Crystal Structure of the Interferon-induced Ubiquitin-like Protein ISG15*§

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The biological effects of the ISG15 protein arise in part from its conjugation to cellular targets as a primary response to interferon-α/β induction and other markers of viral or parasitic infection. Recombinant full-length ISG15 has been produced for the first time in high yield by mutating Cys78 to stabilize the protein and by cloning in a C-terminal arginine cap to protect the C terminus against proteolytic inactivation. The cap is subsequently removed with carboxypeptidase B to yield mature biologically active ISG15 capable of stoichiometric ATP-dependent thiolester formation with its human Ube1L activating enzyme. The three-dimensional structure of recombinant ISG15C78S was determined at 2.4 Å resolution. The ISG15 structure comprises two β-grasp folds having main chain root mean square deviation (r.m.s.d.) values from ubiquitin of 1.7 Å (N-terminal) and 1.0 Å (C-terminal). The β-grasp domains pack across two conserved 3_10 helices to bury 627 Å² that accounts for 7% of the total solvent-accessible surface area. The distribution of ISG15 surface charge forms a ridge of negative charge extending nearly the full-length of the molecule. Additionally, the N-terminal domain contains an apolar region comprising almost half its solvent accessible surface. The C-terminal domain of ISG15 was superimposed on the structure of Nedd8 (r.m.s.d. = 0.84 Å) bound to its AppBp1-Uba3 activating enzyme to model ISG15 binding to Ube1L. The docking model predicts several key side-chain interactions that presumably define the specificity between the ubiquitin and ISG15 ligation pathways to maintain functional integrity of their signaling.

The broad pleiotropic and anti-viral effects of the interferons are mediated through signaling pathways that culminate in the enhanced expression of a temporally coordinated subset of cell type-specific proteins (1, 2). Induction of ISG15 marks the earliest and most universal consequence of exposure to various interferon isoforms, suggesting that elevated levels of this 17-kDa polypeptide are essential for the subsequent response of cells to these cytokines (3, 4) and their ability to mount an effective anti-viral defense (3–6). The ISG15 polypeptide is induced by lipopolysaccharide and double-stranded RNA, both potent markers of parasitic and viral infection (7, 8). Ectopic overexpression of ISG15 directly inhibits human immunodeficiency virus replication by abrogating nuclear processing of unspliced viral RNA precursors, leading to accumulation of nascent transcripts (9). Similarly, influenza B virus replication is blocked by endogenous ISG15 unless its expression is prevented by a specific transcriptional block mediated by the viral NS1 protein (10). Most recently, murine knock-out studies implicate an essential role for ISG15 in innate immunity, a critical defense against infectious agents that guides subsequent host adaptive immune responses (11).

Like most interferon-induced proteins, ISG15 is constitutively present in higher eukaryotes and serves additional function(s) unrelated to its role in anti-viral defenses (4, 12). Recognition that ISG15 harbors tandem ubiquitin-like domains and terminates in a conserved ISG15C78S ubiquitin C-terminal motif suggested some of the biological effects attributed to the polypeptide were directed in part by its conjugation to cellular targets (4, 13). Immunochemical studies confirm that ISG15 conjugates are abundant in eukaryotic cells and increase significantly in response to interferon induction (13, 14). The ISG15 polypeptide was the first example identified of the superfamilly of Class 1 ubiquitin-like proteins, defined by their ability to undergo covalent ligation reactions to specific cellular targets (reviewed in Refs. 15 and 16). Nascent ISG15 is synthesized as an inactive precursor in which the mature glycine C terminus that participates in isopeptide bonds to cellular targets is blocked by an extension peptide that is post-translationally processed by the human ortholog of Ubp1 to yield the active ISG15 protein (17). Interestingly, an extracellular cytokine activity of ISG15 in stimulating interferon-γ production and natural killer cell proliferation also requires an intact mature ISG15 C terminus (18).

Conjugation of ISG15 utilizes a parallel but distinct mechanism to that of ubiquitin (19). The ATP-dependent activation of ISG15 that is required for subsequent conjugation to cellular protein targets is catalyzed by Ube1L1 (10), a 112-kDa paralog

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§ The on-line version of this article (available at http://www.jbc.org) contains Fig. S1.
¶ The abbreviations used are: Ube1L, the ISG15 activating enzyme; AppBp1-Uba3, the heterodimeric Nedd8 activating enzyme; CD, circular dichroism; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Sael-Sae2, human Sse2 homolog; Ub1, ubiquitin activating enzyme; STAT, signal transducers and activators of transcription; E2, ubiquitin carrier protein; r.m.s.d., root mean square deviation.

27356 This paper is available on line at http://www.jbc.org
of the E1/Uba1 ubiquitin-activating enzyme (20). Marked ablation of UbE1L levels in various small cell lung carcinoma lines suggests loss of ISG15 conjugation is a contributing factor in malignant transformation (20, 21). Similarly, induction of UbE1L is directly implicated in retinoic acid-mediated remis- sion during acute promyelocytic leukemia (22), evidence for which includes the observation that either ectopic over expres- sion of UbE1L or its induction by all-trans retinoic acid triggers 26 S proteasome-dependent degradation of promyelocytic leukemia/retinoic acid receptor-α protein and apoptosis in promyelocytic leukemia (23). More recently, human UbcH8 has been identified as the E2 conjugating enzyme for ISG15 and requires the action of UbE1L for ATP-dependent formation of the oblig- ate UbcH8-ISG15 thiolester intermediate for conjugation (24). Cognate isopeptide ligases required for ISG15 conjugation have not been definitively identified to date but are suggested to overlap with a subset of putative ubiquitin-dependant ligases requiring UbcH8 (24). A limited number of protein targets for ISG15 conjugation have been reported, including the murine serpin 2a induced in response to parasite infection (25) and components of the Janus tyrosine kinase-STAT signaling path- way (26). However, observation that disruption of intracellular ISG15 conjugation dynamics by loss of function mutations in the ISG15-specific isopeptidase UBP43 results in severe develop- mental neurological defects hints at more global roles for ISG15 ligation (27).

Although ISG15 was the first ubiquitin-like protein identi- fied (4, 13), much less is known about the functional roles of this polypeptide than for other members of the superfamily. In part this derives from the remarkable absence of ISG15 orthologs in lower eukaryotes that precludes the powerful genetic approaches successfully exploited previously with its more ubiquitously distributed paralogs (13). In addition, the marked instability of ISG15 noted in early work continues as a major impediment to detailed in vitro analysis (28). In the present studies we demonstrate that the inherent instability of recombinant ISG15 derives from disulfide-linked dimerization through Cys76 of the polypeptide, point mutation of which to serine markedly increases the stability of the polypeptide to that approaching ubiquitin. Enhanced stability has allowed the crystallization and structural determina- tion of recombinant ISG15 for the first time. The 2.4 Å crystal structure for ISG15 confirms the tandem ubiquitin-like do- mains of β-grasp folds originally proposed on the basis of sequence analysis (13). The structure for ISG15 reveals a novel surface charge distribution between the β-grasp do- mains and an extensive solvent-exposed surface of low polar- ity encompassing a substantial fraction of the N-terminal β-grasp domain. Finally, docking simulations using the re- cent structure for Nedd8 bound to its cognate activating enzyme, the AppBp1-Uba3 heterodimer (29), suggests the basis for the specificity of UbE1L for ISG15 activation.

**Materials and Methods**

Bovine ubiquitin was purchased from Sigma and purified to apparent homogeneity by fast protein liquid chromatography (30). Human recom- binant proISG15 was that described previously (17). The complete coding sequence for human ISG15-activating enzyme UbE1L (generous gift of Robert M. Krug) was subcloned by PCR from a modified pVL1393 baculovirus transfer vector (10) into the BamH1/EcoRI sites of pGEX to yield pGEX-UbE1L. Human GST-UbE1L was expressed in *Escherichia coli* BL21(DE3) cells harboring pGEX-UbE1L following isopropyl 1-thio-β-D-galactopyranoside induction. The resulting recombinant GST-UbE1L was purified on glutathione-Sepharose following the manu- facturer's instructions.

**Expression and Purification of Recombinant ISG15—Ubiquitin and other type 1 ubiquitin-like proteins are sensitive to proteolytic inacti- vation through cleavage of their C-terminal glycine dipeptide by a widely expressed bacterial periplasmic carboxypeptidase (19). To cir- cuvent this problem in the present studies, a CGT codon for arginine was inserted between the mature human ISG15 C-terminal glycine GCC codon and the TAA STOP codon of pETUCRP (13) by PCR using an appropriately designed 3’-external primer containing a BamHI re- striction site. The resulting full-length PCR-amplified DNA was re-stricted with NcoI/BamH1 and ligated into a similarly restricted pET11d plasmid to yield pET11d-ISG15-R158. Because the bacterial carboxypeptidase that inactivates ubiquitin-like proteins is incapable of removing a C-terminal arginine residue, the presence of Arg555 provided a cap residue to protect the integrity of the mature ISG15 C terminus during purification. In addition, the shift in pI from 6.7 for wild type mature polypeptide to 8.5 for recombinant ISG15-R158 en- hanced resolution of the latter from contaminating proteins that were otherwise difficult to remove without additional chromatographic steps that reduced yield.

Circular dichroism studies described under “Results” demonstrated that recombinant wild-type ISG15 exhibits within its mature ISG15 in- stability resulting from its propensity spontaneously to form a disul- fide-linked dimer during expression and purification. Disulfide-depend- ent instability was addressed by mutating the single Cys78 of human mature ISG15-R158 to serine, yielding ISG15C78S-R158. The Cys78 con- codon within pET11d-ISG15-R178 was mutated by overlap extension PCR using appropriately designed oligonucleotide primers, then the constructed vregion was inserted without restriction into similarly restricted pET11d to yield pET11d-ISG15C88S-R158. In subsequent functional studies, we found that recombinant mature ISG15 and ISG15C78S, from which the protective arginine caps had been proteolytically processed, were poorly radioiodinated by chloro- mine T using a protocol previously successful with ubiquitin and Nedd8 (31, 32). Poor radioiodination presumably resulted from the relative solvent insobility of the tyrosine residues within the ISG15 struc- ture compared with the other two Type 1 ubiquitin-like proteins, ul- timately confirmed by the refined crystal structure of the polypeptide. Overlap extension PCR was exploited to mutate the Asn13 codon of human mature pET11d-ISG15C78S-R158 to tyrosine, yielding pET11d-ISG15N13Y/C78S-R158, to generate a solvent-accessible site for radioiodination, predicted from the subsequent structure for ISG15C78S. Restriction of E. coli strains containing pET11d-GL21(DE3) expressing ISG15-R158, pET11d-ISG15C78S-R158, or pET11d-ISG15N13Y/C78S- R158 were grown at 30 °C to A600 nm of ~0.6 in Luria broth containing 100 μg/ml ampicillin then induced by addition of isopropyl 1-thio-β-p-galactopyranoside to a final concentration of 0.4 mM. After additional 1.5-h incubation at 30 °C, cells were harvested by centrifugation. The resulting cell pellet was suspended in 100 ml of ice-cold 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, then lysed by 12,000 psi in a French pressure cell (all subsequent steps were conducted at 4 °C). crude extract was centrifuged at 10,000 × g for 60 min, after which supernatant proteins precipitating between 30 and 50% saturated ammonium sulfate were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, and dialyzed against 2 × 4 liters of the same buffer. The dialysis solution was then added to a 5 × 20 cm column of DEAE-52 anion exchange cellulose (Whatman) equilibrated with 50 mM Tris-HCl (pH 7.5) con- taining 1 mM DTT. Fractions containing the unadsorbed fraction were pooled then adjusted to 1 M ammonium sulfate and applied to a 3- × 28-cm column of Phenyl-Superose FAST FLOW equilibrated with 20 mM sodium phosphate buffer (pH 7.2) containing 1.0 M ammonium sulfate and 1 mM DTT. Bound proteins were eluted by a 1.0—0 M negative salt gradient (5 mM/ml) at 2 ml/min. Recombinant ISG15- R158, ISG15C78S-R158, and ISG15N13Y/C78S-R158 elute as single peaks at 0.4 mM ammonium sulfate. Fractions containing ISG15-R158 or the two point mutants were pooled and concentrated by ultrafiltration to ~5 ml then resolved at 1 ml/min on a 150- × 30-cm column of Sephacryl-600 FAST FLOW equilibrated with 50 mM Tris-HCl (pH 7.5) containing 25 mM NaCl and 1 mM DTT. Fractions containing the recombinant proteins were pooled then dialyzed against 1 mM DTT using 3.5-kDa exclusion tubing. The protective Arg555 cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit. The protective Arg cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit. The protective Arg cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit. The protective Arg cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit. The protective Arg cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit. The protective Arg cap was removed from purified recombinant ISG15 polypeptides by exploiting the absolute specificity of porcine pancreatic carboxypeptidase B (EC 3.4.17.2) for cleaving C-terminal arginine residues retaining a 3.5-kDa exclusion limit.
dase A-like activity that slowly cleaved the exposed glycine dipeptide following processing. The latter activity could be resolved from the carboxypeptidase B by dissolving the commercial enzyme in 50 mM Tris-HCl (pH 7.5) containing 50 mM NaCl and 1 mM DTT at 1 ml/min flow rate. A mature recombinant ISG15 or its point mutants eluted as single homogeneous peaks at their predicted monomer molecular masses of ~17 kDa. Concentrations of recombinant ISG15 and ISG15C78S were quantitated similarly using an empirical extinction coefficient of 0.98 ml/mg/cm. (13). The concentration of recombinant ISG15N13Y/C78S was quantitated using an empirical extinction coefficient of 0.98 ml/mg/cm.

For measurement of protein unfolding by urea denaturation, equilibrium concentrations of recombinant protein were prepared in 10 mM Tris-HCl (pH 7.5) containing 0.2 mM DTT then mixed with an appropriate concentration of 9 M urea (ultrapure DNA sequencing grade) and 0.2 M MgCl2 and equilibrating against the precipitating solution. The efficacy of this maneuver is demonstrated by the quantitation of final urea concentrations in the transition region about the inflection point when the free energy of denaturation was calculated assuming a two-state model.

RESULTS

Stabilization of Full-length Recombinant ISG15—We have previously reported that expression of recombinant Class 1 ubiquitin-like proteins is accompanied by rapid proteolytic inactivation through the action of a periplasmic carboxypeptidase that is largely refractory to known proteolytic inhibitors (19). In the present work we have exploited the ability of this bacterial protease to excise C-terminal arginyl residues by expressing recombinant ISG15 bearing a protective Arg55 cap. The efficacy of this maneuver is demonstrated by the quantitative recovery in good yield of an intact ISG15 polypeptide of the predicted molecular mass by MALDI-TOF mass spectrometry (not shown). We also noted early in these studies that purification ISG15 was markedly sensitive to slow precipitation over time or upon freezing, particularly at concentrations above ~0.2 mg/ml. Non-reducing SDS-PAGE revealed that the precipitated form of the protein comprised a disulfide-linked dimer of ISG15 (not shown). Inclusion of DTT (10 mM) had little effect in preventing spontaneous precipitation of the ISG15 protein; however, mutation of the single cysteine residue at position 78 abrogated dimerization and precipitation. Subsequent urea denaturation studies have corroborated the enhanced stability of ISG15C78S compared with wild type protein.

Fig. 1A compares the averaged CD spectra for ubiquitin, wild-type ISG15, and ISG15C78S at pH 7.5 and 20 °C. Averaged spectra for the three polypeptides were qualitatively similar. The spectrum for human proISG15 was indistinguishable from that of the mature polypeptide (not shown), indicating that the C-terminal octapeptide extension has negligible effect on the structure of the protein. Secondary structure composi-
non-reducing SDS-PAGE resolution of UbE1L-125I-ISG15 thiolester for-
Solid symbols
represent molar ellipticity of refolded samples after quantita-
tive denaturation in 6 M urea and dilution to 10
open circles
) or ISG15C78S
(open squares).

equal volume of SDS sample buffer in the absence of 2-mercapto-
ethanol
in the presence
(UBE1L were incubate at 37 °C for 5 min in the absence
/H11002

were incubated at 37 °C for 5 min in the absence

P212121 form
I222 form
P-derivative

Resolution (Å)
Cell dimension (Å)
No. of molecule in ASU
No. of observed reflections
No. of unique reflections
Completeness (%)
ε

0.223
30–2.4
55.2, 57.1, 104.2
2
34285
12464
92.2(79.3)
10.5(2.9)
0.071(0.236)

Resolution range (Å)
σ cutoff
No. of protein atoms
No. of water atoms
Rcryst (%)
Rfree (%)
r.m.s.d. bond lengths (Å)
r.m.s.d. bond angles (Å)
Average B-factor
Protein (Å²)
Water (Å²)

30–2.4
0.0
2352
71
23.2(32.2)
28.8(35.3)
1.3
37.7
29.1
30–2.4
0.0
1174
39
20.8(32.1)
21.3(33.4)
1.3
32.0
27.3

No. of water atoms
Completeness (%)
Cell dimension (Å)
Rcryst (%)
σ cutoff

92.2(79.3)
55.2, 57.1, 104.2
23.2(32.2)
0.0

Nos. in parentheses are for the highest resolution shell.

b Determined from the 1.8-Å crystal structure of ubiquitin (41).

c Determined from the 2.4-Å crystal structure of ISG15 (this work).

A
FIG. 1. Characterization of recombinant ISG15. A, averaged (n = 4)
circular dichroism spectra plotted as molar ellipticity (2) for 20 μM
ubiquitin (solid line), 10 μM wild-type ISG15 (dashed line), or 10 μM
ISG15C78S (broken line) at 20 °C in 10 mM Tris-HCl (pH 7.5) containing
0.2 mM DTT. B, urea denaturation curves collected as in A at 222 nm
for fraction denatured (Fp) versus [urea] for wild-type ISG15 (open
circles and solid line) and ISG15C78S (open squares and dashed line).
Solid symbols represent molar ellipticity of refolded samples after
quantitative denaturation in 6 M urea and dilution to 10 μM as
described in the text. Lines represent theoretical sigmoidal fits for the
data. C, secondary plots of ΔGapp versus [urea] for wild-type ISG15
(open circles) or ISG15C78S (open squares). D, autoradiogram following
non-reducing SDS-PAGE resolution of UbE1L-125I-ISG15 thiolester
formation. Reactions of 50 μl of final volume containing 50 mM Tris-HCl
(pH 7.5), 10 mM MgCl₂, 1 mM DTT, 1 mg/ml carrier bovine serum
albumin, 0.57 μM 125I-ISG15N15Y/C78S, and 13 nM recombinant GST-
UBE1L were incubated at 37 °C for 5 min in the absence (+ lane) or
presence (− lane) of 2 mM ATP. Incubations were quenched with an
equal volume of SDS sample buffer in the absence of 2-mercapto-
ethanol then resolved on 10% (w/v) gels without boiling (47). Migration
positions for free 125I-ISG15 and its corresponding thiolester to UbE1L
are shown to the right.

pared with that determined from the published high-resolution
crystal structure of the polypeptide (Table II). Importantly, the
calculated secondary structure composition for wild-type
ISG15 is comparable to that calculated for ubiquitin, suggest-
ing that the two proteins possess similar folds. In addition,
convergence of the spectral fit to unique solutions for the re-
combinant ISG15 forms requires that the protein molecules
possess a uniform structure (33, 34), precluding a significant
fraction of denatured polypeptide in the preparations. The
nearly identical fits for secondary structure compositions for
ISG15, ISG15C78S, and proISG15 indicate that neither the
point mutation at Cys78 nor the C-terminal peptide extension
of the precursor, respectively, have significant effects on overall
structure. Inclusion of the eight additional unordered residues
of the C-terminal propeptide extension accounts for the propor-
tional apparent decrease in secondary structure composition
for proISG15 compared with either wild-type ISG15 or
ISG15C78S (17).

The ellipticity at 222 nm was measured as a function of urea
concentration, and the fraction denatured (Fp) was calculated
assuming a two-state model (35), as described under “Materials
and Methods.” All of the recombinant ISG15 proteins dena-
tured in the range of 3–6 M urea (not shown). By comparison,
the averaged CD spectrum for ubiquitin under the same condi-
tions showed no change even at 9 M urea (not shown), as
found earlier (45). Fig. 1B illustrates that values of Fp for
wild-type ISG15 (open circles and solid line) and ISG15C78S

Values are calculated from the CD measurements on the basis of
Sreerama and Woody (33, 34); for details, see “Materials and Methods.”

ubiquitin
open circles
wild-type ISG15
dashed line
open squares
ISG15C78S (17).

a Soaked with K₂OsCl₆ to convert to the I222 form and treated as the native crystal.
b The Os-soaked crystal was soaked again in a solution containing 3 mM K₂PtCl₄ for 3 h.

TABLE II
Summary of calculated secondary structural elements from CD spectra

| Protein   | α-Helix | β-Sheet | Turns | Unordered |
|-----------|---------|---------|-------|-----------|
| Ubiquitin | 0.149   | 0.367   | 0.367 | 0.117     |
| ISG15     | 0.159   | 0.447   | 0.244 | 0.140     |
| ISG15C78S | 0.132   | 0.457   | 0.280 | 0.159     |
| proISG15  | 0.122   | 0.432   | 0.285 | 0.153     |

TABLE I
Data collection and refinement statistics

Data collection

| P2,2,2, form | Nativea | Pt-derivativeb |
|--------------|---------|----------------|
| Resolution (Å) | 30–2.4(2.44–2.40) | 30–2.4(2.44–2.40) |
| Cell dimension (Å) | 55.2, 57.1, 104.2 | 55.8, 57.0, 103.7 |
| No. of molecule in ASU | 2 | 1 |
| No. of observed reflections | 34285 | 15178 |
| No. of unique reflections | 12464 | 6198 |
| Completeness (%) | 92.2(79.3) | 91.6(70.7) |
| ε | 10.5(2.9) | 9.8(2.5) |
| Rsym | 0.071(0.236) | 0.061(0.192) |

Refinement statistics

| P2,2,2, form | Nativea | Pt-derivativeb |
|--------------|---------|----------------|
| Resolution range (Å) | 30–2.4 | 30–2.4 |
| σ cutoff | 0.0 | 0.0 |
| No. of protein atoms | 2352 | 1174 |
| No. of water atoms | 71 | 39 |
| Rcryst (%) | 23.2(32.2) | 20.8(32.1) |
| Rfree (%) | 28.8(35.3) | 21.3(33.4) |
| r.m.s.d. bond lengths (Å) | 0.008 | 0.006 |
| r.m.s.d. bond angles (Å) | 1.3 | 1.3 |
| Average B-factor | 37.7 | 32.0 |
| Protein (Å²) | 29.1 | 27.3 |

Numbers in parentheses are for the highest resolution shell.

a Determined from the 1.8-Å crystal structure of ubiquitin (41).
b Determined from the 2.4-Å crystal structure of ISG15 (this work).
peptides in 6 M urea then allowing the proteins to refold by diluting the samples to an intermediate urea concentration near the respective inflection points of the unfolding curves. The values for \( F_1 \) observed after dilution matched the predicted values from the nonlinear regression analyses, consistent with the reversibility of the structural transitions (Fig. 1B, solid symbols). The apparent free energies of denaturation, \( \Delta G_{app} \), calculated from \( F_1 \) at each urea concentration followed a linear dependence on denaturant concentration from which the slope, \( m \), and the extrapolated \( y \) intercept corresponding to the free energy of unfolding, \( \Delta G_{H_2O} \), were calculated (35) (Fig. 1C).

Wild-type ISG15 exhibited a \( \Delta G_{H_2O} \) of 5.8 ± 0.6 kcal/mole (\( m = -1.5 \pm 0.1 \) kcal/mole/\( \Delta C_\text{p} \)), whereas that for ISG15C78S was 9.1 ± 0.8 kcal/mole (\( m = -2.0 \pm 0.2 \) kcal/mole/\( \Delta C_\text{p} \)), representing a \( \Delta G_{H_2O} \) for stabilization of -3.3 kcal/mol. Notably, a low concentration of DTT (0.2 mM) was required in the urea denaturation studies to observe sigmoidal unfolding curves and reversibility upon dilution of the denaturant. Presumably, in the absence of DTT the ISG15 irreversibly denatures, yielding non-ideal behavior.

The urea denaturation studies indicated that blocking disulfide-linked dimerization of ISG15 by mutating the single Cys\(^{78} \) residue significantly stabilized the protein. Because Cys\(^{78} \) is absolutely conserved among ISG15 orthologs, we were concerned that the covalent dimer might represent the biologically active form of the polypeptide or that the point mutant might otherwise be inactive. Therefore we tested the ability of ISG15 to activate the reaction of the ISG15-activating enzyme UbE1L (10). Mutation of Asn\(^{113} \) to tyrosine was necessary to provide a surface-accessible tyrosine for iodination, because recombinant ISG15C78S was poorly radiolabeled by chloramine T. The autoradiogram of Fig. 1D demonstrates that ISG15N13Y/C78S forms an ATP-dependent thiolester with UbE1L that is resolved from free radiolabeled polypeptide by non-reducing SDS-PAGE. That the adduct is a thiolester was demonstrated by loss of the band when resolved under reducing conditions (not shown). Therefore, monomeric ISG15 is biologically active in forming the obligate thiolester intermediate to its cognate activating enzyme.

Overall Structure of ISG15—Stabilization of ISG15 allowed the recombinant protein to be concentrated considerably without the denaturation and protein losses observed with wild-type polypeptide. This enabled us readily to obtain crystals of recombinant ISG15C78S suitable for structural determination. The final structural model contains the entire polypeptide chain from Trp\(^{3} \) to Leu\(^{154} \) with the first two N-terminal residues being disordered (Fig. 2A). The I222 structure and those of the two P2\(_2\)

1\(_2\)

structures (in the asymmetric unit) are virtually identical with r.m.s.d. values ranging from 0.4 Å between the two P2\(_2\)

1\(_2\)

structures to 0.7 Å between the I222 and P2\(_2\)

1\(_2\)

structures. Therefore, unless stated otherwise, references to native ISG15 refer to the I222 structure in the remainder of the text. As expected from the sequence similarity (4, 13), the overall structure of ISG15 contains two easily discernable domains, each of which assumes a \( \beta \)-grasp fold that is nearly identical to that found in ubiquitin (41) (Fig. 2). Both domains of ISG15 contain a five-strand mixed \( \beta \)-sheet into which is intercalated a single three-turn \( \alpha \)-helix (Fig. 2). Each domain of ISG15 also retains the two 3\(_{10}\) helices characteristic of ubiquitin, one of which occurs between the \( \alpha \)-helix and the \( \beta \) strand (Ala\(^{50} \)-Gln\(^{43} \) and Asp\(^{119} \)-Leu\(^{121} \)) and the other of which occurs in a turn-rich region between the \( \beta 4 \) and \( \beta 5 \) strands (Pro\(^{59} \)-Ser\(^{62} \) and Gly\(^{138} \)-Gly\(^{141} \)) (Fig. 2). The two \( \beta \)-grasp domains are connected by a six-residue extended linker peptide comprising Asp\(^{76} \)-Pro\(^{81} \) and encompassing (Cys/Ser)\(^{78} \). The two \( \beta \)-grasp domains of ISG15 are remarkably similar in structure, as predicted earlier by their intra-domain sequence conservation (4). The r.m.s.d. between the N-terminal domain (residues 4–76, excluding 49–50) and the C-terminal domain (residues 83–153) of ISG15 is 1.9 Å for the corresponding 70 Cα atoms, and the r.m.s.d. values between ubiquitin and either the N- or C-terminal domains of ISG15 are 1.7 Å and 1.0 Å, respectively. Fig. 2B shows an overlay of the three structures, revealing their marked similarity in folding.

Relative Orientation of the Two Domains—Fig. 2A also shows the relative orientation between the two domains in the ISG15 structure. The N-terminal domain can be superimposed upon the C-terminal domain by rotating the former along the y-axis.
by 45° and by −60° clockwise rotation along the z-axis (the axis perpendicular to the plane of the paper or monitor screen). The two domains are arranged so that the 3_10 element between the α-helix and the β strand of the N-terminal domain (Ala^{145}–Gln^{148}) interacts with the 3_10 element in the turn-rich region between the β4 and β5 strand of the C-terminal domain (Gly^{138}–Gly^{141}). The contact surface between the 3_10 helix segments of the two domains involves mainly van der Waals interactions between His^{39}, Phe^{41}, Pro^{136}, and Gly^{138}. There is also a weak hydrogen bond (3.4 Å) between the Oϵ of Glu^{139} and the main-chain amide of Phe^{41}. The contact area between the two domains is 627 Å², corresponding to 7% of the total solvent-accessible surface area. Because the structures of the I222 and P2_12_1 forms are virtually identical, it is reasonable to conclude that the observed crystal structure of ISG15, in particular, the relative orientation of the two domains is not due to crystal packing, but rather represents the most stable solution structure. However, it is entirely possible that, when ISG15 interacts with other proteins, the molecule might adopt a different relative orientation between the two domains by main-chain bond rotations within the linker peptide connecting them (residues Asp^{76}–Lys^{77}–Cys^{78}–Ser^{79}–Asp^{79}–Glu^{80}). Notably, the linker peptide consists mainly of highly charged hydrophilic residues that might facilitate such a transition.

**Surface Charge Distribution of ISG15**—The distinct functional roles of the Class 1 ubiquitin-like proteins require their respective interacting proteins to recognize unique surface features of the polypeptides. Fig. 3 compares the calculated surface charge distributions for human ISG15 (A) versus human ubiquitin (B) superimposed on their respective solvent-accessible surfaces. The structures for the C-terminal β-grasp domain of ISG15 and for ubiquitin have been positioned in the same orientation to compare similarities and differences in surface charge features. In addition, ribbon diagrams in the same orientation are shown to the right in each panel. The disordered C-terminal four residues are omitted from the structural model for ISG15 in Panel A. The basic and acidic faces of ubiquitin are clearly evident in the left and right sides of the polypeptide shown in Panel B.

Several distinguishing features of the ISG15 surface stand out in this structural comparison. The C-terminal β-grasp domain of ISG15 has a much less pronounced basic face (blue residues) than is present on ubiquitin. This difference is principally due to replacement of Arg^{12} of ubiquitin with Trp^{123} of ISG15, which disrupts an otherwise relatively continuous basic face, the remaining residues of which are conserved between the two proteins. In addition, ISG15 contains a slight bulge in the basic face, owing to a larger loop between β1 and β2 of the C-terminal domain compared with the orthologous region of ubiquitin, which harbors an additional basic residue at Lys^{90} corresponding to Thr^{9} of ubiquitin. The acidic residues of ISG15 are organized into a distinct ridge of negative charge along the molecule that comprise Asp^{119} (Pro^{38}), Asp^{120} (Asp^{39}), Asp^{133} (Asp^{52}), Glu^{132} (Glu^{51}), Glu^{139} (Asp^{58}), and Glu^{27} (Asn^{25}), with paralogous ubiquitin residues in parentheses. Most notably, the sequence in the N-terminal β-grasp domain encompassing the β3 through β5 segments comprise a large hydrophobic region, shown in white, that covers nearly half of the domain. The function of this large apolar region is unclear; however, the extended hydrophobic surface could account for the marked propensity of the purified protein to adsorbed non-
specifically to surfaces in relatively dilute solutions and to undergo dimerization prior to disulfide formation.

A Model For ISG15 Binding to UbE1L—Binding of the different ubiquitin-like proteins to their cognate activating enzymes represents the entry point for these polypeptides into their respective, mechanistically parallel pathways for ligation (46). The fidelity with which these recognition events occurs prevents deleterious cross-talk between pathways and competitive inhibition by the significantly higher steady-state concentrations of free ubiquitin found within cells (47, 48), as discussed previously (13, 19). To identify potential interacting residues that might be important for ISG15 recognition and binding, we modeled the polypeptide onto the recently published structure for human Nedd8 bound to AppBp1-Uba3 (29). The C-terminal ubiquitin-like domain of ISG15 and Nedd8 share 26% identity over 76 residues and 51% side-chain conservation, as defined by the empirical substitution criteria of Bordo and Argos (49). In addition, the r.m.s.d. in Cα positions between human Nedd8 and the C-terminal domain of human ISG15 is 0.84 Å over 67 residues (Fig. 4C) compared with 1.0 Å between human Nedd8 and human ubiquitin (not shown). Therefore, it was a relatively straightforward procedure to overlay the structure for the C-terminal domain of ISG15 onto that of Nedd8 bound to AppBp1-Uba3 (29) to examine potential points of interaction for orthologous positions between Uba3 and Uba1 (Fig. 4, A and B). The area of the interface between the docked molecules for the ISG15 complex is 2077 Å² and is very similar to the corresponding interface area found in the structure of the complex between Nedd8 and AppBp1-Uba3 (2214 Å²). This docking model assumes that the global structural features in the overall interaction surfaces between ubiquitin-like proteins and their cognate activating enzymes are evolutionarily retained. This assumption appears to hold for both the structures for Nedd8 bound to AppBp1-Uba3 (29) and for Sumo bound to Sae1-Sae2 (50). The r.m.s.d. between the two known structures of the E1 paralogs, Uba3 (Nedd8-binding domain) and Sae2 (Sumo-binding domain), is 1.25 Å for 437 Cα atoms, strongly suggesting that the structure of the corresponding domain of UbE1L would also be very similar to those of Uba3 and Sae2.

Within the docked structure, the C-terminal ubiquitin-like domain of ISG15 makes extensive contacts with the cleft defining the adenylate active site of AppBp1-Uba3, whereas the N-terminal ubiquitin-like domain of ISG15 projects well away from the surface of the Uba3 subunit of the activating enzyme (Fig. 4, A and B). The fit of the C-terminal domain of ISG15 into the active site cleft of AppBp1-Uba3 is remarkably good, with few regions of overlap between the solvent-accessible surfaces of the two structures, providing confidence in the general veracity of the docked model. The fit also benefits from the absence of major insertions or deletions in human UbE1L within the sequence segments defining the adenylate active site of Uba3 (49); in addition, human UbE1L is 16.9% identical and 52.4% similar to human Uba3 over residues 406–1011 of human Uba1a. Three of these interactions with UbE1L, involving Arg153, Glu132, and Arg92 of ISG15, should be conserved between ubiquitin and Uba1a (Table III), based on sequence conservation between the interacting pairs. Four other interactions are predicted to be unique to ISG15 and, therefore, probably define the specificity of UbE1L for its cog-
Table III

| ISG15 | HsUbE1L | Ubiquitin | HsUbE1a |
|-------|---------|-----------|---------|
| Arg153 | Glu591/Arg592 | Arg72 | Glu626 |
| Glu132 | Glu593 | Glu51 | Glu946 |
| Arg92 | Glu608 | Lys11 | Glu642 |
| Ly90 | Glu661 | Thr41 | Lys531 |
| Trp123 | Phe505 | Arg32 | Tyr547 |
| Phe149 | Tyr501/Lys502 | His56 | Gly633 |
| Arg97 | Tyr591 | Lys56 | Phe533 |

The predicted interactions between ISG15 and UBE1L

The predicted interactions are based on the "docked" structure of ISG15 with human UbE1L generated by overlaying the ISG15 structure onto that of Nedd8 in the crystal structure of the complex between Nedd8 and human AppBp1-Uba3 (29), as described under "Materials and Methods."

The exposed linker region harbors the single cysteine residue located at position 78 (human ISG15 numbering). Our early experiments using non-reducing SDS-PAGE indicated that concentrated solutions of wild-type mature recombinant ISG15 readily formed a disulfide-linked dimer at neutral pH that subsequently denatured and precipitated from solution (not shown). Similar behavior had been noted previously with ISG15 isolated from natural sources (28, 61). Reversible urea denaturation studies allowed us to graphically measure the free energy of unfolding, ΔG_H2O, quantitatively illustrating that the wild-type mature ISG15 polypeptide is markedly unstable at 20 °C (Fig. 1). Mutation of Cys78 to serine, to maintain the overall polarity at this position, obviated disulfide formation and prevented precipitation of the protein upon concentration. Urea denaturation studies demonstrated that the mutation substantially stabilized the polypeptide as well (ΔΔG_H2O = -3.3 kcal/mol) (Fig. 1). The enhanced stability of this point mutant probably represents a lower limit to the actual effect of the Cys78 mutation, because the inherent insolubility of the oxidized dimer precluded our generating ISG15 quantitatively in this form for analysis in the urea unfolding experiments illustrated in Fig. 1. Instead, we relied on the spontaneous oxidation of wild type polypeptide in these unfolding experiments. The reversibility of the denaturation at low concentrations of DTT and the stabilization of the protein that accompanies mutation of Cys78 suggests a mechanism in which ISG15 first reversibly dimerizes prior to oxidation of Cys78 to yield a metastable disulfide-linked intermediate that is subject to irreversible denaturation in the absence of urea. The instability undoubtedly accounts for precipitation and loss of activity that accompanies concentrating wild-type ISG15 solutions noted earlier (13, 28, 61). Potentially, dimerization could occur through association of the hydrophobic region within the N-terminal domain of ISG15 isolated from natural sources (28, 61). Reversible urea denaturation allowed us to graphically measure the free energy of unfolding, ΔG_H2O, quantitatively illustrating that the wild-type mature ISG15 polypeptide is markedly unstable at 20 °C (Fig. 1). Mutation of Cys78 to serine, to maintain the overall polarity at this position, obviated disulfide formation and prevented precipitation of the protein upon concentration. Urea denaturation studies demonstrated that the mutation substantially stabilized the polypeptide as well (ΔΔG_H2O = -3.3 kcal/mol) (Fig. 1). The enhanced stability of this point mutant probably represents a lower limit to the actual effect of the Cys78 mutation, because the inherent insolubility of the oxidized dimer precluded our generating ISG15 quantitatively in this form for analysis in the urea unfolding experiments illustrated in Fig. 1. Instead, we relied on the spontaneous oxidation of wild type polypeptide in these unfolding experiments. The reversibility of the denaturation at low concentrations of DTT and the stabilization of the protein that accompanies mutation of Cys78 suggests a mechanism in which ISG15 first reversibly dimerizes prior to oxidation of Cys78 to yield a metastable disulfide-linked intermediate that is subject to irreversible denaturation in the absence of urea. The instability undoubtedly accounts for precipitation and loss of activity that accompanies concentrating wild-type ISG15 solutions noted earlier (13, 28, 61). Potentially, dimerization could occur through association of the hydrophobic region within the N-terminal domain of ISG15 that was noted in the charge density map of ISG15 (Fig. 3).

Neither mutation of Cys78 to serine to enhance the stability of the polypeptide nor the subsequent mutation of Asn13 to tyrosine to provide a surface-accessible site for radiiodination qualitatively affects the function of the polypeptide, because ISG15 undergoes ATP-dependent activation and thiolester formation with human recombinant UbE1L, the ISG15-activating enzyme (Fig. 1D) (10). Most important, the ability to protect the C terminus of mature ISG15 from proteolytic inactivation during expression and purification by employing an arginine cap facilitates future detailed mechanistic and functional studies of this ligation pathway.

Earlier sequence analysis accurately predicted the tandem β-grasp folds of ISG15; however, such analyses could not anticipate the potential packing interactions, if any, between the domains. The structure of human ISG15 reveals that the two domains interact largely through hydrophobic interactions between the first and fourth 310 helices (310-1 and 310-4, respectively) (Fig. 5). The interaction surface buries 7% (627 Å²) of the total solvent-accessible surface area (8957 Å²) and represents a considerable stabilizing force for maintaining the overall struct-
tecture depicted in Fig. 2. This suggests that the structure shown in Fig. 2 approximates the stable solution conformation of the polypeptide. This conclusion is consistent with sequence comparison among the known ISG15 orthologs and ubiquitin that show the first and fourth 310 helices are significantly more conserved among ISG15 orthologs than the second and third 310 helices that are solvent-exposed in the structure of Fig. 2 and do not interact (Fig. 5). In addition, the Glu139 residue that participates in a side-chain hydrogen bond with the main-chain amide of Phe41 is also conserved among ISG15 orthologs (Fig. 5).

The FAT10 polypeptide is the only other tandem domain Class 1 ubiquitin-like protein identified (52, 53). The sequences for the FAT10 family of ubiquitin-like proteins have areas of sequence similarity that are distinct from the ISG15 paralogs, particularly in the critical C-terminal sequences, suggesting that they serve different functions (Fig. 5). Induction of FAT10 by Type 2 interferon γ, whereas ISG15 is induced principally by Type 1 interferon α/β, supports a role for these proteins in different cellular processes (4, 52). The pattern of sequence conservation within the two putative β-grasp domains of FAT10, particularly among the key aliphatic residues (red dots), is consistent with the protein assuming a ubiquitin-like fold (Fig. 5). Conservation among the FAT10 sequence segments corresponding to the first and fourth 310 helices of ISG15 compared with the second and third 310 helices are consistent with FAT10 orthologs assuming the same overall domain pack as ISG15 (Fig. 5). We also note that the Glu139 residue that forms a hydrogen bond with the amide of Phe41 at the domain interface of ISG15 is conserved as Asp147 among the FAT10 orthologs (Fig. 5), consistent with this conclusion.

Finally, the overall conservation in fold between Nedd8 and the C-terminal domain of ISG15 allowed us, with some confidence, to model binding of ISG15 within the adenylate active site of the UbE1L-activating enzyme (Fig. 4). The model suggests that the C-terminal domain of ISG15 makes extensive contacts with UbE1L, whereas the amphipathic N-terminal domain (Fig. 3) remains solvent-exposed and not in contact with the enzyme (Fig. 4). This requires that conservation among ISG15 orthologs within the N-terminal domain serves functions distinct from those required for C-terminal activation. The docking model allowed us to identify potential residue critical for binding to and discrimination of ISG15 by UbE1L to the exclusion of other Type 1 ubiquitin-like paralogs, summarized in Table III. Several of these predicted ISG15 binding residues are conserved in ubiquitin (in parentheses), including Arg153 (Arg72), Glu132 (Glu53), and Arg42 (Lys41) (Table III). Notably, Arg72 of ubiquitin serves as a major specificity determinant that allows the ubiquitin (30, 32), Nedd8 (29, 55), and Sumo (50) activating enzymes to distinguish cognate from non-cognate polypeptides. Conservation of the Arg72 residue of ISG15 and ubiquitin, respectively, precludes a similar role in defining the specificity of UbE1L for ISG15. Instead, Lys69, Trp223, and Phe140 of ISG15 appear to provide unique interaction “hot spots” that may allow UbE1L to recognize its cognate substrate to the exclusion of ubiquitin and other paralogs (Table III). We are currently using kinetic approaches with point mutants of ISG15 and UbE1L quantitatively to assess the role of these putative interacting residues in defining the substrate specificity of UbE1L.

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