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Cell Biology

Interferon: Purification and initial characterization from human diploid cells

(antiviral state/glycoprotein/molecular size/human diploid fibroblasts)

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ABSTRACT **Interferon produced by human diploid fibroblast cells in culture has been purified approximately 5000-fold. The purified interferon, when analyzed by electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate, contains only one polypeptide component of 20,000 molecular weight. The interferon activity comigrates with this polypeptide, indicating identity of the activity and the polypeptide. Oxidation of this polypeptide with periodic acid and subsequent staining with Fuchsin base indicates that it contains carbohydrate and suggests that the human fibroblast interferon is a glycoprotein.**

Interferon, a broad-spectrum antiviral protein, has been the subject of numerous biological studies, yet its chemical characterization from any source has remained elusive (see refs. 1 and 2 for reviews). Human leukocyte (3) and human diploid fibroblast (3–5) interferons have been purified by various techniques. This paper reports (*i*) a procedure for purifying human diploid fibroblast interferon and (*ii*) some chemical and physical properties of the purified interferon.

MATERIALS AND METHODS

Production of Crude Interferon. The human diploid fibroblast cells (neonatal foreskin fibroblasts, FS-4) were a gift from Drs. E. Havell and J. Vilcek. The procedures for growing the cells and producing the interferon have been described (6). For production of interferon, cells were grown at 37° in glass roller bottles 20 cm long and 9.5 cm internal diameter. Induction of interferon with poly(I)·poly(C) was performed exactly as previously described (6). Briefly, cell cultures 10–12 days old, 4×10^7 cells per bottle, were induced, then allowed to produce interferon at 34° for 20 hr in serum-free medium, 50 ml per bottle. Crude interferon was collected and stored at 4° until a sufficient quantity was accumulated for purification. Titers of crude interferon varied from 4000 to 20,000 units/ml. The crude interferon was concentrated 10- to 20-fold prior to purification, as described for mouse L cell interferon (7). Interferon was assayed on FS-4 cells with vesicular stomatitis virus as the challenge virus (6). All units are expressed as reference units.

Polyacrylamide Gel Electrophoresis. Preparative electrophoresis was performed in cylindrical separating gels, 1 cm × 20 cm, using the discontinuous buffer system of Laemmli (8) with sodium dodecyl sulfate ($NaDodSO_4$). Stacking gels 2 cm in length of 5% acrylamide were cast on top of the separating gel. The separating gel was an exponential gradient gel (7, 9) of 9–18% acrylamide. The gradient was prepared by mixing 20 ml of 9% acrylamide with 3 ml of 18% acrylamide kept at constant volume, and the ratio of acrylamide to bis-acrylamide was 38:1. All gels and buffers contained 0.1% $NaDodSO_4$. Analytical slab gels, 9–18% acrylamide, were prepared as described (7, 9). Protein in the gels was fixed and stained for 15 min in a solution of methanol:acetic acid:water 50:10:40 (vol/vol/vol) containing 1.25 mg/ml of Coomassie blue R 250. Gels were destained in 7% acetic–5% methanol and dried under reduced pressure without heat. Glycoproteins were stained with a periodic acid-Schiff base (PAS) procedure (10). Stained gels were scanned with a Joyce Loebl microdensitometer Mark IIICS. All analytical gels contained lysozyme, chymotrypsinogen, and ovalbumin as polypeptide standards. The gels to be stained for carbohydrate contained fetuin in addition.

Protein concentrations were determined by the method of Lowry *et al.* (11) or at concentrations of less than 20 μg/ml with a direct spectral method at 190 nm (12) with bovine serum albumin as the standard. The absorbance at 280 nm was used to estimate protein in the P-150 column fractions.

RESULTS

Fractionation with ammonium sulfate

Concentrated crude interferon was brought to pH 2.0 by the addition of 6 M HCl and left overnight at 4° without loss in activity. Solid ammonium sulfate was added at pH 2.0 and 4° to give a 25% saturated solution. The large precipitate was removed by centrifugation and discarded. Ammonium sulfate was added to 55% saturation; the precipitate was collected by centrifugation and dissolved in 25–30 ml of 0.05 M potassium phosphate, pH 6.0. After dialysis against the same buffer, this fraction contained 80–90% of the recoverable interferon activity and varied from 50% to 70% of the total initial units.

Fractionation on Bio-Gel P-150

The interferon in the 25–55% fraction was concentrated by precipitation at pH 6.0 by the addition of solid ammonium sulfate to 90% saturation at 4°. The precipitate was collected by centrifugation and dissolved in 2–3 ml of 0.05 M potassium phosphate, pH 6.0, 0.15 M NaCl, 15% glycerol (column buffer). The interferon was loaded onto a P-150 column at 4° and eluted with column buffer. Fig. 1 shows the elution profiles of the protein and the interferon activity.

Fractionation on carboxymethyl-Sephadex

The pooled P-150 fractions were dialyzed for 24 hr at 4° against 20 volumes of 0.05 M potassium phosphate, pH 6.0, 15% glycerol, then fractionated on a column of carboxymethyl-Sephadex. Approximately 90–95% of the added interferon was bound to the column at pH 6.0, whereas 90% of the protein flowed unimpeded through the column at pH 6.0. The interferon was eluted by increasing the pH stepwise

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid-Schiff base.

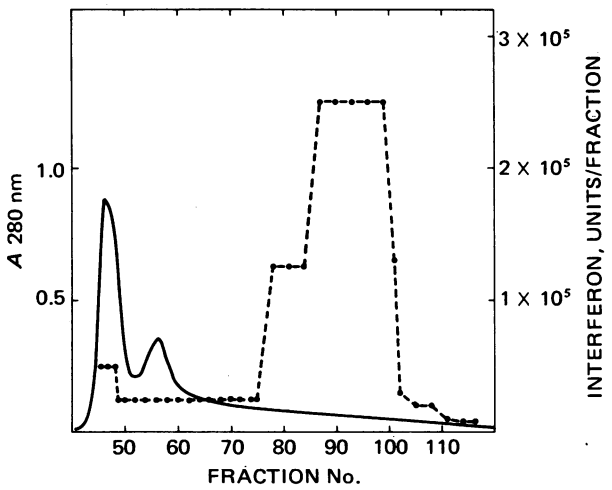

FIG. 1. Fractionations on Bio-Gel P-150. The ammonium sulfate interferon in 2–3 ml of 0.05 M potassium phosphate, pH 6.0, 0.15 M NaCl, 15% glycerol (P-150 buffer) was applied to a Bio-Gel P-150 column, 2.2 × 100 cm, and eluted at 4° at a rate of 2 ml/hr with the same buffer. Two-milliliter fractions were collected. Fractions 85–105 were pooled for fractionation on carboxymethyl-Sephadex.

to pH 8.0. The elution of the interferon from the carboxymethyl-Sephadex column is shown in Table 1.

Fractionation by gel electrophoresis

The interferon eluted from carboxymethyl-Sephadex was made 1% in $NaDodSO_4$ and left at 37° for 1 hr. This interferon was dialyzed for 24 hr at 25° against 100 volumes of 0.01 M Tris·HCl, pH 6.8, 0.05% $NaDodSO_4$, then concentrated by lyophilization to a dry residue. The residue was dissolved in 5% glycerol–H_2O, 1/10 the volume before lyophilization. The interferon was heated at 37° for 1 hr, then loaded onto a cylindrical gel for electrophoresis. After electrophoresis, the gel was fractionated and the interferon eluted from the gel and assayed. The activity profile after elution is shown in Fig. 2.

Table 1. Elution of human fibroblast interferon from carboxymethyl-Sephadex

Fraction	Total units	pH
Original P-150	7.0×10^6	6.0
pH 6 runoff	3.0×10^5	6.0
1	1.0×10^4	6.1
2	20×10^5	6.6
3	15×10^5	7.6
4	5×10^5	7.8
5	1×10^5	7.9
6	1×10^5	7.9
7	4×10^5	9.4
8	1×10^5	9.6
9	1×10^5	9.7
10	1×10^5	9.9

A carboxymethyl-Sephadex column, 1 cm × 3.5 cm, was equilibrated with 0.05 M potassium phosphate, pH 6.0, 15% glycerol at 4°. P-150 fractions 85–105 were passed through the column and the column was washed with 10 ml of the same buffer. The interferon was eluted with 0.1 M potassium phosphate, pH 8.0, 15% glycerol. One-milliliter fractions were collected at a rate of 10 ml/hr. After 6 ml of this buffer, the column was eluted with 0.1 M potassium phosphate, pH 10.0, 15% glycerol. Fractions 2–7 were pooled for fractionation by electrophoresis.

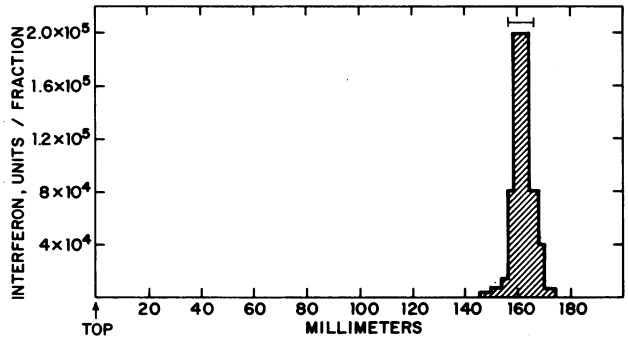

FIG. 2. Preparative polyacrylamide gel electrophoresis. The pooled concentrated fractions of interferon from the carboxymethyl-Sephadex column (Table 1) in 0.5–1.0 ml of 0.1 M Tris·HCl, pH 6.8, 1% $NaDodSO_4$ were loaded onto the stacking gel of a 1 × 20 cm separating gel and subjected to electrophoresis at 25° for 18 hr at 4 mA/gel constant current. The gel was fractionated into 2-mm sections by slicing, and each section was kept at 37° for 24 hr in 1 ml of 0.005 M Tris·HCl, pH 6.8, 0.05% $NaDodSO_4$ to elute the interferon. If the $NaDodSO_4$ concentration is 0.001% or less, there is no effect of $NaDodSO_4$ on the cells or the interferon assay. Peak fractions under the bar were analyzed for polypeptide and glycoprotein content.

A summary of the purification of human fibroblast interferon is shown in Table 2. The interferon, after preparative electrophoresis, is estimated to have a minimum of 2×10^8 units/mg. The yield of original activity has varied from 5% to 15% with three preparations.

Analysis of purified interferon

The peak fractions of interferon activity from the preparative gel (Fig. 2) were pooled, concentrated by lyophilization, and analyzed by electrophoresis for polypeptide components. Analysis was performed on polyacrylamide slab gels containing $NaDodSO_4$. The polypeptides in the interferon fraction from the preparative gel (Fig. 2) are shown in Fig. 3. The interferon from the preparative gel contains only one band, with a mobility between that of lysozyme and chymotrypsinogen (Fig. 3). Additional interferon on an adjacent track of the same gel was eluted after electrophoresis and the activity located. The interferon activity profile is shown in panel A of Fig. 4. Panel B of Fig. 4 shows a densitometer scan of the Coomassie blue stained polypeptide in Fig. 3, and it can be seen that the interferon activity and the polypeptide comigrate.

To determine if the interferon polypeptide contained carbohydrate, we subjected the same interferon to electropho-

Table 2. Purification of human fibroblast interferon

Fraction	Total units ($\times 10^{-6}$)	Total protein mg	Specific activity units/mg	% Recovery
Crude	40	1500	2.7×10^4	100
Ammonium sulfate 25–55%	15	32	3.2×10^5	38
Bio-Gel P-150	7	3.5	2.0×10^6	19
Carboxymethyl-Sephadex	5.0	0.50	1.0×10^7	13
Gel electrophoresis	3	0.020	2×10^8	8

Yield from 190 roller bottles, 8×10^9 cells.

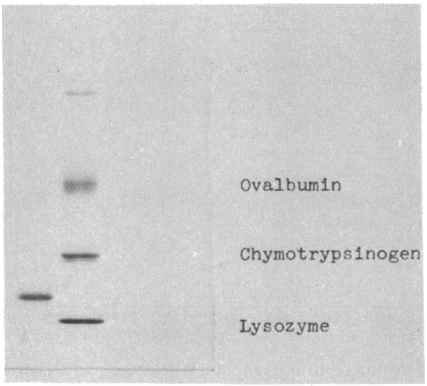

FIG. 3. Electrophoretic analysis of purified interferon in polyacrylamide gels containing $NaDodSO_4$. Fractions of maximum activity from a preparative gel (Fig. 2) were concentrated by lyophilization and dissolved in 0.1 M Tris·HCl, pH 6.8, 1% $NaDodSO_4$. Approximately 0.5 μg of protein was analyzed. The gel was subjected to electrophoresis at 25° for 5 hr at 10 mA/gel constant current. All gel runs contained 1 μg each of ovalbumin, chymotrypsinogen, and lysozyme as polypeptide standards. Gels were stained with Coomassie blue, destained, and then dried by reduced pressure on Whatman 3 MM paper to visualize the polypeptides.

resis as in Fig. 3 and the gel was stained by the PAS procedure. After electrophoresis, glycoprotein carbohydrate was oxidized with periodic acid and the resulting product allowed to react with basic fuchsin to give a red Schiff base (PAS stain). The polypeptide that was stained by Coomassie blue (Fig. 3 and Fig. 4B) is also stained by the PAS procedure; a densitometer scan of the gel is shown in panel C of Fig. 4. This result indicates that the human fibroblast interferon is a glycoprotein. The PAS procedure is specific for glycoproteins since fetuin and ovalbumin were stained but lysozyme and chymotrypsinogen were not.

For molecular weight estimation, the purified interferon was subjected to electrophoresis on a slab gel of 10% acrylamide. The polypeptide was stained and the comigrating interferon activity was eluted. With lysozyme, chymotrypsinogen, ovalbumin, and bovine serum albumin as molecular weight standards, a molecular weight of 20,000 for the interferon has been calculated (13).

Stability of fibroblast interferon

The crude fibroblast interferon is stable for at least 1–2 months when stored at 4°. Purified interferon, 1×10^7 units/mg or greater, is unstable in dilute solutions (less than 10 μg/ml) and loses 50% to 75% of the original activity in 24 hr. This loss is presumed due to the adsorption of the interferon to surfaces. Buffers containing 15% glycerol (Fig. 1, Table 1) did not prevent this loss in activity of purified interferon in dilute solution. The purified interferon is stable, however, at 25° in solutions containing $NaDodSO_4$ of 0.05% to 1.0% and can be lyophilized in the presence of $NaDodSO_4$ without loss of activity.

DISCUSSION

Human fibroblast interferon has been purified to an estimated minimal specific activity of 2×10^8 units/mg. The purified interferon contains a polypeptide of only one molecular size class, as determined by electrophoresis in polyacrylamide gels containing $NaDodSO_4$. The interferon activity comigrates with the polypeptide and has an estimated molecular weight of 20,000 in $NaDodSO_4$. Furthermore, this polypeptide contains carbohydrate, indicating that the fibroblast

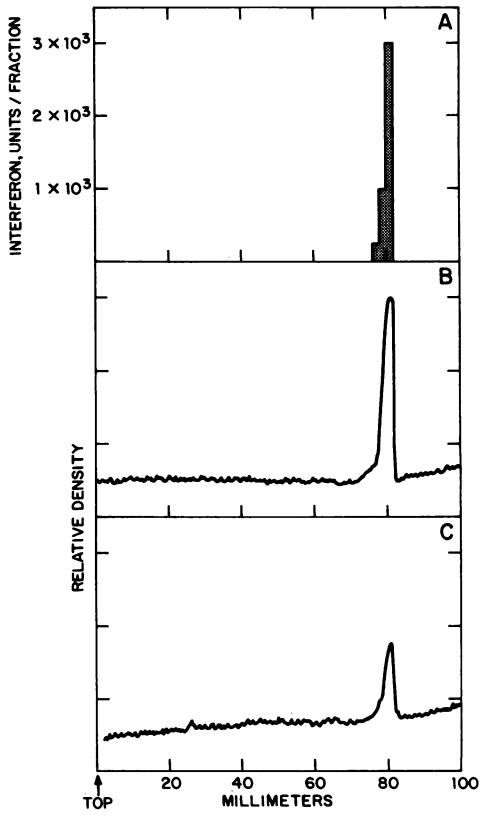

FIG. 4. Electrophoretic profiles of interferon activity, polypeptides, and glycoproteins obtained from purified interferon. Fractions of maximum activity from a preparative gel were analyzed as described in the legend of Fig. 3. (A) Interferon activity eluted after electrophoresis. Units (5×10^3) of interferon, prepared by preparative electrophoresis, applied to the gel and electrophoresis was performed as described in the legend of Fig. 3. After electrophoresis the gel was sliced into 2-mm sections and the interferon was eluted for assay. (B) Densitometer scan of Coomassie blue polypeptide, 0.5 μg of protein. (C) Densitometer scan of PAS polypeptide, 3 μg of protein.

interferon is a glycoprotein. That human fibroblast interferon is a glycoprotein has been previously suggested in experiments where inhibitors of glycosylation reduced substantially the amount of interferon secreted by the cell after induction with poly(I)·poly(C) (14). Coelectrophoresis of a glycoprotein and interferon activity has been obtained with three different preparations of interferon. Although the fibroblast interferon is homogeneous in size, the question of its purity is still valid and should still be investigated. For example, does the purified interferon preparation contain noninterferon glycoproteins of the same molecular size as interferon? The purified interferon, therefore, must be further fractionated by techniques whose separations are not based on size.

If the ultimate specific activity of fibroblast interferon is 2×10^8 units/mg, a molar concentration of interferon can be calculated which induces the antiviral state *in vitro*. Fibroblast interferon at 50 units/ml prevents the replication of vesicular stomatitus virus in human fibroblast cells in culture. From a interferon concentration of 50 units/ml, a molecular weight of 20,000, and a specific activity of 2×10^8 units/mg, an effective antiviral concentration of 1.0×10^{-11} M is calculated. Most polypeptide hormones, like interferon, do not exert their biological effects by direct action, but appear to interact with the surface membrane of a specific receptor cell. The observed biological effects are induced from the membrane receptor site. Some polypeptide hormones that

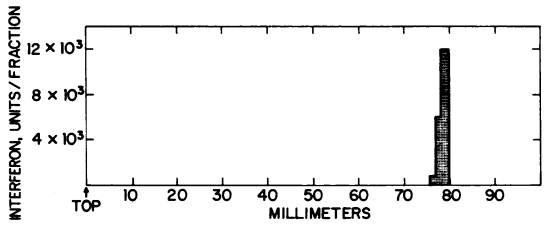

FIG. 5. Electrophoretic mobility of crude interferon. Crude interferon, 3.2×10^5 units/mg, was analyzed on a slab gel containing $NaDodSO_4$. Conditions for electrophoresis and elution of activity were identical to those described for Fig. 3.

have been purified to homogeneity, such as insulin (15), glucagon (16), corticotropin (ACTH) (17), nerve growth factor (12), epidermal growth factor (19), thymin (20), and thyroid stimulating hormone (21), induce their biological effects in tissue slices or cell culture at concentrations from 5×10^{-10} M to 1×10^{-8} M. A concentration for fibroblast interferon of 1×10^{-11} M, calculated from a specific activity of 2×10^8 units/mg, is thus similar to the effective concentrations *in vitro* of a number of homogeneous hormones.

Although the isolated glycoprotein may be homogeneous in interferon activity, it is possible however that within this size class there is more than one glycoprotein with interferon activity. This heterogeneity could manifest itself as charge heterogeneity as a result of (*a*) different amino acid contents, (*b*) different carbohydrate contents, specifically sialic acid content, or (*c*) a combination of (*a*) and (*b*). Any differences could be such that the molecular size of the molecules would be almost identical or at least not resolved by conventional methods. Charge heterogeneity in human fibroblast interferon has been suggested by isoelectric focusing experiments (22) and by affinity chromatography (4) using an antibody to interferon covalently attached to beads of Sepharose. Although charge heterogeneity may suggest more than one polypeptide or glycoprotein with interferon activity, caution should be exercised when interpreting results of experiments where less than homogeneous interferon has been used. Interferon–protein interactions of a noncovalent nature could generate components of heterogeneous charge, all having interferon activity. Recently, a selective binding has been demonstrated of human fibroblast interferon to bovine serum albumin covalently attached to Sepharose beads (23). This binding is noncovalent and can be inhibited by a change in the ionic environment. Moreover, in all fractionations of human interferon on Bio-Gel P-150 we have observed significant amounts of interferon activity emerging at the void volume (Fig. 1). This interferon at the void volume is composed only of the 20,000 molecular weight species when subjected to electrophoresis in $NaDodSO_4$, indicating that noncovalent protein-protein interactions cause the apparent larger molecular size.

We have no evidence that crude fibroblast interferon consists of other molecular size species that are lost during the purification. The data shown in Fig. 5 indicate that crude interferon (3.2×10^5 units/mg) migrates on polyacrylamide gel as a relatively homogeneous activity peak with the same mobility as the purified interferon (Fig. 4A). Since human fibroblast interferon is relatively homogeneous in size compared to mouse L cell interferon (7, 24), it seems to be a good candidate for a detailed study of an interferon.

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