Different Roles for Two Ubiquitin-like Domains of ISG15 in Protein Modification

Received for publication, January 8, 2008, and in revised form, February 20, 2008 Published, JBC Papers in Press, March 20, 2008, DOI 10.1074/jbc.M800162200

Yong-Gang Chang‡‡, Xian-Zhong Yan*, Yuan-Yuan Xie‡§, Xue-Chao Gao‡§, Ai-Xin Song‡, Dong-Er Zhang‖, and Hong-Yu Hu‡‡‡

From the ‡State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China, the §NMR Laboratory, National Center of Biomedical Analysis, Beijing 100850, China, the †Graduate School of the Chinese Academy of Sciences, Beijing 100039, China, and the ‡Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

ISG15 (interferon-stimulated gene 15) is a novel ubiquitin-like (UbL) modifier with two UbL domains in its architecture. We investigated different roles for the two UbL domains in protein modification by ISG15 (ISGylation) and the impact of Influenza B virus NS1 protein (NS1B) on regulation of the pathway. The results show that, although the C-terminal domain is sufficient to link ISG15 to UBE1L and UbcH8, the N-terminal domain is dispensable in the activation and transthiolation steps but required for efficient E3-mediated transfer of ISG15 from UbcH8 to its substrates. NS1B specifically binds to the N-terminal domain of ISG15 but does not affect ISG15 linkage via a thioester bond to its activating and conjugating enzymes. However, it does inhibit the formation of cellular ISG15 conjugates upon interferon treatment. We propose that the N-terminal UbL domain of ISG15 mainly functions in the ligation step and NS1B inhibits ISGylation by competing with E3 ligases for binding to the N-terminal domain.

ISG15 (interferon-stimulated gene 15) is highly induced and conjugated to a large number of cellular proteins upon Type I interferon (IFN) treatment (1, 2). ISG15 protein belongs to the family of ubiquitin-like (UbL) modifiers whose members are capable of forming conjugates to cellular proteins (3). Similar to the ubiquitination pathway, the ISG15 modification or ISGylation process involves the concerted action of a set of enzymes: the activating (UBE1L), conjugating (UbcH8), and ligating enzymes (4–8). However, ISG15 is distinct from other members of the UbLs, such as SUMO and NEDD8 that contain single UbL domain, in that it possesses two tandem UbL domains. Another two-UbL domain-containing protein, FAT10, can modify a very limited number of as-yet-unidentified proteins and has been associated with apoptosis (9, 10). In contrast, ISG15 in response to Type I IFN stimulation is potent in modifying a wide array of cellular proteins that have such diverse roles as RNA splicing, antiviral ability, cytoskeleton regulation, and signal transduction (7, 11, 12). Recently, ISG15 has been shown to be a critical component in IFN-mediated inhibition of human immunodeficiency virus, type 1 release (13). Furthermore, it has also been demonstrated to be a critical antiviral molecule against influenza, herpes, and Sindbis viruses (14, 15). Given that ISG15 possesses the antiviral property, it is not surprising that the Influenza B virus has developed a strategy to block the ISG15 modification of cellular proteins through its nonstructural protein NS1 (NS1B) (4).

To explore the respective roles of the two UbL domains of ISG15, we carried out a series of biochemical experiments to study their specific functions in ISGylation pathway as well as to elucidate the molecular mechanism by which NS1B inhibits ISG15 conjugation to cellular proteins.

EXPERIMENTAL PROCEDURES

Generation of Expression Constructs and Protein Purification—The encoding sequences for UBE1L, NS1B-1–103, NS1B-1–90, NS1B-91–103, ISG15, and its two separate domains, ISG15N (residues 1–80) and ISG15C (82–157), were amplified via PCR and cloned in frame with GST into the pGEX-4T-3 expression vector using BamHI/XhoI cloning sites. The ISG15 sequence was inserted at the N terminus of ISG15 and then subcloned into pCMV-Tag-3B expression vector. The gene coding for NS1B was cloned into pcDNA3-HA expression vector, whereas those genes encoding...
UBE1L and UbcH8 were inserted into pCMV-Tag-2B expression vector. Human HEK 293T and A549 cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), penicillin, and streptomycin and grown at 37 °C in a humidified atmosphere containing 5% CO₂. HEK-293T cells were transfected with the above vectors by using standard calcium phosphate method. A549 cells were transfected by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Four hours after transfection, the media were replaced with fresh media containing 1000 units/ml IFN-α 2a (Roche Applied Science), and the cells were cultured for another 48 h in the presence of IFN-α 2a (1000 units/ml). Forty-eight hours later, the cells were harvested and lysed in the lysis buffer. Western blotting was carried out by detection with anti-His (Invitrogen), anti-FLAG (Sigma), or anti-Myc (Cell Signaling) antibody when needed. The antibodies against endogenous ISG15 and actin were from Santa Cruz Biotechnology (Santa Cruz).

Analytical Gel Filtration Chromatography and GST Pull-down Assay—Protein samples were applied to an analytical Superose-12 HR column for chromatographic analysis. For characterizing the complex formation, NS1B-1–103 was mixed with full-length ISG15 or each of its two domains for 2 h before being loaded onto the above column. The elution volume of each fraction in the profile was obtained by recording the absorbance (A280) from the fast protein liquid chromatography facility. Purified GST or GST fusion proteins were incubated with 20 μl of glutathione-Sepharose 4B beads in an phosphate buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH7.3) for 1 h. The beads were washed three times with the incubation buffer and then incubated for 3 h at 4 °C with the proteins to be tested. The beads were collected by centrifugation and washed three times with the incubation buffer. The samples were made by adding loading buffer into the Eppendorf tube harboring the beads and then subjected to SDS-PAGE, followed by Coomassie staining or Western blotting.

NMR Spectroscopy—15N/13C-Labeled FLAG-ISG15 (~1 mm) was dissolved in a buffer containing 20 mM phosphate, 50 mM NaCl, pH 6.8. All of the heteronuclear spectra were recorded at 25 °C on a Varian INOVA 600 spectrometer. Sequence-specific assignments for FLAG-ISG15 were performed by analyzing two-dimensional heteronuclear single quantum coherence (HSQC) spectra and a set of three-dimensional spectra including HNCACB, CBCA(CO)NH, HNCO, C(CO)NH, and H(CO)NH. For NMR titration experiments, 15N-labeled FLAG-ISG15, FLAG-ISG15N, or FLAG-ISG15C at the molar concentration of 0.2 mM, was used to be titrated with NS1B and its fragments, and then the [3H,15N]HSQC spectra were acquired to monitor the peak intensity changes upon titration.

In Vitro Conjugation Assay—ISG15 conjugation mixture (50 μl) was incubated at 25 °C for 10 min in a buffer containing 25 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 5 mM ATP, and 0.1 mM dithiothreitol. The reactions were stopped by the addition of loading buffer with or without dithiothreitol, and the reaction products were then subjected to SDS-PAGE followed by Western blotting and visualized with ECL development reagent (Pierce).

RESULTS

The C-terminal Domain of ISG15 Is Sufficient to Link to UBE1L and UbcH8 via Thiol Esterification—ISG15 comprises two UbL domains with the N- and C-terminal domains sharing 33 and 32% similarities with ubiquitin, respectively (Fig. 1A) (3, 16). Similar to ubiquitin, full-length ISG15 can form thioester bond with the activating enzyme UBE1L and conjugating enzyme UbcH8 (5, 17). The C-terminal domain contains a classical conjugating motif LRLRGG (Fig. 1A). To understand the thiol esterification properties of the C-terminal domain of ISG15 (ISG15C), we examined the activity of ISG15C during the first two steps of ISGylation, activation and transthiolelation. In vitro experiments were carried out at 25 °C with purified proteins GST-UBE1L, UbcH8, and FLAG-ISG15 or FLAG-ISG15C, and 10 min later the reaction products were detected by Western blotting (see “Experimental Procedures”). Like full-length ISG15, ISG15C can form intermediate adducts with UBE1L and UbcH8, respectively (Fig. 1B, lanes 6 and 8). These products are linked by thioester bonds, as indicated by their disappearance upon the addition of dithiothreitol in the loading buffer to stop the reactions (Fig. 1B, lanes 5 and 7). Furthermore, the amounts of the intermediate adducts of ISG15C with UBE1L and UbcH8 are apparently the same as those of UBE1L~ISG15 and UbcH8~ISG15, suggesting that ISG15C, similar to full-length ISG15, is capable of being activated and transthiolelated by UBE1L and UbcH8 enzymes sequentially (Fig. 1B, lanes 4 and 8). Therefore, the C-terminal domain of ISG15 is sufficient to link via thioester bonds to the activating and conjugating enzymes, whereas the N-terminal UbL domain does not appear essential for activation and transthiolelation in protein ISGylation.

Both the N- and C-terminal Domains of ISG15 Are Required for Its Efficient Conjugation to Cellular Proteins—Because the N-terminal domain is dispensable in the formation of intermediate thioester adducts of ISG15 with UBE1L and UbcH8, we investigated whether it influences the ligation process, the third step of ISGylation. Because the expression of pCMV-Tag-3B-ISG15C is very low as compared with that of the ISG15 construct in HEK 293T cells, we generated two eukaryotic constructs pCMV-Tag-3B-GB1-ISG15 and pCMV-Tag-3B-GB1-ISG15C, which express similarly high level of GB1 fusion proteins Myc-GB1-ISG15 and Myc-GB1-ISG15C, respectively (18). The two constructs were transfected into 293T cells with or without pCMV-Tag-2B-UBE1L and pCMV-Tag-2B-UbcH8 that express the activating enzyme UBE1L and the conjugating enzyme UbcH8, respectively. After 48 h, total cells were lysed and subjected to SDS-PAGE, followed by Western blotting with anti-Myc antibody. ISG15 is capable of being conjugated to a large number of cellular proteins with the assistance of endogenous E3 enzymes, as shown, a smear band dominantly around the molecular mass of 250 kDa (Fig. 1C). As a comparison, ISG15C can also be conjugated to cellular proteins, but the total level of ISG15C conjugates is much lower than that with the full-length ISG15, suggesting that deletion of the N-terminal domain has a great impact on ISG15 conjugation to cellular proteins. Therefore, we infer that both domains of ISG15 are required for efficient conjugation to cellular pro-
teins, although the C-terminal domain of ISG15 possesses the conjugating motif LRLRGG that has been demonstrated to be essential for ubiquitination (19–21). Thus, we propose that although the C-terminal domain of ISG15 is sufficient for ISG15 linkage to UBE1L and UbcH8 via thiol esterification, both domains are required for modification of substrates by attaching ISG15 to the ε-amino groups of lysine residues.

NS1B Inhibits ISG15 Conjugation to Cellular Proteins and Specifically Binds to the N-terminal UbL Domain of ISG15—To define the roles of these respective UbL domains of ISG15, we examined whether NS1B directly interacts with ISG15 protein and how NS1B inhibits the ISGylation level in cultured cells. We generated a eukaryotic expression construct pcDNA3-HA-NS1B, which produces an NS1B protein with an HA epitope at its N terminus, and then transfected it into A549 cells by using Lipofectamine 2000 transfection reagents, followed by 48 h of treatment with IFN-α/β. As shown in Fig. 2A, the cellular level of ISG15 conjugates, detected by anti-ISG15 antibody, is markedly reduced by expression of NS1B as compared with that in mock-treated cells. This result demonstrates that NS1B protein alone is capable of inhibiting the ISG15 conjugation to cellular target proteins.

It was previously postulated that the direct interaction between NS1B and ISG15 blocks the activation of ISG15 by UBE1L and thus ISG15 cannot be recognized by its activating enzyme UBE1L (4). Our above results prompt us to examine whether NS1B binds specifically to the C-terminal domain of ISG15. To this end, we carried out a GST pull-down assay with GST fusion of either full-length ISG15 or each of its two domains. Unexpectedly, ISG15N can pull down NS1B as efficiently as the full-length ISG15, whereas ISG15C cannot (Fig. 2B, lanes 7–9). This observation is further verified by Western blotting with anti-His antibody for detecting ISG15 conjugates but has no effect on thiol esterification of C-terminal domain to UBE1L and UbcH8. A, a schematic representation of the two-UbL domain architecture of ISG15. The two fragments corresponding to the N-terminal and C-terminal domains of ISG15 are designated ISG15N and ISG15C, respectively. Ubiquitin and the C-terminal LRLRGG motif are also highlighted. B, FLAG-ISG15 or FLAG-ISG15C (2 μM) was incubated in a reaction system of 50 μl of solution with GST-UBE1L (0.2 μM) or GST-UBE1L and UbcH8 (2 μM) for 10 min at 25 °C as indicated. The reactions were stopped by adding loading buffer with or without dithiothreitol (DTT). The reaction products were subjected to 15% SDS-PAGE, followed by immunoblotting with anti-FLAG. C, HEK 293T cells were transfected with pCMV-Tag-3B-GB1-ISG15 (lanes 1 and 2) or pCMV-Tag-3B-GB1-ISG15C (lanes 3 and 4) in the presence or absence of pCMV-Tag-2B-UBE1L and pCMV-Tag-2B-UbcH8. After 48 h, the total lysates were harvested and separated in 12% SDS-PAGE, followed by immunoblotting with anti-Myc for detecting ISG15, ISG15C, and their conjugates and with anti-FLAG for UBE1L and UbcH8. The open arrows indicate the nonspecific bands detected by anti-Myc.
NS1B, which contains a C-terminal His$_6$ tag. Taken together, these results clearly demonstrate that NS1B specifically binds to the N-terminal domain of ISG15, suggesting that, besides the C-terminal domain, the N-terminal domain may play a role in promoting E3-mediated transfer of ISG15 to cellular targets in regulating ISG15 modification.

We next corroborated that ISG15 binds with the N-terminal fragment (residues 1–103) of NS1B (supplemental Fig. S1). Then we applied NS1B-1–103 to examine its interaction with the two UbL domains of ISG15. The result shows that both full-length ISG15 and ISG15N can efficiently pull down similar amounts of NS1B-1–103, whereas ISG15C cannot (Fig. 2C, lanes 7–9). Compared with the strong bands for NS1B-1–103 pulled down by ISG15 and ISG15N, the very weak band for NS1B-1–103 by ISG15C pulldown is probably due to the nonspecific adsorption of the NS1B-1–103 protein by the beads. Furthermore, we carried out a gel filtration analysis of the interaction between NS1B-1–103 and either ISG15N or ISG15C. The elution peak for the mixture of NS1B-1–103 and ISG15N shifts toward the higher molecular mass end of the profile (Fig. 2D, left panel), as compared with those for ISG15N and NS1B-1–103 alone, indicating formation of the complex of NS1B-1–103 and ISG15N. In contrast, NS1B-1–103 and ISG15C do not co-elute, suggesting that they do not form a complex (Fig. 2D, right panel). These results demonstrate that NS1B specifically binds to the N-terminal domain of ISG15 through its integrated N-terminal part (residues 1–103), including the three-α-helix domain and the extended region (supplemental Fig. S1).

NS1B-1–103 binds specifically to the N-terminal domain of ISG15: NMR evidence-To directly observe the interaction between NS1B-1–103 with ISG15N in the context of full-length ISG15, we carried out NMR experiments for $^{15}$N-labeled FLAG-ISG15 titrated with GB1-NS1B-1–103. The GB1 fusion protein was applied for the purpose of enhancing the solubility of NS1B-1–103 (22). The sequence-specific assignment for 95% backbone resonances of the amino acid residues of ISG15 was achieved by analyzing a set of two-dimensional and three-dimensional NMR spectra (supplemental Fig. S2). It is worth noting that the FLAG tagging of ISG15 made it possible to acquire NMR data on ISG15 by dramatically enhancing its stability at room temperature.

As shown in Fig. 3, with the increasing amount of GB1-NS1B-1–103 at a molar ratio 1:1 (Fig. 3B) and 2:1 (Fig. 3C) of NS1B-1–103 to ISG15, the intensities (as indicated by peak heights) of the $^1$H-$^{15}$N cross peaks are gradually reduced, indicating the binding of NS1B-1–103 to ISG15. To analyze the peak intensity changes of ISG15 upon titration with GB1-NS1B-1–103, the peak intensity of each amide of free ISG15 is normalized as 1, and the ratios of peak intensities at each titration step to those with free ISG15 are plotted against the residue number of ISG15 (Fig. 3, A–C, left panels). These data show that the N-terminal domain of ISG15 exhibits a more intensive reduction in peak intensities than the C-terminal domain does.
The mean peak intensities are 0.26 and 0.38 for ISG15N and ISG15C, respectively, at the 1:1 molar ratio of GB1-NS1B-1–103 and FLAG-ISG15, and the mean peak intensities are 0.09 and 0.25 at the 2:1 ratio. To verify the specific binding, we labeled these two UbL domains, FLAG-ISG15N and FLAG-ISG15C, and performed titration with GB1-NS1B-1–103. The spectra show that FLAG-ISG15N exhibits a reduction in peak intensities upon the addition of NS1B-1–103 (Fig. 3D), whereas FLAG-ISG15C exhibits no significant change in peak intensities (Fig. 3E), suggesting that NS1B specifically binds to the N-terminal domain of ISG15 but not to the C-terminal domain. The tumbling rate of the NS1B-ISG15 complex that is slower than free ISG15 may also contribute to the peak broadening. Taken together, we conclude that NS1B-1–103 specifically binds to the N-terminal UbL domain of ISG15 in the context of full-length ISG15.

NS1B-1–103 Binds to the N-terminal Domain of ISG15 with a 1:1 Stoichiometry—To further understand the mechanism by which NS1B inhibits the conjugation of ISG15 to cellular proteins, it is necessary to clarify the form of the ISG15-NS1B complex in solution, because the crystal structure of NS1B-1–103 (Protein Data Bank code 1XEQ) shows a dimeric form (23). We performed a gel filtration analysis to characterize NS1B-1–103 and its complex with full-length ISG15. Fig. 4A shows that the elution peaks of the mixture of NS1B-1–103 and ISG15 shift toward the higher molecular mass end of the profile, indicating formation of the complex between NS1B-1–103 (12 kDa) and ISG15 (17 kDa). The constituents of the NS1B-1–103 and ISG15 complex show two bands each for NS1B-1–103 and ISG15, in the SDS-PAGE gel (15%) with Coomassie staining (Fig. 4B, lane 3). To indicate the relative molecular mass of NS1B-1–103 and its complex with ISG15, we chose cytochrome c (13 kDa), UCH-L1 (24 kDa), and GST (55 kDa, dimeric form) as protein markers. The elution volume for NS1B-1–103 is very similar to that for cytochrome c but far from that for UCH-L1, indicating that it exists in a monomeric form in solution (Fig. 4C). Furthermore, the mixture of NS1B-1–103 and ISG15 elutes at a volume similar to that for UCH-L1, suggesting that they form a complex of 1:1 stoichiometry with apparent molecular mass of about 29 kDa (Fig. 4D). With a complex of NS1B-1–103 and ISG15 with a 2:1 or 2:2 manner, the apparent molecular masses would be 41
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and 53 kDa, respectively, but in the elution profile (Fig. 4D), there exist no such peaks. Thus, we conclude that the complex of NS1B-1–103 and ISG15 is formed by a 1:1 stoichiometry.

NS1B Does Not Inhibit Thiol Esterification of ISG15 toward UBE1L and UbcH8—Although NS1B specifically binds to the N-terminal domain of ISG15 and the N-terminal deletion of ISG15 has no effect on its linkage to UBE1L and UbcH8, it remains uncertain whether NS1B binding to the N-terminal domain affects the thiol esterification of the C-terminal domain toward UBE1L and UbcH8. We carried out in vitro competition assays with ISG15, ISG15C, and either UBE1L or UBE1L and UbcH8 in the presence or absence of NS1B-1–103, NS1B-1–145, and full-length NS1B. Increasing the amount of either NS1B or its two fragments has no significant effect on the amounts of the intermediate adducts between UBE1L and ISG15 (Fig. 5, A and C). The amounts of UBE1L~ISG15C, of which the modifier ISG15C has been demonstrated not to interact with NS1B, is not affected by the addition of NS1B and its two fragments (Fig. 5C, lanes 5–8). Similarly, formation of UbcH8~ISG15 adducts is not significantly influenced by NS1B and its two fragments, either (Fig. 5, B and D). Also, the amounts of UbcH8~ISG15C adducts apparently remain the same in the presence or absence of NS1B (Fig. 5D, lanes 5–8).

Thus far, it remains possible that the UbcH8~ISG15 adducts may form at a higher rate in the absence of NS1B than in the presence of NS1B, but the reaction without NS1B can reach the same end point more rapidly than the reaction with NS1B. To exclude this possibility, we performed a time course analysis on the reaction of ISG15 and UbcH8. Fig. 5E shows that during the time course of 0–15 min, the amount of UbcH8~ISG15 adducts steadily increases, indicating that the reaction has not yet reached its end point after a 10-min interval of the reaction. Thus, we conclude that although NS1B interacts with ISG15, it is not likely to inhibit thiol esterification of ISG15 toward UBE1L and UbcH8.

DISCUSSION

We have demonstrated that both UbL domains of ISG15 are required for its efficient conjugation to cellular proteins (Fig. 1C), although the N-terminal domain may not participate in the first two steps of protein ISGylation, that is, ISG15 activation by the activating enzyme UBE1L and ISG15 linkage via a thioester bond to the conjugating enzyme UbcH8. It appears that the N-terminal part plays a regulatory role in enhancing E3-mediated transfer of ISG15 from UbcH8 to ISG15 substrates. In addition, by performing NMR titration and GST pulldown assay, we observed that there is no direct physical interaction between ISG15 and UbcH8 (supplemental Fig. S3B) or several of its substrates including Hsc70, coflin, and destrin (data not shown). Thus, we presume that the regulatory role of the N-terminal UbL domain of ISG15 is somehow associated with ISG15 E3 ligases. The activities of the E3 ligases can be modulated in a variety of ways, such as phosphorylation, modification by ubiquitin-like proteins, binding of inhibitory proteins, and regulation by adaptor proteins that bring together E3s and E2s or substrates (24–30). Therefore, the N-terminal domain of ISG15 represents a distinct way to regulate the conjugation of ISG15 to its substrates probably by enhancing the activities of E3 ligases through specifically interacting with the enzymes or the substrates.

So far, only a limited number of ISG15 E3 ligases, including HERC5, HHARI, and Efp, have been identified (6–8). Of note is that ISG15 has been shown to interact with HERC5, a HECT-type ISG15 E3 ligase that has been demonstrated by two research groups to be required for ISG15 conjugation to a broad spectrum of cellular targets (7, 8). Whether ISG15 interacts with Efp remains to be examined, and other ISG15 E3 ligases are to be identified in the future. Nevertheless, comparing the effect of the N-terminal deletion of ISG15 on the total level of cellular ISG15 conjugates with that caused by small interfering RNA knock-down of HERC5 suggests that it is possible that the N-terminal domain of ISG15 acts in conjunction with HERC5 to ensure the efficient modification of cellular proteins by ISG15. An in vitro reconstituted system is a prerequisite to determine whether HERC5 or other ISG15 E3s could act in such a way as to promote protein ISGylation. However, as noted
by the two groups who identified HERC5 as an ISG15 ligase, the full-length HERC5 expression has not been successful in the bacterial system, although the HECT domain (residues 700–1024) of HERC5 has been expressed in E. coli and purified in our laboratory.4

We have also demonstrated that NS1B alone inhibits in vivo ISG15 conjugation to cellular proteins (Fig. 2A). A set of assays including GST pulldown, gel filtration, and NMR titration have clearly demonstrated that NS1B binds specifically to the N-terminal UbL domain of ISG15 but not to the C-terminal domain and that both the three-α-helix domain and the extended region are required for NS1B binding to the N-terminal domain of ISG15. Yuan and Krug (4) proposed by in vitro GST pulldown with radioactive detection that NS1B blocks protein ISGylation by inhibiting the activation step through interacting with ISG15. Our observation indicates that NS1B does not influence the amount of ISG15 being attached to UBE1L and UbcH8 but does impede ligation of ISG15 to its substrates. This proposal is supported by several lines of evidence that (1) the C-terminal part of ISG15 possesses a similar ability of thiol esterification in the first two steps of protein ISGylation as does the full-length ISG15 (Fig. 1B); (2) deletion of the N-terminal domain significantly reduces the ISG15 conjugates (Fig. 1C); and (3) NS1B specifically binds to the N-terminal UbL domain of ISG15 but not to the C-terminal part (Figs. 2 and 3).

Considering the finding that the C-terminal domain of ISG15 functions in the conjugation to substrates as ubiquitin does in ubiquitination, whereas the N-terminal domain may play a role in the ligation step, we herein propose a putative model for the mechanism of ISG15 conjugation to cellular proteins (Fig. 6). Suppose that an E3 ligase associates with UbcH8 via its adducted ISG15 to promote ligation of ISG15 to the cellular substrates, and NS1B targets the N-terminal domain of ISG15 by competing with the E3 ligase to impede the interaction between UbcH8–ISG15 and the enzyme. Thus, NS1B inhibits ISGylation probably by blocking the E3-mediated transfer of ISG15 from UbcH8 to the substrates. Compared

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4 Y.-G. Chang, X.-Z. Yan, Y.-Y. Xie, X.-C. Gao, A.-X. Song, D.-E. Zhang, and H.-Y. Hu, unpublished data.
with the C-terminal conjugating domain of ISG15 that functions in covalently bonding with E1, E2, E3 (HECT-type), or substrates by its C-terminal LRLRGG motif, the N-terminal regulatory domain (regulatory domain) of ISG15 may play an important regulatory role of promoting the transfer ability of ISG15 E3 ligases in protein ISGylation.

Acknowledgments—We thank Dr. C.-Y. Cao and Y.-H. Zhang for technical help and valuable discussions.

REFERENCES

1. Haas, A. L., Ahrens, P., Bright, P. M., and Ankel, H. (1987) J. Biol. Chem. 262, 11315–11323
2. Knight, E. Jr., Fahey, D., Cordova, B., Hillman, M., Kutny, R., Reich, N., and Blomstrom, D. (1988) J. Biol. Chem. 263, 4520–4522
3. Dao, C. T., and Zhang, D. E. (2005) Front. Biosci. 10, 2701–2722
4. Yuan, W., and Krug, R. M. (2001) EMBO J. 20, 362–371
5. Zhao, C., Beaudenon, S. L., Kelley, M. L., Waddell, M. B., Yuan, W., Schulman, B. A., Huibregtse, J. M., and Krug, R. M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7578–7582
6. Zou, W., and Zhang, D. E. (2006) J. Biol. Chem. 281, 3989–3994
7. Wong, J. J., Pung, Y. F., Sze, N. S., and Chin, K. C. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 10735–10740
8. Dastur, A., Beaudenon, S., Kelley, M., Krug, R. M., and Huibregtse, J. M. (2006) J. Biol. Chem. 281, 4334–4338
9. Raasi, S., Schmidtke, G., and Groettrup, M. (2001) J. Biol. Chem. 276, 35334–35343
10. Feng, X., Wu, H., Grossman, J. M., Hanvivadhakul, P., FitzGerald, J. D., Park, G. S., Dong, X., Chen, W., Kim, M. H., Weng, H. H., Furst, D. E., Gorn, A., McMahon, M., Taylor, M., Brahn, E., Hahn, B. H., and Tsao, B. P. (2006) Arthritis Rheum. 54, 2951–2962
11. Zhao, C., Denison, C., Huibregtse, J. M., Gyg, S., and Krug, R. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10200–10205
12. Giannakopoulos, N. V., Luo, J. K., Papov, V., Zou, W., Lenschow, D. J., Jacobs, B. S., Borden, E. C., Li, J., Virgin, H. W., and Zhang, D. E. (2005) Biochem. Biophys. Res. Commun. 336, 496–506
13. Okumura, A., Lu, G., Pitta-Rowe, I., and Pitta, P. M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 1440–1445
14. Lenschow, D. J., Giannakopoulos, N. V., Gunn, L. J., Johnston, C., O’Guin, A. K., Schmidt, R. E., Levine, B., and Virgin, H. W. T. (2005) J. Virol. 79, 13974–13983
15. Lenschow, D. J., Lai, C., Frias-Staheli, N., Giannakopoulos, N. V., Lutz, A., Wolf, T., Osiak, A., Levine, B., Schmidt, R. E., Garcia-Sastre, A., Leib, D. A., Pekos, A., Knobeloch, K. P., Horak, I., and Virgin, H. W. T. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 1371–1376
16. Narasimhan, J., Wang, M., Fu, Z., Klein, J. M., Haas, A. L., and Kim, J. J. (2005) J. Biol. Chem. 280, 27356–27365
17. Kim, K. I., Giannakopoulos, N. V., Virgin, H. W., and Zhang, D. E. (2004) Mol. Cell. Biol. 24, 9592–9600
18. Bao, W. J., Gao, Y. G., Chang, Y. G., Zhang, T. Y., Lin, Y. J., Yan, X. Z., and Hu, H. Y. (2006) Protein Expr. Purif. 47, 599–606
19. Loeb, K. R., and Haas, A. L. (1992) J. Biol. Chem. 267, 7806–7813
20. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Annu. Rev. Cell Dev. Biol. 22, 159–180
21. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
22. Chang, Y. G., Song, A. X., Gao, Y. G., Shi, Y. H., Lin, X. J., Cao, X. T., Lin, D. H., and Hu, H. Y. (2006) Protein Sci. 15, 1248–1259
23. Yin, C., Khan, I. A., Swapna, G. V., Ertekii, A., Krug, R. M., Tong, L., and Montelon, G. T. (2007) J. Biol. Chem. 282, 20584–20592
24. Gallagher, E., Gao, M., Liu, Y. C., and Karin, M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 1717–1722
25. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
26. Buschmann, T., Fuchs, S. Y., Lee, C. G., Pan, Z. Q., and Ronai, Z. (2000) Cell 101, 753–762
27. Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E. A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., and Kato, S. (2002) FEBS Lett. 580, 4521–4526
28. Ogunjimi, A. A., Briant, D. J., Pece-Barbara, N., Le Roy, C., Di Giugliemo, G. M., Kavsak, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., and Wrana, J. L. (2005) Mol. Cell 19, 297–308
29. Zheng, J., Yang, X., Harrell, J. M., Ryzhikov, S., Shim, E. H., Lykke-Andersen, K., Wei, N., Sun, H., Kobayashi, R., and Zhang, H. (2002) Mol. Cell 10, 1519–1526