Suppression of PACT-induced type I interferon production by herpes simplex virus type 1 Us11 protein

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Herpes simplex virus type 1 (HSV-1) Us11 protein is a double-stranded RNA-binding protein that suppresses type I interferon production through the inhibition of cytoplasmic RNA sensor RIG-I. Whether additional cellular mediators are involved in this suppression remains to be determined. In this study we report on the requirement of cellular double-stranded RNA-binding protein PACT for Us11-mediated perturbation of type I interferon production. US11 associates with PACT tightly to prevent it from binding with and activating RIG-I. The Us11-deficient HSV-1 was indistinguishable from the Us11-proficient virus in the suppression of interferon production when PACT was compromised. More importantly, HSV-1-induced activation of interferon production was abrogated in PACT-knockout murine embryonic fibroblasts. Our findings suggest a new mechanism for viral evasion of innate immunity through which a viral double-stranded RNA-binding protein interacts with PACT to circumvent type I interferon production. This mechanism might also be used by other PACT-binding viral interferon-antagonizing proteins such as Ebola virus VP35 and influenza A virus NS1.
Herpes simplex virus type 1 (HSV-1) commonly infects human causing cold sores, fever blisters and genital sores. It also establishes a lifelong persistent infection in trigeminal ganglion during which only the latency-associated transcript, but not other lytic genes, is expressed. Infection with HSV-1 triggers host innate immune response through the recognition of pathogen-associated molecular patterns by cellular pattern recognition receptors including both Toll-like receptors and RIG-I-like receptors (1). As a result antiviral cytokines such as type I interferons (IFNs) are produced. To facilitate its replication and persistence, HSV-1 has developed various countermeasures to suppress the production and action of type I IFNs and IFN-stimulated genes (2).

Immediate-early protein ICP0, a master regulator of HSV-1 infection and virus-host interaction, is capable of antagonizing innate immunity at multiple levels (2, 3). The delayed-early protein ICP34.5, which is also known as γ34.5 or γ134.5 for its increased expression in later phases of viral infection, is required in certain cell types to prevent an antiviral response where protein synthesis is abruptly inhibited prior to the completion of the virus lifecycle (2-5). ICP34.5 also antagonizes the IFN response (2, 4). Besides ICP0 and ICP34.5, the Us11 gene encodes an additional protein that counteracts IFN production and signaling (5-9). Us11 is a multifunctional double-stranded RNA (dsRNA)-binding protein expressed in the late stage of infection and incorporated to the virion (10-12). Us11 associates with PKR, PACT, 2'-5' oligoadenylate synthetase (OAS), RIG-I and MDA5 in the cell (7, 13-15). It uses its RNA-binding domain to interact with PKR kinase leading to the prevention of eIF2α phosphorylation and the inhibition of autophagy (7, 13, 16). It also binds with OAS, RIG-I and MDA5 to impede their function in IFN induction and IFN response (14, 15). PACT is a cellular dsRNA-binding protein originally identified as a binding partner.
and activator of PKR (17). Although Us11 binds with PACT and inhibits PKR activation, its interaction with PKR but not with PACT is required for the inhibition of PKR activity (13).

RIG-I is a prototypic cytoplasmic sensor of virus-derived RNAs (18, 19). RIG-I-like receptors are critically involved in the sensing of HSV infection (20, 21). Particularly, HSV-1-encoded small non-coding RNAs derived from the latency-associated transcript are sensed by RIG-I (22). Optimal function of RIG-I requires PACT, which interacts with and potently activates RIG-I in a PKR-independent manner (23). Us11 interacts with RIG-I and its homolog MDA5 to suppress their activation of type I IFN production (15). Us11 also interacts with PACT, but the biological function of this interaction is unclear (13). In particular, it remains to be understood whether the interaction between Us11 and PACT might play a role in the perturbation of RIG-I-dependent IFN production.

In the present study we investigated the interaction of Us11 with PACT and determined the requirement of PACT in Us11-induced suppression of innate antiviral response. Our findings reveal a new mechanism for viral evasion of innate immunity, by which a viral dsRNA-binding and IFN-antagonizing protein interacts with PACT to impede the association with and activation of RIG-I.
MATERIAL AND METHODS

Cells and viruses. HEK293 and HEK293T were maintained and propagated in Dulbecco's modified Eagle medium. Mouse embryonic fibroblasts (MEFs) were derived from wild type C57/B6 and PACT^−/− mice (24, 25) kindly provided to Dr. Kuan-Teh Jeang’s laboratory in the National Institutes of Health by Dr. Ganes Sen (Cleveland Clinic, Ohio, USA). Lack of PACT expression in PACT^−/− MEFs was previously verified by Western blotting (26). hTERT-immortalized MEFs were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Sendai virus was obtained from American Type Culture Collection (Manassas, Virginia, USA). Recombinant virus HSV-1-EGFP-Us11, HSV-1-pAUs11, HSV-1-Δ34.5 and HSV-1-Δ34.5-(IE)Us11 were propagated and purified as previously described (27, 28).

Plasmids and siRNAs. Expression vectors for Flag-RIG-I, Flag-PACT, Myc-PACT, Us11 and EGFP-Us11 were described (13, 23, 27). Expression plasmids for N-terminal truncated Us11 (Us11N) and C-terminal truncated Us11 (Us11C) were constructed by PCR. Primers for Us11N are 5'-ATCGAAGCTT TTATGAGCCA GACCCAAACC-3' (forward) and 5'-ATCGCTGCAG CTATGTCCTG GGGATTGTG GC-3' (reverse). Primers for Us11C are 5'-ATCGAAGCTT TTCCGCGTG TTATGTCCTG GGGATTGTG GC-3' (forward) and 5'-ATCGCTGCAG CTATACAGAC CCGCGAGCG-3' (reverse). Expression plasmids of domain 1- and domain 2-deleted PACT mutants (PACTΔD1 and PACTΔD2) were constructed by overlap extension PCR (29). Primers for PACTΔD1 are 5'-ATCGGGATCC ATGTCCCAGACAGGCACGG-3' (forward), 5'-ATCGGGATCC ATGTCCCAGACAGGCACGG-3' (forward), 5'-ATCGAAGCTT TTACTTTCTT TCTGCTAT-3' (reverse), 5'- CAGCTAAGCC AGGGAAAACA AAAGCCAATG CAAGTATTTG CT-3' (forward
overlap) and 5'-AGCAAAATCT TGATTGGCT TTTGTTTTCC CTGGCTTAGC TG-3' (reverse overlap). Primers for PACTΔD2 are 5'-ATCGGGATCC ATGTCCCAGA GCAGGCACG -3' (forward), 5'- AGCAAAATCT TGATTGGCT TTTGTTTTCC CTGGCTTAGC TG-3' (reverse), 5'-AGCAACCAAA GAACCAGCTT AATAGTAATA TTTCTCCAGA GA-3' (forward overlap) and 5'-AAATGTGGTT CTCTG GAGAAC TATATTACTAT TAAGCTGGTT CT-3' (reverse overlap). siRNAs against PACT, si-PACT1 and si-PACT2, were described elsewhere (23).

**Biochemical fractionation.** HEK293T cells were infected with HSV-1-EGFP-Us11 virus. Cells were harvested at 12 h post infection and lysed in fractionation buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 8.0). Cell lysates were clarified and fractionated on a Sephadex 200 column as described (30). Fractions were run on SDS-PAGE and blotted with anti-GFP and anti-PACT from Abcam (Cambridge, Massachusetts, USA).

**Protein and RNA analysis.** Western blotting, co-immunoprecipitation, dual luciferase assay, and quantitative RT-PCR were performed as described (31-33). For immunoprecipitation, mouse anti-Flag (M2) from Sigma-Aldrich (St. Louis, Missouri, USA) and rabbit anti-GFP from Abcam were used. For Western blotting, mouse anti-Flag (M5) from Sigma, mouse anti-GFP (B-2) from Santa Cruz (Dallas, Texas, USA), mouse anti-ICP0 from Santa Cruz and mouse anti-β-actin from Sigma were used. For quantitative RT-PCR, the level of target mRNA was calculated from $2^{-\Delta Ct}$ by the comparative Ct method (31). Primers used in quantitative RT-PCR are:

- 5'-GCAGCATGGCT GAATGAGACT A-3' (human IFN-β forward), 5'-CTCCTTGCC TTCAGGTAAT-3' (human IFN-β reverse), 5'-GCATCTGCC CCCCATATT-3' (human CCL5 forward), 5'-AGCAGCTGCC ACTGTTGATG-3' (human CCL5 forward), 5'-AGCAGCTGCC ACTGTTGATG-3' (human CCL5
reverse), 5'-GACCTGACGG TGAAGATGCT-3' (human ISG15 forward), 5'-
GAAGGTCAGC CAGAACAGGT-3' (human ISG15 reverse), 5'-GCAGCCAAGT
TTACCGAAG-3' (human ISG56 forward), 5'-GCCTTTCTCC GAAGTTTCCT-3'
(human ISG56 reverse), 5'- CACAGCCCTC TCGATCAACT-3' (mouse IFN-β
forward), 5'-GCATCTTCTC CGTCATCTCC-3' (mouse IFN-β reverse), 5'-
TTCTTGGGAC TGATGCTGGT-3' (mouse interleukin-6 or IL-6 forward) and 5'-
GCCATTGCAC AACTCTTTTC T-3' (mouse IL-6 reverse). Primers used to amplify
glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were described
elsewhere (31).
RESULTS AND DISCUSSION

Us11 targeting of PACT. RIG-I can activate IFN production and PACT potently augments this activity (23). Us11 is thought to directly bind RIG-I and MDA5 to inhibit their function (15), but it also physically binds PACT (13). Thus, it will be of interest to see whether the inhibitory effect of Us11 on IFN production is PACT-dependent or not. To shed light on this, we analyzed the inhibitory activity of Us11 in the presence of RIG-I and PACT. In line with our previous findings (23), PACT potentiated the stimulatory effect of RIG-I on luciferase reporter expression driven by IFN-β promoter (IFN-β-Luc) or by IRF3-binding enhancer elements (IRF3-Luc), but not by κB elements (κB-Luc) (Fig. 1, A-C). Interestingly, in HEK293 cells where endogenous PACT is relatively low (23), Us11 did not suppress RIG-I activity on IFN-β-Luc or IRF3-Luc in the absence of ectopically expressed PACT (Fig. 1, A and B, lane 7 compared to lane 3). In other words, RIG-I was functionally intact in the activation of IFN production in the presence of both RIG-I and Us11. In contrast, Us11 almost completely blunted the PACT-augmented activation of RIG-I when both PACT and RIG-I were overexpressed (Fig. 1, A and B, lane 8 compared to lane 4). As reported previously (23) and as verified in Fig. 1, while RIG-I activates both IRF3 and NF-κB, PACT can only stimulate RIG-I-induced activation of IRF3, but not NF-κB. If Us11 targets RIG-I directly, it would probably suppress both IRF3 and NF-κB. In contrast, if Us11 counteracts PACT, it should not affect NF-κB activation. Indeed, Us11 had no effect on the activation of κB-Luc reporter by RIG-I (Fig. 1C). Thus, Us11 apparently inhibited IFN production by antagonizing PACT but not RIG-I directly.
Us11 associates with PACT to impede its interaction with RIG-I. Since both Us11 and PACT are dsRNA-binding proteins (7, 12, 23), their association could be mediated through dsRNA. Although Us11 interacts with PACT in vitro (13), it is not known whether they associate with each other in a dsRNA-independent manner in infected cells. To verify this, we infect HEK293T cells with HSV-1-EGFP-Us11, a recombinant virus expressing an EGFP-Us11 fusion protein (27, 28). Fusion of EGFP with Us11 does not affect biological function of Us11, but provides a useful marker for the tracing of infected cells (27, 28). The expression level of endogenous PACT in HEK293T cells is relatively higher than in HEK293 cells. Biochemical fractionation in the presence of RNase A indicated the presence of PACT and EGFP-Us11 in the same fractions 38-42 of the protein extract of infected cells (Fig. 2A). Reciprocal co-immunoprecipitation and immunoblotting experiments confirmed RNase A-insensitive association of EGFP-Us11 and Flag-PACT in transfected HEK293T cells (Fig. 2B), consistent with the possibility that this interaction is RNA-independent. In particular, both proteins were found in the precipitate prepared with anti-Flag or anti-GFP (Fig. 2B, lane 3 compared to lanes 1 and 2). To verify the digestion of dsRNA by RNase A in our experimental setting, we incubated an in vitro transcribed and annealed dsRNA (S7 dsRNA) with RNase A in the same buffer used in our fractionation and immunoprecipitation experiments. Complete degradation of S7 dsRNA indicated the effectiveness of RNase A in dsRNA digestion under our conditions (Fig. 2C, lane 2 compared to lane 1). Similar results were also obtained when we repeated our fractionation and immunoprecipitation experiments in the presence of RNase III which degrades dsRNA more efficiently (data not shown). Hence, the association between Us11 and PACT was not sensitive to RNase A or RNase III digestion and thus was unlikely mediated through dsRNA. Furthermore,
immunoprecipitation was also performed with lysates of HEK293T cells infected with Us11-proficient HSV-1-EGFP-Us11 and Us11-deficient HSV-1-pAU11 viruses (27). Notably, the Us11 locus of HSV-1-pAU11 is not expressed as depicted in Fig. 2D. Endogenous PACT protein was found in the EGFP-Us11-containing immune complex obtained from EGFP-Us11-overexpressing and HSV-1-EGFP-Us11-infected cells, but not from cells infected with HSV-1-pAU11 (Fig. 2D, lanes 4 and 5 compared to lane 6). Thus, Us11 associates with endogenous PACT in infected cells.

Us11 interacts with PACT (Fig. 2) and RIG-I (15). To perturb the function of RIG-I, Us11 could either impede the interaction between PACT and RIG-I or inhibit the activity of RIG-I in a triple complex containing all three proteins. To explore these two possibilities, we investigated whether PACT and RIG-I are mutually exclusive in the interaction with Us11. Different combinations of Us11, PACT and RIG-I were expressed in HEK293T cells and immunoprecipitation was subsequently performed to examine their interactions. When we overexpressed PACT and Us11, Us11 was detected in the protein complex that contains PACT (Fig. 3A, lane 3; Fig. 3B, lane 2 and Fig. 3C, lane 2). However, Us11 was not found in the RIG-I-containing complex when Us11 and RIG-I were overexpressed (Fig. 3A, lane 2). Although an interaction between Us11 and RIG-I could not be totally excluded; this result did suggest that Us11 might interact with PACT more potently or directly. We next performed reciprocal immunoprecipitation and immunoblotting experiments with HEK293T cells overexpressing RIG-I, PACT and Us11. Only PACT, but not RIG-I was found in the Us11-containing complex, although Us11 was detected in the precipitate that harbors PACT or RIG-I (Fig. 3C, lane 3 compared to Fig. 3B, lane 3). In the absence of Us11, PACT and RIG-I formed a complex (Fig. 3D lane 2). However, when all three proteins were overexpressed, RIG-I disappeared from the complex that
contains PACT (Fig. 3D, lane 3). Meanwhile, Us11 was detected in the PACT-containing complex (Fig. 3D, lane 3). These results were consistent with the model in which Us11 interacts directly with PACT to prevent it from interacting with RIG-I.

To map the interaction domains in Us11 and PACT, truncated or deletion mutants were constructed and analyzed (Fig. 4). Us11C containing the C-terminal RNA-binding domain, but not Us11N carrying the transactivation domain, was capable of interacting with PACT (Fig. 4A, lane 6 compared to lane 5). On the other hand, PACT mutants ΔD1 and ΔD2 with deletion of dsRNA-binding domain 1 and 2 respectively lost their ability to interact with Us11 (Fig. 4B, lanes 5 and 6 compared to lane 4). Thus, generally consistent with previous findings obtained from GST pull-down assay in vitro (13), RNA-binding domains on both proteins are required for the association between Us11 and PACT in vivo.

We demonstrated the importance of the C-terminal dsRNA-binding domain of Us11 in its interaction with PACT (Fig. 4). The same domain was also used in the interaction with RIG-I, MDA5, PKR and OAS (13-15). The mechanism by which Us11 inhibits PACT function remains to be elucidated. Particularly, although we showed that Us11 and RIG-I are mutually exclusive in binding with PACT (Fig. 3), the interplay of Us11, PACT, RIG-I and MDA5 in the activation of type I IFN production merits further investigations. MDA5 has also been implicated in the IFN antagonism of Us11 (15). Since PACT can also activate MDA5 (23), it will be of interest to see whether the suppression of MDA5 by Us11 is also mediated through a direct effect on PACT. A more detailed analysis of the dynamic interaction of Us11 with PACT, RIG-I and MDA5 in infected cells by using biochemical co-fractionation as in Fig. 2A.
will shed significant light on the mechanism by which Us11 suppresses IFN production.

Results from our immunoprecipitation experiments suggested that Us11 associates with PACT more tightly or directly than with RIG-I (Fig. 3). In our setting we were even unable to detect an interaction between Us11 and RIG-I. We therefore argued that Us11 suppresses RIG-I function through direct interaction and inhibition of PACT. We did not exclude the possibility that Us11 might still interact with and inhibit RIG-I and MDA5 as previously reported (15). Plausibly, this could be mediated indirectly through RNA or an adaptor protein. Mapping the interaction domains and comparing the binding affinities between purified recombinant Us11 and PACT, PACT and RIG-I, as well as Us11 and RIG-I proteins using in vitro affinity binding assays, fluorescence anisotropy measurement and surface plasmon resonance technology would clarify whether Us11 indeed interacts with PACT with high affinity to prevent it from interacting with RIG-I and MDA5.

Requirement of PACT for Us11 suppression of IFN production. Sendai virus (SeV) is a potent inducer of type I IFNs. To verify the activity of Us11 to antagonize IFN production, we asked how expression of EGFP-Us11 might affect the induction of IFN and IFN-stimulated genes by SeV. We observed a significant reduction in the levels of IFN-β, CCL5, ISG15 and ISG56 transcripts in HEK293T cells stably expressing EGFP-Us11 (Fig. 5, A to D, lanes 4 compared to lanes 3). Thus, EGFP-Us11 can sufficiently suppress IFN production induced by SeV. With this in mind, we compared Us11-proficient HSV-1-EGFP-Us11 and Us11-deficient HSV-1-pAUs11 viruses in the suppression of SeV-induced IFN production in PACT-compromised cells. The knockdown effect of two siRNAs against PACT mRNA, siPACT1 and siPACT2, in HEK293T cells was verified by Western blotting (Fig. 6A). In the
absence of siPACT1/2, infection with HSV-1-EGFP-Us11 dampened IFN-β induction by SeV (Fig. 6B, lane 4 compared to lane 2). This effect was less pronounced when cells were infected with HSV-1-pAUs11 (Fig. 6B, lane 6 compared to lane 4). However, in PACT knockdown cells transfected with siPACT1, HSV-1-EGFP-Us11 and HSV-1-pAUs11 were equally competent in the suppression of SeV-induced IFN-β production (Fig. 6B, lane 12 compared to lane 10). In this setting, both viruses circumvented IFN production in a PACT-independent manner. The expression of EGFP-Us11 and ICP0, a marker for HSV-1 infection, in infected cells was verified (Fig. 6C). Similar results were also obtained with siPACT2 (data not shown). Thus, the inhibitory role of Us11 on SeV-induced IFN production requires PACT.

Neither HSV-1-EGFP-Us11 nor HSV-1-pAUs11 induced IFN-β in infected cells (Fig. 6B, lanes 3 and 5). This was generally consistent with previous findings on wild-type HSV-1 (2). HSV-1 encodes multiple viral proteins that antagonize IFN production (2-4). The induction of IFNs by either Us11-proficient or Us11-deficient HSV-1 would be efficiently suppressed by virus-encoded IFN-antagonizing proteins such as ICP0 and ICP34.5 (3, 4). In view of this, in the next part of our study we employed ICP34.5-deficient viruses to investigate the IFN induction by HSV-1 and the influence of Us11 on this induction.

Both HSV-1-EGFP-Us11 and HSV-1-pAUs11 viruses were capable of suppressing SeV-induced IFN production in PACT-compromised cells (Fig. 6B, lanes 10 and 12 compared to lane 8). This suppression might be mediated through other IFN-antagonizing proteins such as ICP0 and ICP34.5 in a PACT- and Us11-independent manner. More experiments are required to clarify whether PACT is
dispensable for ICP0- and ICP34.5-mediated suppression of innate antiviral response.

PACT acts upstream of RIG-I and MDA5 in the activation of IFN production (23). That is to say, RIG-I and MDA5 function is severely impaired when PACT is compromised. Although our results did not support a direct interaction between Us11 and RIG-I (Fig. 3), Us11 could still inhibit the activity of RIG-I and MDA5 through PACT or other proteins. Thus, the requirement of RIG-I and MDA5 for Us11-dependent suppression of IFN production should be further investigated in RIG-I\textsuperscript{-/-} and MDA5\textsuperscript{-/-} MEFs.

**Requirement of PACT for HSV-1 induction of IFN production.** Above we used SeV to induce IFN production in HSV-1-infected cells. Since the induction and action of IFNs were inhibited in part by HSV-1 ICP34.5 (2, 4), here we employed an ICP34.5-deleted virus named HSV-1-\(\Delta\)34.5 to induce IFNs. Us11 was not expressed in the early phase of infection when the cells were collected for analysis. Another virus HSV-1-\(\Delta\)34.5-(IE)Us11, from which Us11 is expressed in the immediate early phase of infection (Fig. 7A), was also included in our analysis. This latter virus would allow us to analyze the effect of Us11 expression in the immediate early phase on IFN production (27). These two viruses induced IFN-\(\beta\) expression swiftly after infection of wild type MEFs that are PACT-proficient. As expected, the induction was less pronounced in HSV-1-\(\Delta\)34.5-(IE)Us11-infected cells due to immediate early expression of Us11 that antagonizes IFN production (Fig. 7B, group 3 compared to group 2). To shed light on the role of PACT in HSV-1 induction of type I IFNs, we employed PACT\textsuperscript{-/-} MEFs in which the PACT locus was genetically disrupted. As previously characterized in the literature and by us, no functional PACT protein is
expressed in these MEFs (24-26). In sharp contrast to the results obtained in PACT-proficient cells, no IFN-β was induced when these PACT<sup>−/−</sup> MEFs were infected with either virus (Fig. 7B, groups 5 and 6). That is to say, PACT is required for IFN induction by HSV-1.

We also examined the induction of IL-6, the expression of which is controlled primarily by NF-κB, in MEFs infected with the two mutant HSV-1 viruses. IL-6 expression was activated in PACT-proficient wild type MEFs infected with either virus (Fig. 7C, groups 2 and 3). Consistent with earlier results on the effect of Us11 on NF-κB activation obtained in transfected cells overexpressing Us11 (Fig. 1C), immediate early expression of Us11 did not affect IL-6 induction (Fig. 7C, group 3 compared to group 2). In the absence of PACT, the basal expression of IL-6 was elevated due to unknown reason (Fig. 7C, groups 4-6). In this setting, the two viruses were unable to further activate IL-6 expression. Nevertheless, Us11 was not influential in the activation of IL-6 production.

Exactly how host cells sense HSV-1 to induce type I IFNs remains mysterious (1, 2). RIG-I has been implicated in this process (1, 20, 21). More recent evidence points to the importance of DNA sensors such as IFI16, cGAS and STING (34-36). Our findings that neither HSV-1-Δ34.5 nor HSV-1-Δ34.5-(IE)Us11 virus induced IFN-β in PACT<sup>−/−</sup> MEFs suggested the requirement of PACT for the sensing of HSV-1. It remains to be elucidated as to whether and how PACT cooperates with RIG-I or other sensors to mediate the activation of IFN production by HSV-1. PACT is also known to affect PKR and Dicer function (13, 30). Involvement of PKR and/or Dicer in HSV-1-induced innate immune response could not be excluded but merits further analysis. Theoretically, Us11 and other HSV-1 proteins might also counteract the...
action of PACT in the sensing of HSV-1. To clarify this, the influence of PACT and Us11 on the induction of IFN by HSV-1 DNA should be assessed.

We obtained two lines of evidence concerning the influence of Us11 on NF-κB activation. First, expression of Us11 did not influence RIG-I-induced activation of NF-κB (Fig. 1C). Second, HSV-1-Δ34.5 virus did not induce more IL-6 than HSV-1-Δ34.5-(IE)Us11 (Fig. 7C). Because PACT does not affect RIG-I-dependent activation of NF-κB (23), our results that Us11 did not inhibit NF-κB activation are in keeping with the notion that Us11 directly suppresses PACT but not RIG-I. Us11 was previously found to inhibit NF-κB activation induced by SeV (15). More experiments are needed to clarify whether this might be mediated through a RIG-I-independent mechanism. The induction of IL-6 in mock-infected PACT-/- MEFs was indicative of NF-κB activation (Fig. 7C). PACT is known to affect NF-κB activation through PKR (37). The activation of NF-κB in PACT-/- MEFs might be independent of RIG-I and would be assessed in detail in the next phase of our study.

In summary, we demonstrated suppression of PACT-induced type I IFN production by HSV-1 Us11 protein. PACT-mediated enhancement of RIG-I activation was largely ablated by Us11 (Fig. 1), which interacts with PACT to impedes its interaction with RIG-I (Figs. 2-4). The elevated induction of IFN-β during the infection of PACT-competent cells with Us11-deficient HSV-1-pAUs11 was not seen in PACT-compromised cells (Fig. 5 and Fig. 6). Finally, PACT was indispensable for the activation of type I IFN production by HSV-1 (Fig. 7). Our findings revealed PACT as an essential factor in viral induction of IFNs and a new target of a viral IFN-antagonizing protein.
Other viral IFN-antagonizing proteins such as influenza A virus NS1 (38, 39) and Ebola virus VP35 (40, 41) can also interact with PACT and perturb RIG-I function. Suppression of PACT-augmented activation of RIG-I might therefore represent a viral countermeasure to combat host antiviral response commonly used by other viruses. One recent report on mutual antagonism between VP35 and PACT lent further support to this emerging new concept (42). It remains to be seen whether NS1 and PACT might also antagonize each other (43). In this regard, the influence of PACT on the function of Us11 in the life cycle of HSV-1 also warrants further analysis.
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FIGURE LEGENDS

FIG 1 Us11 inhibits PACT-induced activation of RIG-I. (A) Influence on IFN-β promoter. HEK293 cells were transfected with pIFN-β-Luc reporter and expression plasmids for Flag-PACT, Flag-RIG-I and Us11. Dual luciferase assay was performed at 33 h post-transfection. Results represent the mean ± SD of three independent measurements of firefly luciferase activity normalized to Renilla luciferase activity. Differences between the selected groups were statistically assessed by two-tailed Student’s t test: p1 = 0.002; p2 = 0.087. (B) Influence on IRF3-binding elements. p1 = 0.012. p2 = 0.083. (C) Influence on κB enhancer. p1 = 0.416. p2 = 0.393.

FIG 2 Association of Us11 with PACT. (A) Co-fractionation of Us11 and PACT in HSV-1-infected cells. HEK293T cells were infected with HSV-1-EGFP-Us11 (M.O.I. = 1). Cells were harvested at 14 h post-infection. Protein extract was incubated with 25 µg/ml RNase A for 15 min at 4 °C and then fractionated by Superdex 200 gel filtration. Column fractions were analyzed by Western blotting (WB). (B) Co-immunoprecipitation of ectopically expressed Us11 and PACT. HEK293T cells were transfected with the indicated expression plasmids. Immunoprecipitation (IP) were carried out at 48 h post-transfection with mouse anti-Flag (α-Flag) or rabbit anti-GFP (α-GFP) in the presence of 25 µg/ml RNase A. (C) dsRNA digestion by RNase A. In vitro transcribed and annealed dsRNA of about 1 kb derived from influenza A virus genomic segment 7 (S7 dsRNA) was incubated with 25 µg/ml RNase A for 15 min in the same buffer as above. RNA was then analyzed by agarose gel electrophoresis. (D) Co-immunoprecipitation of Us11 and PACT in HSV-1-infected cells. HEK293T...
cells were infected with HSV-1-EGFP-Us11 or HSV-1-pAUs11 (M.O.I. = 1) or transfected with an EGFP-Us11 expression plasmid. Proteins were analyzed at 14 h after infection or transfection. Diagrams at the top depict the difference between HSV-1-EGFP-Us11 and HSV-1-pAUs11 viruses. Open reading frame of Us11 is disrupted by the upstream poly(A) signal in the HSV-1-pAUs11 virus. Expression of HSV-1 immediate-early protein ICP0, a marker of productive infection, was probed with mouse monoclonal anti-ICP0 (α-ICP0).

**FIG 3** Us11 prevents PACT from binding with RIG-I. HEK293T cells were transfected with plasmids expressing the indicated proteins. Immunoprecipitation (IP) and Western blotting (WB) were performed at 48 h post-transfection.

**FIG 4** Mapping of binding domains. HEK293T cells were transfected with plasmids expressing the indicated proteins. Immunoprecipitation (IP) and Western blotting (WB) were performed at 48 h post-transfection.

**FIG 5** Suppression of IFN-β production by EGFP-Us11. HEK293T cells stably expressing EGFP or EGFP-Us11 were infected with SeV (100 HAU/ml). Relative expression of IFN-β mRNA (A) and IFN-stimulated transcripts CCL5, ISG15 and ISG56 (B-D) was analyzed by quantitative RT-PCR at 14 h post-infection and normalized to the level of GAPDH mRNA. Statistical analysis was performed with two-tailed Student's t test: p1 = 0.006 (A); p1 = 0.008 (B); p1 = 0.0002 (C); p1 = 0.00003 (D). ND: not detected.
FIG 6 Suppression of IFN-β induction by Us11-proficient and Us11-deficient HSV-1 viruses in PACT-compromised cells. (A) Knockdown of PACT expression by siRNAs. HEK293T cells were transfected with siPACT1/2 or scrambled siRNA. Proteins were analyzed at 72 h post-transfection. (B) Comparison of Us11-proficient and Us11-deficient viruses in the suppression of IFN-β induction in PACT-depleted cells. HEK293T cells were transfected with siPACT1 or scrambled siRNA. Cells were infected first with SeV (100 HAU/ml) at 72 h post-transfection and then with HSV-1-EGFP-Us11 or HSV-1-pAUs11 (M.O.I.=1) after another 8 h. Levels of IFN-β mRNA were determined by quantitative RT-PCR at 14 h after HSV-1 infection. Results represent the mean ± SD of three measurements of expression levels normalized to the level of GAPDH mRNA as calculated from $2^{-\Delta\Delta Ct}$. The differences between groups 4 and 6 as well as between groups 8 and 10 are statistically significant ($p_1 = 0.008$ and $p_2 = 0.004$) by two-tailed Student’s t test, whereas the difference between groups 10 and 12 is statistically insignificant ($p_3 = 0.287$). ND: not detected. (C) Verification of protein expression.

FIG 7 IFN-β induction by HSV-1 was abrogated in PACT-/- cells. (A) Diagrams of the genomic structure of recombinant viruses. In HSV-1-Δ34.5 virus, both ICP34.5 alleles have been replaced with β-glucuronidase gene. No Us11 is expressed in the immediate early (IE) phase. In HSV-1-Δ34.5-(IE)Us11 virus, a spontaneous deletion results in the expression of Us11 from the IE promoter of Us12. (B, C) Viral induction of IFN-β and IL-6 in PACT-/- cells. Wild type (WT) and PACT-/- MEFs were infected with HSV-1-Δ34.5 or HSV-1-Δ34.5-(IE)Us11 (M.O.I. = 2.5). Cells were harvested at 4
h post-infection and total RNA was extracted. Expression of IFN-β (B) and IL-6 (C) mRNA relative to GAPDH transcript was analyzed by quantitative RT-PCR. RNA levels were derived from $2^{-\Delta\Delta C_t}$. The difference between groups 2 and 3 in B is statistically significant ($p_1 = 0.009$) by two-tailed Student's t test, whereas the differences between groups 5 and 6 in B as well as between groups 2 and 3 in C are statistically insignificant ($p_2 = 0.324$ in B and $p_1 = 0.957$ in C).
Figure 1
**Figure 2**

- **Part A:** Fraction analysis showing WB: α-GFP and WB: α-PACT signals across different fractions. The fractions are labeled from 31 to 47. The protein bands for EGFP-Us11 and PACT are indicated.

- **Part B:** Western blot analysis with various input and IP conditions. The blots show proteins with the indicated labels: Flag/PACT, EGFP-Us11, and α-Us11. The input conditions are denoted as + or -. The proteins are visualized at different molecular weights.

- **Part C:** Southern blot analysis showing ST dsRNA. The RNase A treatment is indicated with + or -. The bands are labeled from 1 to 2.

- **Part D:** Western blot analysis with input and IP conditions for U12+, U12+, HSV-1-EGFP-Us11, and U12+. The blots show proteins with the indicated labels: EGFP, U11, and U11. The proteins are visualized at different molecular weights.
Figure 3