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The Basis for Selective E1-E2 Interactions in the ISG15 Conjugation System*[S]

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E1 and E2 enzymes coordinate the first steps in conjugation of ubiquitin (Ub) and ubiquitin-like proteins (Ubls). ISG15 is an interferon-α/β-induced Ubl, and the E1 and E2 enzymes for ISG15 conjugation are Ube1L and UbcH8, respectively. UbcH7 is the most closely related E2 to UbcH8, yet it does not function in ISG15 conjugation *in vivo*, while both UbcH7 and UbcH8 have been reported to function in Ub conjugation. Kinetic analyses of wild-type and chimeric E2s were performed to determine the basis for preferential activation of UbcH8 by Ube1L and to determine whether UbcH8 is activated equally well by Ube1L and E1^{Ub} (Ube1). K_m determinations confirmed the strong preference of Ube1L for UbcH8 over UbcH7 (a 29-fold K_m difference), similar to the preference of E1^{Ub} for UbcH7 over UbcH8 (a 36-fold K_m difference). Thioester assays of chimeric E2s identified two structural elements within residues 1–39 of UbcH8 that play a major role in defining Ube1L-UbcH8 specificity: the α1-helix and the β1-β2 region. The C-terminal ubiquitin fold domain (UFD) of Ube1L was required for transfer of ISG15 to UbcH8 and for binding of Ube1L to UbcH8. Replacement of the Ube1L UFD with that from E1^{Ub} resulted in preferential transfer of ISG15 to UbcH7. Together, these results indicate that Ube1L discriminates between UbcH8 and closely related Ub E2s based on specific interactions between the Ube1L UFD and determinants within the N-terminal region of UbcH8.

Ubiquitin (Ub)[3] and ubiquitin-like proteins (Ubls) are covalently conjugated to proteins through amide bonds formed between their terminal carboxyl groups and, in most cases, ε-amino groups of lysine residues of target proteins. Two groups of enzymes, the E1 and E2 enzymes, are essential for all known Ub/Ubl conjugation pathways. These enzymes function cooperatively in reactions that involve enzyme-bound thioester intermediates (1). E1 enzymes catalyze Ub/Ubl activation by first forming an ATP-dependent Ub/Ubl-adenylate, followed by an enzyme-bound Ub/Ubl-thioester at the active site cysteine of the E1. The activated E1 then transfers the Ub/Ubl to the active site cysteine of specific E2 enzymes in a transthiolation reaction, preserving the Ub-thioester linkage. In some cases, the E2 may directly interact with target proteins (*e.g.* Ubc9 in Sumo conjugation, Ref. 2); however, conjugation of Ub and most Ubls requires E3 activities. E3s function minimally as docking or scaffolding proteins, binding both the activated E2 and a substrate protein, orienting them for reaction of the ε-amino group of a lysine side chain of the target protein with the activated carboxyl group of the Ub/Ubl. In the case of the HECT domain E3s, the E2 transfers Ub to the active site cysteine of the E3, with the E3 directly catalyzing the final transfer to the target protein (3).

The E1, E2, and E3 enzymes for conjugation of Ub and Ubls are generally highly specific for function with either Ub or a single Ubl (4); however, potential overlap of the conjugation pathways for Ub and ISG15 was suggested based on identification of the ISG15 E2 enzyme (5). ISG15 is a 17-kDa Ubl that is rapidly and strongly induced by type-1 interferons (IFN-α/β). Over 150 cellular proteins are modified by ISG15 in IFN-β-treated cells (6, 7). The E1 and E2 enzymes for ISG15 are Ube1L and UbcH8, respectively, and like ISG15, expression of both proteins is induced at the transcriptional level by IFN-α/β (5, 8, 9). Depletion of UbcH8 by siRNAs eliminates virtually all ISG15 conjugation in IFN-β-treated cells, while depletion of the most closely related E2, UbcH7 (55% identity, 72% similarity to UbcH8), had no effect on ISG15 conjugation (5). These results strongly suggest that UbcH8 is the only E2 enzyme for the ISG15 pathway. UbcH8 has been reported in several cases to function in Ub conjugation pathways (10–13), often in a manner that is redundant with UbcH7 (5, 14–20), suggesting that UbcH8 might function in both the Ub and ISG15 conjugation systems. Importantly, given the functional redundancy of E2s in the Ub system, it is difficult to unambiguously demonstrate that UbcH8 functions in Ub conjugation *in vivo*. The fact that UbcH8 expression is transcriptionally regulated by IFN-α/β signaling suggests that there may be insufficient amounts of UbcH8 protein present in most cell types in the absence of interferon to significantly influence Ub conjugation.

Structural and biochemical studies on the Sumo and Nedd8 E1s have revealed the basis for interaction of these enzymes with their cognate E2 enzymes (21, 22). Both of these E1s are heterodimeric enzymes (Sae1/Sae2 for Sumo, AppBp1/Uba3 for Nedd8), with the Sae1 and AppBp1 proteins corresponding

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[3] The abbreviations used are: Ub, ubiquitin; ISG15, interferon-stimulated gene, 15 kDa; GST, glutathione *S*-transferase; HECT, homologous to E6AP C terminus; HA, hemagglutinin; DTT, dithiothreitol; Ubl, ubiquitin-like protein; IFN, interferon; UFD, Ub fold domain; PDB, Protein Data Bank.

to the N-terminal domain of monomeric E1s, and the Sae2 and Uba3 proteins corresponding to the C-terminal domain of monomeric E1s. Interestingly, a domain at the C terminus of the Sae2 and Uba3 proteins adopts a structure that resembles ubiquitin (the Ub fold domain; UFD). The UFD is the primary site for interaction of Sae2 and Uba3 with their cognate E2 enzymes (Ubc9 and Ubc12, respectively). Ube1L and $E1^{Ub}$ are also predicted to contain a C-terminal UFD (23). The core region of Ubc12 that interacts with the UFD is primarily the first α-helix and the $\beta1$-$\beta2$ loop (21), and the analogous regions of Ubc9 were identified by mutagenesis as the $E1^{Sumo}$-interacting domain (24). These results were consistent with earlier work that suggested that the N-terminal regions of Ub E2s were critical for interacting with $E1^{Ub}$ (25, 26).

We initiated the current study to determine the basis for specific Ube1L-UbcH8 interactions in the ISG15 system, and in particular, to identify the features that distinguish UbcH8 from UbcH7 in its ability to be activated by Ube1L. Consistent with the studies described above, two primary determinants within the E2 N-terminal region (the $\alpha1$-helix and $\beta1$-$\beta2$ region) were responsible for the differential interaction of UbcH8 and UbcH7 with Ube1L. The UFD of Ube1L bound specifically to UbcH8 and was essential for transfer of ISG15 to UbcH8. In addition, $E1^{Ub}$ was found to discriminate against activation of UbcH8 to a similar degree as Ube1L discriminated against UbcH7, suggesting that UbcH8 may be limited in its capacity to function in Ub conjugation *in vivo*.

EXPERIMENTAL PROCEDURES

Plasmids and Mutagenesis—Plasmids containing Ube1L, UbcH8, Herc5, and ISG15 were described previously (5, 7, 9, 27). Additional pcDNA3 (Invitrogen)-based ISG15 plasmids were made encoding either the HA (YPYDVPDYA) epitope at the N terminus of ISG15 or cloning ISG15 into the pcMV10 vector, which introduces an N-terminal 3×-FLAG epitope (Sigma). The HA epitope was also added to the N terminus of the pcDNA3-Ube1L and pcDNA3-Ube1LΔUFD plasmids. All E2s (chimeric and wild type), $Ube1L^{UFD}$, Ube1LΔUFD, and Ube1L-UFD^{Ub} expression plasmids were constructed by standard PCR ligation methods using pcDNA3 and pFastBac (Invitrogen) as vectors. Sequences of all constructs were verified by DNA sequencing.

Protein Expression and Purification—Recombinant baculoviruses were generated using the Bac-to-Bac Baculovirus Expression System (Invitrogen) for the following: wild-type Ube1L and all Ube1L derivatives, UbcH7, UbcH8, and all chimeric E2 proteins. All proteins were expressed as GST fusion proteins in High Five insect cells. Insect cells were collected 48–72 h post-infection, and lysed in buffer containing 1% Nonidet P-40, 100 mM Tris, pH 7.9, 100 mM NaCl, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 0.3 μM aprotinin. Proteins were affinity-purified using GST-bind resin (Novagen). Ub and ISG15 were expressed as GST fusion proteins using the pGEX6p-1 vector (GE Healthcare) in *Escherichia coli* strain BL21 with an added cAMP-dependent kinase recognition motif (RRASV). Cells were collected and resuspended in 1× phosphate-buffered saline containing 1% Triton and lysed by sonication. Ub and ISG15 were purified on GST-Bind Resin and resuspended in 50 μl of kinase buffer (40 mM Tris, pH 7.5, and 20 mM MgOAc). The proteins were labeled by adding 2 μl of adenosine 5′- [γ-^{32}P]triphosphate (PerkinElmer Life Sciences) and 2 μl of cAMP-dependent protein kinase (Promega), and the reaction mixtures were rotated at room temperature for 1 h. Unincorporated label was removed by washing the beads in kinase buffer.

GST fusion proteins on beads were subjected to site-specific cleavage with PreScission protease (GE Healthcare) to remove GST. All proteins, with the exception of DEAE-purified $E1^{Ub}$, were subjected to SDS-PAGE followed by staining with Coomassie Blue G250 and quantified relative to bovine serum albumin standards using a near-infrared fluorescence scanner (Odyssey, Li-Cor Biosciences). Ube1L and $E1^{Ub}$ enzymes used in K_m assays were purchased from Boston Biochem. $E1^{Ub}$ used in all other assays was expressed using a recombinant baculovirus in High Five insect cells (Invitrogen) and partially purified on DEAE-Sepharose as described previously (28).

Biochemical Assays—All thioester assays were carried out in reactions containing 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT, and 2.25 μM ^{32}P-labeled ISG15 (~9×10^9 cpm/μmol) or 2.7 μM ^{32}P-labeled ubiquitin (~4×10^9 cpm/μmol). All reactions were initiated with the addition of [^{32}P]Ub/[^{32}P]ISG15, incubated at room temperature, terminated with SDS-PAGE loading buffer lacking DTT, and analyzed by SDS-PAGE and autoradiography or by Bio-Rad Phosphorimager with Quantity One Software. $E1^{Ub}$/Ube1L activity was determined in an end-point assay using [^{32}P]Ub/[^{32}P]ISG15 and minimal amounts of E1. UbcH7/UbcH8 activity was determined in a similar manner using excess $E1^{Ub}$/Ube1L and minimal UbcH7/UbcH8. All concentrations listed are of active enzyme. Except where indicated, all thioester assays used 0.5 μM wild type and chimeric E2s. Assays in Fig. 2 were incubated for 5 or 75 min and contained 0.5 μl DEAE purified $E1^{Ub}$/13.2 nM Ube1L. Assays in Fig. 4 contained 0.5 μl of DEAE-purified $E1^{Ub}$ or 4.8 nM Ube1L and ISG15 samples were incubated 4 min, while Ub samples were incubated for both 1 and 10 min. The reactions in Fig. 5*A* contained 4.4 nM Ube1L or Ube1LΔUFD and were incubated for 10 min, while the reactions in Fig. 5*D* contained 4.4 nM Ube1L/Ube1L-UFD^{Ub} and were incubated for 5 min and 30 min, respectively. For the $Ube1L^{UFD}$ competition assay (Fig. 5*C*), 0.5 μM UbcH8 was incubated with either 0, 1, 2, or 4 μM $Ube1L^{UFD}$ for 3 min. A reaction mix containing 4.4 nM Ube1L, 25 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mM DTT, and 2.25 μM [^{32}P]ISG15 was added to each of the UbcH8 reactions for 4 min before the reaction was terminated. For the K_m and k_{cat} values in Table 1, initial velocity conditions were determined for each E2 so that the E1 concentration and incubation time resulted in linear product formation, where less than 10% of the E2 was converted to E2~Ubl. Preliminary K_m assays using 0.23 nM $E1^{Ub}$/3.4 nM Ube1L, and the proper incubation time were performed to determine the appropriate range of E2 concentrations for each wild-type or chimeric E2 protein. A minimum of three K_m assays were performed and known amounts of [^{32}P]Ub or [^{32}P]ISG15 were included to convert counts to a concentration value. After quantitation using the Bio-Rad Phosphorimager and Quantity One software, kinetic constants

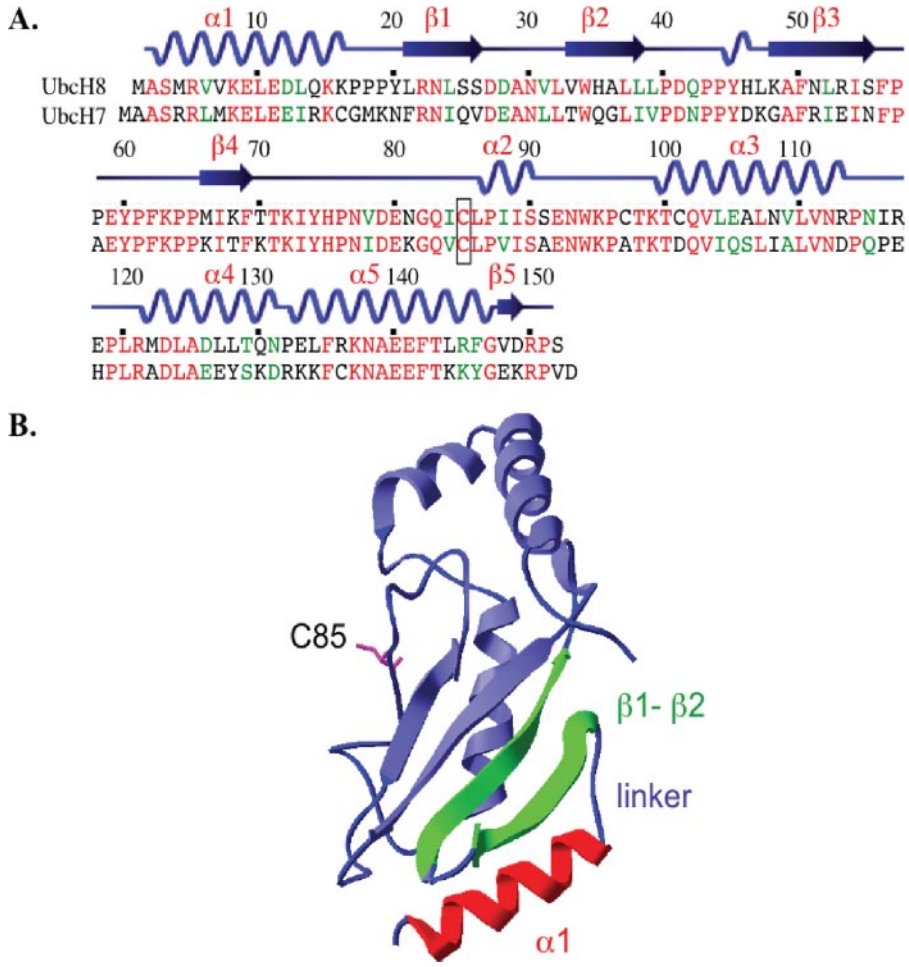

FIGURE 1. *A*, alignment of the UbcH8 and UbcH7 sequences, with secondary structure elements of UbcH8 indicated. Numbering is according to UbcH8 residues. The active site cysteine residues are *boxed*. Residues in *red* represent identical residues, *green* represent similar residues. *B*, structure of UbcH8 (PDB 1WZW, Footnote 4). The α1-helix (*red*) and β1-β2 region (*green*) are indicated, along with the linker connecting these elements and the active site cysteine (C85, *pink*).

were determined using nonlinear regression of Michaelis-Menten plots with Graphpad Prism software. All kinetic constants reported include the S.E.

Transfection Assays—Human HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Plasmid DNA transfections were performed with cells at 80% confluence using Lipofectamine transfection reagent (Invitrogen). For the experiment shown in Fig. 5*B*, plasmids expressing Herc5 (0.5 μg), 3× FLAG-ISG15 (0.5 μg), and UbcH8 (.25 μg) were transfected with HA-Ube1L, HA-Ube1LΔUFD (0.25 μg), or no E1. Cells were harvested and lysed 48 h post-transfection or post-IFN-β treatment in lysis buffer containing 1% Nonidet P-40, 100 mM Tris, pH 7.9, 100 mM NaCl, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 0.3 μM aprotinin. 30 μg of total cell proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-FLAG antibody (Sigma) to detect ISG15-conjugated proteins and anti-HA antibody (Covance) to detect E1 expression.

RESULTS

E2~ISG15 Thioester Formation in Vitro—UbcH7 is 55% identical and 72% similar to UbcH8 and is the most closely related E2 to UbcH8 among all human E2 enzymes. Fig. 1*A* shows an alignment of the UbcH8 and UbcH7 protein sequences, along with their common secondary structure elements as determined from x-ray crystal structures (UbcH8: PDB 1WZW, UbcH7: PDB 1D5F (29)).[4] Both proteins belong to the subgroup of E2s defined, in part, by a conserved sequence motif within the N-terminal α-helix (α1): *X*Rϕ*XX*(D/E)*X* (where *X* is any residue and ϕ is a hydrophobic residue) (30). This motif constitutes residues 4–10 of UbcH8 and represents the most common motif found in the α1-helix among all ubiquitin E2s (30). With the exception of UbcH8, none of the E2s in this subgroup have been reported to function with Ubls other than Ub. UbcH7 and UbcH8 both contain a conserved phenylalanine residue (Phe-63 in UbcH7, F62 in UbcH8) that is a key contact for interaction of these proteins with HECT and RING E3s (29, 31), and both proteins consist solely of the ~150 amino acid common core E2 structure with no N- or C-terminal extensions. Comparing the sequences of the two proteins, the longest contiguous stretch of non-conserved residues is the six-residue random coil linker between the first α-helix and the first β-strand (α1-β1 linker; residues 16–21 of UbcH8), where the UbcH8 sequence is KPPPYL and UbcH7 is CGMKNF. The α1-helix, the linker, and the β1-β2 region are highlighted in the UbcH8 *ribbon structure* shown in Fig. 1*B*.

Both UbcH7 and UbcH8 have been reported previously to cooperate with human $E1^{Ub}$ (Ube1; for clarity referred to here as $E1^{Ub}$) in catalyzing protein ubiquitination *in vitro* (5, 13, 17) and *in vivo* (11), while only UbcH8 functions in ISG15 conjugation *in vivo* (5). To determine if these results are consistent with biochemical characteristics of $E1^{Ub}$ and Ube1L, we performed a preliminary examination of E1-E2 interactions using *in vitro* thioester assays and incubation times of either 5 or 75 min. As shown in Fig. 2, at the 5-min time point, $E1^{Ub}$ preferentially transferred Ub to UbcH7 compared with UbcH8. At the 75-min time point, the differences in UbcH7 and UbcH8 Ub thioester formation were minimized. Similar results were seen with ISG15 thioester assays, where UbcH8 activation was detected at the 5-min time point, while UbcH7 activation was almost undetectable, but at the 75-min time point the differences between

[4] T. Mizushima, M. Suzuki, N. Teshima, T. Yamane, S. Murata, and K. Tanaka, unpublished data.

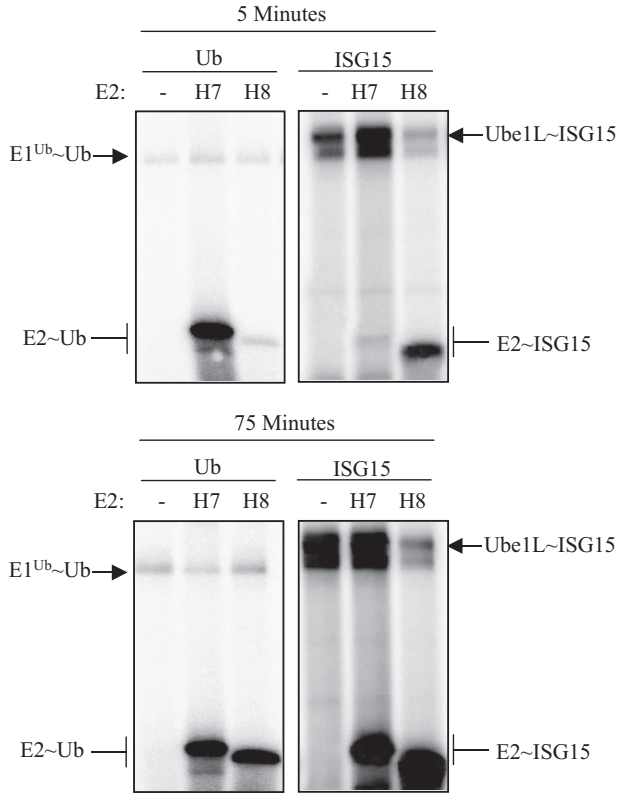

FIGURE 2. **E1-E2 thioester assays with wild-type UbcH7 and UbcH8.** Thioester complex formation was analyzed after incubation with $E1^{Ub}$ or Ube1L for either 5 min (*top panel*) or 75 min (*bottom panel*) with wild-type UbcH7 or UbcH8. The Ub and ISG15 were labeled with ^{32}P and thioester adducts were detected by autoradiography.

TABLE 1
Kinetic constants of $E1^{Ub}$ and Ube1L for formation of E2~ubiquitin and E2~ISG15 thioesters

E1	E2	k_{cat}	K_m	k_{cat}/K_m
		s^{-1}	nM	$s^{-1}M^{-1}$
$E1^{Ub}$ (Ub)	UbcH7	0.0115 +/− 0.001	185 +/− 26	65500 +/− 13000
$E1^{Ub}$	UbcH8	0.000340 +/− 0.0001	6650 +/− 140	50.7 +/− 19
Ube1L (ISG15)	UbcH7	0.000620 +/− 0.00001	1890 +/− 370	372 +/− 180
Ube1L	UbcH8	0.00265 +/− 0.0003	66.4 +/− 8.3	42600 +/− 790
Ube1L	A	0.000778 +/− 0.0004	86.1 +/− 33	8560 +/− 670
Ube1L	D	0.00218 +/− 0.0001	1770 +/− 150	1240 +/− 53
Ube1L	H	0.00282 +/− 0.0009	86.9 +/− 19	33900 +/− 10000
Ube1L	J	0.00106 +/− 0.0001	1940 +/− 220	573 +/− 130
Ube1L	K	0.00181 +/− 0.0002	1750 +/− 290	1210 +/− 370

UbcH8 and UbcH7 activation were minimized. These results indicate that both UbcH7 and UbcH8 can be charged with both Ub and ISG15 to varying degrees. They also demonstrate that *in vitro* experimental conditions may lead to inaccurate conclusions regarding E1 and E2 cooperativity, and suggested the need for more quantitative kinetic analyses.

The K_m values of $E1^{Ub}$ and Ube1L for both UbcH7 and UbcH8 (Table 1) were determined by quantifying E2~Ub/ISG15 thioester formation under initial rate conditions, using ^{32}P-labeled Ub and ISG15 (Table 1). The K_m of Ube1L for UbcH8 was determined to be 66.4 ± 8.3 nM and for UbcH7 it was ~29-fold higher (1890 ± 370 nM). This difference was consistent with the fact that neither endogenous UbcH7 nor any other Ub E2 can substitute for UbcH8 in ISG15 conjugation in interferon-treated cells (5). Similarly, the K_m of $E1^{Ub}$ for UbcH7

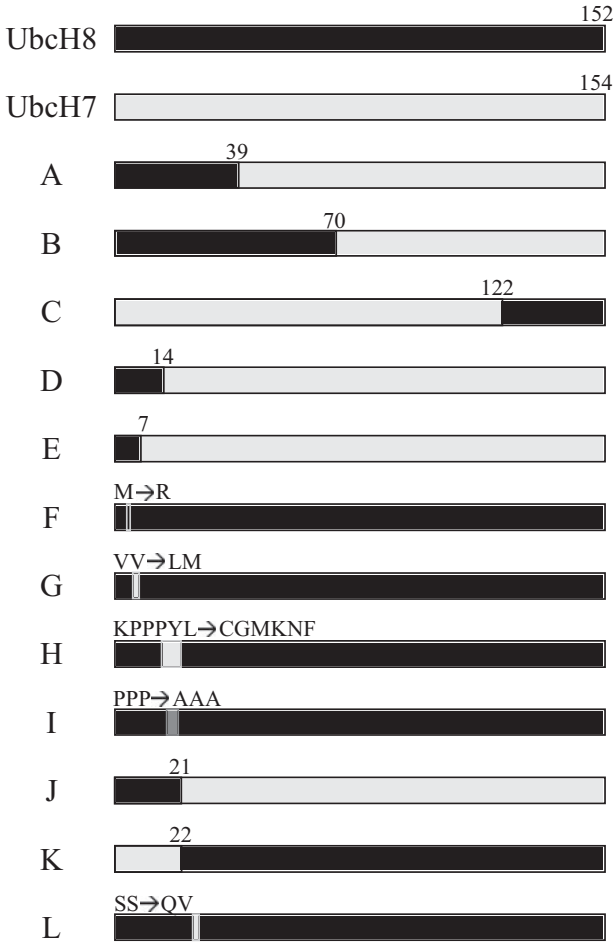

FIGURE 3. **Schematic of chimeric and mutant E2 proteins.** UbcH8 sequences are shown in *black* and UbcH7 sequences are shown in *gray*. Numbering at chimera junctions represents the first residue (if the chimera contains UbcH8 in its C terminus) or the last residue (if the chimera contains UbcH8 in its N terminus) of the UbcH8 sequence present in the chimera. Specific amino acid changes are shown for some chimeras and mutants.

was determined to be 185 ± 26 nM and for UbcH8 it was ~36-fold higher (6650 ± 140 nM). The ratio of k_{cat}/K_m is an indicator of the specificity of an enzyme for a substrate, and this value for $E1^{Ub}$ was ~1,300-fold greater with UbcH7 than with UbcH8 (65,500 *versus* 50.7 s^{-1} M^{-1}; Table 1). For Ube1L, k_{cat}/K_m was ~114-fold greater with UbcH8 than with UbcH7 (42,600 *versus* 372 s^{-1} M^{-1}). Together, these kinetic parameters are consistent with previous demonstrations (5) that no other endogenous E2 proteins can substitute for UbcH8 in the ISG15 system *in vivo*.

Residues 1–39 Are Critical for UbcH8 Interaction with Ube1L in Vitro—To identify the determinants of UbcH8 that confer specificity for Ube1L, we expressed and purified a set of chimeric UbcH8-UbcH7 proteins (Fig. 3). These proteins were assayed for ISG15 thioester formation with purified Ube1L and ^{32}P-labeled ISG15, as well as for Ub thioester formation with $E1^{Ub}$ and ^{32}P-labeled Ub (Fig. 4). To ensure incubation times were within the initial velocity period, reaction progress curves were examined for UbcH8 with Ube1L and UbcH7 with $E1^{Ub}$. Two time points were used for Ub thioester assays, as UbcH8~Ub thioester formation was nearly undetectable after 1 min. Chimeras A and B, containing either the N-terminal 39 or 70 residues of UbcH8, functioned similar to UbcH8 in ISG15

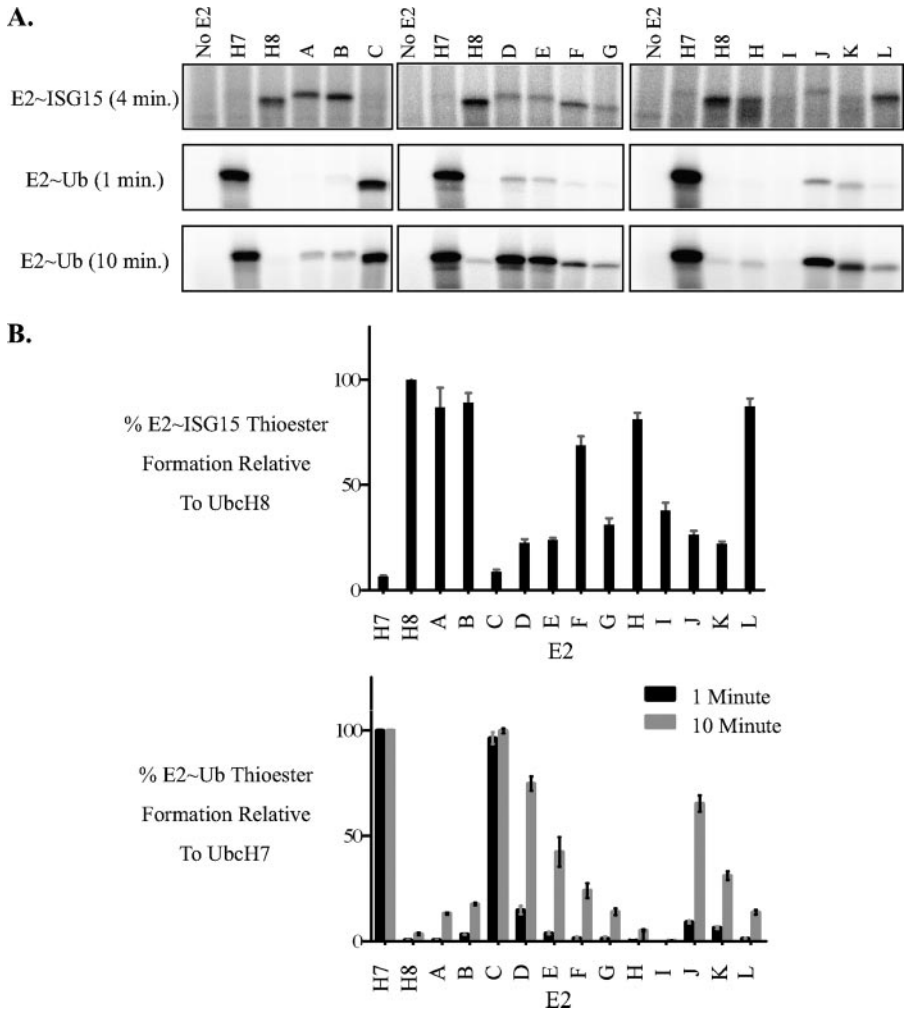

FIGURE 4. ***In vitro* Ub and ISG15 thioester assays with chimeric E2s.** *A*, equivalent amounts of the indicated E2 proteins were incubated with [^{32}P]ISG15 and Ube1L for 4 min (*top panel*), [^{32}P]Ub and E1^{Ub} (DEAE-purified) for 1 min (*middle panel*), or [^{32}P]Ub and E1^{Ub} (DEAE-purified) for 10 min (*bottom panel*). Reaction products were analyzed by SDS-PAGE without reducing agent. *B*, E2 thioester adducts were quantitated and are represented as a percentage relative to UbcH8 (for ISG15 thioesters; *upper panel*) or relative to UbcH7 (for Ub thioesters; *lower panel*).

thioester formation (87 and 89%, respectively, relative to UbcH8), while thioester formation with chimera C (containing residues 1–122 of UbcH7) was undetectable. These results suggested that the N-terminal 39 residues of UbcH8 contain the major determinants for productive interaction with Ube1L. Interestingly, chimera C formed a Ub thioester with similar efficiency as wild-type UbcH7 in reactions programmed with E1^{Ub}, suggesting that the determinants of E1^{Ub}-UbcH7 specificity correspond, at least broadly, to the determinants of Ube1L-UbcH8 interaction. Chimeras A and B also formed Ub thioesters at a relatively low efficiency, similar to wild-type UbcH8, further suggesting that the N-terminal regions of UbcH8 and UbcH7 direct specificity for Ube1L and E1^{Ub}, respectively.

Additional chimeras (Fig. 3) were made to further localize the determinants of UbcH8 required for functional interaction with Ube1L. Surprisingly, chimeras D and E, containing only the first 14 or first 7 residues of UbcH8, were positive for ISG15 thioester formation (at 23 and 24%, respectively, of level of UbcH8; Fig. 4*B*). Chimera D contains the complete α1-helix, while E contains the N-terminal half of the α1-helix, which includes the conserved E2 sequence motif described above (*X*Rϕ*XX*(D/E)*X*, where R is residue 5 of UbcH8). One significant difference between UbcH8 and UbcH7 within this region is that UbcH8 contains a methionine at residue 4, while UbcH7 contains an arginine at the analogous position. The M_4R mutant of UbcH8 (chimera F) was diminished in thioester formation by 31% relative to UbcH8. In addition to residue 4, UbcH8 contains VV at residues 6–7, whereas UbcH7 contains LM at the analogous positions. ISG15 thioester formation was decreased by 69% when the LM sequence replaced the VV sequence of UbcH8 (chimera G). Furthermore, chimeras F and G functioned much better with Ub than wild-type UbcH8 when incubated for 10 min. These results indicate that the α1-helix of UbcH8 is an important determinant, but not the sole determinant, of specificity for Ube1L.

As noted above, linker residues between the α1-helix and the β1-sheet (UbcH8 resides 16–21) are very divergent between UbcH8 and UbcH7, and we therefore addressed whether these residues contributed to the specificity of UbcH8 for Ube1L *in vitro*. A chimera was constructed with the linker residues from UbcH7 replacing those of UbcH8 (chimera H). This resulted in a 19% decrease in thioester formation relative to UbcH8. A more dramatic decrease of 62% was observed when UbcH8 residues 17–19 (PPP) were mutated to AAA (chimera I). Ub thioester formation with chimera H was comparable to UbcH8 while no Ub thioester formation was observed with chimera I. This suggests that the linker sequence might not be a direct determinant of specificity for Ube1L, but rather that alterations of this sequence might have deleterious structural effects on the orientation of the α1-helix or the β1-β2 region (discussed further below). Consistent with this possibility, the crystal structure of Ubc12^{core} with a fragment of Uba3 revealed no interaction of the UFD with the corresponding Ubc12^{core} α1-β1 linker (21).

The third region within the N-terminal 39 residues with the potential to influence Ube1L interactions was the β1-β2 region (residues 21–39). ISG15 thioester formation of chimera K (containing residues 22–152 of UbcH8) was 22% of that of UbcH8, while thioester formation of chimera J (containing residues 21–154 of UbcH7) was 27% of UbcH8. This indicates that the UbcH8 β1-β2 region contributes to ISG15 thioester formation,

but that it is not sufficient for full activation. There are few amino acid differences between UbcH7 and UbcH8 within the $\beta1$-$\beta2$ region; however, UbcH8 contains SS at resides 25–26 while UbcH7 contains QV at the analogous positions. When these residues were exchanged in UbcH8 (chimera L), ISG15 thioester formation was reduced by 13%, and this chimera functioned 4-fold better than UbcH8 with $E1^{Ub}$. These results are consistent with the $\beta1$-$\beta2$ region of UbcH7 and UbcH8 being an additional determinant of E1 recognition.

Kinetic analyses of select chimeras were used to further analyze the role of structural elements within the first 39 residues of UbcH8. Replacement of the $\alpha1$-helix or $\beta1$-$\beta2$ region of UbcH8 with UbcH7 residues (chimeras D, K, or J) resulted in a 27–29-fold increase in the K_m of Ube1L compared with wild-type UbcH8 (Table 1). These chimeras also showed a large decrease in k_{cat}/K_m compared with UbcH8. In contrast, residues in the linker region (chimera H) had a K_m and k_{cat} similar to that of wild-type UbcH8. This was reflected in a k_{cat}/K_m ratio that was 80% that of UbcH8 compared with ratios ~1–3% of UbcH8 for chimeras D, K, and J. Finally, the K_m of Ube1L for chimera A, containing the first 39 residues of UbcH8, was very similar to that of wild-type UbcH8, although k_{cat}/K_m for chimera A was ~5-fold lower than UbcH8. This suggests that chimera A contains the determinants necessary for efficient Ube1L interaction, but that it may be partially defective for accepting ISG15 from Ube1L. Overall, these results are consistent with the UbcH8 $\alpha1$-helix and $\beta1$-$\beta2$ regions being the primary elements recognized by Ube1L.

Interaction of UbcH8 with the UFD of Ube1L—The Sae2 and Uba3 proteins, components of the Sumo and Nedd8 E1 enzymes, respectively, contain a C-terminal Ub fold domain (UFD). This is the primary site for interaction with the core domains of their appropriate E2 enzymes (21, 22). It was proposed that the C terminus of Ube1L is also likely to contain a UFD based on structural propensities of residues conserved with Uba3 (21). To determine whether the UFD of Ube1L has a similar role as in Sae2 and Uba3, a C-terminal deletion mutant of Ube1L (Ube1LΔUFD) was constructed, lacking the last 102 amino acids of the protein (residues 911–1012). If the UFD is the site of interaction with UbcH8 then the Ube1LΔUFD would be predicted to be able to form an ISG15 thioester, but be unable to transfer ISG15 to UbcH8. As shown in Fig. 5*A*, this was the case. In addition, the Ube1LΔUFD mutant did not support ISG15 conjugation when co-transfected with ISG15, UbcH8, and Herc5 into non-interferon-treated 293 cells (Fig. 5*B*).

The purified UFD fragment of Ube1L (consisting of residues 902–1013) was predicted to compete with full-length Ube1L for binding to UbcH8, and as shown in Fig. 5*C*, the UFD inhibited UbcH8~ISG15 thioester formation in a concentration-dependent manner. Finally, a chimeric Ube1L protein was created in which the UFD of Ube1L was replaced with the UFD from $E1^{Ub}$ (Ube1L-UFD^{Ub}; replaces residues 910–1013 of Ube1L with residues 951–1059 of $E1^{Ub}$). *In vitro*, Ube1L-UFD^{Ub} would be expected to transfer ISG15 preferentially to UbcH7, rather than UbcH8, and this was indeed the case (Fig. 5*D*). The chimeric Ube1L-UFD^{Ub} protein was much less stable and less active than wild-type Ube1L, and therefore the absolute effi-

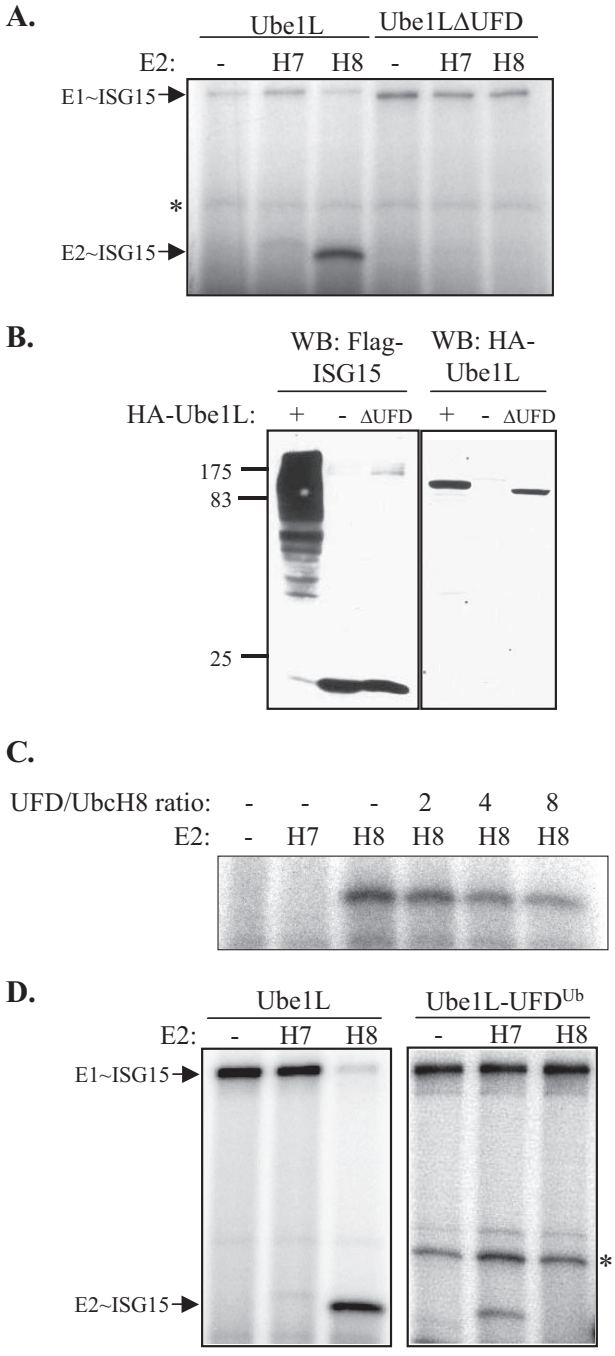

FIGURE 5. **The UFD of Ube1L is required for the interaction with UbcH8.** *A*, [^{32}P]ISG15 was incubated for 10 min with Ube1L or Ube1LΔUFD, with the indicated E2 proteins, and reactions were analyzed by SDS-PAGE without reducing agent. An E2-independent background band is indicated (*). *B*, ISG15 conjugation in transfected 293 cells. 293 cells were transfected with plasmids expressing 3× FLAG-ISG15, UbcH8, Herc5, and either HA-Ube1L, no Ube1L, or HA-Ube1LΔUFD. Cell extracts were prepared and analyzed by immunoblotting with anti-FLAG antibody to detect ISG15 conjugates (*left panel*). Expression of HA-Ube1L and HA-Ube1LΔUFD was confirmed using anti-HA antibody (*right panel*). *C*, purified UFD of Ube1L is a competitive inhibitor of UbcH8~ISG15 thioester formation. UbcH8 thioester formation was analyzed as in *A*, with increasing amounts of purified UFD protein present in the reaction (expressed as the molar ratio of UFD to UbcH8 protein). *D*, a chimeric Ube1L protein containing the UFD of $E1^{Ub}$ (Ube1L-UFD^{Ub}) preferentially transfers ISG15 to UbcH7. [^{32}P]ISG15 was incubated with Ube1L or Ube1L-UFD^{Ub} and either no E2, UbcH7, or UbcH8. Reaction products were analyzed by SDS-PAGE without reducing agent. An E2-independent background band is indicated (*).

ciencies of UbcH7~ISG15 thioester formation in the presence of the chimeric and wild-type Ube1L enzymes were not directly comparable. Nevertheless, the fact that the chimeric Ube1L preferentially transferred ISG15 to UbcH7 over UbcH8 is consistent with a model where the primary determinants of E1-E2 interactions in the ISG15 system are specified by the UFD of Ube1L with the α1-helix and β1-β2 region of UbcH8.

DISCUSSION

The inherent similarities between Ub and Ubls and the enzymes of their conjugation systems leads to important questions about whether all Ub/Ubl pathways are separate and distinct, and if so, how specificity is determined. We have shown here that the basis for Ube1L-UbcH8 specificity is similar to that described previously in the Sumo and Nedd8 systems: interactions between the UFD of the Ube1L and the α1-helix and β1-β2 regions of UbcH8 are the major specificity determinants. Subtle differences in these regions between UbcH8 and UbcH7 are sufficient to allow effective discrimination against this very closely related Ub E2. Furthermore, the degree to which Ube1L discriminates against UbcH7 (based on K_m values) is similar to the degree to which $E1^{Ub}$ discriminates against UbcH8, raising the question of whether UbcH8 functions in the Ub system. Similar results and conclusions concerning the role of UbcH8 in Ub conjugation have been discussed previously (32).

A UbcH8-UbcH7 chimeric E2-containing residues 1–39 of UbcH8 (chimera A) could interact efficiently with Ube1L. Within this N-terminal region, both the α1-helix and the β1-β2 region contributed to Ube1L specificity. Kinetic assays with chimeras containing either the α1-helix or the β1-β2 region of UbcH7 resulted in K_m and k_{cat}/K_m values similar to those of UbcH7. Within the α1-helix, there are only three non-conserved residues and alteration of these residues in UbcH8 to those found in UbcH7 decreased *in vitro* E2~ISG15 thioester formation significantly, while E2~Ub thioester formation was correspondingly increased. Two of the three residues, M_4 and V_7, correspond in position to residues of Ubc12 that make key interactions with the Uba3 component of the Nedd8 E1 (21, 24, 33).

The β1-β2 region also contributed to Ube1L-UbcH8 specificity (comparing, for example, chimeras A and J in ISG15 thioester formation); however it is likely that multiple subtle differences between UbcH8 and UbcH7 within this region contribute to this specificity. SS_{25-26} is the most divergent dipeptide in this region of UbcH8 (QV_{26-27} in UbcH7), and the residue corresponding to UbcH8 S_{26} in the Ubc9p and Ubc12 structures was previously shown to be important for SUMO and Nedd8 thioester formation, respectively (24, 33). Exchange of SS_{25-26} in UbcH8 for QV_{26-27} led to a small but significant decrease in ISG15 thioester formation. Interestingly, this mutant formed a Ub thioester with an ~4-fold increased efficiency relative to wild-type UbcH8. It is therefore possible that SS_{25-26} may serve less as a specificity determinant for Ube1L-UbcH8 interaction than as a barrier to $E1^{Ub}$-UbcH8 interaction. A role for such barriers in establishing E1-E2 specificities has recently been proposed in an analysis of the Nedd8 E2, Ubc12, where it was shown that certain surface residues of Ubc12 appear to function more in preventing mischarging by $E1^{Ub}$ than in specifying charging by the Nedd8 E1 (34).

The third structural element within residues 1–39 of UbcH8 is the linker between α1 and β1, and it is the most divergent region of sequence over the entire length of UbcH7 and UbcH8. A direct swap of UbcH7 linker into UbcH8 however, had little effect on the K_m of chimera H compared with wild-type UbcH8. Furthermore, comparison of chimeras J (UbcH8 α1-helix and linker) and D (UbcH8 α1-helix only) revealed only a minor difference in thioester formation, suggesting that the linker sequence *per se* is not a determinant of Ube1L-UbcH8 specificity. This is consistent with the fact that the corresponding element in Ubc12 does not make contact with the Uba3 UFD in the co-crystal structure (21).

As in the Sumo and Nedd8 E1s, the C-terminal UFD of Ube1L is essential for transfer of ISG15 to UbcH8 and a chimeric Ube1L containing the UFD from $E1^{Ub}$ preferentially transferred ISG15 to UbcH7 over UbcH8. The fact that the chimeric Ube1L-UFD^{Ub} protein had very low activity compared with wild-type Ube1L precluded analyses to determine whether the UFD was the sole determinant of E2 specificity, and based on detailed structural studies of Ubc12 with the Nedd8 E1 complex it is likely that there is an additional surface(s) involved in the Ube1L-UbcH8 interaction (34). Nevertheless, the results presented here strongly support a model in which the primary basis for preferential transfer of ISG15 to UbcH8 is the ability to recruit the E2 via the UFD.

Interestingly, before UbcH8 was shown to be E2 for the ISG15 system (5, 8, 9), it was reported to be an E2 for the Ub system (10–14, 16, 17, 20, 35). However, endogenous UbcH8 expression levels are very low in most non-interferon human cell lines, including HeLa, A549, and 293 cells, where it is virtually undetectable by immunoblotting (see supplemental data). While interferon-β treatment of HeLa cells leads to the induction of UbcH8, transient transfection of a CMV promoter-based UbcH8 expression vector led to an ~60-fold higher level of UbcH8 over the interferon-induced level of endogenous UbcH8 (supplemental data). Combined with the relatively high K_m of $E1^{Ub}$ for UbcH8, these observations suggest that: 1) UbcH8 is unlikely to function in Ub conjugation in many commonly utilized cell lines (at least in the absence of interferon stimulation), and 2) that experimental overexpression may lead to such high levels of UbcH8 that the relatively high K_m of $E1^{Ub}$ for UbcH8 might be overcome, allowing it to function in Ub conjugation and leading to potentially erroneous conclusions regarding the participation of UbcH8 in Ub-dependent processes. Alternatively, there may be cell or tissues types where UbcH8 expression is sufficient to allow its utilization in the Ub system. For example, global microarray gene expression profiling suggests that UbcH8 may be preferentially expressed in certain cells of the immune system (36). Finally, a second E1 enzyme for Ub has been recently described, Uba6/E1-L2 (37–39), raising the possibility that UbcH8 might normally be activated with Ub through Uba6 rather than $E1^{Ub}$ (Ube1). However, in end-point thioester assays, UbcH8 was not activated with Ub any more efficiently by Uba6 than $E1^{Ub}$ (Ube1) (39).

If UbcH8 does not function in Ub-dependent processes, why has the ISG15 system evolved to utilize an E2 that is so similar to

UbcH7 (as well as other related E2s, such as the UbcH5 family of E2s)? Why has UbcH8 not diverged more extensively from E2s of the Ub system? One possibility may be related to the fact that the major E3 for the ISG15 system is a HECT E3, Herc5 (27). Herc5 is the only HECT E3 known to function with a modifier other than Ub, and because of inherent HECT E3 structure and/or the unique mechanism of HECT E3s, Herc5 might place considerable constraints on the how far the primary sequence of UbcH8 can diverge from other human E2s that function with HECT E3s (*e.g.* UbcH5a, b, c, UbcH6, UbcH7).

Interestingly, there are other features of the ISG15 system that more closely resemble features of the Ub system than other Ubl systems. For example, human Ube1L is the most closely related E1 enzyme to human Ube1/E1^{Ub} (38), and ISG15 is the only Ubl where the last six residues of the protein (LRLRGG) are identical to that of Ub. Are these similarities indicative of functional or regulatory overlap between these pathways? As with UbcH8, it is clear that no other E3 can substitute for the broad effect of Herc5 in ISG15 conjugation (27); however, it is not known whether Herc5 might also function in Ub conjugation. There may be mechanistic or structural features of Herc5 that distinguish it from Ub HECT E3s, or Herc5 might simply preferentially recruit UbcH8 over other E2s. These unique problems make the ISG15 system of interest for addressing general mechanism and design of Ubl conjugation systems. In addition, understanding the biochemistry of ISG15 conjugation may ultimately aid in the elucidation of the biochemical function of ISG15 conjugation and the basis of its antiviral activity.

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