A 15-kDa Interferon-induced Protein Is Derived by COOH-terminal Processing of a 17-kDa Precursor*

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An interferon-induced 15-kDa protein is synthesized from a precursor of higher molecular weight; the precursor contains 165 amino acids (17 kDa), whereas the stable product (15 kDa) contains 156 amino acids. The stable 15-kDa form is derived from the precursor 17-kDa form by the removal of eight amino acids from the COOH terminus and the methionine from the NH2 terminus. The existence of the precursor 17-kDa protein can be demonstrated after brief periods of in vivo labeling with [35S]methionine and by translation of mRNA in vitro.

An interferon-induced 15-kDa protein has been purified from Daudi cells, and its DNA has been characterized. The molecular weight calculated for the deduced protein was 15,815, in good agreement with the 15,000 estimated from SDS-PAGE (1). Amino acid sequencing of protein-derived peptides confirmed more than 80% of the DNA sequence, but because no peptide from the COOH-terminal region was recovered, confirmation of the cDNA in this region was not possible. Recently, the sequence for the genomic DNA of the 15-kDa protein has been determined (2). This work indicates that the stop codon comes immediately after amino acid 165 rather than after 145, resulting in a deduced protein 20 amino acids longer than originally reported (1). A re-evaluation of the cDNA nucleotide sequence also places the stop codon after position 165. A summation of the molecular weights of the 165 amino acids yields a protein (designated 17 kDa) of 17,890, which differs significantly from 15,000 estimated from SDS-PAGE for the protein isolated from Daudi cells. Either the isolated protein has the 165 amino acids specified by the DNA but runs anomalously fast in SDS-PAGE, or the initially translated 17-kDa protein is subsequently processed to the smaller 15-kDa protein. Sequencing of the NH2 terminus of the protein and its NH2-terminal peptides showed that the well known post-translational removal of the Met-1 had occurred (3). However, more extensive NH2-terminal processing seemed unlikely, because no NH2-terminal signal peptide sequence is predicted by either the cDNA (1) or genomic DNA (2). Hence, we chose to investigate the possibility of post-translational COOH-terminal processing. We report here that the 15-kDa protein isolated from IFN-treated cells is synthesized as a 17-kDa precursor and is rapidly converted to the 15-kDa form by removal of eight amino acids from the COOH terminus and the NH2-terminal methionine.

EXPERIMENTAL PROCEDURES

Materials—All reagents and materials were of the highest quality available and were purchased from standard suppliers. IFN-β specific activity 2 × 106 units/mg, was prepared as described (4).

Cyanogen Bromide Cleavage of the 15-kDa Protein—A solution of 2.17 nmol of reversed-phase HPLC-purified 15-kDa protein (5) in 0.10 ml of 70% formic acid was treated with 5 mg of cyanogen bromide for 24 h in the dark at 25 °C. The solution was evaporated to dryness twice in vacuo; the residue was dissolved in 10% formic acid, and a portion was fractionated by reversed-phase HPLC on a 4.5 × 50-mm Vydac C4 column equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a gradient: of acetonitrile, 0-60% (60 min), 0.5 ml/min, 25 °C, and were detected by monitoring at 220 nm.

Amino Acid Sequencing and FAB Mass Spectroscopy—Amino acid sequencing was performed with an Applied Biosystems gas phase sequenator, model 470. FAB mass spectroscopy was performed using a JEOL HX100HF double focusing magnetic sector mass spectrometer scanning the mass range 100–1500.

Cell Culture—Daudi cells were grown as previously described (5). Human osteosarcomas, human WISH, and human diploid fibroblast cells were grown in minimal essential medium supplemented with 10% fetal calf serum.

In Vivo and In Vitro Labeling of Proteins—For long-term labeling (3 h) of Daudi cells, 5 × 106 cells, untreated cells, or IFN-β-treated cells (50 units/ml, 16 h) were washed once with RPMI 1640 growth medium minus methionine containing 10% dialyzed fetal calf serum and resuspended in 2 ml of the same medium containing 250 μCi/ml [35S]methionine, 7400 Ci/mmol. After 3 h cytoplasmic extracts were prepared as previously described (6). Human diploid fibroblast, human WISH, and human osteosarcoma cells in plastic culture flasks were treated with 50 units/ml IFN-β for 16 h, washed once with minimal essential medium minus methionine containing 10% dialyzed fetal calf serum, and the proteins were labeled for 3 h in 3 ml of the same medium containing 100 μCi/ml [35S]methionine. Cytoplasmic extracts were prepared as described above for Daudi cells after detaching the cells by scraping. For short-term labeling of Daudi proteins 1 × 106 cells were treated with IFN-β, 100 units/ml, for 16 h, washed twice with RPMI 1640 growth medium minus methionine, resuspended in the same medium at 1.5 × 106 cells/200 μl, and incubated for 10 min at 37 °C. To initiate labeling, 125 μCi of [35S]methionine was added to each 200 μl of cells. The 200-μl aliquots were incubated at 37 °C for designated time periods, and the labeling was terminated by adding each 200 μl of cells to 10 ml of ice-cold phosphate-buffered saline. For a chase, 800 μl of cells were labeled as above for 5 min, then 100 μl of 10 μM methionine was added, and the 800 μl of cells were diluted to 15 ml with RPMI 1640 growth medium containing 15% fetal calf serum and incubated at 37 °C for the designated chase times. To terminate the chase, 5 ml of the labeled cells were added to 10 ml of ice-cold phosphate-buffered saline. Cytoplasmic extracts were prepared as described above and used immediately for immunoprecipitation or stored at −70 °C. Proteins were labeled in vitro by translating poly(A⁺) RNA from IFN-treated cells in rabbit reticulocyte and wheat germ lysate systems. RNA was prepared by the guanidinium isothiocyanate-cesium chloride method (6), and poly(A⁺) RNA was prepared from total RNA by chromatography on oligo(dT) (7). Each reaction mixture in 50-μl total volume contained either rabbit reticulocyte or wheat germ lysate, 5 μg of

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poly(A)* RNA from IFN-β-treated Daudi cells, and 50 μCi of [35S]methionine. Chases were initiated by adding 5 μl of 10 mM methionine. Protein synthesis was terminated by adding SDS to 0.1% and placing the sample on ice. [35S]Methionine-labeled proteins were then analyzed by immunoprecipitation and SDS-PAGE.

Immunoprecipitation—A polyclonal antisera to homogeneous 15-kDa protein was raised in a New Zealand White rabbit. Four μg of purified 15-kDa protein was initially injected intradermally in complete Freund’s adjuvant. Subsequently, 4 μg was injected at day 21 and at day 36; 20 μg was injected at day 50 and at day 61, all in incomplete Freund’s adjuvant. For immunoprecipitations [35S]methionine-labeled cytoplasm from 1 × 10^6 cells was added to 200 μl of antibody buffer (0.5% Triton X-100, 0.15 M NaCl, 0.005 M EDTA, 0.002% NaN3, 0.05 M Tris-HCl, pH 7.4, 1 mg/ml bovine serum albumin) followed by 20 μl of Protein A-Sepharose. After 5 min at 4°C the Protein A-Sepharose was removed by centrifugation and discarded. To the supernatant 0.1-1.0 μl of antiserum was added. After 30-60 min at 4°C, 20 μl of Protein A-Sepharose was added, and the mixture was left at 4°C for 30 min with periodic mixing. The Protein A-Sepharose was washed three times with antibody buffer and once with 0.01 M Tris-HCl, pH 7.4. Proteins were eluted from the Protein A-Sepharose by boiling for 2 min in loading gel buffer containing 2% SDS and 2% β-mercaptoethanol, analyzed by SDS-PAGE on slab gels using the buffer system of Laemmli (8), and then visualized by autoradiography.

RESULTS

Since the molecular weight of the IFN-induced protein predicted from the genomic and cDNAs (17,890) did not agree with that of the purified protein estimated by SDS-PAGE (about 15,000), we suspected that post-translational modification to reduce the mass from 17 to 15 kDa might be occurring. We excluded amino-terminal processing from consideration, since the cDNA and genomic DNA do not predict a signal sequence prior to the NH2 terminus of the 15-kDa protein (1, 2). Furthermore, the NH2-terminal sequence predicted from the DNA is the same as that found for the protein with the exception that the protein does not contain the initial methionine (1). Another possible mechanism for reducing the molecular weight, although not as frequently studied as NH2-terminal processing, is COOH-terminal processing, i.e. removal of COOH-terminal amino acids post translationally. It therefore became essential to determine if the COOH-terminal amino acid sequence of the protein is different from that predicted from the genomic and cDNAs. We used two techniques to determine the carboxyl terminus of the 15-kDa protein: (a) amino acid sequencing of the peptides generated by cyanogen bromide cleavage; and (b) fast atom bombardment mass spectrometry (FAB-MS) to determine the molecular mass of the cyanogen bromide-generated peptides. Fig. 1B shows the amino acid sequence of the 15-kDa protein deduced from the genomic and cDNAs and confirmed by amino acid sequencing of peptides constituting 80% of the protein (1). This revised sequence contains 20 more amino acids at the COOH terminus than deduced originally from the cDNA (1). Cyanogen bromide cleavage at the methionines should generate four peptides with the COOH-terminal peptide containing 15 amino acids if there is no processing. Cyanogen bromide cleavage was performed; the peptides were separated by reversed phase HPLC (Fig. 1A) and subjected to FAB-MS analysis. Peak fraction 1 showed a protonated molecular ion [(M+H)+] of 785 daltons, which is identical to that calculated for a COOH-terminal peptide beginning at Asn-151 and ending with Gly-157 (Fig. 1C). Amino acid sequencing of this peptide gave the sequence: Asn-Leu-Arg-Leu-Arg-Gly-Gly. This confirms that the 15-kDa protein isolated from IFN-induced cells ends with glycine and contains 156 amino acids (157 minus Met-1). Since the isolated 15-kDa protein contains nine amino acids fewer than the 165 predicted from the DNA, it became of interest to determine if a larger precursor could be detected. In order to detect the 15-kDa protein with maximum sensitivity, a polyclonal antiserum was prepared in a rabbit against a polyclonal antiserum to homogeneous 15-kDa protein.

![Fig. 1. Analysis of the COOH terminus of the 15-kDa protein. A, reversed phase HPLC separation of cyanogen bromide peptides. Conditions are described under "Experimental Procedures." Peak 1 is the COOH-terminal peptide. B, amino acid sequence of the 15-kDa protein deduced from the genomic and cDNA. Methionine cleavage sites for cyanogen bromide are designated MET. The COOH terminus of the 15-kDa protein is designated by an arrow. C, molecular ion mass and amino acid sequence of the cyanogen bromide-generated COOH-terminal peptide, HPLC peak 1.](image)

![Fig. 2. SDS-PAGE analysis of immunoprecipitated 15-kDa protein from extracts of human cell cytoplasts. Lane 1, control Daudi cells; lane 2, IFN-β-treated Daudi cells; lanes 3–5, IFN-β-treated diploid fibroblast, WISH, and osteosarcoma cells, respectively.](image)
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**Fig. 3. In vivo and in vitro synthesis of a 17-kDa precursor protein.** A, SDS-PAGE analysis of immunoprecipitated protein after a pulse-chase experiment with IFN-β-treated Daudi cells. Lanes 1, 2, 3, and 4, 0.5, 1, 2, and 5 min of [35S]methionine pulse, respectively. Lanes 5–8, pulse-chase of [35S]methionine in IFN-β-treated Daudi cells; lane 5, 5-min pulse only; lanes 6, 7, and 8, 5-min pulse then 5, 10, and 20 min of chase, respectively. B, in vitro pulse-chase experiment in rabbit reticulocyte and wheat germ lysate. Lanes 1, 2, 3, poly(A) RNA from IFN-β-treated diploid fibroblast cells, rabbit reticulocyte lystate; lane 1, 5-min [35S]methionine pulse; lane 2, 5-min pulse, 60-min chase; lane 3, 5-min pulse, 120-min chase. Lanes 5 and 6, poly(A) RNA from IFN-β-treated Daudi cells, wheat germ extract; lane 5, 5-min pulse, 60-min chase; lane 6, 5-min pulse, 115-min chase. Lane 7, 15-kDa protein from IFN-β-treated Daudi cells, 3-h [35S]methionine pulse. Lanes 8–12, poly(A) RNA from IFN-β-treated Daudi cells, rabbit reticulocyte lystate; lane 8, 5-min pulse only; lanes 9–12, 5-min pulse and then 55-, 115-, 240-, and 360-min chase, respectively.

an excess of nonradioactive methionine. The 17-kDa protein disappeared after 5 min of the chase (Fig. 3A, lanes 6–8), whereas the 15-kDa protein increased and then became constant after 5–10 min of the chase. These data indicate that the 15-kDa protein is first synthesized as a 17-kDa precursor that is rapidly converted to the stable 15-kDa form. The 17-kDa protein is, we believe, the 165-amino acid protein predicted from the DNA, and the stable 15-kDa protein is the result of the removal of eight amino acids from the COOH terminus and one methionine from the NH₂ terminus of the 17-kDa precursor. The relative timing of these two events has not been determined.

Further evidence for a 17-kDa precursor was obtained from in vitro translation of mRNA from IFN-β-treated Daudi cells. Fig. 3B shows that only the 17-kDa protein is synthesized in 5 min by the rabbit reticulocyte system. The 15-kDa protein appears after a 25-min chase, increases after a 55-min chase, and remains stable thereafter (Fig. 3B, lanes 8–12). The 17-kDa precursor, however, disappears after a 55-min chase. The same result was obtained when mRNA from IFN-β-treated diploid fibroblast cells was translated by the rabbit reticulocyte system (Fig. 3B, lanes 1–3). Both results demonstrate that the reticulocyte system can synthesize and process the 17-kDa protein. Although the wheat germ system also synthesizes the 17-kDa protein, this system cannot process it (Fig. 3B, lanes 4–6).

**DISCUSSION**

The results of the in vivo pulse-chase and in vitro synthesis experiments, taken together with the data showing that the 15-kDa protein isolated from Daudi cells has eight fewer amino acids at its COOH terminus than predicted from the DNA, strongly suggest that the 15-kDa protein is derived by COOH-terminal processing of a 17-kDa precursor. COOH-terminal processing of proteins has only recently been reported (9), and its biological role is still unclear. It has been suggested that COOH-terminal sequences may serve as a signal for the sorting of proteins within a cell, possibly within the lysosome (9, 10). Furthermore, a specific COOH-terminal sequence, Lys–Asp–Glu–Leu, has been shown to be involved in the accumulation of proteins in the lumen of the endoplasmic reticulum (11). The role of COOH-terminal processing of the 17-kDa protein is unknown, because the biological function of the 15-kDa protein remains unknown. Recently an interesting sequence homology has been found between the 15-kDa protein and ubiquitin (12). When the sequences of two ubiquitin molecules are aligned head to tail along the 156-amino acid sequence of the 15-kDa protein, several regions of similarity are evident. Furthermore, the same authors report isolation of an IFN-induced ubiquitin cross-reactive protein whose mass is 15 kDa (12). They suggest that this similarity and cross-reactivity indicate that the 15-kDa protein is a functionally distinct isofrom of ubiquitin. Further studies will be needed to substantiate this interesting hypothesis.

Our results demonstrate that the 15-kDa protein is derived from a 17-kDa precursor by COOH-terminal processing and removal of the NH₂-terminal methionine. This processing alone is probably responsible for the apparent mass change of 2000 estimated by SDS-PAGE. To investigate the biological role of the 15-kDa protein and the enzymes involved in the COOH-terminal processing, larger amounts of the 15- and 17-kDa proteins will be needed than are currently available from cell culture. To accomplish these goals, we have expressed the 17-kDa precursor in Escherichia coli and are modifying its cDNA so as to obtain expression of the 15-kDa protein. Both proteins will then be purified for use in biological studies.

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