Supporting Information

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SI Results

ISG15 Modification of the NS1A Protein in Transfection Assays Requires All Three ISG15 Conjugation Enzymes. Pilot transfection experiments in 293T cells showed that plasmid-expressed Ud NS1A protein was conjugated to ISG15 when plasmids expressing ISG15 conjugation enzymes were cotransfected (Fig. S1*A*). Maximal ISG15 modification of the NS1A protein, as well as maximal overall ISG15 conjugation, required cotransfection of plasmids expressing all three ISG15 conjugation enzymes (Ube1L, UbcH8, and Herc5) (Fig. S1*A*, lanes 4 and 8). However, significant ISG15 conjugation of the NS1A protein, as well as of other proteins, occurred when the Herc5-expressing plasmid was omitted (Fig. S1*A*, lanes 3 and 7). A previous study using HeLa cells showed that the ISG15 conjugation that occurs under these conditions is dependent on Herc5 that is endogenously expressed in these cells (1). To determine whether this is also the case in 293T cells, we transfected these cells with an siRNA directed against Herc5 before the transfection of the plasmids expressing F-NS1A, HA-ISG15, and the E1 and E2 enzymes (Fig. S1*B*). The H5-specific siRNA eliminated ISG15 conjugation of NS1A, as well as ISG15 conjugation of other proteins (Fig. S1*B*, lane 5), whereas a control siRNA did not decrease ISG15 conjugation (Fig. S1*B*, lane 4). We conclude that 293T cells contains active Herc5 enzyme and that the ISG15 conjugation of the NS1A protein requires all three ISG15 conjugation enzymes.

SI Materials and Methods

Cells and Viruses. A549, 293T, and HeLa Tet-on cells were maintained in DMEM containing 10% FBS. Influenza A virus stocks were grown in 10-day fertilized eggs, and virus titers were determined by plaque assays in MDCK cells. Cells were infected with the indicated virus, and the infected cells were incubated in DMEM containing 0.5 μg/mL N-acetylated trypsin in the absence of serum. Recombinant influenza A viruses were generated using plasmid-based reverse genetics, as described previously (2, 3). The negative-strand viral genomic RNAs are encoded in eight pHH21 plasmids under the control of a polymerase I promoter. To generate Ud viruses expressing FLAG-tagged NS1A proteins, a FLAG sequence was inserted between the 5′ UTR and the second codon of the NS1A reading frame in the pHH21 plasmid. The indicated K-to-R mutations were introduced into the NS1A protein using standard oligonucleotide mutagenesis methods. Where indicated, a pHH21 plasmid encoding the WSN NS1A protein (WT or K41A mutant) was substituted for the pHH21 plasmid encoding the Ud NS1A protein. All eight genomic RNA segments of the recombinant viruses were sequenced.

Detection of ISG15 Modification of NS1A in Transfected Cells. Mammalian vectors expressing His-HA-ISG15 and its conjugation enzymes (Ube1L, UbcH8, and Herc 5) were described previously (1, 4). The NS1A mammalian expression vectors were constructed by fusing full-length NS1A proteins to a Flag or 3×Flag tag at their N-termini using the pCMV10 or pCMV14 vector, respectively. A mutated 3′ splice site was introduced into the NS1A ORF to block production of spliced NS2 mRNA (5). The plasmid expressing the indicated NS1A protein was transfected into 293T cells using Mirus transfection reagent, with or without plasmids expressing HA-tagged ISG15 and its three conjugation enzymes. Cells harvested 48 h after transfection were extracted in 0.5% Nonidet P-40 lysis buffer (50 mM Tris 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) supplemented with protease inhibitor. To identify NS1A-ISG15 conjugates, extracts were immunoprecipitated with anti-Flag M2 agarose, followed by elution with 3×Flag peptide and then immunoblotting with either anti-NS1A or anti-HA antibody.

Assays for the Interaction of the NS1A Protein with Herc5. For in vitro GST binding assays, purified GST-Ud NS1A protein or a GST-Ud NS1A protein mutant was mixed with ^{35}S-labeled Herc5 protein, Herc5 protein truncations, Herc4, Herc6, or Nedd4, and the mixture was subjected to glutathione-Sepharose affinity selection (6). To prepare these radiolabeled proteins, the DNA encoding each of these proteins was subcloned into pcDNA3 and translated using a Promega TnT Coupled Transcription/Translation Kit in the presence of (^{35}S) methionine. For the detection of NS1A-Herc5 association in virus-infected cells, 293T cells were transfected with a plasmid expressing Flag-tagged Herc5, followed 36 h later by infection with Ud virus. Extracts from cells collected 8 h after infection were immunopurified using anti-Flag M2 agarose followed by immunoblotting with anti-NS1A antibody.

1. Dastur A, Beaudenon S, Kelley M, Krug RM, Huibregtse JM (2006) Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J Biol Chem* 281:4334–4338.
2. Takeda M, Pekosz A, Shuck K, Pinto LH, Lamb RA (2002) Influenza a virus M2 ion channel activity is essential for efficient replication in tissue culture. *J Virol* 76:1391–1399.
3. Twu KY, Noah DL, Rao P, Kuo RL, Krug RM (2006) The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. *J Virol* 80:3957–3965.
4. Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM (2005) Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc Natl Acad Sci USA* 102:10200–10205.
5. Qian XY, Alonso-Caplen F, Krug RM (1994) Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J Virol* 68:2433–2441.
6. Nemeroff ME, Qian XY, Krug RM (1995) The influenza virus NS1 protein forms multimers in vitro and in vivo. *Virology* 212:422–428.

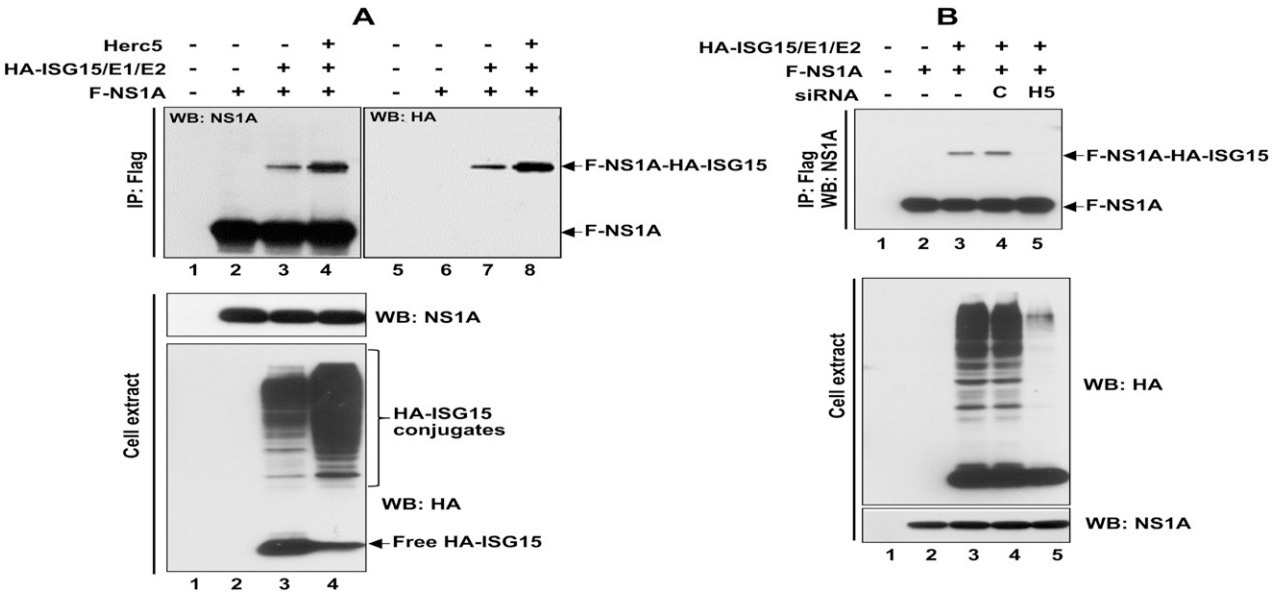

Fig. S1. The NS1A protein is ISG15 modified in transfection assays, and this modification requires all three enzymes of the ISG15 conjugation system. (*A*) 293T cells were transfected with a plasmid expressing Flag-tagged Ud NS1A (F-NS1A) alone (lanes 2 and 6), or along with plasmids expressing HA-ISG15 and the ISG15 E1 and E2 enzymes (lanes 3 and 7), or along with plasmids expressing HA-ISG15 and the ISG15 E1, E2, and E3 enzymes (lanes 4 and 8). To identify NS1A-ISG15 conjugates, transfected cell extracts were immunoprecipitated using anti-FLAG M2 agarose, followed by immunoblots using either anti-NS1A or anti-HA antibody. Equal aliquots of the cell extracts were immunoblotted with anti-NS1A antibody (verifying equal NS1A protein expression) or with anti-HA antibody (showing overall ISG15 conjugation). (*B*) 293T cells were first transfected with either a control siRNA (lane 4) or an siRNA targeting Herc5 (H5) (lane 5). Twelve hours later, the cells were cotransfected with plasmids expressing Flag-tagged NS1A, HA-ISG15, the E1 enzyme (Ube1L), and the E2 enzyme (UbcH8). As further controls, cells were not transfected with an siRNA and were transfected with a plasmid expressing F-NS1A alone (lane 2), or along with plasmids expressing HA-ISG15 and the ISG15 E1 and E2 enzymes (lane 3). Cell extracts were prepared 36 h after plasmid DNA transfection and were immunoprecipitated using anti-Flag M2 agarose, followed by immunoblotting using anti-NS1A antibody. Equal aliquots of the cell extracts were also directly immunoblotted with either anti-NS1A or anti-HA antibody.

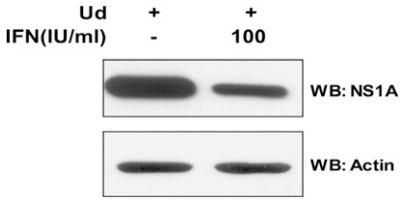

Fig. S2. Pretreatment of A549 cells with 100 U/mL of IFN-β inhibits the synthesis of the NS1A protein ≈10-fold. A549 cells were treated with 100 units (IU)/mL of IFN-β for 24 h, or were left untreated, and were then infected with 5 pfu/cell of Ud virus. Cells were collected 8 h after infection, and equal amounts of the extracts were analyzed by immunoblotting with either anti-NS1A antibody or antibody against β-actin. The aliquot of the extracts used in this analysis is one tenth of that used in the immunoblots shown in Fig. 1 in main text.

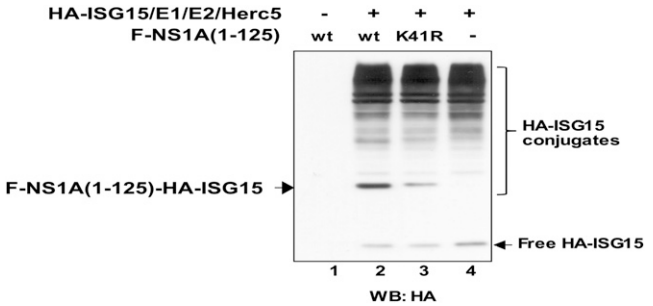

Fig. S3. The NS1A(1-125) containing a K41R mutation does not inhibit overall ISG15 conjugation. The same aliquots of the extracts used in Fig. 3*A* in main text were analyzed by immunoblots using anti-HA antibody to detect overall ISG15 conjugation.

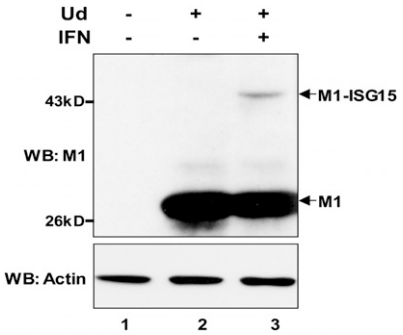

Fig. S4. Influenza M1 protein is conjugated to ISG15 in infected cells. A549 cells were treated with or without IFN-β for 24 h and were then infected with 5 pfu/cell of Ud virus. Cell extracts were prepared 8 h after infection and immunoblotted with either anti-M1 antibody or antibody against β-actin.

Table S1. Mass spectrometry analysis of purified NS1A-ISG15 protein

dCn	XCorr	NS1A residue	Peptide sequence
0.7879	4.3257	20–35	R.KQVVDQELGDAPFLDR.L
0.8308	5.6739	20–35	**R.K#QVVDQELGDAPFLDR.L**
0.8613	4.6262	21–35	K.QVVDQELGDAPFLDR.L
0.4838	2.065	38–44	R.RDQKSLR.G
0.7273	3.0879	38–44	**R.RDQK#SLR.G**
0.2481	0.7987	39–41	R.DQK.S
0.5484	2.1589	39–44	**R.DQK#SLR.G**
0.6748	4.6001	45–62	R.GRGSTLGLNIEAATHVGK.Q
0.599	2.6418	47–67	R.GSTLGLNIEAATHVGKQIVEK.I
0.8008	4.0723	47–62	R.GSTLGLNIEAATHVGK.Q
0.0956	1.6919	63–70	**K.QIVEK#ILK.E**
0.5627	1.4354	63–67	K.QIVEK.I
0.2605	3.5221	68–78	K.ILKEESDEALK.M
0.5456	3.3231	68–78	**K.ILK#EESDEALK.M**
0.5344	1.7844	71–78	K.EESDEALK.M
0.6693	3.2692	71–88	K.EESDEALKM*TM*ASTPASR.Y
0.2561	2.7047	79–88	K.MTM*ASTPASR.Y
0.3482	2.4832	79–88	K.M*TMASTPASR.Y
0.7176	2.9207	79–88	K.M*TM*ASTPASR.Y
0.7186	2.3223	79–88	K.MTMASTPASR.Y
0.6833	3.4625	89–100	R.YITDM*TIEELSR.D
0.7302	3.144	89–100	R.YITDMTIEELSR.D
0.3119	2.7642	101–108	R.DWFM*LMPK.Q
0.4554	2.354	101–108	R.DWFMLM*PK.Q
0.6975	2.6975	101–108	R.DWFMLMPK.Q
0.7624	3.0195	101–108	R.DWFM*LM*PK.Q
0.5506	2.6123	109–118	**K.QK#VEGPLCIR.I**
0.6581	2.7992	109–118	K.QKVEGPLCIR.I
0.7074	2.2817	111–118	K.VEGPLCIR.I
0.0451	4.1458	119–131	**R.IDQAIMDK#NIM*LK.A**
0.09	3.6413	119–131	**R.IDQAIM*DK#NIMLK.A**
0.135	3.5591	119–131	**R.IDQAIM*DK#NIM*LK.A**
0.1595	2.0003	119–131	R.IDQAIM*DKNIMLK.A
0.3156	3.4164	119–131	R.IDQAIMDKNIM*LK.A
0.3833	3.4829	119–131	R.IDQAIM*DKNIM*LK.A
0.6784	2.817	119–126	R.IDQAIM*DK.N
0.6832	2.9751	119–126	R.IDQAIMDK.N
0.0455	1.418	127–131	K.NIM*LK.A
0.1745	1.5068	127–131	K.NIMLK.A
0.7642	4.8931	132–148	K.ANFSVIFDRLETLILLR.A
0.7887	3.01	132–140	K.ANFSVIFDR.L
0.7956	2.6745	141–148	R.LETLILLR.A
0.6846	5.5052	149–184	R.AFTEEGAIVGEISPLPSFPGHTIEDVKNAIGVLIGR.L
0.7712	7.0251	149–175	R.AFTEEGAIVGEISPLPSFPGHTIEDVK.N
0.8026	2.4241	176–184	K.NAIGVLIGR.L
0.6234	2.4249	185–193	R.LEWNDNTVR.V
0.7395	2.4227	194–200	**R.VSK#TLQR.F**
0.4114	1.0288	197–200	K.TLQR.F
0.022	2.9404	201–219	**R.FAWGSSNENGRPPLTPKQK#.R**
0.6581	3.5543	201–217	R.FAWGSSNENGRPPLTPK.Q
0.0506	0.7342	222–224	R.MAR.T
0.1309	1.0134	228–231	R.SKVR.R
0.4902	1.2619	233–238	R.DKM*AD.-
0.5122	1.2344	233–238	R.DKMAD.-

Peptide recognition was based on cross-correlation value (XCorr) and delta-cross correlation (dCn) values as indicated. M* represents oxidized methione, and K# represents lysine residue modified by a Gly-Gly remnant derived from the C terminus of ISG15. The peptides containing identified ISG15 attachment sites are highlighted in bold type.