

Biologic activity in a fragment of recombinant human interferon α

(thermolysin/molecular sizes/partial amino acid sequence/antiviral activity)

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Communicated by Christian B. Anfinsen, November 2, 1983

ABSTRACT To attempt to locate functionally important regions of the interferon (IFN) molecule, recombinant human IFN- α_2 was subjected to proteolytic digestion. The bacterial proteinase thermolysin produced two major complementary fragments, HuIFN- α_2 -(1-110) and HuIFN- α_2 -(111-153). After reduction with 2-mercaptoethanol and separation of the two major fragments on NaDodSO₄/polyacrylamide gel electrophoresis, antiviral activity persisted in the larger, M_r 12,000, fragment consisting of the amino-terminal 110 amino acids.

The availability of large amounts of recombinant DNA-derived interferon (IFN) has fostered investigation of IFN's structure-function relationships; however, the specific structural features required for biologic activity remain largely unknown. Despite disulfide bond assignment (1), secondary structure measurements (2), and investigation of novel recombinant IFNs, (3, 4), little information is available as to which areas of the protein may be functionally important. Modification of the structure of IFNs by using a variety of enzymes and chemicals has yielded varied results. Incubation of IFNs with glycosidic enzymes in general does not appear to alter the antiviral activity (5). Cleavage of IFNs with endopeptidases such as trypsin or with chemicals such as cyanogen bromide has thus far been unsuccessful in generating active fragments (6, 7). In contrast, some active species were obtained after treatment with exopeptidases (8), pepsin (9), and periodate (10); however, reductions in size were modest with the active species of M_r 15,000 or larger. To attempt to identify small functional portions of the IFN molecule we have studied proteolytic fragments of a recombinant human IFN, HuIFN- α_2 , produced by the bacterial proteinase thermolysin. We report here that proteolysis of this IFN with thermolysin generates a biologically active fragment originating from the amino-terminal end of the molecule.

MATERIALS AND METHODS

Thermolysin was obtained from Calbiochem. HuIFN- α_2 (1.5×10^8 units/mg of protein) was the gift of Schering. HuIFN- α_A (2.0×10^8 units/mg of protein) was the gift of Hoffmann-La Roche. ¹²⁵I-labeled HuIFN- α_A (¹²⁵I-HuIFN- α_A) was prepared as described (11).

Preparative NaDodSO₄/polyacrylamide gel electrophoresis (NaDodSO₄/PAGE) was performed according to the method of Laemmli (12). The separating gels were 16% acrylamide/*N,N'*-methylene bisacrylamide, 0.75 mm in thickness and 12 cm in length. Electrophoresis was performed at 15 mA per slab gel. The gels were stained and destained as described (13). The stained gel bands were sliced from the gel and eluted as reported (13). The samples were then assayed for biological activity or dialyzed and lyophilized for

amino acid analysis and amino acid sequence determination as described (14). Continuous elution of IFN fragments was done by the method of Hunkapiller (unpublished data).

IFN antiviral assays were performed by using Madin-Darby bovine kidney (MDBK) cells or human foreskin fibroblasts with vesicular stomatitis virus as reported (15). Competitive receptor binding assays using ¹²⁵I-HuIFN- α_A and MDBK cells were performed as described (11).

RESULTS AND DISCUSSION

The effects of thermolysin on the antiviral and receptor binding activities of IFN are shown in Fig. 1. HuIFN- α_2 (0.4 mg/ml) was exposed to thermolysin (0.012 mg/ml) at 37°C in 0.1 M Tris-HCl buffer with 1 mM CaCl₂ at pH 8.0. After 1, 2, and 4 hr, aliquots were removed for antiviral and competitive binding assays. Half of each sample was reduced with 2-mercaptoethanol, and both reduced and unreduced portions were tested for antiviral activity and ability to compete for binding to the IFN receptor. As indicated by the NaDodSO₄/polyacrylamide gel (Fig. 1 *Inset*, left portion), digestion over 4 hr resulted in a small reduction of the molecular weight of unreduced IFN. No remaining native IFN band could be identified in the 4-hr digest; calculations based on the minimal amount of IFN visible on gels (0.3 μ g) and on the amount of sample applied (8-10 μ g) indicated that at least 96% of the starting material was cleaved. In spite of this, no change was seen in either the antiviral activity or binding activity (Fig. 1). These results are compatible with those of Wetzel *et al.*, who reported that thermolysin cleaves the carboxyl-terminal 13 amino acids without loss of biological activity (6).

Reduction of thermolysin-digested IFN with 2-mercaptoethanol prior to electrophoresis showed (Fig. 1 *Inset*, right portion) that this single major enzymatic product contained two predominant disulfide-linked fragments, which we have designated "A" and "B." Reduced digest was assayed for antiviral and receptor binding affinity. After 4 hr of proteolysis, $\approx 30\%$ of the antiviral activity (0.5 log₁₀ unit) and $\approx 80\%$ of the binding activity of reduced undigested IFN remained. These activities were not due to residual intact IFN because, as noted above, digestion was at least 96% complete. Rather, they reflected biological activity in one or more of the reduced IFN fragments. When the antiviral effects of the reduced fragments were tested with human foreskin fibroblasts as the target cell, only 12% of the activity remained after proteolysis (data not shown). The greater loss in this system is compatible with prior studies indicating that human foreskin fibroblasts may be more sensitive than bovine kidney cells to modifications of the IFN molecule (18).

To attempt definitive identification of the two fragments, digestion and NaDodSO₄/PAGE were performed on a preparative scale. Each of the two major fragments (Fig. 2) was

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Abbreviations: IFN, interferon; MDBK, Madin-Darby bovine kidney.

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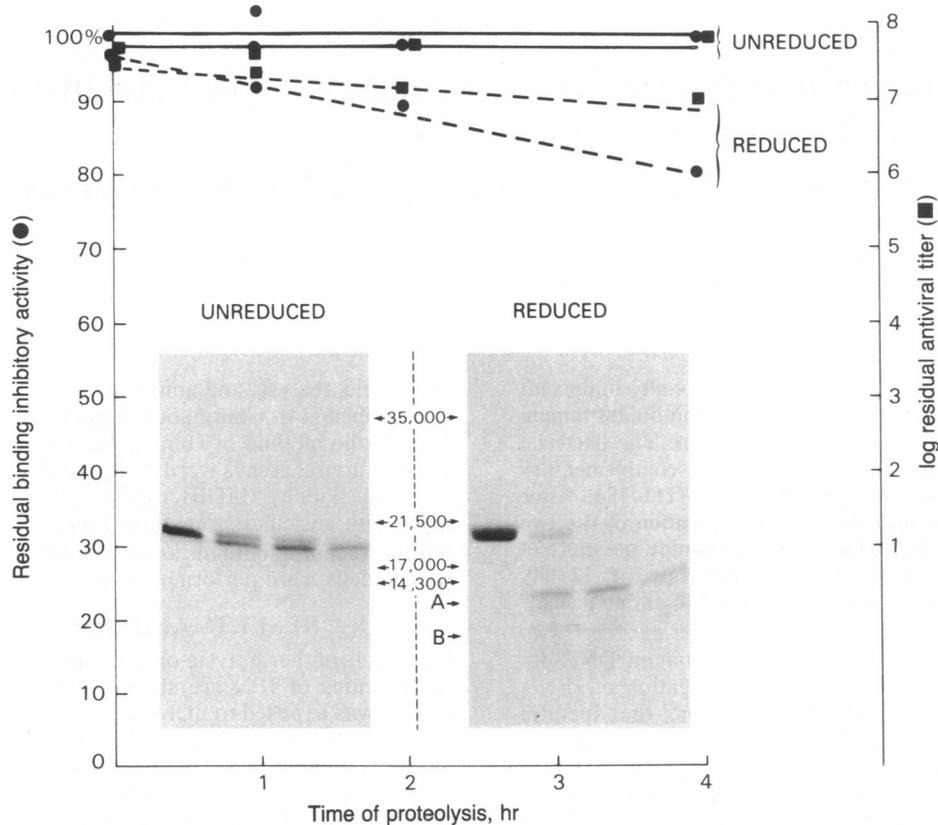


FIG. 1. Effect of thermolysin treatment on antiviral and receptor binding activities of IFN. (*Inset*) Effect of thermolysin on NaDodSO₄/polyacrylamide gel pattern of unreduced (four left lanes) and reduced (four right lanes) IFN. HuIFN- α_2 (0.4 mg/ml) was incubated at 37°C with 0.012 mg of thermolysin per ml in 0.1 M Tris-HCl with 1 mM CaCl₂, at pH 8 (reaction volume, 100 μ l) (R. Wetzel, personal communication). After 1, 2, and 4 hr, aliquots were removed and divided into two portions, one assayed without further treatment for antiviral and competitive receptor binding activities and one reduced by treatment with 10% 2-mercaptoethanol at 0°C for 1 hr prior to assay. For receptor studies, confluent monolayers of MDBK cells were exposed to ¹²⁵I-HuIFN- α A at 0.01 μ g/ml for 2½ hr at 4°C in the presence of unlabeled IFN or proteolytic fractions. Binding inhibitory activity was computed as: [cpm with ¹²⁵I-HuIFN- α A alone - cpm with ¹²⁵I-HuIFN- α A plus digested HuIFN- α_2]/[cpm with ¹²⁵I-HuIFN- α A alone - cpm with ¹²⁵I-HuIFN- α A plus excess native HuIFN- α A]. For each point, the final concentration of digested IFN was 0.2 μ g/ml and the excess unlabeled IFN was 0.65 μ g/ml. When inhibitory activity was close to 100%, studies were repeated with a dilution (0.02 μ g/ml) of the digest to confirm the sensitivity of the binding curve to changes in digest concentration. HuIFN- α A differs from HuIFN- α_2 by one amino acid at position 23 (16, 17).

eluted from the gel, dialyzed, lyophilized, and then subjected to sequence analysis through the first 20 residues using gas-phase sequence analysis (14). Each eluted band contained an apparent single sequence. The larger fragment corresponded to the amino-terminal portion of the intact molecule. The sequence of the smaller fragment began with the leucine at position 111. Thus, the major proteolytic cleavages are between residues 110 to 111 and 153 to 154. Molecular weights for fragments A and B calculated from the amino acid sequence of IFN- α_2 are 12,490 and 5,420, respectively.

To determine which fragment showed biologic activity, IFN was digested, reduced with 2-mercaptoethanol, and applied to a NaDodSO₄/polyacrylamide gel system allowing continuous elution of material from the bottom of the gel during electrophoresis (unpublished data). Approximately 35 fractions were collected in this way. Aliquots of each fraction were run on gradient analytical gels to determine the presence and molecular weights of eluted fragments, and another portion of each fraction was assayed for antiviral activity. Apparent molecular weights estimated from the mobilities in this system were 9,000–10,000 and 6,000 for the two major fragments. As shown in Fig. 3, activity is identified in the fractions corresponding to uncleaved IFN and also in fragment A, the larger of the two major proteolytic fragments corresponding to the amino terminus of the IFN molecule. Based on an average recovery of protein of 50% in this system (unpublished observations), a minimal specific activ-

ity of 4×10^6 units/mg for the fragment was calculated. A similar calculation applied to the uncleaved IFN peak recov-

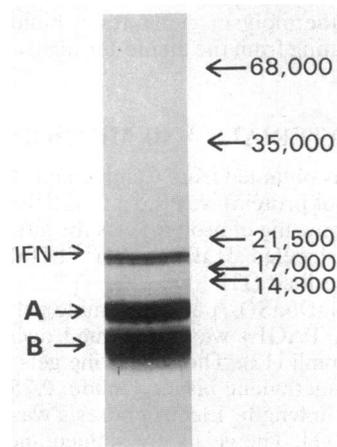


FIG. 2. Preparative NaDodSO₄/PAGE of IFN fragments. HuIFN- α_2 (4.0 mg/ml) was incubated at 37°C with 0.12 mg of thermolysin per ml (reaction volume, 100 μ l). Under these conditions, $\approx 90\%$ digestion required only 30 min of incubation. Aliquots were then reduced with electrophoresis solubilizing buffer containing 3% 2-mercaptoethanol and applied to 16% polyacrylamide gels containing 0.1% NaDodSO₄.

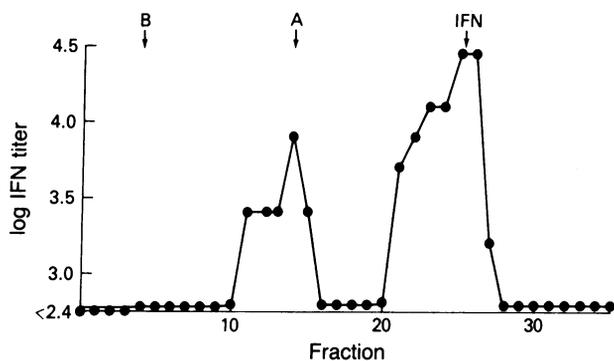


FIG. 3. Continuous elution of IFN fragments from a NaDodSO₄/polyacrylamide gel. IFN was digested as in Fig. 2 (reaction volume, 86 μ l); then 20 μ g was applied to the continuous gel elution system. Fractions of 100 μ l were collected continuously from the bottom of the gel during electrophoresis. Positions of IFN and of fragments A and B were determined by subsequent analytical NaDodSO₄/PAGE of aliquots of each sample.

ered from the gel gave a specific activity of 10^8 units/mg, which is comparable to that of the starting material, 1.5×10^8 units/mg. In addition to its antiviral effect, the amino-terminal fragment also inhibited growth of Daudi cells (result not shown). In further experiments, we have eluted active fragments from stained polyacrylamide gels, according to procedures devised for intact IFN (13). Using this method, we have detected antiviral activity in fragment A and, in some experiments, in the smaller fragment as well. It is our present belief that optimal and stable antiviral activity of the fragment(s) will depend critically on isolation conditions, including appropriate disposition of disulfide bonds (1).

The orientation of IFN on its cell surface receptor has been investigated in many laboratories using several approaches, including recombinant DNA techniques, monoclonal antibodies against IFN, and the generation of fragments by chemical synthesis or proteolytic cleavage. HuIFN- α s, with deletions of the first 15 amino-terminal amino acid residues and the last 10–13 carboxyl-terminal amino acid residues, have been shown to exhibit virtually the same antiviral activity as the parent molecules (3, 6, 19, 20). Studies with a monoclonal antibody, which recognizes the last 16 carboxyl-terminal amino acid residues of HuIFN- α A, have confirmed that the carboxyl-terminal region of the HuIFN- α A molecule is not involved in interaction with the receptor, for this antibody is capable of binding to HuIFN- α A bound to its receptor (21, 22). Studies with recombinant DNA-derived HuIFN- α A and HuIFN- α D and their hybrid derivatives have shown that the amino-terminal portion of the HuIFN- α s appears to influence the antiviral activity on human cells, whereas their carboxyl-terminal portion appears to influence their activity on mouse cells (3, 23, 24). Of interest, both the parent and hybrid molecules appear to elicit the antiviral response equally well on bovine kidney cells (3, 23, 24). Therefore, it appears the bovine receptor for HuIFN- α can accommodate more structural variants than either the human or mouse HuIFN- α receptors and thus may have the ability to recognize multiple sequences or conformations of the HuIFN- α s. Our studies are consistent with this proposal, for HuIFN- α (1–110) in general exhibits greater antiviral activity on bovine cells than human cells.

Availability of an active fragment of IFN will hopefully

facilitate studies on a variety of questions related to the mechanism of IFN action. For example, although previous attempts to chemically synthesize active fragments have been unsuccessful (21, 25), knowledge of a smaller peptide with activity may help to target areas of the molecule favorable for synthesis. Also, the roles of different areas of the molecule in IFN's various cellular effects may be investigated.

We thank Dr. N.-Y. Nguyen for amino acid analysis and Dr. R. Wetzel for helpful discussion.

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