NOTES

Species-Specific Antagonism of Host ISGylation by the Influenza B Virus NS1 Protein[▽]

Gijs A. Versteeg,^{1†} Benjamin G. Hale,^{1†} Sander van Boheemen,^{1‡} Thorsten Wolff,⁴
Deborah J. Lenschow,^{5,6} and Adolfo García-Sastre^{1,2,3*}

Department of Microbiology,¹ Department of Medicine,² and Global Health and Emerging Pathogens Institute,³ Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029; Robert Koch-Institute, FG17, 13353 Berlin, Germany⁴; and Departments of Internal Medicine⁵ and Pathology & Immunology,⁶ Washington University School of Medicine, St. Louis, Missouri 63110

Received 12 November 2009/Accepted 25 February 2010

Interferon-stimulated expression and conjugation of the ubiquitin-like modifier ISG15 restricts replication of several viruses. Here, we established complete E1-activating, E2-conjugating, and E3 ligase-dependent expression systems for assaying both human and mouse ISGylation. We confirm that human HerC5, but not human HerC6, has ISG15 E3 ligase activity and identify mouse HerC6 as a bona fide ISG15 E3 ligase. Furthermore, we demonstrate that influenza B virus NS1 protein potently antagonizes human but not mouse ISGylation, a property dependent on B/NS1 binding the N-terminal domain of human but not mouse ISG15. Using chimeric human/mouse ISG15 constructs, we show that the B/NS1:ISG15 interaction is both necessary and sufficient to inhibit ISGylation regardless of the ligation machinery used. Inability to block ISGylation in certain species may contribute to limiting influenza B virus host range.

Type I interferons (IFNs) are induced in response to infection and mediate the expression of >300 IFN-stimulated genes (ISGs), many of which confer an antiviral state within cells (19, 20). One of earliest and most abundantly expressed ISGs is IFN-stimulated gene 15 (ISG15). The ISG15 protein is a ubiquitin-like modifier consisting of two ubiquitin-like domains connected by a “hinge” region (16). Like ubiquitin, ISG15 is conjugated to lysines on numerous target proteins through its conserved C-terminal LRLRGG sequence. This reaction is mediated by specific E1-activating, E2-conjugating, and E3-ligase enzymes, all of which are also IFN-inducible (8).

ISG15 exhibits potent antiviral activity against several viruses, including influenza viruses, herpes simplex virus type 1 (HSV-1), murine gammaherpesvirus, Sindbis virus, Ebola virus, human immunodeficiency virus type 1 (HIV-1), and vaccinia virus (6, 10, 13, 14, 17, 18). Conjugation of ISG15 to target proteins is essential for its antiviral function (4, 5), and several viruses (e.g., Crimean Congo hemorrhagic fever virus [CCHFV], equine arteritis virus, porcine respiratory and reproductive syndrome virus, and the severe acute respiratory syndrome [SARS] coronavirus) counteract host ISGylation by encoding ISG15-deconjugating enzymes (4,

15). In contrast to such catalytic antagonists, the NS1 protein of influenza B virus (B/NS1) and the E3L protein of vaccinia virus appear to prevent ISGylation by binding unconjugated ISG15 (6, 24). For B/NS1, the precise mechanism is unclear, but specific inhibition of ISG15-E1 adduct formation (24) and/or a steric block of the ISG15-E3 interaction have been proposed (1).

Our recent *in vivo* study demonstrated that both mouse-adapted (B/Lee/40) and non-mouse-adapted (B/Yamagata/16/88) influenza B viruses are sensitive to the antiviral activities of mouse ISG15 conjugation (12). This was surprising given the ability of B/NS1 to counteract ISG15 function in human cells (1, 24). Thus, we investigated the possibility that B/NS1 limits host cell ISGylation in a species-specific manner. To this end, we reconstituted human and mouse ISGylation systems in tissue culture and used them to test the ability of B/NS1 to bind and inhibit human or mouse ISG15.

Cloning of murine HerC6 and establishment of human and mouse ISGylation assays. Human and mouse Ube1L proteins have been identified as E1 ISG15-activating enzymes, and the human UbcH8 and mouse UbcM8 proteins have E2 ISG15-conjugating enzyme activity (11, 24). HerC5 is considered the major human ISG15 E3 ligase identified to date (23) and is found only in primates (3, 9). Expression plasmids for human and mouse E1 and E2 enzymes, as well as for human HerC5 (hHerC5) were reported previously (11, 23, 24). To complete a full set of equivalent mouse ISGylation components, we cloned a mouse homologue of the closely related hHerC6 in order to test the enzyme for E3 ligase activity. Sequence analysis indicated that the cloned open reading frame (ORF) is 100% identical to GenBank sequence XM_001478484 and encodes a

* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Phone: (212) 241-7769. Fax: (212) 534-1684. E-mail: adolfo.garcia-sastre@mssm.edu.

† These authors contributed equally to this work.

‡ Present address: Department of Virology, Erasmus Medical Center, 3015GE Rotterdam, The Netherlands.

[▽] Published ahead of print on 10 March 2010.

A

hHerC5	1	WERRSRRKSRRN-GRSTAGKAAATQPAKSPGAQLWLFPSAAGLHRAALLRRVEVTRDLCCSPGRRLAVLERGGAGVQVHQLAGSGGARTPKCIKLGKNNM	97
hHerC6	1	MYFCWAGADSRELQRRRTAGSPGAELLQAASGERHSLLLLTN--HRVLSGCDNSRGQLGRR--GAQRGELPEPIQALETLIIDLVSCGKEHSLAVCHKG	94
mHerC6	1	MYFSWAAGSRKP-RRLLKAGTSGIELLQAASGEHHSLLLSFN--HRVYSCGDNSSWGQLGQR--RDQSTERPEPIQALNDLHVDLVSCGKEHSAVCHKG	93
hHerC5	98	KIHSVDQGAEHMILSSDGKPFYDNYSMKHLRFESILQEKKIQTICGDYHSLALSKGGELFAWGQNLHGQLGVGRKFPSTTTTPIVEHLAGVPLAQ	195
hHerC6	95	RVFAWGAGSEGLGIG-----EFKEISFTPKKIMTLND-IKIQVSCGHYHSLALSKDSQVFSWGKNSHGQLGLGKEFPSSQASPQRVRSLEGIPLAQ	185
mHerC6	94	KVFAWGAGSEGLGIG-----EFKEISFMPTKIKALAG-IKIQVSCGHYHSLALSEDGHVFSWGRNSEGQLGLGKNSRSQAIPQKWKSLLEGIPLAQ	184
hHerC5	196	ISAGEAHSTALSMGNIYSWGKNECGQLGLGHTESKDD--PSLIEGLDNQKVEFVACGSSHALLTQDGLLFTFGAGKHGQLGHNSTQNELRPLCLVA	290
hHerC6	186	VAAAGAHSFALSLCGTSFGWGSNSAGQLALSGRNVVPQSNKPLSVGALKNLGVVYISCGDAHTAVLTQDGKVFTFGDNRSQQLGYSPTEKRGQPQLVE	283
mHerC6	185	VAAAGTHSFALSLTGTSTFGWGSNRSQQLALSGNKVKEQIYKPHSIGALKNLVSIYISCGYEHTAVLTEEGQVFTFGGNSSQQLQSPRSRGQRPQLIE	282
hHerC5	291	ELVGYRVTQIACGRWHTLAYVSDLGKVFSGSGKDGQLGNGGTRDQLMPLPYKVSSEELKLESHTSEKELIMIAGGNQSI LLWI KKENSYVNLKRTI	388
hHerC6	284	RIDG-LVSQIDCGSYHTLAYVHTTGQVVSFGHGSPD--TSKPTHPAALTENFDISCLISAEDFVDVQVKHIFAGTYANFVTH--QDTSSTRAPGKTL	376
mHerC6	283	GIGG-RVSQIECASYHTIAYVYTTGQVVSFLGRGPSH--TSNPTHQEAPAENSIDITCLLAEDLVDIEVKDI FAGAHANFVTTTRVSHTRSTGVSMKIL	377
hHerC5	389	PTLN--EGTVKRWIAADVET-KRWQSTKREIQEISSPACLTSFSLRKRRTTEMMPPVYLDLNKARNIFKELTQKDWITNMITTCLKDNLLKRLPFHSP	482
hHerC6	377	PEISRIQSMAEKWIAVKRRSTEHEMAKSEIRMISSPACLTSFSLKRGRTGETTSDVDLEMAEDTFKKLTKEWISSMITTCEDDLLRALPCHSP	474
mHerC6	378	PEIIRINQSLVKKWRANKR-KDREGAKREISLIFSSACLTSFSLKRGRTGETTSDVDLEMAEDTFKKLTKEWISSMITTCEEYLLRDLPLYSSP	474
hHerC5	483	PQEALEIFFLLPCECPVMHISNNWESLVVPFTKVVCKMSDQSSLVLEEYWATLQESTFSKLVQMFKTAVICQLDYWDESAEENGNVQALLEMLKHLRV	580
hHerC6	475	HQEALESVFLLLPCECPVMHDSKNWKNLVVPFAKAYCEMSKQSLQVLKKCWAFQLESSLNLIQMLKAAIISQLLHQTKTEQDHCNVKALLGMMKELHKV	572
mHerC6	475	HQEAALLVFLLLPECSIMQDPKNWKTLAFAFAKAIHKMGPSQLAFIRTCWASLEVSSSLNLIQMLKAAIISQIQYGVATEQYITNIVKLLLEVIKEVH--	570
hHerC5	581	NQVKCQLPESIFQVDELLHRLNFFVEVCCRRLWKMTVDASENVQCCVIFSHFPPIFNNSLSKIKLLHTDTLLKIESKKHKAYLRSAIEEERESEFALR	678
hHerC6	573	NKANGRLPENTFINELSNLLNFYIDRGRQLFRDNHLIPAE-TPSPVIFSDFPPIFNNSLSKIKLLQADSHIKMQMSEKKAYMLMHETILQKKDEFPPS	669
mHerC6	571	KANGCQLPESAFIINELSGIFNFDAEAGRMFIRHNDLCTE-SSDMVVFSDFLFVEFDLPISKIKLMKCDSFVKLMS-----EVMAFPEKMSPP	655
hHerC5	679	PTFDLTVRRNHLIEDVLNQLSQFENEDLRKELWVYSFGEIYDLDGGVKEFFYCLFAEMIQPEYGMFMYPEGASCMWFPVKPKFEKKRYFFFGVLGCL	776
hHerC6	670	PRFILLRVRRSRVLKDALRQLSQAEATDFCKVLVVEFINELCPESGGVSSEFFHCFEEMTKPEYGMFMYPEMGSCMWFPAPKPFEKKRYFFFGMLCGL	767
mHerC6	656	PYLILKVRRLSHLVEDTLRQLRQVEDFLRKQLSVGFINEIRPEAGGVSEFFHCIFEEMTDPKYEMFIYPEKGSMMWFPVNPKEKSSYFFLFGILCGL	753
hHerC5	777	SLFNCNVANLPFPLALFKKLLDQMPSLEDLKELSPDLGKNLQTLDDDEGDNFEVYFIHFNHWDNRDNTNLPNGSSIIVNQTNKRQDYVSKYINIFYN	874
hHerC6	768	SLFNLNVANLPFPLALYKKLLDQKPSLEDLKELSPRLGKSLQEVLDADAADDIGDALCIRFSIHWDQNDVDLIPNGSIPVVDQTNKRQDYVSKYIDIFYN	865
mHerC6	754	SLHNLKVINLPFPLALYKKLLNQKPSLEDLKELSLPLGRNLQEVLNCEAGDI-EELHMYFSIYWDQKDVDLIPDGISVPVNETNKRQDYVSKYVDIFYN	850
hHerC5	875	DSVKAVYEEFRGFFYKMCDEDIKLFHPEELKDVIVGNTDYDWKTFEKNARYEPGYNSSHPTIVMFWKAFHKLTLDEKKKFLVFLTGTDRQLQMKDLNN	972
hHerC6	866	VSVKAVYEEFQRGFFYRVCEKEILRHFYPEELMTAIGNTDYDWKQFEQNSKYEQGYQKSHPTIQLFWKAFHKLTLDEKKKFLFLTGTDRDLHARGIKQ	963
mHerC6	851	ISIKTIYEEFHRGFFYKVCNWDITRQFQPEELMTAIGNATCDWKQFENNISKYKDYDKSHPTILLFWKAFHDLTLDEKKKFLFLTGTCDRLHVKGKLN	948
hHerC5	973	MKTIFFCCPESWNERDPIRALTCSFVFLPKYSTMETVEEALQEAINNRRGFG-----	1024
hHerC6	964	MEIVFRCPETFSERDHTPSITCHNIIISLPKYSTMERMEEALQVAINNRRGFVSPMLTQS	1022
mHerC6	949	EGIVFRCSETFSEEDNPSRLTCHRMIDLPKYSSMRMKEALQVAINNSTGFVSQE----	1003

RCC1 domain

HECT domain

B

FIG. 1. Alignment of cloned mHerC6. (A) The amino acid sequence of cloned mHerC6 was aligned to sequences of hHerC5 and hHerC6 using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>). (B) Dendrograms calculated from alignment based on the full-length sequences (top) or on only the RCC1 or HECT domains (middle and bottom, respectively). Branch lengths are indicated at the base of each branch. Residues conserved in all aligned sequences, dark blue; residues conserved in the majority of aligned sequences, light blue; nonconserved residues, unmarked.

protein with 41.5% amino acid identity to hHerC5 and 66.1% identity to hHerC6 (Fig. 1A). A phylogenetic tree calculated from the alignment (BLOSUM62, neighbor joining) using Jalview (2) also indicated that mHerC6 was more similar to hHerC6 than to hHerC5. This was the case for alignment of both the full-length sequences and individual functional domains (Fig. 1B).

Human HerC6 is reported to contribute little to global ISGylation in humans (3). Nevertheless, we tested mouse HerC6 (mHerC6) for ISG15 E3 ligase activity and compared it to hHerC5 in a tissue culture expression-based assay. 293T cells in 35-mm dishes (50% confluent) were cotransfected with 250 ng of each species-specific E1/E2/E3 expression plasmid as well as 6H-tagged ISG15 (FuGENE6 transfection reagent; Roche). To ensure that the assay was dependent on each single component, control transfections in which one of the plasmids was replaced by a GFP plasmid were included. Cells were harvested 24 h posttransfection in 6 M urea, 2 M β -mercaptoethanol, and 4% sodium dodecyl sulfate (SDS). After sonication and boiling, lysates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting using a monoclonal antibody recognizing the 6H tag on ISG15 (H1029; Sigma) or a β -actin-specific polyclonal antibody (A2103; Sigma). As shown in Fig. 2A, when all four ISGylation components were expressed together, ISG15-conjugated proteins were readily detectable in both mouse and human conjugation systems (lanes 5 and 10). Substitution of any component with GFP prevented formation of ISG15 conjugates (Fig. 2A, lanes 1 to 4 and 6 to 9). These results indicate that all three E1/E2/E3 enzymes plus ISG15 are required for ISGylation in our system and demonstrate that the transfection procedure alone does not stimulate aberrant stress-mediated upregulation of the ligation machinery.

To assess the specificity of our system, we also generated and tested HA-tagged versions of hHerC5, hHerC6, mHerC6, and a mutant mHerC6 with its HECT domain catalytic cysteine changed to alanine (C970A). Each intraspecies pair was expressed at similar levels in transfected 293T cells (Fig. 2B). However, hHerC6 had minimal effects on stimulating human ISGylation in our assays (Fig. 2C), confirming the previous observations of others (3). Furthermore, although the HA-tagged mHerC6 construct had an ISGylation capability similar to that of untagged mHerC6, the C970A catalytic mutant mHerC6 failed to efficiently stimulate ISGylation (Fig. 2D). These results indicate that both hHerC5 and mHerC6 are bona fide ISG15 E3 ligases. Although hHerC6 may be only a minor ISG15 E3 ligase (3), we show here that its mouse counterpart confers potent global ISG15 conjugation. Thus, given the absence of a HerC5 gene in the mouse genome, mHerC6 could be one of the major ISG15 E3 ligases in mice and a potential functional counterpart to hHerC5. Future studies to determine the overall contribution of mHerC6 to endogenous mouse ISGylation are clearly warranted.

B/NS1 inhibits human, but not mouse, ISGylation. The development of component-dependent human and mouse ISGylation systems allowed us to directly assess the effect of influenza B/NS1 expression on human and mouse ISG15 conjugation. As previous data indicated that B/NS1 antagonizes ISGylation by binding ISG15 rather than enzymatically removing it from target proteins (1, 24), we first titrated the amount

of hISG15 plasmid to determine a concentration at which B/NS1 could significantly inhibit global ISGylation. As a positive control, we used the ovarian tumor (OTU) domain of CCHFV L protein, which removes ISG15 conjugates catalytically (4) and should thus function efficiently irrespective of the amount of ISG15 expressed. 293T cells were transfected as before with equal amounts (200 ng) of plasmids encoding the three conjugation enzymes, V5-hISG15, and either an empty parental plasmid, a plasmid encoding the NS1 protein of influenza B/Yamagata/16/88 virus (Yam88/NS1; B/NS1), or a plasmid encoding the OTU domain of CCHFV-L (4). After 24 h, cell lysates were analyzed by SDS-PAGE and Western blotting for V5-hISG15 conjugation using a horseradish peroxidase (HRP)-coupled anti-V5 antibody (MCA1360P; Serotec). With these equimolar DNA concentrations, expression of B/NS1 had little effect on ISG15 conjugation (Fig. 3A, lanes 1 to 3). However, when the ratio of V5-hISG15 plasmid to B/NS1 plasmid was decreased, we observed dose-dependent inhibition of ISGylation by B/NS1 (Fig. 3A, lanes 4 to 12). This is consistent with the notion that B/NS1 must sequester ISG15 to inhibit it and cannot act catalytically like CCHFV-OTU. Subsequent experiments were therefore performed using the non-ISG15-saturating conditions of 40 ng of ISG15 expression plasmid, 360 ng of B/NS1 expression plasmid, and 200 ng of all other ISGylation component plasmids.

To investigate if conjugation of mISG15 is also affected by B/NS1, both the complete mouse and human ISGylation systems were separately cotransfected into 293T cells with the B/NS1 plasmid. As controls, an empty parental plasmid or plasmids encoding the NS1 protein of an influenza A virus (A/Puerto Rico/8/34, A/NS1) or CCHFV-OTU were used. Lysates were prepared 24 h posttransfection and analyzed by Western blotting for ISG15 conjugation. Specific antibodies were also used to detect β -actin, HA-tagged OTU (H9658; Sigma), A/NS1, and B/NS1. B/NS1 potentially limited formation of hISG15 conjugates yet strikingly had minimal effects on mISG15 conjugation (Fig. 3B, lanes 2 and 6). As expected, expression of OTU completely abrogated ISGylation irrespective of the species background (Fig. 3B, lanes 4 and 8). Surprisingly, we observed a very minor (yet reproducible) effect on both human and mouse ISGylation by the A/NS1 protein. Although A/NS1 is unable to bind ISG15 (24), we cannot rule out possible indirect effects caused by the interaction of A/NS1 with the two HerCs (25), or even nonspecific effects of this protein on the host mRNA nuclear export machinery (21), which may have resulted in decreased expression of the transfected ligation components. Nevertheless, our results clearly indicate that B/NS1 is capable of limiting only human, not mouse, ISGylation.

B/NS1 interacts with human, but not mouse, ISG15. We next determined whether differential binding of B/NS1 to human or mouse ISG15 could explain the inability of B/NS1 to efficiently inhibit mISG15 conjugation. To this end, expression plasmids with glutathione *S*-transferase (GST) fused in frame to human or mouse ISG15 were generated and coexpressed in 293T cells with plasmids encoding B/NS1. Protein lysates were subjected to GST pull-down, and precipitated proteins were analyzed by Western blotting using B/NS1- and GST-specific antibodies (RPN1236; GE Healthcare). Despite similar expression levels of B/NS1 in the whole-cell lysates (Fig. 4A,

FIG. 2. E1-, E2-, and E3-dependent conjugation of human and mouse ISG15. (A) 293T cells were transfected for 24 h with equal amounts of plasmids encoding human or mouse 6H-ISG15 together with the respective E1, E2, and E3 ISG15 conjugation machinery. In separate wells, each component was also replaced with an equal amount of GFP plasmid as a negative control. Cell lysates were analyzed by Western blotting using 6H tag-specific antibodies. Nonspecific bands are indicated by asterisks. (B) Relative expression of different HA-tagged HerC5 and HerC6 constructs transfected into 293T cells for 24 h and analyzed by Western blotting using an anti-HA antibody. (C) 293T cells were transfected for 24 h with equal amounts of plasmid encoding HA-tagged hHerC5, hHerC6, or vector only together with conjugatable (GG) or unconjugatable (AA) V5-tagged hISG15 and the human E1/E2 enzymes. Western blotting with an anti-V5 antibody was used to detect ISG15 conjugates. (D) Assay as described for panel C except that the mouse ISG15 and E1/E2 machinery was used. The specific mouse E3 constructs used are indicated. In all blots, detection of β -actin was used as a loading control.

lower left) and equal pull-down of the GST fusions, only GST-hISG15, not GST-mISG15, precipitated B/NS1 (Fig. 4A, top left). We also tested the ability of a B/NS1 protein derived from a mouse-adapted influenza B virus strain (influenza B/Lee/1940 virus; which is 88% identical to the NS1 protein of influenza B/Yamagata/16/88 virus) to interact with human and mouse ISG15. However, even this NS1 protein failed to interact with mISG15 and coprecipitated only with hISG15 (Fig. 4A, right).

Human and mouse ISG15 proteins are 65% identical at the

amino acid level. To more precisely map the interaction between B/NS1 and hISG15, we generated GST fusions with chimeric ISG15 proteins containing the N-terminal ubiquitin-like domain of hISG15 and the C-terminal ubiquitin-like domain of mISG15 (H_NM_C) or *vice versa* (M_NH_C). In GST pull-down experiments we found that B/NS1 interacted only with the H_NM_C ISG15 chimera, not the M_NH_C variant (Fig. 4B and C). These data confirm that B/NS1 specifically interacts with the N-terminal ubiquitin-like domain of hISG15 (1) and further indicate that residues within this domain (or part of the

FIG. 3. B/NS1 inhibits human, but not mouse, ISGylation. 293T cells were transfected with the indicated plasmids for 24 h, and cell lysates were analyzed by Western blotting for V5-ISG15 conjugation using a V5 tag-specific antibody. (A) Human E1, E2, E3, and V5-hISG15 plasmids were cotransfected with plasmids encoding B/NS1 or CCHFV-OTU. The concentration of conjugation machinery plasmids was kept constant, while the relative amounts of hISG15 plasmid were lowered and hISG15 was replaced by B/NS1. (B) Complete human or mouse ISGylation systems, cotransfected with B/NS1, A/NS1, or CCHFV-OTU. In all blots, detection of β -actin was used as a loading control.

FIG. 4. B/NS1 interacts with human, but not mouse, ISG15. 293T cells were transfected with the indicated plasmids; cell lysates were subjected to GST pull-down and subsequent Western blot analysis of precipitated proteins using GST- and B/NS1-specific antibodies. (A) GST, GST-hISG15, or GST-mISG15 cotransfected with B/NS1 from influenza B/Yamagata/88 virus (Yam88/NS1) or from influenza B/Lee/40 virus (Lee/NS1). (B) GST-H_NM_C or GST-M_NC_C chimeric ISG15 proteins were cotransfected with Yam88/NS1. Molecular mass markers (kDa) are shown to the right. (C) Graphic representation of human ISG15 (PDB ID: 1Z2M) (16), rendered using PyMol. The N-terminal ubiquitin-like domain is shown in green; the C-terminal ubiquitin-like domain is shown in blue. Amino acids in the N-terminal domain differing between human and mouse ISG15 are indicated in purple. Sequence alignment of the two ISG15s and differing amino acids are shown above the structures.

ISG15 interdomain hinge [22]) determine the species specificity of B/NS1.

B/NS1 inhibits conjugation of a chimeric H_NM_C ISG15 protein in both human and mouse ISGylation assays. We used

our component-dependent ISGylation assay (and the knowledge that B/NS1 interacts only with a chimeric ISG15 molecule containing the N-terminal ubiquitin-like domain of hISG15) to test the hypothesis that binding of B/NS1 to ISG15 is required

FIG. 5. The hISG15 N-terminal domain is required for inhibition of ISGylation by B/NS1. 293T cells were cotransfected for 24 h with plasmids encoding V5-tagged $H_N M_C$ or $M_N H_C$ chimeric ISG15 proteins together with either the human ISGylation system (left) or the mouse ISGylation system (right). Cell lysates were analyzed for ISG15 conjugation using V5 tag-specific antibodies. In all blots, detection of β -actin was used as a loading control.

to block ISGylation. 293T cells were transfected with the human or mouse E1/E2/E3 conjugating enzymes, B/NS1, or the empty parental plasmid and with a plasmid encoding one of the chimeric ISG15 proteins. Both the human and mouse E1/E2/E3 enzymes mediated conjugation of the chimeric mouse/human ISG15s. However, with the human ligation machinery B/NS1 decreased conjugation only of the $H_N M_C$ ISG15 chimera, not the $M_N H_C$ variant (Fig. 5, lanes 1 to 4). Furthermore, B/NS1 potentially reduced conjugation of the $H_N M_C$ chimera in the presence of the mouse E1/E2/E3 machinery (lanes 5 to 8). Given that B/NS1 could not block ISGylation of full-length mISG15 in the same mouse system (Fig. 3B), these data suggest that binding of B/NS1 to ISG15 is both necessary and sufficient to inhibit ISGylation and that the species-specific inhibition of ISGylation by B/NS1 is dependent on ISG15 and not the E1/E2/E3 conjugation components.

Concluding remarks. Failure of viruses to counteract specific components of innate immunity in select species is well-recognized as playing a role in host restriction (19). Here, we determined that the influenza B virus NS1 protein is unable to block mouse ISGylation due to an inability to bind mouse ISG15. Mice are not a natural host for influenza B viruses, although they have been used as an *in vivo* infection model (7, 12, 13). Thus, lack of this B/NS1 inhibitory function in mouse cells may be one contributing host restriction factor. Given that mouse-adapted B/Lee/40 NS1 protein does not bind mouse ISG15, our work implies that rationally designed mutant influenza B viruses expressing NS1 with the ability to block mouse ISGylation (or perhaps the generation of mice with “human-

ized” ISG15) may be better *in vivo* model systems of influenza B virus infection. Assessing the ability of B/NS1 to inhibit ISGylation in other species (either naturally infected or not) may provide further insights into the biology of this virus, or lead to the development of new models (22).

We are grateful to Richard Cadagan and Osman Lizardo for excellent technical assistance.

Research in the A.G.-S. laboratory is partly supported by NIH funding (grants R01 AI46954, U19 AI62623 [Center for Investigating Viral Immunity and Antagonism], U54 AI57158 [North East Biodefense Center], and U19 AI83025) and by CRIP (Center for Research on Influenza Pathogenesis, NIAID contract HHSN266200700010C).

REFERENCES

- Chang, Y. G., X. Z. Yan, Y. Y. Xie, X. C. Gao, A. X. Song, D. E. Zhang, and H. Y. Hu. 2008. Different roles for two ubiquitin-like domains of ISG15 in protein modification. *J. Biol. Chem.* **283**:13370–13377.
- Clamp, M., J. Cuff, S. M. Searle, and G. J. Barton. 2004. The Jalview Java alignment editor. *Bioinformatics*. **20**:426–427.
- Dastur, A., S. Beaudenon, M. Kelley, R. M. Kelley, and J. M. Huibregtse. 2006. Herc5, an interferon-induced HECT E3 enzyme, is required for conjugation of ISG15 in human cells. *J. Biol. Chem.* **281**:4334–4338.
- Frias-Staheli, N., N. V. Giannakopoulos, M. Kikkert, S. L. Taylor, A. Bridgen, J. Paragas, J. A. Richt, R. R. Rowland, C. S. Schmaljohn, D. J. Lenschow, E. J. Snijder, A. Garcia-Sastre, and H. W. Virgin. 2007. Ovarian tumor domain-containing viral proteases evade ubiquitin- and ISG15-dependent innate immune responses. *Cell Host. Microbe* **2**:404–416.
- Giannakopoulos, N. V., E. Arutyunova, C. Lai, D. J. Lenschow, A. L. Haas, and H. W. Virgin. 2009. ISG15 Arg151 and the ISG15-conjugating enzyme Ube1L are important for innate immune control of Sindbis virus. *J. Virol.* **83**:1602–1610.
- Guerra, S., A. Caceres, K. P. Knobeloch, I. Horak, and M. Esteban. 2008. Vaccinia virus E3 protein prevents the antiviral action of ISG15. *PLoS Pathog.* **4**:e1000096.
- Hai, R., L. Martinez-Sobrido, K. A. Fraser, J. Ayllon, A. Garcia-Sastre, and

- P. Palese. 2008. Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. *J. Virol.* **82**:10580–10590.
8. Harty, R. N., P. M. Pitha, and A. Okumura. 2009. Antiviral activity of innate immune protein ISG15. *J. Innate Immun.* **1**:397–404.
9. Hochrainer, K., H. Mayer, U. Baranyi, B. Binder, J. Lipp, and R. Kroismayr. 2005. The human HERC family of ubiquitin ligases: novel members, genomic organization, expression profiling, and evolutionary aspects. *Genomics* **85**:153–164.
10. Hsiang, T. Y., C. Zhao, and R. M. Krug. 2009. Interferon-induced ISG15 conjugation inhibits influenza A virus gene expression and replication in human cells. *J. Virol.* **83**:5971–5977.
11. Kim, K. I., N. V. Giannakopoulos, H. W. Virgin, and D. E. Zhang. 2004. Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. *Mol. Cell. Biol.* **24**:9592–9600.
12. Lai, C., J. J. Struckhoff, J. Schneider, L. Martinez-Sobrido, T. Wolff, A. Garcia-Sastre, D. E. Zhang, and D. J. Lenschow. 2009. Mice lacking the ISG15 E1 enzyme Ube1L demonstrate increased susceptibility to both mouse-adapted and non-mouse-adapted influenza B virus infection. *J. Virol.* **83**:1147–1151.
13. Lenschow, D. J., N. V. Giannakopoulos, L. J. Gunn, C. Johnston, A. K. O'Guin, R. E. Schmidt, B. Levine, and H. W. Virgin. 2005. Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. *J. Virol.* **79**:13974–13983.
14. Lenschow, D. J., C. Lai, N. Frias-Staheli, N. V. Giannakopoulos, A. Lutz, T. Wolff, A. Osiak, B. Levine, R. E. Schmidt, A. Garcia-Sastre, D. A. Leib, A. Pekosz, K. P. Knobeloch, I. Horak, and H. W. Virgin. 2007. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc. Natl. Acad. Sci. U. S. A.* **104**:1371–1376.
15. Lindner, H. A., N. Fotouhi-Ardakani, V. Lytvyn, P. Lachance, T. Sulea, and R. Menard. 2005. The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme. *J. Virol.* **79**:15199–15208.
16. Narasimhan, J., M. Wang, Z. Fu, J. M. Klein, A. L. Haas, and J. J. Kim. 2005. Crystal structure of the interferon-induced ubiquitin-like protein ISG15. *J. Biol. Chem.* **280**:27356–27365.
17. Okumura, A., G. Lu, I. Pitha-Rowe, and P. M. Pitha. 2006. Innate antiviral response targets HIV-1 release by the induction of ubiquitin-like protein ISG15. *Proc. Natl. Acad. Sci. U. S. A.* **103**:1440–1445.
18. Okumura, A., P. M. Pitha, and R. N. Harty. 2008. ISG15 inhibits Ebola VP40 VLP budding in an L-domain-dependent manner by blocking Nedd4 ligase activity. *Proc. Natl. Acad. Sci. U. S. A.* **105**:3974–3979.
19. Randall, R. E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J. Gen. Virol.* **89**:1–47.
20. Sadler, A. J., and B. R. Williams. 2008. Interferon-inducible antiviral effectors. *Nat. Rev. Immunol.* **8**:559–568.
21. Satterly, N., P. L. Tsai, D. J. van, D. R. Nussenzweig, Y. Wang, P. A. Faria, A. Levay, D. E. Levy, and B. M. Fontoura. 2007. Influenza virus targets the mRNA export machinery and the nuclear pore complex. *Proc. Natl. Acad. Sci. U. S. A.* **104**:1853–1858.
22. Sridharan, H., C. Zhao, and R. M. Krug. 2010. Species specificity of the NS1 protein of influenza B virus: It binds only human and non-human primate ubiquitin-like ISG15 proteins. *J. Biol. Chem.* doi:C109.095703.
23. Wong, J. J., Y. F. Pung, N. S. Sze, and K. C. Chin. 2006. HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. *Proc. Natl. Acad. Sci. U. S. A.* **103**:10735–10740.
24. Yuan, W., and R. M. Krug. 2001. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J.* **20**:362–371.
25. Zhao, C., T. Y. Hsiang, R. L. Kuo, and R. M. Krug. 2010. ISG15 conjugation system targets the viral NS1 protein in influenza A virus-infected cells. *Proc. Natl. Acad. Sci. U. S. A.* **107**:2253–2258.