
10
0
50
100
150
200
ICP0(ng)
anti-ICP0
anti-β actin
c
Hela cell
Fold induction
0
5
10
15
0
50
100
150
200
ICP0(ng)
anti-ICP0
anti-β actin
d
293T cell
Fold induction
0
5
10
15
0
50
100
150
200
ICP0(ng)
anti-ICP0
anti-β actin

centrifugation at 4°C, 10,000 rpm for 30 min. The supernatant was used in immunoblotting, immunoprecipitation and pull-down experiments.

Coimmunoprecipitation

Exogenous HA-JNK was immunoprecipitated with antibody of anti-HA coupled onto protein A beads. Exogenous Myc-TAK1 was immunoprecipitated with antibody of anti-Myc coupled onto protein A beads. Flag-tagged protein was immunoprecipitated with anti-Flag beads (Sigma F-2426) directly. Soluble cell lysates containing equal amounts of whole proteins were incubated on a rotor with the indicated beads at 4°C for 2–4 h. The beads were then washed extensively with cell lysis buffer for 3–4 times and TBS for 1 time. The protein was eluted by adding SDS-PAGE loading buffer, subsequently run on SDS-PAGE gel, transferred to Immobilon-P membranes (Millipore) and visualized by Western blot using indicated antibodies. Or the beads were used in the subsequent reactions, such as kinase assay.

In vitro kinase assay

To monitor JNK activity, exogenous HA-JNK was immunoprecipitated by anti-HA protein A beads from cell extracts transfected for 24 h with indicated plasmids. Part of the beads were then incubated with 2 μg bacterially expressed GST-ATF2 (1–109) in 20 μl of kinase buffer containing 25 mM Tris–HCl (pH7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 5 mM β-glycerophosphate, 0.1 mM Na_3VO_4 together with 0.5 μCi of γ^{32}P-labeled ATP (3000 Ci/mmol). After incubating at 30°C for 30 min, the reaction products were resolved by 12% SDS-PAGE. Gels were then dried and exposed at −70°C to XAR film with intensifying screens. For TAK1 kinase assay, part of the TAK1 immunoprecipitates were incubated with 1 μg of bacterially expressed MKK6 (K82A) in 20 μl of kinase buffer containing 10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 1 μCi of γ^{32}P-labeled ATP at 25°C for 5 min. Samples were then subjected to 9% SDS-PAGE, and autoradiograph was obtained. For the entire kinase assay, part of each immunoprecipitate was subjected to Western blot to show the equal efficiency of immunoprecipitation.

UV irradiation of 293T cells

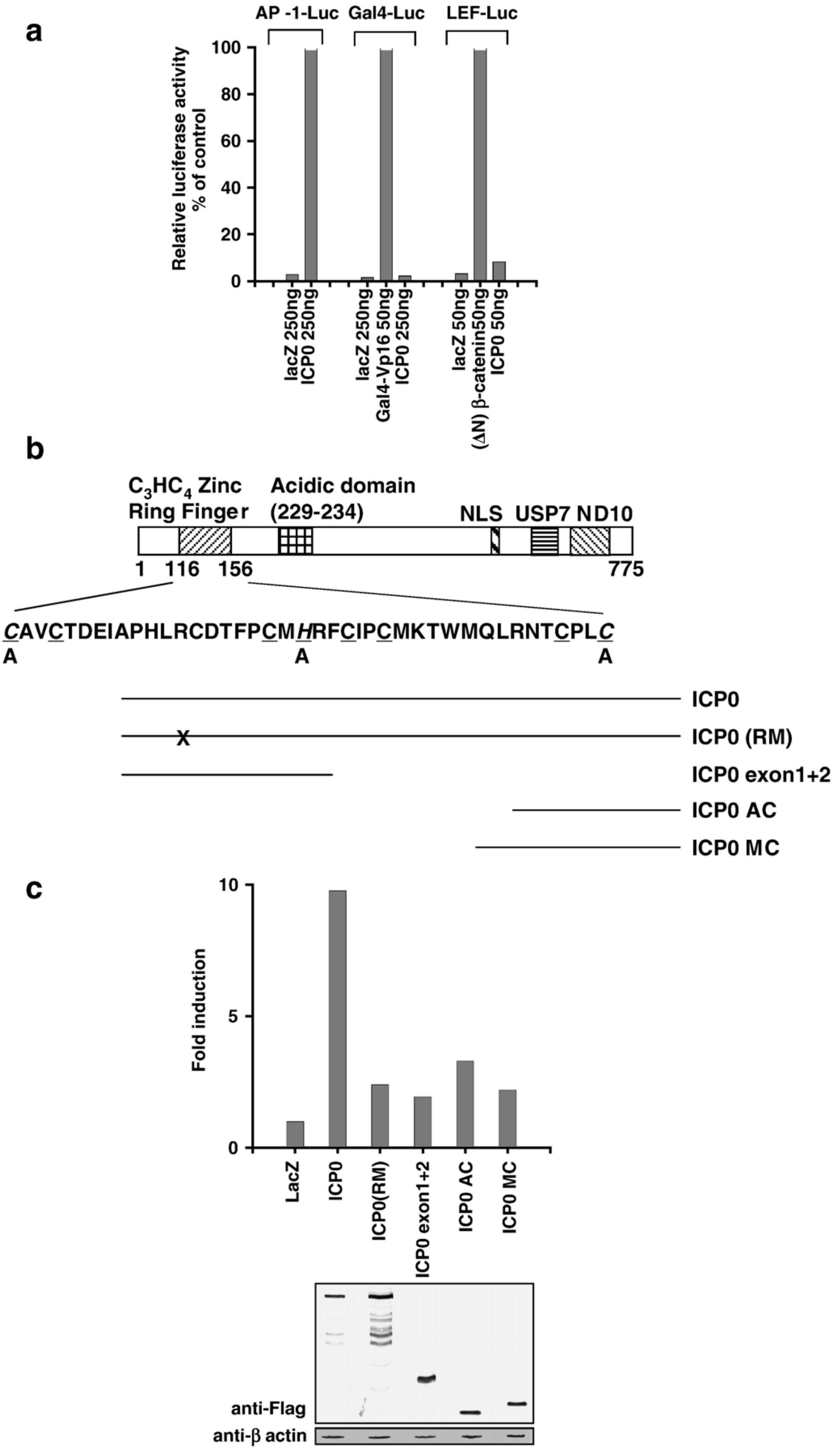
The irradiation was conducted 36 h after transfection with an uncovered 6-well plate containing 80–90% confluence 293T cell with 1.5 ml DMEM plus 10% FBS in each well. We conducted UV irradiation in the presence of culture medium to avoid the cellular responses to nutritional shock (for example, withdrawal of growth factors in serum). After 30-min exposure to 40 mJ/cm^2 in a UV cross-linker, the cells were returned to the incubator for 30 min before harvesting.

Results

ICP0 specifically stimulates AP-1-dependent transcription in vivo

We initially set out to characterize bovine immunodeficiency virus 1 (BIV-1) enhancer LTR that is inducible by BICP0. It came into our attention that this induction was impaired when the AP-1 consensus motif within the LTR was mutated (Fig. 1). Additionally, it was found that HSV-1 infection stimulated c-Jun N-terminal mitogen-activated protein kinase pathway [24,25]. These led us to speculate whether HSV-1 ICP0 stimulated gene transcription via promoting AP-1 activity. To explore this hypothesis, human neuroblastoma SH-SY5Y cells were transiently transfected with increasing amounts of an ICP0 expression plasmid together with an AP-1-Luc reporter construct, which consists of a luciferase reporter gene under the control of a minimal promoter and several tandem repeats of AP-1 binding sites (Fig. 2a). A *Renilla* luciferase vector was included in all transfection experiments as an internal control to correct for transfection efficiency. As a negative control, a plasmid expressing LacZ was used in parallel experiments. The expression of ICP0 could lead up to a 9-fold induction of AP-1-driven transcription, and the folds of stimulation were in direct proportion to the amount of ICP0 expressed (Fig. 2b). As a positive control, expression of the JNK1 also induced AP-1-dependent luciferase activity in SH-SY5Y cells. In contrast, no stimulation was detected when LacZ was expressed (data not shown). Since HSV-1 infects other cell lineages besides neuron, we undertook the same luciferase assay experiments to check whether ICP0 could still do so in

Fig. 3. The RING domain of ICP0 is important for its selective activation of AP-1. (a) ICP0 activates AP-1-Luc, but not LEF-Luc or Gal4-Luc reporter genes. ICP0 or control plasmids were co-transfected into 293T cells along with the indicated reporter genes. (ΔN)-β-catenin and Gal4-VP16 are the cognate activators of LEF-Luc and Gal4-Luc, respectively. (b) Schematic diagram of ICP0 is indicated at the top. Positions of the C_3HC_4 Zinc Ring finger domain and the other domains were described. NLS—nuclear localization signal; USP7—sequences required for binding to the ubiquitin-specific protease USP7; ND10—sequences required for efficient localization at ND10 domains. Amino acid sequence of the C_3HC_4 Zinc Ring finger domain (aa 116–156) was shown. The consensus Zinc Ring finger sequence is underlined, and the mutations in this domain are in italics (aa 116 was changed from C to A, aa 136 from H to A and aa 156 from C to A). Diagrams of the ICP0 mutants are shown at the bottom. Black lines indicate the sequences retained in each mutant construct. ICP0 (RM): the Zinc Ring finger domain is site-mutated, 'X' indicates the mutations; ICP0 exon1+2: N-terminus of ICP0 harboring the RING domain; ICP0 AC and ICP0 MC: C-terminal parts of ICP0 that do not contain the RING domain (see details of these plasmids in Materials and methods). (c) AP-1-Luc activation induced by truncations of ICP0. Flag-tagged-variants of ICP0 were co-transfected into 293T cells along with the AP-1 reporter gene and internal control respectively. 48 h later, cells were collected, and dual-luciferase assay was performed.


a
AP -1-Luc
Gal4-Luc
LEF-Luc
Relative luciferase activity % of control
100
80
60
40
20
0
lacZ 250ng
ICP0 250ng
lacZ 250ng
Gal4-Vp16 50ng
ICP0 250ng
lacZ 50ng
(ΔN) β-catenin50ng
ICP0 50ng
b
C3HC4 Zinc Ring Finger
Acidic domain (229-234)
NLS
USP7
ND10
1
116
156
775
CAVCTDEIAPHLRCDTFPCMHRFCIPCMKTWMQLRNTCPLC
A
A
A
ICP0
ICP0 (RM)
X
ICP0 exon1+2
ICP0 AC
ICP0 MC
c
Fold induction
10
5
0
LacZ
ICP0
ICP0(RM)
ICP0 exon1+2
ICP0 AC
ICP0 MC
anti-Flag
anti-β actin

Hela and 293T cells. As was shown in Figs. 2c and d, ICP0 expressions did lead to a strong induction of the transcription factor AP-1, suggesting that ICP0 could activate AP-1 in the cell lines which could be infected by HSV-1.

Previously, ICP0 was proposed to activate gene transcriptions by interfering with ND10 substructure. To rule out the possibility that AP-1 activation by ICP0 was due to indiscriminate potentiation of transcription factors in the nucleus, we transfected Gal4-Luc, LEF-Luc or AP-1-Luc reporter genes individually into 293T cell together with ICP0. As was expected, AP-1-Luc transcription was markedly increased by ICP0. However, Gal4-Luc and LEF-Luc gene transcriptions remained constant in the presence or absence of ICP0. In contrast, Gal4-Luc was activated by its cognate activator Gal4-VP16. The same was true for LEF-Luc in response to its cognate activator (ΔN) β-catenin [26]. These results indicated that ICP0 specifically activated AP-1-dependent gene transcription (Fig. 3a).

It was reported that the N-terminal part of ICP0 contained a Zinc Ring finger domain characteristic of ubiquitin E3. Previous study showed that the cysteine residues at position 116 and 156 and the histidine residue at position 136 were critical in substrate-independent polyubiquitination reaction (data not shown). To test whether the RING domain is essential for activating AP-1, ICP0, ICP0 (RM) and other truncations of ICP0 were constructed (Fig. 3b). As was shown in Fig. 3c, ICP0 (RM) achieved little stimulation above background, while ICP0 could induce robust activation of AP-1, implicating that the RING domain was absolutely necessary for AP-1 activation, and ubiquitin was possibly involved in the process. Either ICP0 exon1+2 or ICP0 AC, MC alone could not activate AP-1-dependent transcription. These indicated that ubiquitin ligase activity alone was not sufficient to activate AP-1, although ICP0 exon1+2 alone could catalyze substrate-independent poly-ubiquitination reaction (data not shown). Taken together, these results established that ICP0 could specifically activate AP-1-dependent transcription in different cell lines, which required the RING domain.

The upregulation of AP-1 activity by ICP0 is attenuated by c-Jun (S73A), c-Jun (S63/73A), but not by p38 (AF), ERK1 (K71R) and ERK2 (K52R)

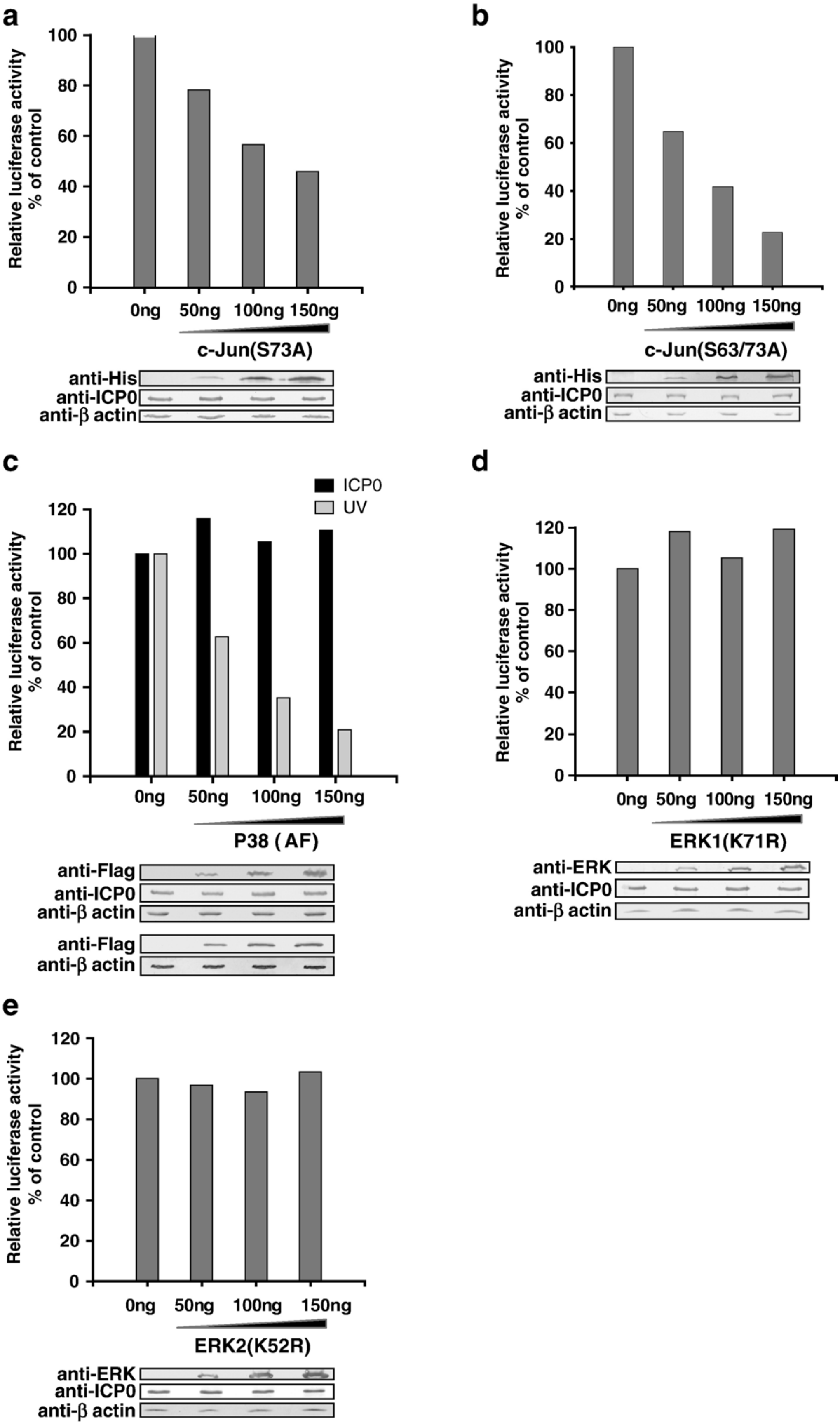
The AP-1 transcription factor c-Jun is a prototypical nuclear effector of the JNK signal transduction pathway. The integrity of JNK phosphorylation sites at serines 63/73 in c-Jun is essential for signal-dependent target gene activation. When serines 63/73 of c-Jun are all mutated into alanines, this mutant c-Jun (S63/73A) becomes a repressor that could inhibit AP-1 activation by many stimuli [27,28]. We explored whether c-Jun (S63/73A) had the inhibitory effect on AP-1 activation mediated by ICP0. When ICP0 and AP-1-Luc reporter gene were co-transfected into 293T cell, AP-1 activation by ICP0 was significantly inhibited in the presence of c-Jun (S63/73A) (Fig. 4b). The c-Jun (S63A) had a relatively slight inhibition (Fig. 4a). These suggested that c-Jun phosphorylations were essential for ICP0-mediated AP-1 activation and ICP0 may be acted upstream of c-Jun.

Three major groups of MAPK have been identified in mammals [29], p38, the ERK (extracellular signal-regulated protein kinases) and the JNK (also known as stress-activated MAP kinases, SAPK). Each of these groups of MAPK is activated by specific protein kinase cascades, and some of the MAPK pathways have been suggested to play a role in the phosphorylation of AP-1 members [30,31]. To test the possible involvement of p38 signaling pathway in ICP0-induced AP-1 activation, the dominant-negative mutant p38 (AF), which cannot be phosphorylated since the TGY dual phosphorylation site has been changed to AGF [31], and AP-1-Luc reporter gene were introduced into 293T cells together with ICP0 or LacZ respectively. As a positive control, AP-1 activation in response to UV [32] was investigated too. As was shown in Fig. 4c, the dominant-negative mutant p38 significantly reduced UV-induced AP-1 activation, while it had no inhibitory effect on AP-1 activation by ICP0. The expressions of Flag-tagged p38 (AF) were checked to be normal in both cases, suggesting that p38 was not involved in AP1 activation in response to ICP0. To determine whether ERK has any effect on ICP0-induced AP-1 activation, kinase-deficient mutants ERK1 (K71R) and ERK2 (K52R) [33] were used. Luciferase assay revealed that the ICP0-induced AP-1 activation remained constant in the presence or absence of the mutants, although these mutants had lost their kinase activities too (Figs. 4d and e). These data indicated that JNK but not p38 MAPK or ERK might participate in the ICP0-induced AP-1 activation.

JNK is activated during ICP0-mediated AP-1 activation

To determine whether JNK has any effect on ICP0-induced gene expression, we performed the luciferase

Fig. 4. ICP0-mediated AP-1 activation is inhibited by c-Jun (S73A), c-Jun (S63/73A), but not by p38 (AF), ERK1 (K71R) and ERK2 (K52R). Activation of AP-1 by ICP0 is inhibited by c-Jun (S73A) (a) and c-Jun (S63/73A) (b). ICP0 and AP-1-Luc together with the internal control were transfected into 293T cells without or with his-c-Jun (S73A) (a) or his-c-Jun (S63/73A) (b). Cells were pelleted, lysed and assayed for relative luciferase activity 48 h after transfection. Lower panels: expression of the indicated constructs. (c) The dominant-negative mutant p38 (AF) significantly reduced UV-induced AP-1 activation, while it had no inhibitory effect on AP-1 activation by ICP0. Flag-p38 (AF), AP-1-Luc reporter gene and internal control were introduced into 293T cells together with ICP0 or LacZ respectively. 36 h post transfection, cells transfected with LacZ and reporter gene were exposed to UV irradiation. After 30 min of exposure and 30 more minutes of incubation, the irradiated and unirradiated cells were collected for dual-luciferase assay. Activation of AP-1 by ICP0 is not inhibited by ERK1 (K71R) (d) or attenuated by ERK2 (K52R) (e). Upper panel: AP-1-Luc activation by ICP0 in the presence of gradient amounts of ERK1 (K71R) or ERK2 (K52R). Lower panels: expression of the indicated constructs.


a
Relative luciferase activity % of control
100
80
60
40
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0
0ng 50ng 100ng 150ng
c-Jun(S73A)
anti-His
anti-ICP0
anti-β actin
b
Relative luciferase activity % of control
100
80
60
40
20
0
0ng 50ng 100ng 150ng
c-Jun(S63/73A)
anti-His
anti-ICP0
anti-β actin
c
ICP0
UV
Relative luciferase activity % of control
120
100
80
60
40
20
0
0ng 50ng 100ng 150ng
P38 (AF)
anti-Flag
anti-ICP0
anti-β actin
anti-Flag
anti-β actin
d
Relative luciferase activity % of control
120
100
80
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40
20
0
0ng 50ng 100ng 150ng
ERK1(K71R)
anti-ERK
anti-ICP0
anti-β actin
e
Relative luciferase activity % of control
120
100
80
60
40
20
0
0ng 50ng 100ng 150ng
ERK2(K52R)
anti-ERK
anti-ICP0
anti-β actin

reporter assays using the specific JNK pathway inhibitor SP600125 [34]. As shown in Fig. 5a, treatment of cells with SP600125 alone did attenuate the luciferase activity in dose-dependent mode. On the other hand, treatment with PD98059 (an ERK inhibitor [35]) could only slightly inhibit the AP-1 activation by ICP0, while treatment with SB202190 (a p38 MAPK inhibitor [36]) slightly augment ICP0-mediated AP-1 activation. These suggested that JNK played an important role in modulating the ICP0-induced AP-1 stimulation. To further investigate the relevancy of p38 and JNK in ICP0-induced AP-1 activation, we determined the activation of p38 MAPK in response to ICP0 by measuring the phosphorylation of p38 with phospho-specific p38 antibody. As shown in Fig. 5b, Co-expression of TAB1 (TAK1-binding protein 1) with p38 did promote phosphorylation of p38 as previously reported [37]. However, ICP0 and its various mutants failed to stimulate phosphorylation of p38.

It was known that residues Thr-69 and Thr-71 in ATF2 could be dual-phosphorylated upon JNK activation. To examine whether ICP0 could activate JNK, 293T cells were co-transfected with ICP0 and HA-JNK. A GST fusion protein harboring the transactivation amino terminus of ATF2 (residues 1–109) was used as a substrate, and the kinase activity of JNK was assessed after it was immunoprecipitated. Cells exhibited a marginal activity in the phosphorylation of GST-ATF2 8 h after transfection when ICP0 started to appear in very small amount, and JNK activity increased dramatically (about 6-fold) 24 h after transfection when ICP0 was produced in considerably large amount (data not shown). So, we carried out kinase assay 24 h after transfection. As was shown in Fig. 5c, it barely observed any JNK activity in 293T cells co-transfected with control vector. In contrast, exogenous expression of ICP0 resulted in a robust increase in JNK activity just like the positive control TRAF6, which is known to activate JNK [38–40]. The plasmid expressing LacZ was used as a negative control, and it did not influence JNK activity too. In addition, we observed similar positive regulation of JNK activity in SH-SY5Y and Hela cells transfected with ICP0 (data not shown), confirming that ICP0 was responsible for the induction of JNK activity. When the kinase-deficient mutant JNK (K55M) and the phosphorylation-defective mutant JNK (T183A/Y185F) were introduced, ICP0 failed to upregulate JNK activity (Fig. 5d, lanes 3 and 4).

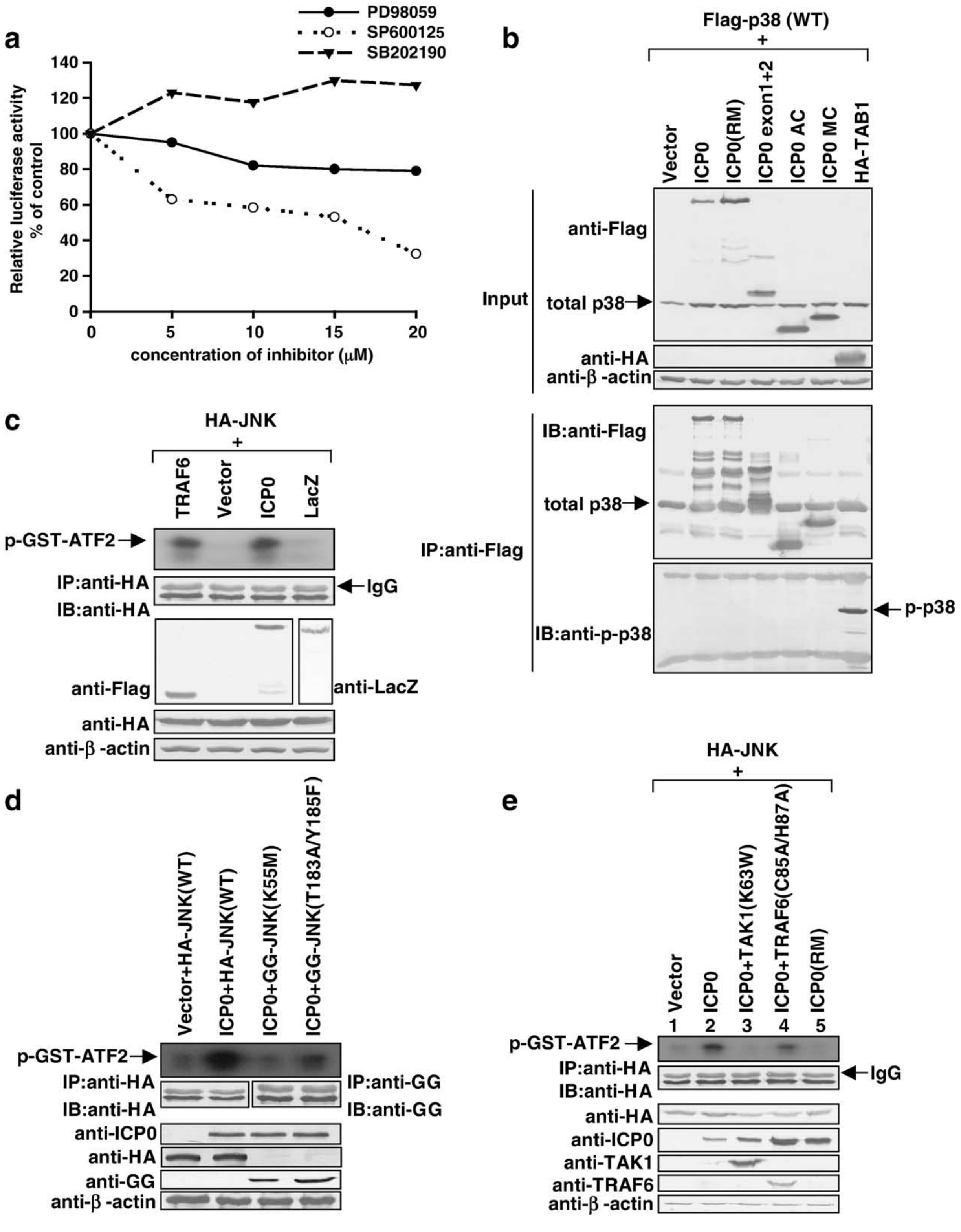
TAK1 is the mitogen-activated protein kinase kinase kinase (MAP3K) involved in ICP0-mediated JNK activation

So far, it was well established that ICP0 had the ability to activate JNK. We therefore went on to investigate how it activated this signaling pathway. ICP0 or triplet-point mutant ICP0 (RM) was transfected into 293T cells together with HA-JNK. 24 h after transfection, cells were collected, and the kinase activity of JNK was assessed following immunoprecipitation. Consistent with the results of luciferase assay, mutants in Zinc Ring finger domain deprived ICP0 of the ability to induce JNK activation (Fig. 5e, lanes 2 and 5). This suggested that RING domain played a crucial role during JNK activation by ICP0.

Based on the previous functional analysis, it is very likely that ICP0 may be integrated into the JNK signaling pathway upstream the TAK1 site. It is well known that the TRAF proteins serve as cytoplasmic adapters that can interact directly with the intracellular domains of cell surface receptors and mediate signaling [41]. Among these TRAF molecules, TRAF6 has been shown to be a pivotal component that activates JNK in response to pro-inflammatory mediators such as interleukin-1 (IL-1) and lipopolysaccharides (LPS) [38,39]. It can mediate JNK activation via the formation of a TRAF6–TAB2–TAK1 complex [40]. TAK1 is an upstream MAP3K that can phosphorylate MKK6 which in turn activates the JNK pathway [42]. Two mutations in the highly conserved cysteine and histidine residues of the RING domain of TRAF6 abolished its ability to promote TAK1 kinase activity, and a point mutation in the ATP-binding domain

Fig. 5. JNK is activated during ICP0-mediated AP-1 activation. (a) JNK pathway is involved in ICP0-induced AP-1 stimulation. ICP0 or control plasmid was co-transfected into 293T cell along with the AP-1 reporter gene and internal control. 24 h later, PD98059, SP600125 and SB202190 were added into the culture medium to the indicated final concentrations. Cells were harvested for dual-luciferase assay after one more hour of incubation. Only SP600125 has a dose-dependent inhibitory effect on ICP0-induced AP-1 activation. Data are means of at least three independent experiments. (b) Expression of ICP0 and its various mutants can not stimulate phosphorylation of p38. After transfecting 293T cells with the indicated constructs for 24 h, cells were collected, and lysates were analyzed by immmunoblotting using specific antibody as indicated. Input: Flag-tagged ICP0 variants, Flag-p38 and HA-TAB1 were detected. IP: Exogenous p38 was then immunoprecipitated with anti-Flag beads, and the immunoprecipitates were subjected to immunoblotting using anti-Flag or anti-phospho-p38 MAPK antibodies. (c) ICP0 stimulates JNK activity. After transfecting 293T cells with the indicated constructs for 24 h, cells were harvested and exogenous HA-JNK was immunoprecipitated. Upper panel: JNK activity was checked by standard kinase assay using bacterially expressed GST-ATF2 (1–109) as substrate. The equal immunoprecipitation efficiency of HA-JNK was detected. Lower panel: equal expression efficiency of the indicated protein. Total protein was immunoblotted and probed with anti-Flag antibodies (TRAF6 and ICP0), anti-LacZ antibodies (LacZ), anti-HA antibodies (HA-JNK) and anti-β-actin antibodies for loading control. (d) The kinase-deficient mutant JNK (K55M) and the phosphorylation-defective mutant JNK (T183A/Y185F) impair the JNK activity induced by ICP0. After transfecting 293T cells with the indicated constructs for 24 h, cells were collected, and exogenous JNK (anti-HA antibodies for wide type JNK and anti-GG antibodies for mutant JNK) was immunoprecipitated for standard kinase assay using GST-ATF2 (1–109) as substrate. The equal efficiency of immunoprecipitation and expression of the indicated protein were also detected. (e) The RING domain of ICP0 acts a crucial role during JNK activation, and this activation is attenuated by TAK1 (K63W) (Lane 3) not TRAF6 (C85A/H87A) (lane 4). 293T cells were transfected with HA-JNK and indicated constructs. 24 h later, cells were harvested, and exogenous HA-JNK was immunoprecipitated for standard kinase assay using GST-ATF2 (1–109) as substrate. The equal immunoprecipitation efficiency of HA-JNK and the equal expression efficiency of the indicated protein were detected.

of TAK1 abrogated its ability to stimulate JNK. To examine the possible role of TRAF6 and TAK1 in mediating ICP0-induced JNK activation, the dominant-negative TRAF6 (C85A/H87A) or TAK1 (K63W) was transfected into 293T cells with ICP0 and AP-1-Luc. As was shown in Figs. 6a and b, AP-1 activation by ICP0 was not affected at all even in the presence of a large amount of TRAF6 (C85A/H87A), which suggested that TRAF6 was not essential for ICP0-mediated AP-1 activation and ICP0 acted downstream of TRAF6. When the kinase-dead mutant of TAK1 was transfected into 293T cells together with ICP0 and AP-1-Luc, it produced a significant reduction of AP-1 activation.



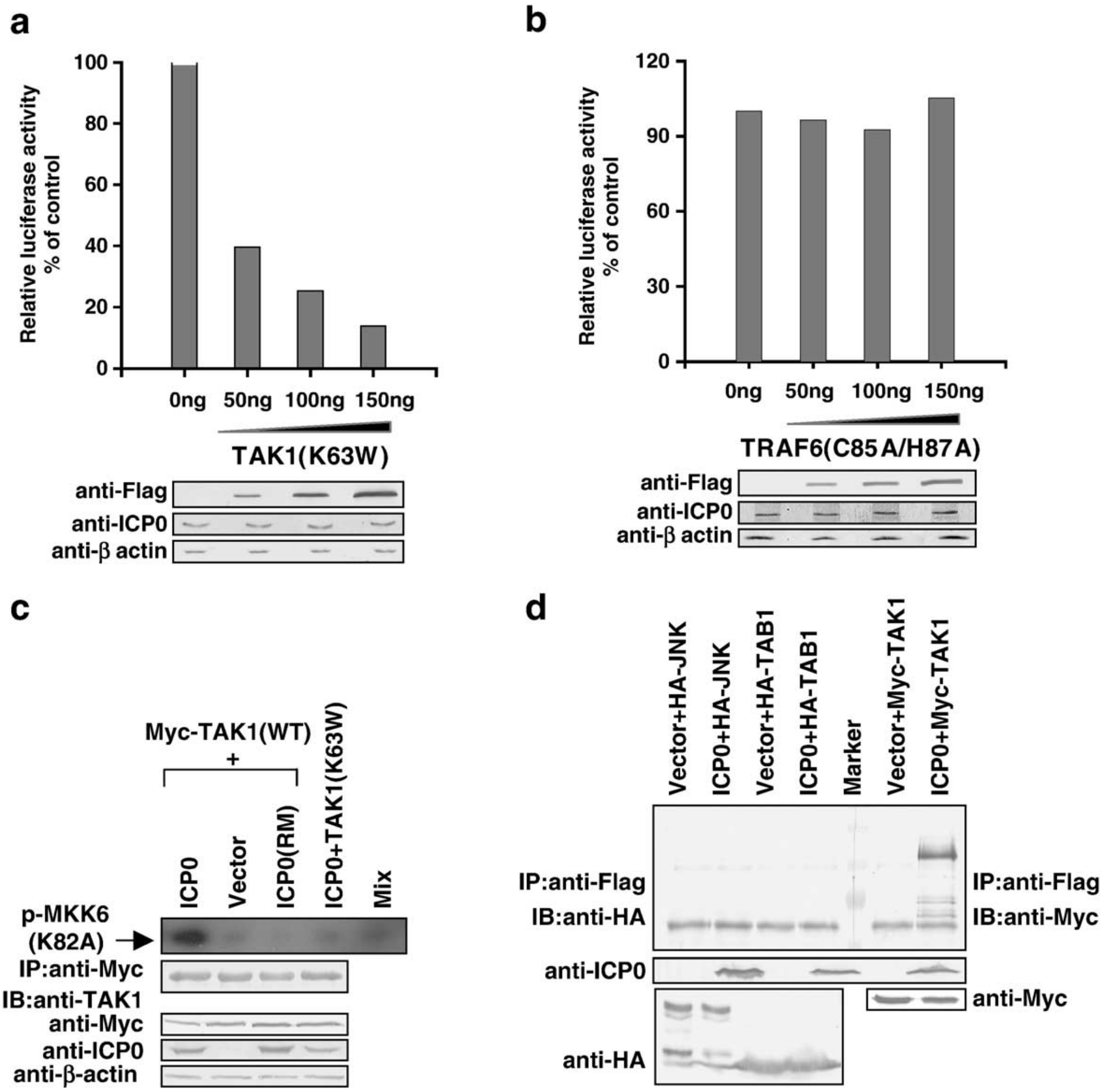


Fig. 6. TAK1 is involved in ICP0-mediated JNK activation. Activation of AP-1 by ICP0 is inhibited by Flag-TAK1 (K63W) (a) but not inhibited by Flag-TRAF6 (C85A/H87A) (b). (c) ICP0 stimulates TAK1 activity. An empty vector, ICP0, or ICP0 (RM) was transfected into 293T cells together with Myc-TAK1 (WT), and Myc-TAK1 (K63W) was co-transfected with ICP0. 24 h post transfection, Myc-tagged TAK1 variants were immunoprecipitated with anti-myc antibodies for kinase assay. The catalytically inactive MKK6 (K82A) was used as the substrate. The equal immunoprecipitation efficiency was detected with anti-TAK1 antibodies, and the equal expression efficiency of the indicated protein was detected in the three lower panels. (d) ICP0 physically interacts with TAK1. ICP0 or vector plasmid was co-transfected with HA-JNK, HA-TAB1 or Myc-TAK1 respectively. 24 h later, cells were lysed, and ICP0 was immunoprecipitated by anti-flag beads. The beads were then checked with anti-HA or anti-Myc antibodies. The equal expression efficiency of the indicated protein was shown in the three lower panels.

This proved that TAK1 was required during AP-1 activation in response to ICP0. In addition, we found that co-expression of this dominant-negative TAK1 (K63W) with ICP0 significantly reduced ICP0-induced JNK activity, while TRAF6 (C85A/H87A) could not lead to this reduction (Fig. 5e, lanes 3 and 4). Taken together, these data demonstrated that exogenous expression of ICP0 in 293T cells could lead to activation of JNK and ICP0 acted upstream of JNK, probably targeting TAK1.

Since activation of TAK1 could lead to the activation of JNK [42], we next examined whether ICP0 could stimulate TAK1 kinase activity. A control vector, ICP0 or ICP0 (RM) was separately transfected into 293T cells together with Myc-TAK1 (WT). 24 h post transfection, TAK1 was immunoprecipitated for kinase assay. The catalytically inactive MKK6 (K82A) instead of the wild type one was used as the substrate to prevent autophosphorylation of MKK6. As was shown in Fig. 6c, exogenous expression of ICP0 resulted in a robust increase in TAK1 activity, while almost none of MKK6 phosphorylation was observed in 293T cells co-transfected with the control vector. When the integrity of the Zinc Ring finger domain was destroyed, the activation of TAK1 by ICP0 was reduced to the basal level too. Consistent with the inhibitory effects observed in JNK kinase assay (Fig. 5e) and luciferase assay (Fig. 6a), TAK1 (K63W) also abolished the ICP0-induced TAK1 activation (Fig. 6c). These results strongly supported the notion that ICP0 might target TAK1 directly. To explore this possibility, we co-transfected flag-ICP0 with HA-JNK, HA-TAB1 or Myc-TAK1 respectively. Then, flag-ICP0 immunoprecipitates were analyzed to check which protein was pulled down by ICP0. It turned out that full-length TAK1 interacted specifically with ICP0, while JNK and TAB1 did not show any affinity to ICP0 (Fig. 6d). Taken together, these data convincingly established that ICP0 was able to manipulate the JNK signaling pathway, and it fulfilled this function by directly interacting with TAK1 and stimulating the kinase activity of the latter.

Discussion

As the 'pirates of the cell', viruses have been known to hijack cellular signaling pathways and transcription factors and adapt them to their own advantage. In particular, the cellular activator protein 1 (AP-1) pathway appears to be an attractive target for common human viral pathogens. The fine tuning of AP-1 activity is important for flexible regulation of cell growth, gene expression, differentiation and development in response to different stimuli [17]. Several viral proteins, such as polyoma virus middle-sized tumor antigen [43], hepatitis B virus HBx [44], Epstein–Barr virus latent membrane protein-1 [45], HCMV IE1 [46], HSV-2 ICP10 PK [20,21] and SARS coronavirus nucleocapsid protein [47], have been shown to activate AP-1. However, the underlying mechanism appears to be different among them. Adding to the list, our study demonstrated that ICP0 could also trigger the AP-1 activity in a unique way. Since ICP0 belongs to the IE proteins which are required for the activation of later classes of viral genes [48] and this ICP0 plays a key role in regulating the balance between the lytic and latent states [7], we suggest that activation of AP-1 by ICP0 may represent a mechanism by which the virus manipulates cellular processes to facilitate viral lytic cycle. It might be of some significance for viral growth and/or DNA replication. In addition, the upregulation of AP-1 activity possibly transactivates a subset of AP-1-dependent cellular genes whose protein products are required for viral life cycle. Alternatively, stimulation of this JNK signaling pathway could represent a defense mechanism of the host cell against virus invasion, with the aim of abrogating virus replication by programmed cell death or apoptosis [49,50]. Confirmation of these hypotheses requires a full understanding of the biological mechanisms activated by viral infection and a detailed analysis of the host cell responses during the viral invasion. However, whatever its precise significance in the viral life cycle, it is clear that activation of AP-1 by ICP0 is one of the importantly stimulatory effects of HSV infection on cellular biology. This effect is likely to play a significant role in the interaction between HSV and the cells it infects.

In this paper, we show that ICP0 can induce AP-1-dependent transcription via the TAK1→JNK→AP-1 cascade. This notion is supported by several lines of evidence in the present study. First, ICP0 was able to significantly stimulate gene expression of a luciferase reporter construct that contained several tandem repeats of authentic AP-1 sequence at the promoter region. In addition, ICP0 could only activate this AP-1-Luc reporter construct and had no stimulatory effect on either LEF-Luc or Gal4-Luc reporter construct. Therefore, a functional connection could be drawn between ICP0 and the AP-1 signaling pathway. One possible mechanism is that ICP0 might interact with c-Fos or c-Jun and thereby modulate AP-1 activity directly. However, we failed to detect any direct association between ICP0 and AP-1 (data not shown). Consequently, it is likely that activation of MAPK is involved during ICP0-induced AP-1 activation. In support of this, AP-1 induction by ICP0 was blocked efficiently by the repressor c-Jun (S73A) and c-Jun (S63/73A), suggesting that phosphorylation of c-Jun was essential for the process. Second, to find out the identity of the MAPK involved, the phosphorylation-defective mutants were introduced. It turned out that the dominant-negative mutant p38 (AF) significantly reduced UV-induced AP-1 activation, while it had no inhibitory effect on ICP0-driven AP-1 activation. Furthermore, neither ERK1 (K71R) nor ERK2 (K52R), the two dominant-negative repressors for ERK pathway, inhibited ICP0-induced AP-1 activation. These data indicated that JNK, but not p38 MAPK or ERK, might participate in the ICP0-induced AP-1 activation. Third, ICP0-induced activation of AP-1 was specifically attenuated by the selective JNK inhibitor, SP600125. In parallel experiments, PD98059 (an ERK inhibitor) and SB202190 (a p38 MAPK inhibitor) did not show any significant effect, suggesting a role of JNK in modulating the ICP0-induced AP-1 stimulation. This hypothesis was further substantiated by the finding that overexpression of ICP0 could lead to a robust increase in JNK activity, but not in any phosphorylation of p38. Fourth, the dominant-negative MAP3K inhibitor TAK1 (K63W) greatly attenuated ICP0-induced AP-1 reporter gene activation, while the upstream inhibitor TRAF6 (C85A/H87A) could not. Consistent with this observation, TAK1 (K63W), but not TRAF6 (C85A/H87A), was found to significantly reduce ICP0-induced JNK activity. Last, by means of in vitro kinase assay, we demonstrated that expression of ICP0 per se could stimulate TAK1 kinase activity. In addition, coimmunoprecipitation assay showed that ICP0 interacted specifically with TAK1, but not with JNK or TAB1. Taken together, we concluded that ICP0 activated AP-1 pathway through interacting with TAK1 and promoting the kinase activity of the latter.

Albeit we show that the TAK1/JNK/c-Jun cascade can mediate AP-1 induction by ICP0, a gap remains in the signaling cascade between ICP0 and TAK1. How ICP0 activates TAK1 remains elusive. A recent study showed that the ubiquitinated TRAF6 and the formation of K63-

linked polyubiquitin chains could directly activate the endogenous TAK1 complex [51]. Given that ICP0 is an ubiquitin ligase E3, we hypothesize that ICP0 may activate TAK1 by mimicking TRAF6. This is supported by several lines of evidence. First, the RING domain of ICP0 was necessary for AP-1 activation, and the triplet-point mutant of ICP0 (116A/136A/156A) was deprived of the ability to stimulate AP-1 reporter gene (Fig. 3c). In addition, the RING domain mutant of ICP0 also failed to activate TAK1 (Fig. 6c) as well as JNK (Fig. 5e). All these demonstrated that the RING domain was indispensable and implicated that ICP0 might serve as an ubiquitin ligase E3 in the process. Second, ICP0, just like TRAF6, can also catalyze the synthesis of unique polyubiquitin chains linked through lysine-63 (K63) of ubiquitin with ubiquitination enzymes such as E1 and Ubc13ev1A (data not shown). Third, the self-ubiquitination of BICP0, the functional counterpart of ICP0, is also detected and proved to be important during its effective transactivation of AP-1-dependent transcription (data not shown). Thus, we would like to suggest that the K63-linked polyubiquitin chains attached to ICP0 may reach into TAK1 to activate it as a result of complex formation among TAB1, TAB2 and TAK1. It is also formally possible that there is a transient but undetectable level of poly-ubiquitination on TAK1, and this dynamic ubiquitination of TAK1 leads directly to its activation. Verification of this hypothesis requires a biochemically well-defined system in vitro, which we are currently trying to establish in this laboratory.

In summary, we propose a model as follows (Fig. 7): The gD envelope glycoprotein of HSV binds to its cellular receptor, the HSV entry mediator A (HveA) [52], which is a member of the TNFR superfamily and whose cytoplasmic region binds to TRAFs. HSV-1 infection leads to the synthesis of IE viral proteins including ICP0. In turn, ICP0 catalyzes the K63-linked polyubiquitin chains with the help of Ubc13ev1A. Then, it specifically recognizes and interacts with the TAK1. Notwithstanding this uncertainty concerning the detailed mechanism of TAK1 activation, our data demonstrate the activation of TAK1 induced by ICP0. Activated TAK1 then phosphorylates MKK6, leading to the activation of JNK kinase pathways. Induction of this pathway by ICP0 may lead to the activation of cellular genes important for HSV itself and also can cause the activation of other viruses residing in the same cells, such as human immunodeficiency virus (HIV). Indeed, a direct effect of HSV superinfection on HIV-1 replication has been demonstrated [53]. In addition, ICP0 was once reported to stimulate the HIV-1 LTR enhancer, which consequently enhanced gene expressions and replication of HIV-1 per se [54]. Furthermore, increased activity of AP-1 may disrupt normal cellular functions and lead eventually to uncon-

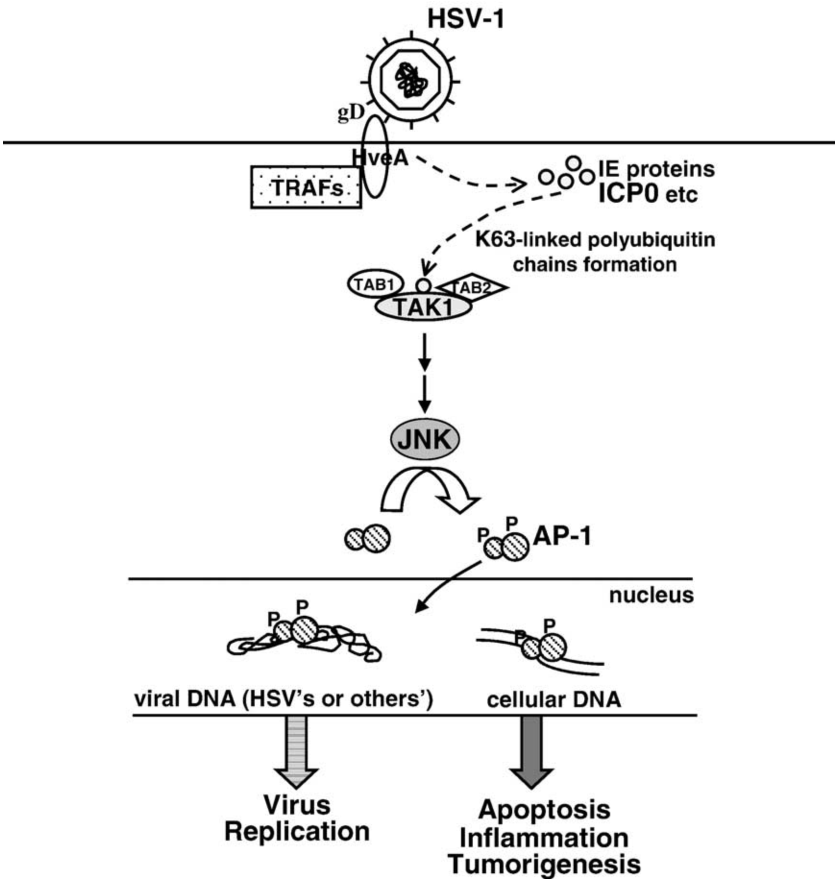


Fig. 7. Model for the signaling pathway that ICP0 hijacks to activate a myriad of viral and cellular genes. See text for details.

trolled cell growth, inflammation and tumorigenesis. Because HSV is prevalent in the human population and we have shown ICP0 to be a potent activator of the cellular transcription factor AP-1, it will be important to define the exact roles played by the JNK pathway in ICP0-mediated cellular transformation. The intermediary signaling components we have identified could be used as potential drug targets to interfere with the ICP0-mediated JNK pathway. Such a reagent would be of potential therapeutic benefit in the treatment of HSV-associated diseases.

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