Precursor Processing of Pro-ISG15/UCRP, an Interferon-β-induced Ubiquitin-like Protein*

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Induction of the 17-kDa ubiquitin-like protein ISG15/UCRP and its subsequent conjugation to cellular targets is the earliest response to type I interferons. The polypeptide is synthesized as a precursor containing a carboxyl-terminal extension whose correct processing is required for subsequent ligation of the exposed mature carboxyl terminus. Recombinant pro-ISG15 is processed in extracts of human lung fibroblasts by a constitutive 100-kDa enzyme whose activity is unaffected by type I interferon stimulation. The processing enzyme has been purified to apparent homogeneity by a combination of ion exchange and hydrophobic chromatography and found to be stimulated 12-fold by micromolar concentrations of ubiquitin. Analysis of the products of pro-ISG15 processing enzyme demonstrates specific cleavage exclusively at the Gly157-Gly158 peptide bond to generate a mature ISG15 carboxyl terminus. Irreversible inhibition of pro-ISG15 processing activity by thiol-specific alkylating agents and a pH rate dependence conforming to titration of a single group of pH 8.1 indicate the 100-kDa enzyme is a thiol protease. Partial sequencing of a tryptic-derived peptide indicates the enzyme is either the human ortholog of yeast Ubp1 or a Ubp1-related protein. As yeast do not contain ISG15, these results suggest that a ubiquitin-specific enzyme was recruited for pro-ISG15/UCRP processing by adaptive divergence.

Type I interferons (interferons-α and -β) induce a specific subset of proteins responsible for mediating the antiviral and broad pleiotrophic effects of these cytokines, reviewed in Ref. 1. One of the earliest responses to type I interferons is the increased transcription of the ISG15 gene, which is controlled by a 5′ interferon-stimulated response element (1, 2). The sequence of the resulting 17.1-kDa ISG15 protein, also known as the ubiquitin cross-reactive protein (UCRP), is composed of two ubiquitin-like domains that retain the canonical LRLRGG sequence required for conjugation of ubiquitin to intracellular targets as the committed step for 26 S proteasome-dependent degradation (3–5). The biological effects of ISG15 are similarly mediated by its covalent conjugation to a small subset of cellular proteins through an enzyme pathway that is distinct from that of ubiquitin ligation (5, 6). Both free and conjugated ISG15 pools are significantly induced following exposure to interferon-α/β; however, in contrast to the homeostatic mechanism that maintains a constant ratio of free to conjugated ubiquitin (7), the two pools of ISG15 exhibit a characteristic biphasic induction during which free polypeptide increases early during the interferon response and then undergoes increased conjugation from 12 to 72 h (5). The ISG15 polypeptide acts in trans to noncovalently bind associated target proteins and model chimeric constructs to intermediate filaments; in contrast, free ISG15 shows only marginal affinity for binding to these cytoskeletal components (8). Neither free nor conjugated ubiquitin levels are affected by interferon treatment, indicating that up-regulation of ISG15 ligation is a specific and independently regulated response (4, 5).

ISG15 was the first example of a small class of ubiquitin-like proteins that includes SUMO-1, 3, Nedd8, and Ubl1 (reviewed in Refs. 9 and 10). These proteins exhibit significant sequence similarity to ubiquitin and, with the exception of ISG15, contain single ubiquitin domains (11). Although SUMO-1 and Nedd8 polypeptides are found throughout eukaryotes (9, 10), search of the complete yeast genome has identified no candidate ISG15 ortholog, indicating that this regulatory pathway represents a relatively recent functional divergence (5). Ubiquitin and ubiquitin-like proteins are synthesized as precursors bearing carboxyl-terminal extensions, which range in size from short polypeptides to complete protein domains (12). Expression of the carboxyl-terminal GTEPGGRS extension peptide of pro-ISG15 minimizes degradation of the nascent polypeptide, presumably through a kinetic folding effect (2). Although the function(s) of the unrelated extension peptides on newly synthesized chains of ubiquitin and the other ubiquitin-like proteins is unknown, their presence requires processing of the precursor protein to expose the carboxyl-terminal glycine required in isopeptide bond formation to their respective targets. In addition, correct processing is required for a novel extracellular cytokine function for ISG15 involving induction of CD56+ natural killer cell proliferation, augmentation of non-major histocompatibility complex-restricted cytotoxicity, and T-cell interferon-β induction (13). Processing of the pro-ISG15 carboxyl-terminal extension occurs rapidly without significant ac-

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The abbreviations used are: ISG15, interferon-β-stimulated gene (15 kDa); FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; UCH, ubiquitin carboxyl-terminal hydrolase; Ubp, ubiquitin-specific protease; UCRP, ubiquitin cross-reactive protein; DTT, dithiothreitol; HPLC, high performance liquid chromatography; TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, t-1-tosylamido-2-phenyl ethyl chloromethyl ketone; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; CAPS, 3-cyclohexylaminopropanesulfonic acid.

2 S. N. Twigger and A. L. Haas, submitted for publication.

3 Alternate names and orthologs of SUMO-1 include SMT3, GMPI, Sentrin, and PIC1.

4 The plant ortholog of Nedd8 is termed Rub1.

5 A. L. Haas, unpublished observations.
cumulation of the precursor (4, 14), as is generally characteristic for processing of the entire class.

A large family of enzymes has been described that act proteolytically at the carboxyl terminus of ubiquitin to generate the free polypeptide from fusion proteins, ubiquitin-protein conjugates, mult ubiquitin chains that serve as degradation signals for 26 S proteasome specificity, and small molecule adducts arising by reaction of cellular nucleophiles with activated intermediates of ubiquitin (15). These enzymes collectively belong to the family of ubiquitin carboxyl-terminal hydrolases (UCH), which have been arbitrarily divided into two subfamilies based on molecular weight, sequence homology, and leaving group specificity (15, 16). Family 1 is composed of low molecular mass (~30 kDa) enzymes, of which three tissue-specific mammalian isoforms (L1, L2, and L3) have been described (15, 17) that have related orthologs in yeast and Dro sophila (16). These enzymes are believed to function in generating mature ubiquitin from the precursor fusion proteins and in salvaging ubiquitin carboxyl-terminal adducts, based on observations that this class prefers small leaving groups or extended polypeptide chains (15). Family 2 encompasses the ubiquitin-specific proteases and the deubiquitinating enzymes, which exhibit considerable heterogeneity in size (50–300 kDa) and show little sequence homology except for conserved cysteine and histidine motifs containing the active site residues (16). Over 11 members of this family have been described in yeast with orthologs identified in mammals and Dro sophila (16). This family of ubiquitin proteases functions to release free ubiquitin from ubiquitin-protein conjugates and their polyubiquitin degradation signals (16). The large number of UCH enzymes suggests a wide variety of cellular functions and/or potential roles with other ubiquitin-like proteins.

In the present work, we identify two pro-ISG15 processing activities in cell extracts of the human lung carcinoma line A549. A minor activity (~1%) is probably a member of the UCH isozyme family, based on molecular weight and competitive inhibition by free ubiquitin. In contrast, free ubiquitin is a positive allosteric effector of the major activity, which shares features of family 2 with respect to size and apparent catalytic mechanism. Partial sequencing following purification of the latter processing enzyme to apparent homogeneity suggests this activity is either the human ortholog of the yeast family 2 processing enzyme Ubp1 or a Ubp1-like protein. These observations suggest that an existing ubiquitin-specific enzyme has been recruited for pro-ISG15 processing by adaptive divergence.

**MATERIALS AND METHODS**

Bovine ubiquitin was purchased from Sigma and purified to apparent homogeneity (18). Human recombinant interferon-β containing a C17s mutation to enhance stability was supplied by Trikon BioSciences.

**Recombinant Pro-ISG15 Expression and Purification—**Recombinant pro-ISG15 was expressed in Escherichia coli BL21 (DE3) cells harboring the pET11d-ISG17 plasmid. Purification of pro-ISG15 was carried out as described previously for mature recombinant ISG15 (6), with the exception that inclusion of CoCl2 during isolation was omitted inasmuch as carboxypeptidase inactivation by cleavage of the carboxyl terminal glycine dipeptide from ISG15 was precluded by the absence of the carboxyl-terminal extension peptide present on the precursor. The resulting recombinant pro-ISG15 was >99% pure by SDS-PAGE when visualized by silver staining. The concentration of pro-ISG15 was determined spectrophotometrically using the empirically determined ε280 nm = 0.79 mg/ml/cm for mature ISG15 (6) corrected for the molecular weight difference. Typical yields of pro-ISG15 were 2–3 mg/liter culture using a 19-mer reverse primer encompassing the T7 promoter of the pET11d vector and a 39-mer forward primer in which a tyrosine codon was engineered between the carboxyl-terminal serine of pro-ISG15 and the STOP codon followed by a BamHI restriction site. The resulting polymerase chain reaction product was digested with NcoI and BamHI, then ligated into NcoIBamHI-digested pET11d. After transformation into E. coli DH5α, colonies containing the mutant protein were isolated and the sequence was confirmed by the dideoxy method (19). Expression and purification of the pro-ISG15-Y mutant was identical to that for the wild type protein, yielding 2 mg/liter culture (A280 nm = 1.0) that was >99% pure by SDS-PAGE analysis and silver staining. The presence of the tyrosine residue in the purified mutant protein was confirmed by amino acid analysis by the Wisconsin College of Physicians and Nuclear Acid Shared Facility and laser desorption time of flight mass spectrometry. The absolute quantity of pro-ISG15-Y was determined spectrophotometrically using a calculated extinction coefficient of ε280 nm = 0.85 ml/mg/cm. Control studies demonstrated that pro-ISG15-Y was processed at the same rate as the wild type pro-ISG15, indicating no significant structural differences between the two proteins. This has been independently confirmed by the quantitatively similar circular dichroism spectra for mature and precursor forms of ISG15.

The mutant protein was radiolabeled with IODGEN (Pierce) as described previously (6), except that the iodination reaction was carried out for 15 min to preferentially label the additional carboxyl-terminal tyrosine residue rather than the two internal tyrosines present in ISG15. The radiolabeled activities ranged from 160 to 1600 cpm/pmol/2% of the total pro-ISG15-Y bearing the radiolabel. Based on the sequence and secondary structure similarities between ubiquitin and ISG15, the carboxyl terminus of pro-ISG15 is assumed to be solvent-exposed whereas the two internal tyrosine residues are buried and thus less accessible to radiiodination. Product analysis after the processing of 1-pro-ISG15-Y revealed no detectable signal present in mature ISG15, confirming that the label was exclusively at the carboxyl-terminal tyrosine.

**Activity Assay—**The pro-ISG15 processing activity was monitored either by a SDS-PAGE gel-shift assay exploiting the molecular weight difference between pro-ISG15 and the mature polypeptide or by the release of the radiolabeled trichloroacetic acid-soluble carboxyl-terminal extension peptide from 132I-pro-ISG15-Y. For the gel shift assay, reactions of 20 μl final volume containing 50 mM Tris-HCl (pH 7.6), 1 mM DTT, and 5–25 μM pro-ISG15 were incubated at 37 °C in the presence of A549 105 × g supernatant. At the indicated times, incubations were quenched by adding an equivalent volume of 2× Laemmli sample buffer containing 4% (v/v) β-mercaptoethanol and boiled for 5 min. Unprocessed pro-ISG15 and the mature ISG15 product were resolved by SDS-PAGE and then electrophoretically transferred to BA83 nitrocellulose (Schleicher & Schuell). Following sequential incubation of blots with affinity-purified anti-ISG15 or anti-ubiquitin antibodies (10 μg/ml) and 125I-pro-ISG15-Y revealed no detectable signal present in mature ISG15, confirming that the label was exclusively at the carboxyl-terminal tyrosine.

The addition of a tyrosine residue at the carboxyl terminus of pro-ISG15 allowed the processing reaction to be followed by the appearance of trichloroacetic acid-soluble radioactivity representing the cleaved peptide bearing the labeled tyrosine residue. Incubations were carried out as described for the gel shift assay but were quenched by addition of 0.2 ml each of 5 mg/ml carrier bovine serum albumin and 20% (v/v) ice-cold trichloroacetic acid. Quenched reactions were incubated on ice for 10 min and then centrifuged at 14,000 × g for 10 min. The supernatant was quantitatively transferred to another tube and the pellet washed with 0.2 ml of ice-cold 2% (v/v) trichloroacetic acid, which was combined with the supernatant. Both the pellet and the supernatant were counted. Radioactivity present in the supernatant was corrected for label present in a 0-min blank that generally represented <10% of the total radioactivity.

Subsequent studies to be described below demonstrated that the major pro-ISG15 processing activity exhibited a linear dependence on substrate concentration below 0.6 μM and was stimulated by the presence of free ubiquitin. Therefore, a unit of processing activity was defined as the quantity required to produce 1 pmol/min mature ISG15 at pH 7.5 and 37 °C in the presence of 5 μM pro-ISG15, 25 μM ubiquitin, and 1 mM DTT.

**Cell Culture—**Human A549 lung carcinoma cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 2 mM l-glutamine and 10% (v/v) fetal calf serum. Monolayer cultures were maintained in...
Processing of a Ubiquitin-like Protein

Pro-ISG15 Processing Activity Is Not Induced by Interferon-β—Incubation of recombinant pro-ISG15 with an A549 postribosomal supernatant obtained from uninduced confluent cultures results in the appearance of an anti-ISG15 immunoreactive band comigrated with authentic mature ISG15 when analyzed by Western blotting as described under “Materials and Methods” (Fig. 1). Endogenous free ISG15 present in the cell extract is not detected at the exposure time chosen for the autoradiogram of Fig. 1. An absolute rate for pro-ISG15 processing of 4.2 pmol/min/10⁶ cells was calculated by quantitating mature ISG15 associated ¹²⁵I radioactivity by counting and comparing to signal obtained from an authentic recombinant mature ISG15 standard (Fig. 1, left lane). That equivalent amounts of mature and precursor forms of ISG15 yield similar autoradiographic intensities (Fig. 1, left lanes) indicates that the presence of the carboxyl-terminal extension peptide does not significantly affect binding of the affinity purified rabbit polyclonal antibodies.

Similar pro-ISG15 processing is observed for A549 extracts harvested after 24 h of treatment in the presence of 10³ IU/ml interferon-β, a concentration previously shown to yield maximum rates of ISG15 induction (4) (Fig. 1). Elevated levels of endogenous mature ISG15 are evident in the blank sample (lanes B) obtained prior to addition of exogenous pro-ISG15 and in the 0-min lane quenched prior to addition of the substrate (Fig. 1). Quantitation of the absolute amounts of mature ISG15 in the interferon-β-induced incubations and correction for that introduced with the A549 extract yielded a linear rate for pro-ISG15 processing of 4.4 pmol/min/10⁶ cells. These results indicate that pro-ISG15 processing activity is constitutively present in uninduced cells and is not significantly affected by interferon treatment, confirming earlier qualitative results from pulse-chase experiments (3).

When incubations similar to those of Fig. 1 are extended to longer times, a progressive loss of pro-ISG15 and appearance of the mature product is observed when probed with anti-ISG15 antibody (Fig. 2A). Approximately 5% of the initial pro-ISG15 remained at the longest times examined (Fig. 2B). The residual unreacted pro-ISG15 was not due to inactivation of the processing activity inasmuch as no further loss of pro-ISG15 was observed when an additional aliquot of fresh A549 cell extract was added (data not shown). Inability of the processing enzyme to convert the remaining fraction of substrate likely represents denaturation of pro-ISG15, consistent with the complete absence of processing by fresh extracts in the presence of heat denatured pro-ISG15 (data not shown). Both the decline in pro-ISG15 and appearance of the mature product displayed rigorous first order kinetics over 10 half-lives (solid lines in Fig. 2B) for which first order rate constants of 0.53 h⁻¹ and 0.58 h⁻¹, respectively, were determined from nonlinear least-squares fitting of the data in Fig. 2B. Quantitation of the Western blot showed that appearance of mature ISG15 accounted for 78% of the pro-ISG15 lost, the remaining 22% likely resulting from incomplete degradation of the polypeptide by the extracellular products.

Identical samples from the upper panel of Fig. 2A were also probed with affinity-purified anti-ubiquitin antibodies, which displayed a reduced recognition for pro-ISG15 compared with the mature polypeptide. Previous work has shown that the anti-ubiquitin antibodies are directed against an epitope(s) in the carboxyl terminus of the polypeptide, accounting for the
Purification of the Pro-ISG15 Processing Activity—Resolution of the A549 post-ribosomal supernatant on a Mono Q HR10/10 anion exchange FPLC column results in two peaks of activity (Fig. 3). The smaller peak consistently represented a member of the UCH family 1; however, the size discrimination by SDS-PAGE of fractions spanning the 125I-pro-ISG15-Y processing activity revealed a single band of 100-kDa relative molecular mass by Coomassie Blue staining (Fig. 5A). Fig. 5B compares the initial rates of formation of mature ISG15 versus the quantity of 100-kDa protein determined densitometrically (arbitrary units). That the 100-kDa protein band corresponds to the pro-ISG15 processing activity is indicated by the excellent correspondence between activity and protein, confirmed by the constant relative specific activity across the protein peak (Fig. 5C). Fractions from the Phenyl-Sepharose activity peak were pooled and concentrated, then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT. The pooled Phenyl-Sepharose activity represented a 250-fold purification with a 65% yield relative to the post-ribosomal supernatant. Table I summarizes the three-step purification of the pro-ISG15 processing activity to apparent homogeneity.

Pro-ISG15 Processing Occurs at the Predicted Site—The data of Fig. 2 suggest processing of pro-ISG15 occurs at the correct site between glycine 157, representing the carboxyl terminus of the mature polypeptide, and glycine 158 of the carboxyl-terminal octapeptide extension; however, the size discrimination by SDS-PAGE was not adequate to confirm this cleavage position unambiguously. In a larger processing reaction similar to that of Fig. 2 but containing tracer levels of 125I-pro-ISG15-Y to monitor product formation and recovery, quantitation revealed that 2 nmol of total pro-ISG15 was processed in 30 min at 37 °C using 60 units of the partially purified enzyme from the Mono Q HR10/10 column. The trifluoroacetic acid-soluble supernatant of the A549 post-ribosomal supernatant on a Mono Q chromatography (data not shown). At higher concentrations of sample, the activity eluted at apparent molecular masses that were approximately multiples of the 100-kDa mass found at low protein concentrations, suggesting that the enzyme undergoes a weak concentration-dependent oligomerization.

We consistently observed a significant loss in total recovered activity when cytosolic A549 extracts were resolved by Mono Q chromatography. This was not due to instability of the activity, inasmuch as control samples not loaded onto the column retained complete activity. Nor was the loss the result of denaturing interactions with the column, inasmuch as all activity was recovered when the sample was loaded in high NaCl to block binding. This technical problem was resolved when we observed that 5 μM free ubiquitin significantly stimulated the processing activity (Fig. 4), in contrast to the expected competitive inhibition by free ubiquitin if the activity were a ubiquitin-specific enzyme. The significant loss in activity during Mono Q FPLC is explained by resolution of the processing activity from free endogenous ubiquitin, which appears in the unbound fraction at pH 7.5. In the gel shift assay of Fig. 4, approximately the same stimulation of pro-ISG15 processing is observed at 5 and 50 μM, indicating that the effect of ubiquitin is saturated at the lower concentration. The experiment does not distinguish whether ubiquitin acts as an allosteric activator of the processing enzyme or stabilizes ISG15 against proteolytic degradation in the extract. The latter alternative is less likely, as exogenous ISG15 was relatively stable in the cell extracts when followed by quantitative Western blotting; in addition, bovine serum albumin had no effect on the rate of pro-ISG15 processing, suggesting a specific effect of free ubiquitin (Fig. 4).

Fractions encompassing peak 2 were pooled and adjusted to 1 mM ammonium sulfate then applied to a 2.5 × 36-cm preparative Phenyl-Sepharose Fast Flow column equilibrated with 25 mM sodium phosphate buffer (pH 7.5) containing 1 mM ammonium sulfate and 1 mM DTT. Bound proteins were eluted from the column with a 1–0 M negative linear ammonium sulfate gradient (−5.5 mM/ml) at a flow rate of 2 ml/min. The 125I-pro-ISG15-Y processing activity eluted as a single symmetric peak at 0.22 M ammonium sulfate. Resolution by SDS-PAGE of fractions spanning the 125I-pro-ISG15-Y processing activity revealed a single band of 100-kDa relative molecular mass by Coomassie Blue staining (Fig. 5A). Fig. 5B compares the initial rates for formation of mature ISG15 versus the quantity of 100-kDa protein determined densitometrically (arbitrary units). That the 100-kDa protein band corresponds to the pro-ISG15 processing activity is indicated by the excellent correspondence between activity and protein, confirmed by the constant relative specific activity across the protein peak (Fig. 5C). Fractions from the Phenyl-Sepharose activity peak were pooled and concentrated, then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT. The pooled Phenyl-Sepharose activity represented a 250-fold purification with a 65% yield relative to the post-ribosomal supernatant. Table I summarizes the three-step purification of the pro-ISG15 processing activity to apparent homogeneity.

Fig. 2. Time course for pro-ISG15 processing. A reaction of 250 μl final volume containing 1.5 mg of A549 105 × g supernatant and 5 μM recombinant pro-ISG15 was incubated at 37 °C as described in the legend to Fig. 1. At the indicated times, 25-μl aliquots were removed and analyzed by Western blotting as described under “Materials and Methods.” Panel A, replicate blots were probed with either affinity-purified anti-ISG15 or anti-ubiquitin (anti-Ub) antibodies. The left two lanes contain 10 pmol of either recombinant pro-ISG15 or mature ISG15, respectively. Panel B, bands corresponding to either pro-ISG15 (open circles) or mature ISG15 (closed circles) were excised from the anti-ISG15-stained blots in panel A and the associated radioactivity quantitated by γ counting. Absolute amounts of the precursor and product were determined by comparing the associated radioactivity to a series of standards resolved in parallel. Solid lines represent theoretical first order plots based on non-linear least-squares fitting of the data as described in the text.
Fig. 3. Mono Q anion exchange resolution of two pro-ISG15 processing activities. Fifteen mg of a supernatant from A549 cells were resolved by Mono Q HPLC anion exchange chromatography as described under “Materials and Methods.” Processing activity (dotted line) was monitored by a Western blot gel shift assay similar to that of Fig. 2 stained with anti-ubiquitin antibody to improve the signal to noise ratio.

Fig. 4. Free ubiquitin stimulates pro-ISG15 processing. Incubations similar to those of Fig. 1 were conducted with $10^5 \times g$ supernatant in the absence or presence of $5 \mu M$ recombinant pro-ISG15 for the indicated times. Incubations additionally contained the indicated concentrations of free ubiquitin (Ub) or $50 \mu M$ bovine serum albumin (BSA). Incubations were resolved by 16% SDS-PAGE and analyzed by Western blot using anti-ubiquitin antibody. As in Fig. 2, the anti-ubiquitin probed underestimates the amount of pro-ISG15 relative to the mature product of processing but improves the signal to noise ratio for the poorly resolved bands. Migration positions for pro-ISG15, ISG15, and ubiquitin are shown to the left.

tant from this incubation was resolved by C18 reverse phase chromatography, as described under “Materials and Methods.” Control reactions conducted in the absence of either processing enzyme (Fig. 6A) or pro-ISG15/$^{125}$I-pro-ISG15-Y (data not shown) yielded comparable HPLC elution profiles. Quantitation of the acid-soluble radioactivity indicated complete recovery of the 2 nmol of processed extension peptide, half of which was applied to the HPLC column. The resulting elution profiled showed a new peak at 23 min (Fig. 6B). The peak of radioactivity eluted slightly earlier than the new absorbance peak at 23 min (data not shown), presumably due to differences in binding between unlabeled and iodopeptide. The absolute amount of $^{125}$I-nonapeptide present was below the limit of detection in trace B.

That the new peak eluting at 23 min was the expected octapeptide product was demonstrated by coelution with 1 nmol of synthetic unlabeled octapeptide (Fig. 6C). In addition to retention of elution position (Fig. 6, insets), the integrated peak area in panel C agreed well with that expected for the sum of processed peptide and exogenous standard. In addition, the predicted molecular mass for the HPLC-purified peptide peak was confirmed by mass spectrometry (data not shown). Therefore, the 100-kDa processing enzyme catalyzes the specific cleavage of the extension peptide to yield mature ISG15, confirmed by the ability of the resulting ISG15 to support ubiquitin activating enzyme-catalyzed ATP:PPi exchange (data not shown).

Characterization of the Processing Enzyme—The kinetics of the purified enzyme was quantitated by following the processing of radiolabeled pro-ISG15-Y. Preliminary isotope dilution experiments confirmed that $^{125}$I-pro-ISG15-Y was identical to wild type pro-ISG15 in rates of processing (data not shown). At pH 7.5 the initial velocity for $^{125}$I-pro-ISG15-Y processing was linear to 0.6 m M polypeptide, the highest concentration tested (data not shown), which sets a lower limit for $K_m$ of $\sim 2$ m M based on the sensitivity of the assay to detect deviations from linearity with respect to substrate concentration that would be associated with saturable binding. Addition of 50 m M ubiquitin increased $V_m/K_m$ 12-fold ($5.9 \times 10^{-4} \text{ min}^{-1}$) without altering the linearity of the concentration dependence (data not shown). In the presence of 25 m M each of $^{125}$I-ISG15-Y and ubiquitin, the initial rate of processing measured by the formation of trichloroacetic acid-soluble radioactivity increased nonlinearly from pH 6 to 9 (Fig. 7, closed circles) and then decreased abruptly at higher pH values (Fig. 7, open circles). The sharp decrease in initial rate above pH 9 likely results from alkaline denaturation of the processing enzyme inasmuch as the loss of activity was irreversible (data not shown). Below pH 9, the pH dependence for initial rates of processing could be best fit to a single titration of $pK_a$ 8.1 using a non-linear curve fitting algorithm (GraFit, Erithacus Software, Ltd.). Similar pH dependence for processing was observed in the absence of added ubiquitin (data not shown), indicating that the $pK_a$ 8.1 titration observed in Fig. 7 was not contributed by the polypeptide.

The effect of selected protease inhibitors on $^{125}$I-pro-ISG15-Y processing was determined at pH 7.5 (37 °C) by measuring the pseudo first order rate constants for inactivation. With the exception of EDTA and EGTA, all other inhibitors listed in Table II exhibited first order kinetics for the quantitative inactivation of processing activity when corrected for the slow rate of spontaneous inactivation in the absence of inhibitors. Iodoacetic acid, iodoacetamide, and TLCK exhibited the greatest rates for the irreversible inactivation of $^{125}$I-pro-ISG15-Y processing (Table II). Differences in reactivities among the thiol-selective alkylating reagents probably reflect steric constraints and the active site microenvironment. In contrast, the serine protease-selective inhibitor PMSF exhibited an ~20-fold lower rate constant for inactivation relative to iodoacetamide. The relative difference in rates of reaction between the thiols and serine-selective inhibitors suggests the processing enzyme is a thiol protease, although the data do not rule out modification of an essential but noncatalytic sulfhydryl group. Measurable inhibition was also exhibited by the chymotrypsin and trypsin inhibitors TPCK and TLCK, respectively, suggesting the processing enzyme has a reactive nucleophile that reacts with the carbonyl groups present on both inhibitors. The $-2$ fold greater effect of TLCK may reflect the lysyl residue of the inhibitor mimicking arginine 155 of pro-ISG15, similar to the inhibition of ubiquitin activating enzyme by TLCK.$^5$ Neither EDTA or EGTA had any measurable effect on processing activity at concentrations of 10 m M, suggesting the absence of a metal requirement for octapeptide cleavage.

Microsequencing of the Pro-ISG15 Processing Enzyme—We
Fig. 5. Phenyl-Sepharose resolution of the pro-ISG15 processing enzyme. Fractions containing peak 2 from the Mono Q HR 5/10 anion exchange column were pooled and resolved by Phenyl-Sepharose Fast Flow hydrophobic chromatography as described under “Materials and Methods.” Panel A, aliquots of each fraction encompassing the peak of processing activity were resolved by SDS-PAGE on 10% (w/v) gels and visualized by Coomassie Blue staining to reveal a single band of 100 kDa. Panel B, enzyme activity (solid circles) is plotted versus the band density in arbitrary units (open circles) for the 100-kDa Coomassie-stained band of panel A. Panel C, plot of relative specific activity across the peak of 100-kDa processing activity.

Table I

| Step | Protein | Units | Specific activity | Yield | Purification |
|------|---------|-------|-----------------|-------|--------------|
|      | mg | units/mg | %  | fold |
| $10^3 \times g$ supernatant | 660 | 10,700 | 16 | 100 | 1 |
| Mono Q FPLC | 5.6 | 8,150 | 1,454 | 76 | 91 |
| Phenyl-Superose | 1.8 | 7,000 | 3,989 | 65 | 250 |

*One unit of activity equals the processing of pro-ISG15 at a rate of 1 pmol/min in 50 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 25 µM ubiquitin, and 5 µM pro-ISG15 at 37 °C.

were unable directly to sequence the apparently homogeneous pro-ISG15 processing enzyme from Fig. 5, presumably due to a blocked amino terminus; however, sequencing from trypsin-digested immobilized protein following SDS-PAGE resolution, as described under “Materials and Methods,” was successful. One well resolved major peak from reverse phase resolution of the resulting trypsin-digested peptides was chosen for partial sequencing. Although the amount of peptide (1.4 pmol) was near the limit of detection, unambiguous peaks could be assigned for 8 of 11 cycles (0.8 pmol signal in final cycle). Comparison to the GenBank and SwissProt data bases yielded significant homology to only two proteins: the 100-kDa Saccharomyces cerevisiae Ubp1 and the 33-kDa predicted product of human clone KIAA0161 (GenBank accession no. D79983) (Fig. 8). As processing activity correlated with the 100-kDa polypeptide band identified by SDS-PAGE in Fig. 5, the predicted molecular mass of the KIAA0161 gene product precludes it as the candidate processing enzyme in the absence of possible sequencing artifacts. In contrast, agreement between the partial sequence of the pro-ISG15 processing enzyme and Ubp1 is consistent with the relative molecular mass of 100 kDa (Fig. 5); the pH dependence for processing, which suggests the observed pKₐ 8.1 corresponds to the active site cysteine present in Ubp1 (Fig. 7); and the inhibitor profile, which also suggests the activity is a cysteine protease (Table II). The partial peptide sequence of the processing enzyme corresponds to an inserted segment within the His box motif common to all class II ubiquitin-specific proteases that is unique to yeast Ubp1 (Fig. 8). Therefore, the pro-ISG15 processing enzyme is either the human ortholog of yeast Ubp1 or a Ubp1-related protein.

DISCUSSION

The constitutive and interferon-β-induced biological effects of ISG15 require correct processing of the octapeptide extension from the precursor protein in order to expose the mature carboxyl terminus required for subsequent conjugation to intracellular targets and for extracellular cytokine activity (5, 13). Processing of carboxyl-terminal extensions from nascent precursors is a requirement shared by all ubiquitin and ubiquitin-like proteins identified to date (11), although the enzyme(s) responsible for such activation has not been identified. We were initially interested in distinguishing whether pro-ISG15 processing occurred through existing ubiquitin-specific enzyme(s), as an alternate substrate, or through a pro-ISG15-specific enzyme activity.

The present studies reveal two pro-ISG15 processing activities in A549 cell extracts (Fig. 3), both of which appear to be ubiquitin-specific enzymes for which pro-ISG15 serves as an alternate substrate. A minor component, representing 10% of total recovered activity after Mono Q anion exchange chromatography, likely results from the action of a UCH. This conclusion is supported by the native molecular mass of 31 kDa determined by gel filtration chromatography and competitive inhibition of pro-ISG15 processing by exogenous free ubiquitin. However, the activity is not human UCH-L3 as an authentic recombinant sample of this enzyme failed to process pro-ISG15 yet rapidly cleaved tyrosine 77 from a 125I-UbY77 construct (data not shown). Instead, our attention focused on the major component, comprising 90% of the total recovered activity following Mono Q chromatography (Fig. 3). Although the latter activity exhibited trace inhibition at low ubiquitin concentrations (data not shown), the effect was masked by the marked stimulation of processing activity by free ubiquitin at physiological concentrations (Fig. 4). Stimulation of pro-ISG15 processing by free ubiquitin accounted for the marked loss of activity during Mono Q FPLC chromatography; in addition, when activity was adjusted for the effect of ubiquitin, this enzyme accounts for greater than 99% of total pro-ISG15 processing activity in A549 extracts.

The major processing activity was purified to apparent homogeneity and quantitatively correlated with a protein band of 100 kDa (Fig. 5). Partial microsequencing of this enzyme yielded a peptide sequence showing significant similarity to S. cerevisiae Ubp1 (GenBank accession no. M63484) when physical and catalytic characteristics of the family 2 ubiquitin hydrolase are compared with those of the pro-ISG15 processing enzyme. The peptide sequence obtained in Fig. 8 corresponds to...
an insert within the otherwise conserved His box of the family 2 ubiquitin-specific proteases that is unique to yeast Ubp1 (21); therefore, it is probable that the pro-ISG15 processing enzyme corresponds to the human ortholog of yeast Ubp1 or a Ubp1-related enzyme. This assignment is supported by the molecular weight correlation between Ubp1 and the processing enzyme, a pH rate profile corresponding to titration of a single group of $pK_a$ 8.1 presumably corresponding to the active site cysteine of the ubiquitin-specific proteases (Fig. 7), and sensitivity to inactivation by thiol selective alkylating reagents (Table II).

Ubiquitin-specific proteases also contain a conserved histidine that serves as a general base catalyst to extract the proton from the nucleophilic cysteine during the catalytic cycle (16). Preliminary experiments show that pro-ISG15 processing is blocked by preincubation with diethylpyrocarbonate (data not shown), a histidine-specific modifying reagent. Although the properties of the pro-ISG15 processing enzyme resemble those of another family 2 isopeptidase, isopeptidase T (22–24), an authentic sample of isopeptidase T was unable to process 125I-pro-ISG15 even though it effectively cleaved free lysine 48-linked polyubiquitin chains (data not shown). This observation, together with the absence of the partial sequence (Fig. 8) within isopeptidase T, precludes this ubiquitin-specific protease as the processing enzyme.

Yeast Ubp1 is a non-essential enzyme for which null mutants exhibit a decreased rate of ubiquitin/26 S proteasome-dependent degradation of $\beta$-galactosidase polyubiquitinated conjugates (21). As ISG15 is absent from yeast, the normal role for Ubp1 must be as an isopeptidase involved in polyubiquitin chain metabolism. Interestingly, yeast Ubp1 is not active against pro-ISG15 because the latter is not processed in cell-free yeast extracts or when transiently expressed from an appropriate plasmid. This suggests that, if the processing enzyme is the human ortholog of yeast Ubp1, activity against pro-ISG15 must represent an adaptive divergence in specificity to accommodate a low affinity alternate substrate. Such adaptation of an existing activity may reflect the relatively recent emergence of ISG15-dependent regulation compared with the more ancient systems of SUMO-1 and NEDD8. Interestingly, Li and Hochstrasser (25) have very recently shown that processing of the SUMO-1 precursor in yeast occurs through a
specific enzyme apparently not required in the ubiquitin system. This interpretation is consistent with the low affinity of the processing enzyme for pro-ISG15 revealed by the linear concentration dependence for the latter.

Although substrate recognition by the processing enzyme is of relatively low affinity, it must require specific interactions because denatured pro-ISG15 is not a substrate (Fig. 2 and Footnote 5). This conclusion is also supported by the observation that only one major activity, when adjusted for the stimulatory effect of ubiquitin, is active with pro-ISG15 despite the large number of constitutive UCH and Ubp isozymes present in this and other eukaryotic cell lines (16). The rate of ISG15 synthesis in interferon-β-induced A549 cells can be estimated as 0.02 pmol/min per 10^6 cells based on earlier steady state quantitation of total ISG15 accumulation after 10 h of induction (10 pmol/10^6 cells) (5), a cell volume of 4 μl/10^6 cells (26), and the reasonable assumption that ISG15 accumulation is linear. Therefore, the rate of pro-ISG15 processing in A549 extracts from Fig. 1 is >100-fold faster than the estimated rate of pro-ISG15 synthesis, consistent with the absence of pro-ISG15 accumulation within intact cells (3, 4).

Several lines of evidence indicate that the processing enzyme cleaves the precursor at the correct Gly^{157}-Gly^{158} bond to generate active mature polypeptide and the carboxyl-terminal octapeptide extension. The product of this reaction, when resolved by SDS-PAGE, results in migration of ISG15 with a relative molecular weight identical to an authentic mature polypeptide (Figs. 1, 2, and 4). More important, the putative mature ISG15 product is capable of supporting ubiquitin activating enzyme-catalyzed ATP:PPi exchange (data not shown), unlike des-Gly-Gly-ISG15 in which the carboxyl-terminal glycine dipeptide is absent (6). In addition, the carboxyl-terminal extension peptide released during incubation with purified processing enzyme coelutes with an authentic synthetic peptide by C18 reverse phase HPLC (Fig. 6) and corresponds to the expected product by mass spectrometry (data not shown).

The most unexpected property of the pro-ISG15 processing enzyme is its marked stimulation by free ubiquitin (Fig. 4). Similar stimulation of a ubiquitin-specific protease by free ubiquitin has been observed for isopeptidase T, a 100-kDa family 2 ubiquitin-specific protease involved in the disassembly of free polyubiquitin chains released following degradation of the target protein by the 26 S proteasome (22–24). Detailed kinetic studies of isopeptidase T show that the effect of free ubiquitin is in stabilizing a catalytically competent conformation of the enzyme (23, 24). It is possible that an analogous conformational effect accounts for the stimulation of pro-ISG15 processing, and we are currently expanding the kinetic analysis of pro-ISG15 processing to examine this question.

The present studies are the first to identify the cellular processing activity responsible for generating active ISG15 from the carboxyl-terminal blocked precursor. Identification of the pro-ISG15 processing enzyme as the human ortholog of yeast Ubp1 or a Ubp1-like protein indicates that with the emergence of the ISG15 regulatory pathway, the critical step of precursor processing was assumed by an existing ubiquitin-specific enzyme, perhaps by selective mutation of selected residues to accommodate the new low affinity alternate substrate.

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REFERENCES
1. Kalvakolanu, D. V., and Borden, E. C. (1996) Cancer Invest. 14, 25–53
2. Reich, N., Evans, B., Levy, D., Fahey, D., Knight, E., and Darnell, J. E. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6394–6398
3. Korant, B. D., Blomstrom, D. C., Jenak, G. J., and Knight, E., Jr. (1984) J. Biol. Chem. 259, 14835–14839
4. Haas, A. L., Ahrens, P., Bright, P. M., and Ankel, H. (1987) J. Biol. Chem. 262, 11315–11323
5. Loeb, K. R., and Haas, A. L. (1992) J. Biol. Chem. 267, 7806–7813
6. Narasimhan, J., Potter, J. L., and Haas, A. L. (1996) J. Biol. Chem. 271, 324–330
7. Haas, A. L., and Bright, P. M. (1987) J. Biol. Chem. 262, 345–351
8. Loeb, K. R., and Haas, A. L. (1984) Mol. Cell. Biol. 14, 8408–8419
9. Saitoh, H., Pu, R. T., and Dasso, M. (1997) Trends Biochem. Sci. 22, 374–376
10. Hochstrasser, M. (1996) Genes Dev. 12, 901–907
11. Haas, A. L., and Siepmann, T. J. (1997) FASEB J. 11, 1257–1268
12. Jentsch, S., Seufert, W., and Hauser, H. P. (1991) Biochim. Biophys. Acta 1089, 127–139
13. D’Cunha, J., Knight, E. J., Haas, A. L., Truitt, R. L., and Borden, E. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 211–215
14. Blomstrom, D. C., Fahey, D., Kutny, R., Korant, B. D., and Knight, E., Jr. (1986) J. Biol. Chem. 261, 8811–8816
15. Larsen, C. N., Krantz, B. A., and Wilkinson, K. D. (1998) Biochemistry 37, 3358–3368
16. Wilkinson, K. D. (1997) FASEB J. 11, 1245–1256
17. Pickart, C. M., and Rose, I. A. (1985) J. Biol. Chem. 260, 7903–7910
18. Baboshina, O. V., and Haas, A. L. (1996) J. Biol. Chem. 271, 2823–2831
19. Sanger, M. L., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
20. Haas, A. L., and Bright, P. M. (1985) J. Biol. Chem. 260, 12464–12473
21. Tobias, J. W., and Varshavsky, A. (1991) J. Biol. Chem. 266, 12021–12028
22. Hadari, T., Warms, J. V., Rose, I. A., and Hershko, A. (1992) J. Biol. Chem. 267, 719–727
23. Stein, R. L., Chen, Z. J., and Melandri, F. (1995) Biochemistry 34, 12616–12623
24. Wilkinson, K. D., Tashayev, V. L., O’connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995) Biochemistry 34, 14535–14546
25. Li, S.-J., and Hochstrasser, M. (1999) Nature 398, 246–251
26. Mitraka, B. M., and Rawlins, J. P. (1981) Clinical Biochemical and Hematological Reference Values in Normal Experimental Animals, Masson, New York