Herc5, an Interferon-induced HECT E3 Enzyme, Is Required for Conjugation of ISG15 in Human Cells*  

Received for publication, December 1, 2005  Published, JBC Papers in Press, December 28, 2005, DOI 10.1074/jbc.M512830200  

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ISG15 is an interferon (IFN)-α/β-induced ubiquitin-like protein that is conjugated to cellular proteins during innate immune responses to viral and bacterial infections. A recent proteomics study identified 158 human proteins targeted for ISG15 conjugation, including the ISG15 E1 and E2 enzymes (Ube1L and UbcH8, respectively) and a HECT E3 enzyme, Herc5. Like the genes encoding Ube1L and UbcH8, expression of Herc5 was also induced by IFN-β, suggesting that Herc5 might be a component of the ISG15 conjugation system. Consistent with this, small interfering RNAs targeting Herc5 had a dramatic effect on overall ISG15 conjugation in human cells, abrogating conjugation to the vast majority of ISG15 target proteins in vivo. In addition, co-transfection of plasmids expressing ISG15, Ube1L, UbcH8, and Herc5 resulted in robust ISG15 conjugation in non-IFN-treated cells, while the active-site cysteine mutant of Herc5 or a mutant lacking the RCC1 repeat region did not support ISG15 conjugation. These results demonstrate that Herc5 is required for conjugation of ISG15 to a broad spectrum of target proteins in human cells.

Type 1 interferons play an essential role in innate immunity (1). One of the many genes strongly activated by IFN-α/β encodes ISG15, a 15-kDa ubiquitin-like protein (Ubi) (2, 3). Like ubiquitin (Ub), UbIs are linked to target proteins via isopeptide bonds between their terminal carboxyl group and lysine side chains of target proteins (4). The fact that ISG15 is expressed and conjugated in IFN-α/β-stimulated cells and lipopolysaccharide-stimulated cells implies that ISG15 conjugation is likely to mediate an important component of the innate immune response. This is supported by the finding that the influenza B virus NS1B protein specifically blocks ISG15 conjugation (5).

The biochemical effect of ISG15 on target proteins is unknown; however, the recent identification of a large number of target proteins (6) provides opportunities for determining both the function of ISG15 and its role in the innate immune response. Also essential for functional studies is the identification of the complete set of enzymes required for ISG15 conjugation. As with Ub conjugation, it is presumed that a cooperating set of E1, E2, and E3 enzymes, in addition to possible accessory factors, will be required for ISG15 conjugation. The ISG15 E1 and E2 enzymes have been identified. Ube1L is a single-subunit enzyme 62% similar to the Ub E1 enzyme (5), and UbcH8 is the major, if not exclusive, E2 enzyme for ISG15 (7, 8). The genes encoding both Ube1L and UbcH8 are, like ISG15, transcriptionally activated by IFN-α/β (5, 7–9), suggesting that the entire conjugation system might be coordinately regulated. A candidate E3 enzyme for ISG15 conjugation emerged from mass spectrometry-based identification of ISG15 target proteins (6). Proteomics analyses of SUMO- and Ub-conjugated proteins have shown that enzymatic components of Ub/Ubl conjugation pathways are often auto-conjugated (10, 11), and consistent with this, Ube1L and UbcH8 were identified as ISG15-modified proteins. In addition, a single HECT E3, Herc5, was identified as an ISG15-modified protein, suggesting that this enzyme might also be a component of the ISG15 conjugation pathway. We show here that Herc5 is required for the conjugation of ISG15 to a broad spectrum of target proteins in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, Antibodies, and siRNAs—HeLa and 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Constructs for expression of Ube1L, UbcH8, and ISG15 were described previously (6). A plasmid containing the complete Herc5/CBE1 open reading frame (12) was provided by M. Ohtsuo (Hiroshima University). The Herc5 open reading frame and mutantx were subcloned into a pcDNA3 vector containing an aminoterminal TAP (Tandem Affinity Purification) tag (13) or an aminoterminal HA tag. Anti-p56 antibody was provided by Ganes Sen (Cleveland Clinic), anti-MxA antibody by Otto Haller (University of Freiburg), and ISG15 antibody by Ernest Borden (Cleveland Clinic). siRNAs were supplied by Dharmaco, Inc., and sequences are shown for those targeting human Herc5 (5A, 5B, 5C), Herc6 (6A, 6B), and both Herc5 and Herc6 (5/6): 5A, GAGAAGUAAGAUAACUGUCAUU; 5B, GACACAACUUAAUUCUCCAUUU; 5C, UAAAGACUGACAGUUGUUUUU; 6A, UACCCGAGAUUAUACUAAUU; 6B, GAAGUCGCUAGUUAAGAAG; 5/6, GAGACUAUGUUAUCUAGUUUA. E6AP (14) and UbcH8 (7, 8) siRNAs were validated previously.

Reverse Transcriptase (RT)-PCR and Microarray Analyses—Total RNA for RT-PCR was isolated using the PARIS kit (Ambion, Inc.). SuperscriptII reverse transcriptase (Invitrogen) was used for cDNA synthesis, which was used as a template in subsequent PCR reactions. Initially, a range of PCR cycles was performed using cDNA from IFN-β-treated or untreated cells to determine the linear range of amplification for each gene, and these parameters were used for PCR shown in Figs. 1A and 2A. Microarray analyses were performed as described previously (14). Elements chosen for analysis were screened for several data quality standards, including minimum intensity and pixel consistency.

Transfection Experiments—siRNAs transfections were carried out using Oligofectamine (Invitrogen), at a final concentration of 100 nM siRNA. Cells were treated with IFN-β (1,000 units/ml; Berlex) for the indicated times. Protein extracts were made in buffer containing 0.1 M Tris (pH 7.0), 0.1 M NaCl, 1% Nonidet P-40, and 1 mM diethiothreitol. To
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RESULTS

Herc5 is one of six human Herc proteins (HECT and RCC1), defined by a carboxyl-terminal HECT domain and by one or more RLDs (RCC1-like domains) (15). Herc5 was a candidate for being a component of the ISG15 conjugation pathway based on the fact that it was identified as an ISG15-modified protein (6), and it belongs to the HECT family of E3 enzymes (16), some of which can interact with UbcH8 (8, 17). To determine whether Herc5 plays a significant role in overall ISG15 conjugation, two synthetic double-stranded siRNAs (designated 5A and 5B) were designed to target Herc5 mRNA. The Herc6 protein is 49% identical to Herc5, and two siRNAs (6A and 6B) were therefore designed to target Herc6, as well as one siRNA that would simultaneously target both Herc5 and Herc6 (5/6). RT-PCR confirmed that transfection of the Herc5- and Herc6-specific siRNAs reduced Herc5 and Herc6 mRNA levels, respectively, in IFN-β-treated HeLa cells (Figs. 1A, 5/6 reduced both Herc5 and Herc6 mRNAs, and as shown below (Fig. 3C), all three siRNAs against Herc5 efficiently knocked down expression of Herc5 at the protein level. HeLa cells were transfected with the Herc5 or Herc6 siRNAs or, as a negative control, an siRNA against the E6AP HECT E3 (14), and 6 h after transfection, IFN-β was added for an additional 48 h. Total cell extracts were prepared and ISG15 conjugates were analyzed by immunoblotting with an antibody against ISG15. Induction of ISG15 and high molecular weight ISG15 conjugates was observed in IFN-β-treated cells (Fig. 1B, compare lanes 1 and 2). Both of the Herc5-specific siRNAs and the siRNA that targeted Herc5 and Herc6 (5/6) resulted in a dramatic decrease in overall ISG15 conjugates (lanes 3–5), while neither of the Herc6-specific siRNAs or the E6AP siRNA led to a discernible decrease in ISG15 conjugates (lanes 6–8). Similar results were observed in 293 cells (data not shown).

Combinations of Herc5 and Herc6 siRNAs were also transfected together, on the premise that potential Herc6-dependent effects might be more evident following reduction of Herc5 activity (Fig. 1C). For these experiments, a third Herc5 siRNA (5C) was used that elicited only a partial reduction in Herc5 activity (compare lanes 1–3). Co-transfection of Herc6 siRNA 6A with either Herc5 siRNA 5A or 5C did not elicit any further decrease in ISG15 conjugates relative to the Herc5 siRNAs alone. The effect of Herc5 and Herc6 siRNAs on conjugation of ISG15 to two specific target proteins (6), p56 and MxA, was also examined (Fig. 1D). The identification and validation of the IFN-β-induced ISG15-conjugated forms of both of these proteins was described previously (6). Consistent with the effect of Herc5 siRNAs on total ISG15 conjugates, siRNAs that targeted Herc5 blocked conjugation to both p56 and MxA, while Herc6-specific siRNAs did not. We conclude that Herc5 plays a major role in mediating overall ISG15 conjugation to a broad spectrum of target proteins. We cannot rule out that Herc6 might also function in the ISG15 conjugation pathway, but if so, it clearly plays a minor role relative to Herc5.

We determined whether expression of Herc5 and/or Herc6 was regulated by IFN-β by microarray gene expression analyses, comparing IFN-β-treated cells to untreated cells at various time points following addition of IFN-β (3, 6, and 21 h). Fig. 2A shows representations of the microarray elements corresponding to ISG15, Ube1L, UbcH8, Herc5, and Herc6 cDNAs, along with elements corresponding to three genes not expected to be affected by IFN-β treatment (E11α, UbcH7, and E6AP). The induction of ISG15, Ube1L, and UbcH8 was evident, along
with both Herc5 and Herc6, with maximal induction over this time course at 21 h post-IFN-β treatment. This corresponds to the beginning of maximal accumulation of ISG15 conjugates (6, 18). Expression of E1Ub, UbcH7, and E6AP was not affected by IFN-β treatment. Fig. 2B shows that the time course and magnitude of Herc5 and Herc6 induction was similar to that of Ube1L and UbcH8. The regulation of Herc5 and Herc6 expression by IFN-β is consistent with the demonstrated importance of Herc5 in ISG15 conjugation, as well as a potential minor role for Herc6.

Co-transfection of plasmids expressing ISG15, Ube1L, and UbcH8 leads to ISG15 conjugation in non-IFN-treated HeLa cells, although at a lower level than observed in IFN-β-treated cells (6). To determine whether this level of conjugation was due to a significant basal level of Herc5 expression, we transfected Herc5 siRNAs prior to co-transfection of plasmids expressing ISG15, Ube1L, and UbcH8. As shown in Fig. 3A, transfection of Herc5 siRNAs (5A, 5B, or 5/6) did indeed block ISG15 conjugation, while Herc6 siRNAs did not, indicating that a basal level of Herc5 expression is responsible for ISG15 conjugation in this context.

We also determined whether co-transfection of a Herc5-expressing plasmid would further boost the level of ISG15 conjugates seen in non-IFN cells. In HeLa cells, transfection of a Herc5-expressing plasmid boosted ISG15 conjugation ~3-fold over that seen with transfection of ISG15, Ube1L, and UbcH8 plasmids (Fig. 3B). Importantly, the Herc5 active-site mutant (C994A) did not stimulate ISG15 conjugation, indi-

FIGURE 2. Herc5 and Herc6 expression is induced by IFN-β. A, microarray gene expression analysis was performed, comparing HeLa cells treated with IFN-β for the indicated time periods (0, 3, 6, and 21 h) to untreated HeLa cells. cDNA from IFN-β-treated and untreated cells were labeled with Cy5 (red) and Cy3 (green), respectively. Microarray elements corresponding to the indicated genes are shown. B, bar graph representation of the microarray data shown in A, illustrating the time course and magnitude of induction for each of the indicated genes.
cating that the catalytic activity of Herc5 is required for its function in the ISG15 system. In 293 cells, co-transfection of wild-type Herc5 had a stronger effect on ISG15 conjugation, with a 10–20-fold stimulation.

Because a Herc5-specific antibody was not available, we took advantage of our epitope-tagged (HA and TAP) Herc5 expression vectors to confirm that all three Herc5 siRNAs effectively knocked-down Herc5 at the protein level (Fig. 3C). The 5A, 5B, and 5/6 siRNAs resulted in a dramatic reduction of HA-Herc5 protein, while neither of the Herc6 siRNAs, an siRNA against E6AP nor a UbCH8-specific siRNA, affected HA-Herc5 protein levels. Identical results were seen using a TAP-tagged Herc5 expression vector (data not shown). Only the E6AP-specific siRNA affected the level of the endogenous E6AP HECT E3.

The 293 cell transfection system was used to analyze the effect of two additional Herc5 mutants. Herc5 deleted of the first 381 amino acids, which includes all of the RCC1 repeats, did not support robust ISG15 conjugation, although some stimulation of high molecular weight conjugates was still detected (Fig. 3C, compare lanes 5 and 6). This indicates that the RCC1 repeats are important for overall ISG15 conjugation but that they are perhaps not required for modification of all target proteins.

While the HECT domains of Herc5 and Herc6 represent the most conserved region of the two proteins (67% identity), they differ in the position of a conserved phenylalanine residue found at the fourth position from the carboxyl terminus of most HECT E3s (−4F). This residue was previously shown to play a role in promoting ubiquitin conjugation (19). In Herc5, this phenylalanine is at the second from last position (−2F), while in Herc6 it is at the ninth from last position (−9F). We tested whether this difference might be related to the ability of Herc5 to function in the ISG15 pathway by replacing the last residue of Herc5 (FG) with the last eight residues of Herc6 (FVSPMLTG5). Fig. 3C shows that this mutant (C5–6; lane 7) stimulated ISG15 conjugation as efficiently as wild-type Herc5. The divergent carboxyl-terminal tails are therefore not the basis for the functional difference between Herc5 and Herc6 in the ISG15 system.

UbCH8 is the only E2 enzyme known to function in ISG15 conjugation (8), and as expected, Herc5-dependent stimulation of ISG15 conjugation was not evident upon co-transfection with UbCH7 (Fig. 3E, lanes 2 and 5). In addition, a single amino acid mutation at residue 62 of UbCH8 (F62A) abrogated Herc5-dependent ISG15 conjugation. F62 of UbCH8 represents a conserved residue among E2 proteins that have been shown to interact with HECT E3s (e.g. UbCH5 isoforms, UbCH6, UbCH7, UbCH8), and both biochemical and structural experiments have indicated that this residue is critical for the ability of E2s to interact with the HECT domain (20, 21). The fact that the F62A mutation of UbCH8 abrogated Herc5-dependent ISG15 conjugation is consistent with a model in which UbCH8 directly interacts with Herc5 in mediating ISG15 conjugation.

DISCUSSION

The results presented here show that the Herc5 HECT E3 enzyme is required for conjugation of ISG15 to a broad range, and potentially the complete range, of natural ISG15 target proteins. The coordinate induction by IFN-β of ISG15, UbE1L, UbCH8, and Herc5 is also consistent with a central role for Herc5 in the conjugation system. The most straightforward model for the involvement of Herc5 in the ISG15 system is that it participates directly as an ISG15 ligase, as part of an ISG15 transshiolation cascade from UbE1L to UbCH8 to Herc5, with Herc5 catalyzing ISG15-target protein conjugation. This model is supported by our proteomic analysis of ISG15-conjugated proteins, where UbE1L, UbCH8, and Herc5 were all identified as ISG15-conjugated proteins (6). In addition, a HECT E3 enzyme that can interact with UbCH8 (Saccharomyces cerevisiae Rsp5) was shown to form an ISG15 thioester intermediate and catalyze ISG15 conjugation in vitro (8). This indicates that there are no inherent structural impediments that prevent at least some HECT E3s from catalyzing ISG15 conjugation.

One approach to prove that Herc5 participates directly as an ISG15 ligase would be to establish an in vitro system in which Herc5 forms an ISG15 thioester and conjugates ISG15 to a natural ISG15 target protein. However, we have not yet succeeded in preparing purified recombinant Herc5 protein or protein fragments (e.g. the HECT domain alone) that can be activated with either ubiquitin or ISG15 in vitro. In the absence of such an in vitro system, we cannot rule out alternative models for the role of Herc5 in ISG15 conjugation. For example, it is conceivable that Herc5 might catalyze a ubiquitination event that is a prerequisite for ISG15 conjugation. However, siRNA-mediated depletion of the E1Ub enzyme did not result in any decrease in Herc5-dependent ISG15 conjugation activity in non-IFN-treated cells (data not shown). Regardless of the mechanism, the results presented here demonstrate that Herc5 plays a central and necessary role in ISG15 conjugation in vivo.

A proteomics-based approach identified 158 ISG15 target proteins at a very high confidence level (6). If Herc5 acts directly as an ISG15 E3 enzyme, this would imply that this single E3 has the capacity to recognize, either directly or indirectly, a large number of target proteins. SUMO conjugation utilizes a relatively small number of E3s (two major PIAS family proteins in S. cerevisiae); however, in this case a small consensus site on target proteins is often a determinant for recognition by the SUMO conjugation machinery (22). Analysis of target proteins identified to date has not revealed a motif common to target proteins that might be directly recognized by Herc5. An alternative is that additional cellular proteins or factors might mediate the interaction of Herc5 with individual target proteins or subsets of target proteins. Our previous demonstration that at least some HECT E3s have the capacity to catalyze ISG15 conjugation in vitro (8) raises the question of what might limit the participation of HECT E3s other than Herc5 from participating in the ISG15 conjugation system. The involvement of accessory or "licensing" factors might explain how other HECT E3s are prevented from catalyzing ISG15 conjugation.

Of the six human Herc proteins (Herc1–6), Herc6 is the most similar to Herc5 (50% identical), and its expression is also induced by IFN-β. While we did not detect an effect of Herc6 siRNAs on overall ISG15 conjugation, it is possible that Herc6 plays a minor role in ISG15 conjugation, perhaps targeting a limited set of proteins compared with Herc5. Further biochemical comparisons of Herc5 and Herc6 will be important for identifying the determinants of Herc5 that confer its dominant function in the ISG15 system.

While several mammalian species appear to have direct Herc5 homologs (primates, cow, dog), rodents (mice, rats) do not. It was speculated that Herc5 is evolutionarily the most recent Herc family member and resulted from duplication of the physically adjacent Herc6 gene (15). It is conceivable that Herc6 plays a major role in ISG15 conjugation in rodents. In addition to Herc5 and Herc6, our microarray analysis identified several TRIM (tripartite motif) proteins that were induced by IFN-β, some of which have been shown to function as Ub E3s (23, 24) and at least one of which (TRIM25/Efp) has been reported to interact with UbCH8 (23). siRNAs that targeted two of these proteins (TRIM22 and TRIM25/Efp) had no effect on the overall pattern or accumulation of ISG15 conjugates nor did transfection of TRIM22 or TRIM25 expression vectors boost or alter the overall pattern of ISG15 conjugates (data not shown). We conclude that these ligases do not play a broad role in ISG15 conjugation or, alternatively, function downstream of Herc5.

The demonstration that Herc5 is required for ISG15 conjugation to a
broad spectrum of target proteins in human cells will facilitate analysis of effects of ISG15 conjugation on target proteins and elucidation of the role of ISG15 conjugation in anti-viral and anti-microbial responses.

Acknowledgment—We thank Chen Zhao for critical reading of the manuscript and helpful discussions.

REFERENCES

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J. Biol. Chem. 2006, 281:4334-4338. doi: 10.1074/jbc.M512830200 originally published online December 28, 2005

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