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The ISG15 Conjugation System Broadly Targets Newly Synthesized Proteins: Implications for the Antiviral Function of ISG15

Larissa A. Durfee,¹ Nancy Lyon,¹ Kyungwoon Seo,¹ and Jon M. Huibregtse^{1,*}

¹Section of Molecular Genetics and Microbiology, Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA

*Correspondence: huibreg@mail.utexas.edu

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SUMMARY

ISG15 is an interferon-induced and antiviral ubiquitin-like protein (Ubl). Herc5, the major E3 enzyme for ISG15, mediates the ISGylation of more than 300 proteins in interferon-stimulated cells. In addressing this broad substrate selectivity of Herc5, we found that: (1) the range of substrates extends even further and includes many exogenously expressed foreign proteins, (2) ISG15 conjugation is restricted to newly synthesized pools of proteins, and (3) Herc5 is physically associated with polyribosomes. These results lead to a model for ISGylation in which Herc5 broadly modifies newly synthesized proteins in a cotranslational manner. This further suggests that, in the context of an interferon-stimulated cell, newly translated viral proteins may be primary targets of ISG15. Consistent with this, we demonstrate that ISGylation of human papillomavirus (HPV) L1 capsid protein has a dominant-inhibitory effect on the infectivity of HPV16 pseudoviruses.

INTRODUCTION

ISG15 is a 17 kD ubiquitin-like protein (Ubl) that is rapidly induced by type 1 interferons (IFN- α and β). Induction by interferon implied, more than 20 years ago, that ISG15 was a component of the innate immune response (Farrell et al., 1979); however, it has only been recently confirmed that ISG15 has antiviral activity against several types of viruses, including influenza, sindbis, herpes, human immunodeficiency, and ebola (Hsiang et al., 2009; Lenschow et al., 2005; Okumura et al., 2006, 2008). In addition, several viruses have evolved mechanisms for interfering with ISG15 function: the NS1 protein of influenza B blocks ISG15 conjugation (Chang et al., 2008; Yuan and Krug, 2001), SARS coronavirus encodes an ISG15 deconjugating enzyme (Lindner et al., 2007), and the vaccinia E3 protein binds to ISG15 and blocks its antiviral activity (Guerra et al., 2008). The ability of ISG15 to be conjugated to other proteins has been shown in some cases to be essential for its antiviral activity (Giannakopoulos et al., 2009; Lai et al., 2009). The

biochemical function of ISG15 conjugation and the basis of the antiviral activities of ISG15 conjugation remain unknown.

Whereas ISG15 is a very rapidly induced IFN-stimulated protein (Farrell et al., 1979), conjugation does not become apparent until 18–24 hr after IFN stimulation (Loeb and Haas, 1992), corresponding with the delayed induction of the ISG15 E1, E2, and E3 enzymes (Dastur et al., 2006). The human ISG15 E1 enzyme is Ube1L (Yuan and Krug, 2001), and the E2 enzyme is UbcH8/Ube2L6 (Durfee et al., 2008; Kim et al., 2004; Zhao et al., 2004). The major E3 for human ISG15 is Herc5, a HECT domain ligase that contains N-terminal RCC1 repeats (Dastur et al., 2006; Wong et al., 2006). Herc5 depletion results in a dramatic decrease in ISG15 conjugation, affecting conjugation to the vast majority of cellular target proteins (Dastur et al., 2006), and coexpression of ISG15, Ube1L, UbcH8, and Herc5 in non-IFN-stimulated cells reconstitutes broad and robust ISG15 conjugation (Dastur et al., 2006; Takeuchi et al., 2006; Wong et al., 2006). Herc5 has also been shown to have antiviral activity against influenza A virus (Zhao et al., 2010). EFP, a RING E3, has been reported to be an additional E3 for ISG15; however, only a single target has been reported (Zou and Zhang, 2006). Therefore, whereas additional proteins may play a role in ISG15 conjugation, Ube1L, UbcH8, and Herc5 represent the core IFN-induced components of this conjugation system in human cells.

Proteomics studies have identified more than 300 cellular proteins that are targeted for ISGylation (Giannakopoulos et al., 2005; Malakhov et al., 2003; Takeuchi et al., 2006; Wong et al., 2006; Zhao et al., 2005). These targets were present in many cellular compartments, and no functional classes of proteins were particularly overrepresented, although 12 IFN-induced human proteins were identified (Zhao et al., 2005). A common structural or primary sequence element that confers ISG15 conjugation has not been identified, nor have poly-ISG15 chains been observed. Importantly, only a small fraction of the pool of a given target protein is generally modified (Zhao et al., 2005). These observations raise the following questions: (1) how can a single ISG15 ligase be responsible for targeting such a large and diverse set of proteins, (2) what is the biochemical effect of ISG15 conjugation on target proteins, and (3) how can modification of a small fraction of any individual target protein have a significant effect on the overall activity of that protein? The findings presented here lead to a model that accounts for broad substrate targeting in the ISG15 system

and provide a basis for understanding the antiviral activity of ISG15 conjugation.

RESULTS

The ISG15 System Targets a Broad Range of Proteins

ISG15 conjugation can be observed experimentally by treating HeLa cells with IFN- β , preparing total cell lysate 24–48 hr posttreatment, and immunoblotting with anti-ISG15 antibody (Figure 1A). Surprisingly, when such lysates were immunoblotted with antibodies against previously identified target proteins, it was difficult to observe their modification. This is seen in Figure 1B for two such proteins, IQGAP1 and Ube1. Whereas modification of both proteins could be detected on very long exposures, modification of Hsc70 (Figure 1B) and four other constitutively expressed target proteins could not be detected (Table S1 available online). The success rate for validation of IFN-induced target proteins was higher, with three out of five targets confirmed, as shown for p56 (Figure 1C) and summarized in Table S1. As negative controls, five proteins not previously identified as targets of ISG15 modification were examined, and none of these were detectably modified in IFN- β -stimulated cells (Table S1).

ISG15 conjugates can also be generated by expressing the core components of the conjugation system (ISG15 or FLAG-ISG15, Ube1L, UbcH8, and Herc5) by a four-plasmid transfection of non-IFN-stimulated cells. As seen in Figure 2A (top), robust ISG15 conjugation was observed in HEK293T cells subjected to this four-plasmid transfection. However, it was again difficult to detect ISGylation of individual endogenously expressed target proteins, as shown for IQGAP1, Moesin, and Ube1 (Figure 2A), although, as in IFN-stimulated HeLa cells, a low level of modification of IQGAP1 and Ube1 could be detected on very long exposures (data not shown). Modification of only two of seven previously identified targets could be validated by this method (Table S1).

To further investigate the problem of target protein validation, non-IFN-stimulated cells (HEK293T) were transfected with plasmids expressing individual epitope-tagged target proteins, along with plasmids encoding the four core ISG15 conjugation components (a five-plasmid transfection). Surprisingly, in this scenario, ISG15 modification was detected for all previously identified target proteins tested (nine out of nine proteins), as shown in Figures 2B and S1 and summarized in Table S2. These results prompted us to assay, as presumptive negative controls, several epitope-tagged human proteins that had not been previously identified as ISG15 targets. Five out of seven of these were also found to be ISGylated, including E6AP, p53, Dlg, Herc4, and Herc6 (Figures 3A and S2 and Table S2). Several nonhuman proteins were also assayed, including *E. coli* β -galactosidase, *Shigella flexneri* OspG, *Salmonella typhimurium* SopA, the TAP epitope tag (two copies of the protein A sequence and the calmodulin-binding protein), and two viral proteins (HPV18 L1 and HIV integrase). All of these were modified when expressed along with the conjugation components (Figures 3B and S2 and Table S2). Three proteins were identified that were consistently not ISGylated in this assay system: GFP, human Wbp2, and a 35 kD carboxy-terminal fragment of paxillin. We

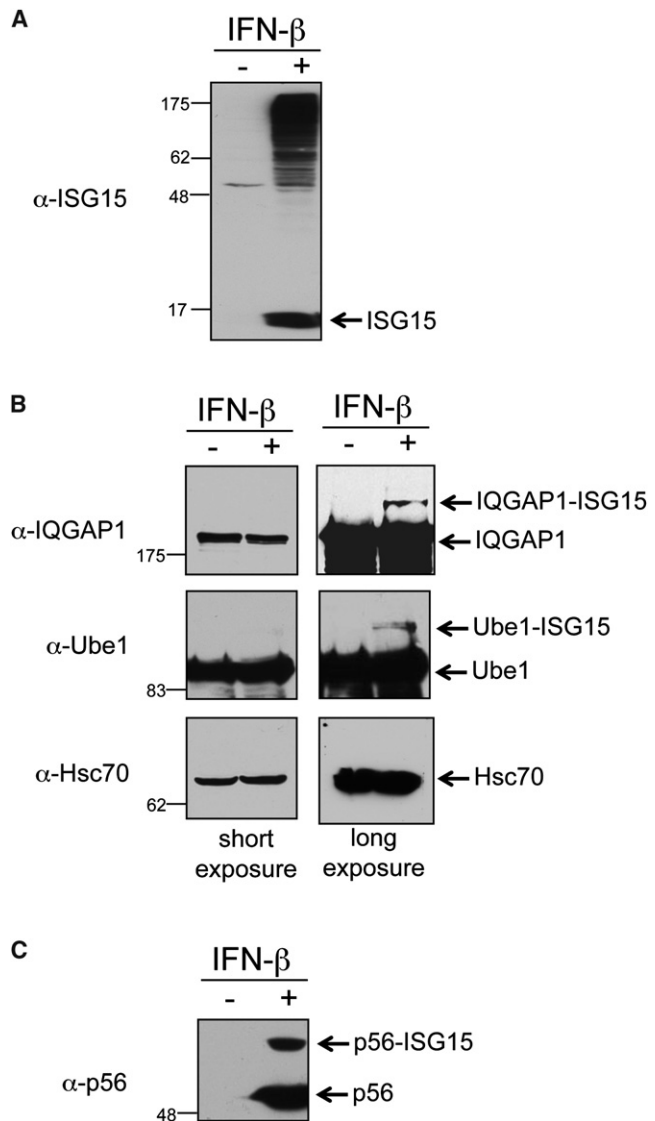


Figure 1. Detection of ISG15 Conjugates in IFN- β -Treated HeLa Cells

(A) Total ISG15 conjugates in HeLa cells. Cells were either untreated or treated with IFN- β for 48 hr. Cell extracts were analyzed by SDS-PAGE and immunoblotting with anti-ISG15 antibody.

(B) ISG15 modification of constitutively expressed target proteins. Cells were treated and extracts analyzed as in (A), using the indicated antibodies. Short (left) and long exposures (right) are shown for each immunoblot.

(C) ISG15 modification of p56, an interferon-induced protein. Same as described in (A), with immunoblot analyzed with anti-p56 antibody.

also confirmed that the nature of the epitope tag did not influence modification (see Figures 2 and S2 for modification of TAP-, HA-, and V5-Ube1 and Table S2 for others).

In an attempt to map a domain on a target protein that was recognized by the ISGylation enzymes, three nonoverlapping fragments of IQGAP1 were assayed for modification (Figure 3C). Surprisingly, all three fragments of the protein were ISGylated. Similar results were seen with two nonoverlapping fragments

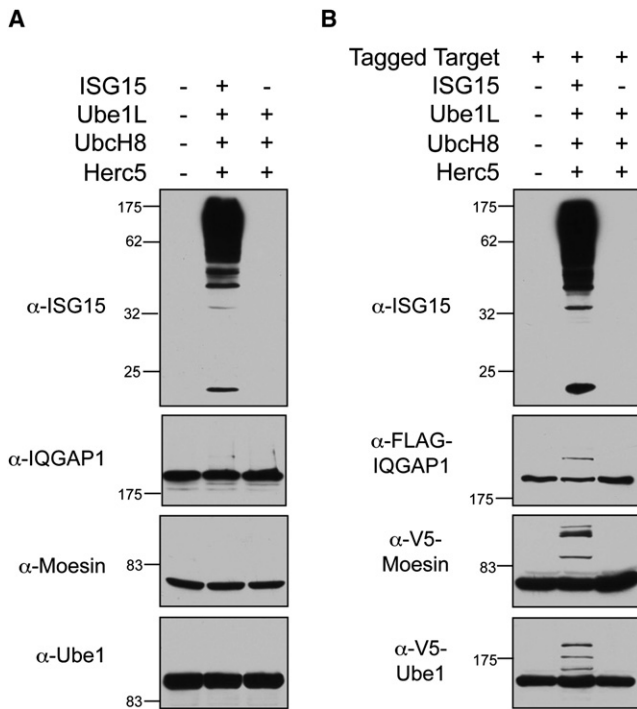


Figure 2. ISG15 Conjugation to Endogenously Versus Exogenously Expressed Target Proteins

(A) ISGylation of endogenously expressed target proteins. HEK293T cells were either mock transfected (lane 1); transfected with plasmids expressing Ube1L, UbcH8, Herc5, and ISG15 (lane 2); or transfected with Ube1L, UbcH8, and Herc5 (lane 3). Cell extracts were prepared 48 hr posttransfection and analyzed for ISG15 conjugation by immunoblotting using the indicated antibodies.

(B) ISGylation of exogenously expressed target proteins. HEK293T cells were transfected with a plasmid expressing an epitope-tagged target protein (FLAG-IQGAP1 in top two panels, V5-moesin, or V5-Ube1) either alone (lane 1), combined with Ube1L, UbcH8, Herc5, and ISG15 (lane 2), or combined with Ube1L, UbcH8, and Herc5 (lane 3). Cell extracts were prepared 48 hr posttransfection and analyzed by immunoblotting using the indicated antibodies.

of MxA (Figure S3), indicating that the ISGylation machinery does not recognize a single epitope even within an individual protein. Together, these results suggested that, at least in the five-plasmid transfection assay, the ISGylation machinery recognizes target proteins in a broad and relatively nonspecific manner.

To address whether the broad target recognition in the five-plasmid transfection assay was related to overexpression of the transfected target proteins, we monitored, at several time points after transfection, the protein levels and modification of two exogenously expressed target proteins (TAP-Ube1 and TAP-IQGAP1) relative to the corresponding endogenously expressed proteins. Figure 4A (left panels) shows the relative levels of exogenously and endogenously expressed proteins in the absence of ISG15 (expression of target protein with Ube1L, UbcH8, and Herc5, without ISG15), using an antibody that detects both proteins. The exogenously expressed proteins could be detected by 6 hr posttransfection for both TAP-Ube1 and TAP-IQGAP1. For Ube1, the levels of the exogenous protein did not exceed that of endogenous Ube1, even at 30 hr post-

transfection, whereas, for IQGAP1, the levels of exogenous protein were similar and perhaps slightly higher than endogenous IQGAP1 at 24 and 30 hr posttransfection (correcting for the ~50% transfection efficiency). However, at the 18 hr time point, when the exogenously expressed proteins were clearly less abundant than the endogenously expressed proteins, ISGylation of the exogenously expressed proteins was detected when the target protein was expressed with all four conjugation components (Figure 4A, middle). In contrast, modification of the endogenous proteins was not detectable at any time point (Figure 4A, right). Therefore, the preferential modification of exogenously expressed proteins was not due to a higher steady-state level of the exogenously expressed target proteins.

Newly Synthesized Proteins Are Targeted for ISGylation

To account for the preferential modification of exogenously expressed target proteins, we considered that, in the five-plasmid transfection assay, the entire pool of the target protein was synthesized in the same window of time that the ISG15 conjugation machinery was active. In contrast, in all assays in which endogenously expressed proteins were examined, only a fraction of the target protein was synthesized while the conjugation machinery was active (a function of the rate of synthesis of that protein). Also, in the five-plasmid transfections, the mRNA levels encoding the exogenously expressed proteins were ~10-fold higher than the corresponding endogenously expressed mRNAs (based on three examples, Figure S4A), suggesting that the exogenously expressed proteins, though not present at a higher steady-state level, were translated at a higher rate. These distinctions suggested that ISG15 conjugation might be limited to the newly synthesized pool of any given target protein. An additional observation consistent with this was the relatively high success rate for validation of IFN-induced ISG15 targets (e.g., p56, MxA) in IFN-stimulated cells (see Table S1), wherein the entire pool of these proteins is, by definition, newly synthesized.

We tested the hypothesis that ISGylation is limited to newly synthesized proteins in three ways. In one approach (Figure 4B), an intracellular pool of a target protein was first established by plasmid transfection. Twenty-four hours later, the ISG15 conjugation machinery was expressed by a four-plasmid transfection, with or without cotransfection of a siRNA targeting the mRNA encoding the plasmid-expressed target protein. Extracts were prepared 22 hr after the second transfection, and the target protein was analyzed for ISGylation. Our hypothesis predicted that, if ISG15 modification was dependent on protein synthesis, then siRNA-mediated destruction of the mRNA would result in a loss of ISGylation of the target protein. That is, the pre-existing pool of the target protein, synthesized in the first 24 hr, would not be modified. In contrast, in the absence of a specific siRNA or in the presence of an off-target siRNA, the target protein would continue to be synthesized, and ISGylation would be observed. This experiment was dependent on the rate of degradation of the target protein, and for the targets analyzed, protein levels did not decline more than 2-fold after siRNA transfection (Figure S4B). RT-PCR also confirmed that the specific siRNAs led to > 80% knockdown of the target siRNAs by 12 hr posttransfection (data not shown). Figure 4C

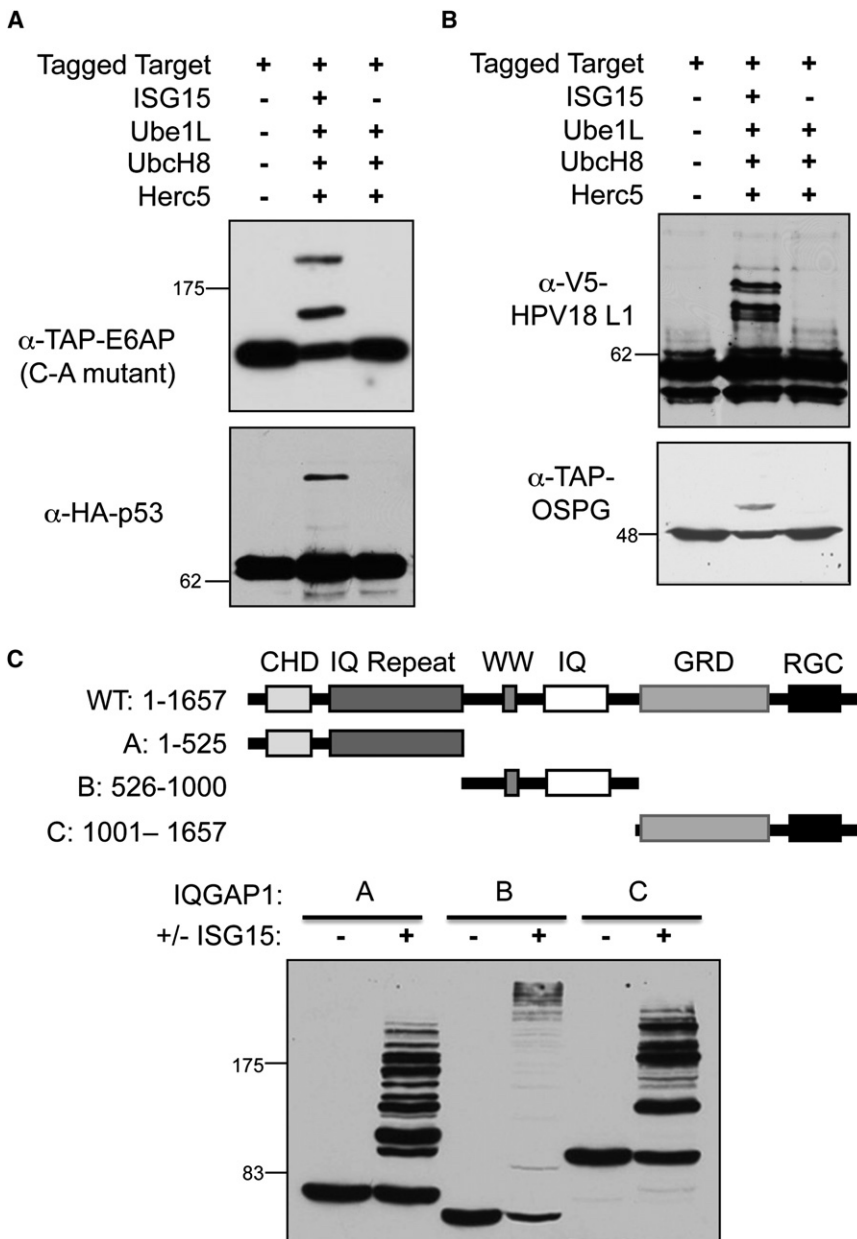


Figure 3. ISG15 Conjugation to Proteins Not Previously Identified as ISG15 Targets

(A) Modification of human proteins not previously identified as ISG15 targets. HEK293T cells were transfected with plasmids expressing either TAP-E6AP or HA-p53 alone (lane 1), combined with Ube1L, UbcH8, Herc5, and ISG15 (lane 2), or combined with Ube1L, UbcH8, and Herc5 (lane 3). Cell extracts were prepared 48 hr posttransfection and analyzed by immunoblotting with anti-TAP or anti-HA antibodies. See also Figure S2.

(B) ISG15 modification to foreign proteins. HEK293T cells were transfected with plasmids expressing the indicated proteins and either V5-HPV18 L1 protein or TAP-tagged *Shigella* OSPG protein and analyzed as in (A) with anti-V5 or anti-TAP antibodies.

(C) Modification of nonoverlapping fragments of IQGAP1. A schematic of IQGAP1 is shown (top), along with three nonoverlapping fragments, A–C, with amino acid numbering shown. Plasmids expressing TAP-tagged fragments A–C were transfected into HEK293T cells and analyzed as in (A) using anti-TAP antibody.

labeled with ^{35}S -labeled cysteine either before conjugation was occurring (6 hr posttransfection) or while conjugation was occurring (22 hr posttransfection). Total cell lysates were collected 24 hr posttransfection, and an anti-FLAG immunoprecipitation was analyzed by SDS-PAGE and autoradiography. Our hypothesis predicted that ^{35}S -containing proteins labeled at the early time point would not be ISGylated, whereas proteins labeled at the late time point, while conjugation was occurring, would be susceptible to ISGylation. A broad range of labeled proteins were conjugated to ISG15 in cells labeled at the 22 hr time point, whereas the pattern of labeled immunoprecipitated proteins from the early labeling period was only slightly above that seen in control untransfected cells (Figure 5B, compare

lane 2 to lanes 1, 5, and 6). The slight signal over background in the lanes corresponding to the early labeling period may be due to either a secondary mode of ISG15 conjugation (e.g., a minor Herc5-independent conjugation pathway) or due to turnover of the ^{35}S -cysteine and re-incorporation into newly translated proteins at later time points. This experiment was also performed using a FLAG-ISG15 expression plasmid encoding an ISG15 mutant lacking the N-terminal ubiquitin-like domain (FLAG-ISG15- ΔN), which is conjugated similarly to wild-type ISG15. As above, ^{35}S -labeled proteins were coimmunoprecipitated with FLAG-ISG15- ΔN when cells were labeled at the 22 hr time point, but not when cells were labeled at the 6 hr time point (Figure 5B, lanes 3 and 4). An anti-FLAG western

shows the results for analysis of TAP-IQGAP1 and TAP-p53. ISGylation of both proteins was observed in the absence of any siRNA or in the presence of off-target siRNAs but was greatly diminished in the presence of a specific siRNA. These results were consistent with the hypothesis that a protein must be actively synthesized in order for it to be modified by ISG15.

A pulse-labeling scheme was used to globally determine whether ISGylation was limited to proteins synthesized in the same window of time that the conjugation machinery is active. In preliminary experiments, we determined that ISG15 conjugates became evident ~ 12 hr after a four-plasmid transfection (FLAG-ISG15, Ube1L, UbcH8, and Herc5). Therefore, 293T cells were transfected with the four plasmids and then pulse-

labeled with ^{35}S -labeled cysteine either before conjugation was occurring (6 hr posttransfection) or while conjugation was occurring (22 hr posttransfection). Total cell lysates were collected 24 hr posttransfection, and an anti-FLAG immunoprecipitation was analyzed by SDS-PAGE and autoradiography. Our hypothesis predicted that ^{35}S -containing proteins labeled at the early time point would not be ISGylated, whereas proteins labeled at the late time point, while conjugation was occurring, would be susceptible to ISGylation. A broad range of labeled proteins were conjugated to ISG15 in cells labeled at the 22 hr time point, whereas the pattern of labeled immunoprecipitated proteins from the early labeling period was only slightly above that seen in control untransfected cells (Figure 5B, compare

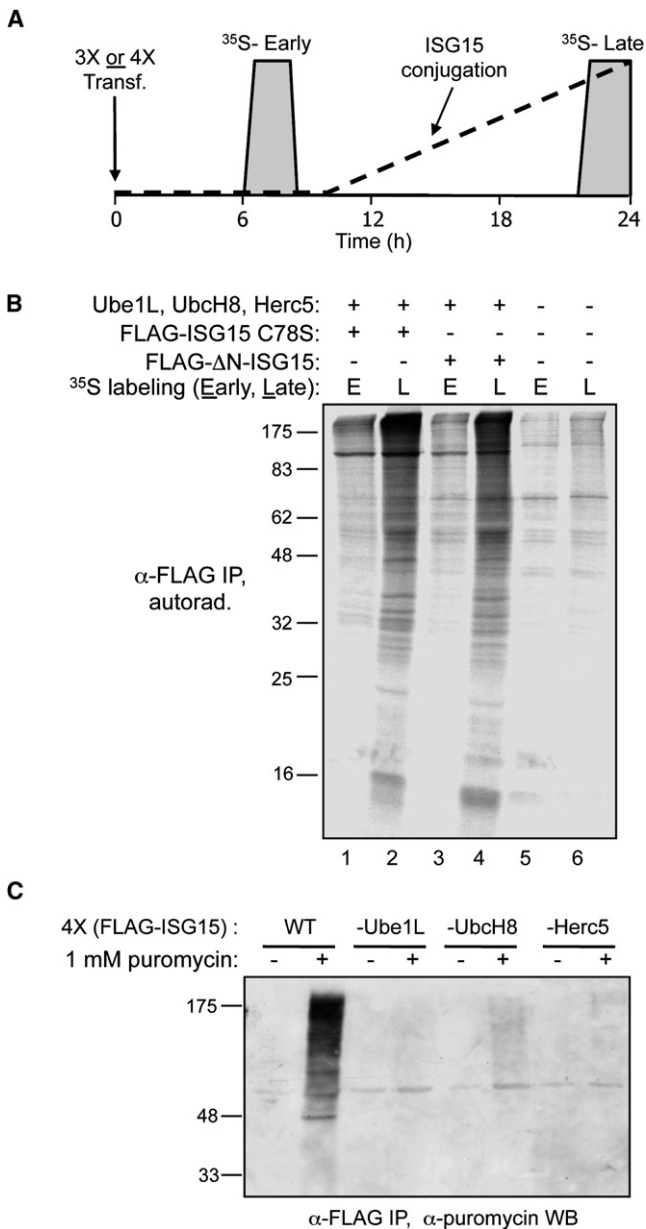


Figure 5. ³⁵S-Cysteine Metabolic Labeling Demonstrates that Pre-Existing Pools of Cellular Proteins Are Not Subject to ISG15 Conjugation

(A) Schematic of the experimental design. HEK293T cells were transfected with plasmids expressing either Ube1L, UbcH8, and Herc5 (3x) or Ube1L, UbcH8, Herc5 and either FLAG-ISG15 C78S or FLAG-ΔN-ISG15 (4x). Cells were labeled for 2 hr with ³⁵S-cysteine beginning either 6 or 22 hr posttransfection (gray boxes), corresponding to periods before or during which ISG15 conjugation was occurring (approximate time course of ISG15 conjugation indicated by dashed line). Cell extracts were prepared 24 hr posttransfection. (B) Immunoprecipitation of labeled cellular proteins conjugated to ISG15. ISG15 conjugates were immunoprecipitated with anti-FLAG beads, and ³⁵S-labeled cellular proteins that were conjugated to ISG15 were analyzed by SDS-PAGE and autoradiography. (Lanes 1 and 2) Immunoprecipitation of extracts after 4x transfection (with FLAG-ISG15 C78S), labeled with ³⁵S-cysteine either early (E; 6 hr) or late (L; 22 hr). (Lanes 3 and 4) Immunoprecipitation of extracts after 4x transfection (with FLAG-ΔN-ISG15), labeled with

Such polypeptides were not detected in cells that were not treated with puromycin. Cells lacking Ube1L, UbcH8, or Herc5 had a very small amount of conjugates, corresponding to the low basal expression of these enzymes in non-IFN-treated cells (data not shown). These results were therefore consistent with the model that newly synthesized proteins are targeted for ISGylation.

Herc5 Is Associated with Polysomes

The ISGylation of newly synthesized proteins suggested that modification might occur cotranslationally. We therefore determined whether endogenously expressed Herc5 cofractionated with ribosomes and/or polysomes. HeLa cells were treated with IFN-β for 24 hr, and cell extracts were fractionated by sucrose gradient sedimentation. Ribosome- and polysome-containing fractions, including the 40S and 60S ribosomal subunits, were identified by characteristic absorbance profiles at 254 nm and confirmed by immunoblotting with antiribosomal subunit antibodies. As shown in Figure 6A, the vast majority of Herc5 protein was within the polysome-containing fractions. Herc5 cofractionated with 80S ribosomes when extracts were treated with RNase (Figure 6B), which destroys the polysomes and leads to a large increase in the number of 80S ribosomes. This suggested that Herc5 was associated with polysomes via ribosomes, rather than mRNA. Consistent with this, Herc5 cofractionated with 60S ribosomes when extracts were treated with EDTA (Figure 6C), which dissociates polysomes and 80S ribosomes into 40S and 60S subunits. EDTA also releases nascent polypeptides from the 60S subunit (Ullers et al., 2004; Valent et al., 1997), suggesting that Herc5 was unlikely to be tethered to ribosomes via nascent polypeptides. Herc4 is a HECT domain ligase that has 57% primary sequence similarity to Herc5 (Hochrainer et al., 2005), but it is not IFN induced and does not function in ISG15 conjugation. Neither endogenously expressed Herc4 nor another unrelated HECT E3, E6AP/Ube3A, cofractionated with polysomes (Figure 6A).

Herc5 contains four well-defined RCC repeats between residues 150 and 370 and three less well-conserved repeats in the first 150 amino acids (Hadjebi et al., 2008). Deletion of this entire region (ΔRCC) abrogates ISGylation of target proteins (Dastur et al., 2006), as does deletion of amino acids 2–100 (Δ100); however, both proteins retain catalytic activity based on autoconjugation activity (Figure S6). Full-length HA-tagged Herc5 expressed by transfection was present in the polysome fractions (Figure 6D), although it was also present in earlier ribosome-containing fractions of the gradient, probably due to the ~5-fold overexpression of HA-Herc5 relative to the IFN-induced levels

³⁵S-cysteine either early (E) or late (L). (Lanes 5 and 6) Immunoprecipitation of extracts from untransfected cells, labeled with ³⁵S-cysteine either early (E) or late (L).

(C) Cells treated with puromycin contain polypeptides that are modified with both puromycin and ISG15. HEK293T cells were transfected with either four plasmids (FLAG-ISG15, Ube1L, UbcH8, and Herc5) or three plasmids (same, without Ube1L, UbcH8, or Herc5). Twenty-four hours posttransfection, cells were harvested or treated with 1 mM puromycin for 2 min. Cell extracts were prepared, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-puromycin antibody.

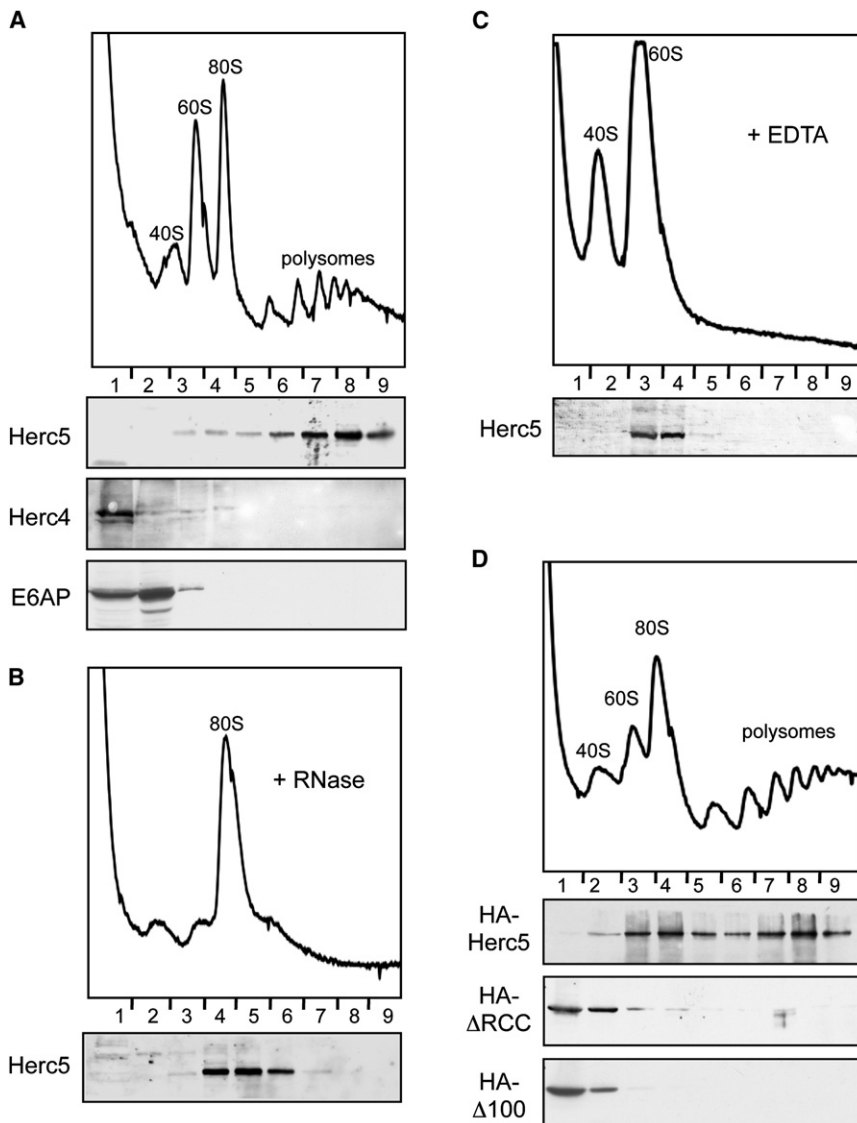


Figure 6. Herc5 Fractionates with Polysomes

(A) Herc5 fractionates with polysomes in HeLa cell extracts. HeLa cells were treated with IFN- β for 24 hr and subjected to polysome analysis (Experimental Procedures). The A_{254} profile shows the positions of the 40S, 60S, 80S, and polysome fractions. Fractions were analyzed by immunoblotting with the indicated antibodies.

(B) Herc5 fractionates with the 80S ribosomes after RNase treatment. HeLa cells were treated and analyzed as described in (A); however, RNase was added to the cell lysate after harvest. Fractions were analyzed by immunoblotting with anti-Herc5 antibody.

(C) Herc5 fractionates with the 60S ribosomal subunit after EDTA treatment. HeLa cells were treated and analyzed as described in (A), with addition of EDTA to the cell lysate after harvest. Fractions were analyzed by immunoblotting with anti-Herc5 antibody.

(D) The RCC1 repeats of Herc5 are required for cosedimentation with polysomes. HEK293T cells were transfected with either HA-Herc5-, HA- Δ RCC-, or HA- Δ 100-expressing plasmids and harvested after 24 hr. Cell extracts were fractionated as above and analyzed by anti-HA immunoblotting.

of Herc5 in HeLa cells. In contrast, both the HA- Δ RCC and HA- Δ 100 Herc5 proteins were present almost exclusively in the earliest fractions of the gradient. Therefore, the RCC1 repeat region of Herc5 is essential for both ISGylation and polysome association.

A Model for an Antiviral Function of ISG15

The findings presented here have implications for the identity of the biologically relevant targets of ISG15. Type 1 IFNs are secreted by virus-infected cells to establish an antiviral state in uninfected surrounding cells. As these cells are likely to be the next sites of virus infection, we propose that the ISGylation system is designed to target newly synthesized viral proteins. However, even among newly synthesized proteins, ISGylation is inefficient, raising the question of how modification of a small fraction of a viral protein could have a significant antiviral effect. The answer may lie in the fact that structural proteins are not only among the most actively synthesized of viral proteins, but must

often assemble into precise repeating geometric configurations to form infectious virus particles (e.g., capsid proteins). Therefore, ISGylation of even a small fraction of the total pool of a viral structural protein might have a dominant-negative effect on production of infectious virus.

The HPV16 L1 capsid protein was ISGylated in transfection experiments (Figure 7A). We therefore employed an HPV pseudovirus (PsV) system to measure

the effect of ISGylation on infectivity (Buck et al., 2004; Buck and Thompson, 2007). Papillomavirus virions are composed of 360 molecules of the L1 capsid protein and up to 72 molecules of L2 (Buck et al., 2008). When L1 and L2 are expressed in the presence of an \sim 8 kbp GFP expression plasmid, the plasmid is packaged as if it were viral genomic DNA; these PsV can then be isolated on velocity-density gradients and their infectivity measured by quantitating the delivery of the GFP reporter plasmid to naive cells. HPV16 pseudoviruses were generated in 293TT cells in which ISG15 conjugation was either occurring (cotransfection of ISG15 and E1, E2, E3 enzymes) or not occurring (cotransfection of ISG15 and inactive mutant forms of the E1, E2, and E3 enzymes). The presence of assembled PsV at the expected gradient fractions (Buck et al., 2004) was confirmed by transmission electron microscopy (data not shown). In the ISGylated PsV fractions, \sim 10% of the total L1 protein was ISGylated (Figure 7B). The total amount of L1 protein in ISGylated PsV fractions was \sim 30% less than in

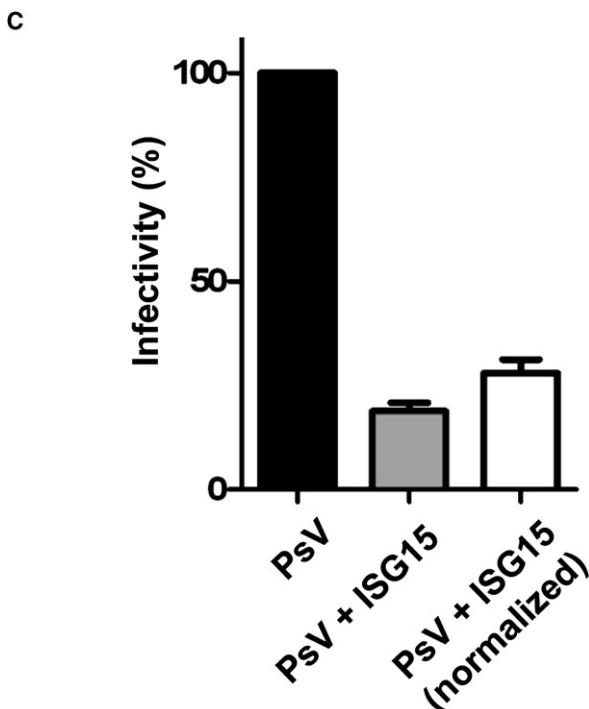
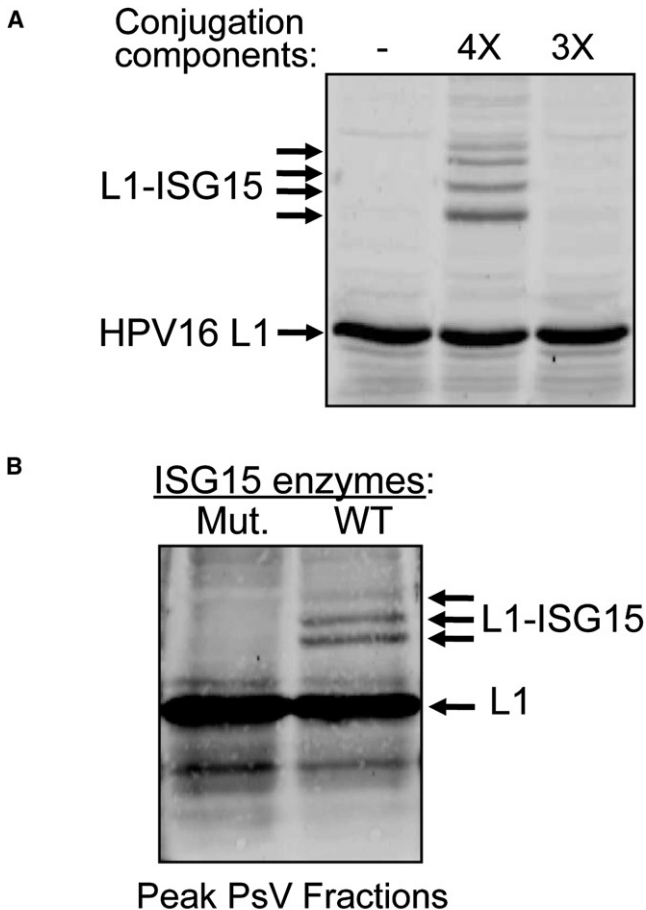


Figure 7. ISGylation of HPV16 L1 Has a Dominant-Negative Effect on Infectivity of HPV Pseudovirus

(A) ISG15 Modification of HPV16 L1. 293TT cells were cotransfected with p16shell, alone, or with plasmids expressing ISG15, Ube1L, UbcH8, and Herc5 (4x), or this set of plasmids without ISG15 (3x). Cells extracts were analyzed by SDS-PAGE and immunoblotting with anti-L1 antibody.

(B) ISGylated HPV16 L1 is detected in fractions containing HPV pseudovirus. Equal volumes of PsV-containing fractions, prepared in cells expressing active ISGylation enzymes (WT) or inactive mutants (Mut.), were separated by SDS-PAGE and immunoblotted with anti-L1 antibody.

(C) ISGylation of HPV16 L1 decreases the infectivity of HPV pseudovirus. 293T cells were infected with equal volumes PsV from the fractions shown in Figure 7B, formed either in the absence (PsV) or presence (PsV + ISG15) of the ISG15 conjugation system. GFP-positive cells were counted 60 hr postinfection by FACS. The infectivity of wild-type PsV was set to 100%. Results are presented both without (middle) and with (right) normalization for total L1 protein concentration. Error bars indicate standard error of the mean of three independent experiments performed in triplicate.

the control PsV fractions. Together, these observations indicated that ISGylated L1 was incorporated into PsV particles but that the overall yield of PsV was decreased. To examine the infectivity of ISGylated and non-ISGylated PsV, the peak gradient fractions were added to the culture media of 293T cells, followed by quantitation of GFP-positive cells by FACS analysis. Without normalization for total L1 concentration, the infectivity of ISGylated PsV was decreased ~80% relative to the non-ISGylated PsV in three independent experiments (Figure 7C). With normalization, the infectivity of ISGylated PsV was ~70% lower than non-ISGylated PsV (Figure 7C). These results establish that low-level ISGylation of a viral structural protein can have a dominant-negative effect on virus infectivity.

DISCUSSION

We have shown here that the range of ISG15 target proteins is extremely broad, that newly synthesized proteins are the targets of ISGylation, and that Herc5 is associated with the translational machinery. These findings suggest that the ISG15 system is designed to target newly synthesized proteins, with limited target protein selectivity. These findings imply that, in the context of an interferon response, viral proteins, rather than cellular proteins, may be the principal targets of the ISG15 conjugation system.

Herc5 protein in extracts of interferon-stimulated HeLa cells cofractionated with polysomes and further analyses suggested that it is associated with a core component of the 60S ribosomal subunit. As the exit tunnel for nascent polypeptides is on the 60S subunit (Kramer et al., 2009), an attractive model is that Herc5 modifies newly synthesized polypeptides cotranslationally as they emerge from the exit tunnel. Of interest, other protein modification enzymes have been identified that are ribosome associated and function near the exit tunnel, including methionine isopeptidase (Raue et al., 2007; Vetro and Chang, 2002), N-terminal acetyltransferases (Green et al., 1978; Pestana and Pitot, 1975), and chaperones that aid in cotranslational folding of proteins (Kramer et al., 2009). Polysome association of Herc5 suggests that the inclusion of a translation system will be a requirement for reconstitution of *in vitro* ISGylation, which,

in turn, is likely to be required for a direct test of the cotranslational model. Also, it is important to note that, though the range of ISG15 target proteins appears extremely broad, not all newly synthesized proteins were ISGylated in our assays. Factors that might influence susceptibility to modification include rates of translation, cotranslational folding rates, and the sequence or secondary structure context of lysine acceptors.

Interferon-stimulated cells are primed to defend against an impending viral infection, which suggests that newly translated viral proteins might be biologically relevant targets of the ISG15 system. The low degree of target protein selectivity of the ISG15 system is consistent with the requirement that the innate immune response protect cells against a wide range of pathogens. An implication of this model is that modification of cellular target proteins may be simply collateral damage in the attempt to target viral proteins. This, in turn, may be tied to the observation that ISGylation is relatively inefficient: an inefficient ISGylation system might protect against excessive damage to cellular proteins while at the same time still being an effective antiviral due to dominant-negative effects on abundantly expressed virus structural proteins, as shown here with the HPV pseudovirus system. These results are consistent with results showing that mutant forms of viral structural proteins can, themselves, have a dominant-negative effect on infectivity (Lee et al., 2009). In addition, potential dominant-negative effects of ISGylation are not necessarily limited to effects on virus structural proteins; any viral protein that oligomerizes would be a potential biologically relevant target in our model.

The biochemical function of ISG15 conjugation remains unknown. The two most general possibilities are that ISG15 signals to another protein or protein complex (e.g., in the way that ubiquitin signals to the proteasome) or that it simply disrupts the function of proteins to which it is conjugated. There is evidence to support the latter in studies that have examined individual target proteins (Jeon et al., 2009; Zou et al., 2005), and this may also be inferred by ISG15 protein sequence comparisons between mammalian species. Unlike ubiquitin and most other UbIs, ISG15 protein sequences are very divergent. Only 72 out of 157 residues (46%) are identical between the human, mouse, rat, dog, cow, and sheep ISG15 protein sequences. We suggest that these divergent mammalian ISG15 proteins are unlikely to have retained a common signaling function and that the similarity retained reflects that which is required for maintaining the ubiquitin-like folds of ISG15 and for productive interaction with the conjugation enzymes. We therefore suggest that the function of ISG15 is to generally disrupt target protein function and, in particular, the functions of viral proteins.

The HPV pseudovirus system provides a proof of principle that low-level ISGylation of a virus structural protein can have dominant-negative effects on virus infectivity. The precise step in the infectivity of HPV pseudoviruses that is affected by ISGylation of HPV capsids is not yet known but could range from receptor recognition to a defect in endocytosis or release from endocytic compartments to the delivery of the packaged DNA to the nucleus. The effect of type 1 interferons on HPV replication is known to be complex (Beglin et al., 2009); however, multiple studies have indicated that HPVs interfere with expression and/or function of components of the interferon response

(Barnard and McMillan, 1999; Chang and Laimins, 2000; Nees et al., 2001; Ronco et al., 1998). In addition, topical IFN- α is an approved treatment for certain HPV lesions (Slade et al., 1998). The results presented here warrant an examination of the role of the ISG15 system in the response of HPV lesions to type 1 IFNs and, more broadly, of the effect on ISG15 on proteins of many other classes of viruses.

EXPERIMENTAL PROCEDURES

Antibodies, Plasmids, and siRNAs

All antibodies and plasmids used in this study are listed in Table S3. The *IQGAP1* SMARTpool siRNA was supplied by Dharmacon. Additional siRNA sequences: *p53*, 5'-GACTCCAGTGGTAATCTACTT-3' and *hTfr*, 5'-AAGGTGT AGTGGAAAGTATC-3'.

Cell Culture, IFN- β Treatment, Transfections, and Immunoblotting

Maintenance, transfection, harvest, and immunoblotting of HeLa and HEK293T cells were as described previously (Durfee et al., 2008). 293TT cells were provided by John Schiller (NCI, Bethesda, MD) and maintained as described (Buck et al., 2004). ISG15 conjugation was induced by treating HeLa cells with 1000 units/ml IFN- β (Betaseron) for either 24 or 48 hr. For the experiment shown in Figure 5B, Lipofectamine 2000 was used in the initial transfection, and XtremeGENE siRNA Transfection Reagent (Roche) was used for the cotransfection of DNA and siRNA.

³⁵S-Labeling and Puromycin Labeling Experiments

HEK293T cells in 35 mm dishes were transfected with the indicated plasmids, as described above, and labeled for 2 hr with 80 μ Ci of ³⁵S-cysteine (1000 Ci/mmol). The FLAG-ISG15 construct used in this experiment contained the C78S mutation, eliminating the only cysteine residue in ISG15. The C78S mutant is fully functional for conjugation, and the use of this mutant ensured that the ³⁵S-cysteine-containing proteins detected in the immunoprecipitation represented labeled cellular proteins that had been conjugated to unlabeled ISG15, as opposed to unlabeled proteins being conjugated to ³⁵S-labeled ISG15. Similarly, the Δ N-ISG15 construct lacked C78. Cells were washed with PBS and either harvested immediately (late labeling period) or replenished with fresh media. Cell lysates were collected and immunoprecipitations were performed with anti-FLAG M2 agarose beads (Sigma). Immunoprecipitates were separated by SDS-PAGE, and labeled proteins were detected by autoradiography. For puromycin experiments, cells were transfected with the indicated plasmids and treated, 24 hr posttransfection, with puromycin (1 mM final concentration) for 2 min. Total cell extracts were prepared and FLAG-ISG15 conjugates were precipitated with anti-FLAG M2 agarose beads (Sigma). The immunoprecipitates were then analyzed by immunoblotting with an antibody recognizing puromycin.

Sucrose Gradient Fractionation

HEK293T or HeLa cells were harvested 24 hr after transfection or IFN- β stimulation, as described (Huang et al., 2007), with the exception that heparin (200 μ g/ml) was added to the polysome lysis buffer and RNasin was omitted. Lysates were centrifuged for 10 min at 16,300 \times g at 4°C, and supernatants were loaded onto linear 7%–47% (w/v) sucrose gradients containing cycloheximide (200 μ g/ml). For EDTA treatment, cells were lysed in polysome lysis buffer containing 50 mM EDTA (MgCl₂ and heparin omitted) before applying to the sucrose gradient (supplemented with 10 mM EDTA instead of MgCl₂). For the RNase treatment, RNase (Sigma) was added to the lysate (heparin omitted) at a final concentration of 10 μ g/ml and incubated at 30°C for 15 min before applying to the sucrose gradient. Gradients were centrifuged at 222,000 \times g for 90 min at 4°C in a Beckmann SW41Ti rotor. Polysome profiles were monitored by absorbance at 254 nm, and gradient fractions were collected on an ISCO density gradient fractionator. Trichloroacetic acid (TCA) was added to each sucrose gradient fraction to a final concentration of 10% (v/v) for protein precipitation. Precipitated proteins were then prepared for SDS-PAGE and immunoblotting analyses.

HPV Pseudovirus Production and Infectivity Assays

293TT cells were cotransfected with p16shell (HPV16 L1 and L2 expression vector), pcVM10-GFP, and either the four ISGylation plasmids or a set of four plasmids that expressed ISG15 plus the inactive mutant forms of the E1 (Ube1L-ΔUFD), E2 (UbcH8 F62A), and E3 (Herc5 C994A) enzymes. After 48 hr, 293TT cells were collected, and pseudovirus was purified according to the previously described protocols (Buck et al., 2004; Buck and Thompson, 2007). After ultracentrifugation using Optiprep (Sigma) gradients, fractions were collected and peak L1 content was determined via SDS-PAGE and immunoblotting for HPV16 L1. Relative amounts of L1 (ISGylated and non-ISGylated) were determined using a fluorescent secondary antibody and the Odyssey system (Li-Cor). L1 protein concentration was determined by quantitation with BSA standards on colloidal Coomassie-stained SDS-PAGE gels using the Odyssey system as well. For the infection assay, 293T cells were grown in 12-well plates and infected with equal amounts of PsV-containing fractions. Sixty hours postinfection, cells were trypsinized and subjected to FACS analysis using a FACSCalibur machine (Becton Dickinson). CellQuest Pro v5.2.1 (Becton Dickinson) was used for data acquisition and analysis of 10,000 live cell events.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and three tables and can be found with this article online at doi:10.1016/j.molcel.2010.05.002.

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