MECHANISM OF INTERFERON ACTION
PARTIAL PURIFICATION AND CHARACTERIZATION OF A LOW-MOLECULAR-WEIGHT INTERFERON-MEDIATED INHIBITOR OF TRANSLATION WITH NUCLEOLYTIC ACTIVITY

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SUMMARY: A low-molecular-weight interferon-mediated ribosome-associated inhibitor of reovirus mRNA translation was purified from the 0.5 M KCl ribosomal salt-wash fraction of mouse L_{929} cells. The inhibitor possessed nucleolytic activity with reovirus [^{3}H]mRNA as a substrate. Loss of translational inhibitory activity correlated with the thermal inactivation of the nuclease. A low-molecular-weight ($\leq$10K) component present in the Bio-Gel P150 chromatography fractions which contained the interferon-mediated nucleolytic activity was labeled *in vivo* with [^{14}C]valine; a smaller component present in the same fractions was phosphorylated *in vitro* with [γ-^{32}P]ATP. The $\leq$10K components were resolved from ~50K, ~30K and ~20K phosphorylatable proteins associated with ribosomes that possess the interferon-mediated inhibitor(s) of viral mRNA translation.

Interferon treatment of many species of animal cells in culture greatly reduces the ability of the treated cells to support the multiplication of a wide range of unrelated DNA and RNA viruses (1,2). For many viruses, including reovirus (3), inhibition of virus-specified protein synthesis is the primary level of genome expression inhibited *in vivo* as the result of interferon treatment (1,2). The interferon-mediated inhibition of viral mRNA translation observed *in vivo* has also been demonstrated *in vitro*. Cell-free extracts prepared from interferon-treated murine cells catalyze the translation of a variety of exogenously added viral mRNAs much less efficiently than extracts prepared from untreated cells (4-7). In several systems, the interferon-mediated inhibition of viral mRNA translation is facilitated by a ribosome-associated inhibitor(s) that can be separated from ribosomes either by

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washing with high-salt buffers (5,7) or by preincubation (6). Even though certain interferons are relatively species specific, the interferon-mediated ribosome-associated inhibitor(s) does not appear to be species specific (8).

Recent studies indicate that interferon treatment of murine cells also mediates an enhanced nucleolytic degradation of viral mRNA (9-11) and an enhanced phosphorylation of at least three proteins (12-14). Nuclease activity associated with ribosomal salt-wash fractions, as well as protein phosphorylation, was maximally enhanced after 12 hr of interferon treatment (15). We have now purified an interferon-mediated inhibitor of translation from the ribosomal salt-wash fraction prepared from mouse L_{929} fibroblasts; this inhibitor preparation catalyzed the degradation of reovirus mRNA and, in addition, it contained a low-molecular-weight component which was phosphorylated *in vitro*.

RESULTS AND DISCUSSION

Purification of interferon-mediated ribosome-associated translational inhibitor. An interferon-mediated inhibitor of reovirus mRNA translation was resolved by Bio-Gel P150 chromatography from the 0.5 M KCl ribosomal salt-wash fraction obtained from interferon-treated mouse L_{929} cells (Fig. 1, fractions 32-43). The apparent molecular weight of the inhibitor was near 12,000 daltons when estimated by gel filtration with P150. The ribosomal salt-washes prepared from both untreated and interferon-treated cells each contained chromatographically common components that either stimulated (fractions 14-17) or inhibited (fractions 22-32) translation *in vitro*. The recovery after P150 chromatography of the total inhibitory and stimulatory activities, as well as the [^{14}C]valine-labeled protein, is summarized in Table 1. Increases in the apparent inhibitory and stimulatory units recovered as compared to those applied (Table 1) were most likely due to the chromatographic separation of inhibitory factors from stimulatory

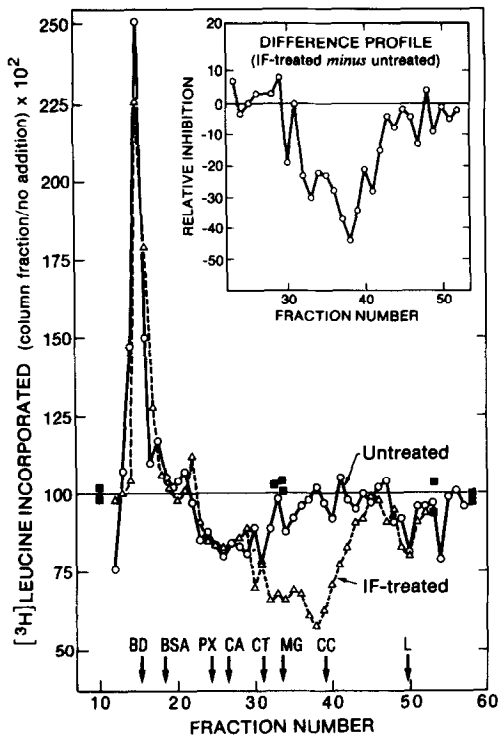


Fig. 1. Chromatography of 0.5 M KCl ribosomal salt-wash fractions prepared from untreated and interferon-treated L_{929} cells on Bio-Gel P150. Cells in roller culture were treated with 300 units mouse interferon/ml in the presence of [^{14}C]valine (2.5 μ Ci/ml), and combined in the ratio of 1:3 with cells treated with interferon (no [^{14}C]valine) for 24 hr. Ribosomal salt-wash fractions were prepared as previously described (8), except the final dialysis buffer contained 5 mM $Mg(OAc)_2$. The ribosomal salt-wash fraction from either untreated (6.5 mg protein) or interferon-treated (7 mg protein) cells was chromatographed at 4° on a 0.7 X 64 cm column of Bio-Gel P150 (100-200 mesh) equilibrated with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.5, 120 mM KCl, 4 mM $Mg(OAc)_2$, 1 mM DTT and 5% (v/v) glycerol. Fractions (0.5 ml) were collected into polystyrene tubes, and bovine serum albumin (BSA) was added to 0.5 mg/ml. The effect of a 20 μl aliquot of each fraction (O—O, untreated; Δ---Δ, interferon-treated) on the incorporation of [^{3}H]leucine into protein in response to methylated reovirus mRNA (17), catalyzed by the ascites protein synthesizing system prepared from untreated cells was determined (8). Incubation was at 25° for 60 min; 40 μl aliquots were processed (7). ■ , buffer controls (90,000 cpm untreated, or 134, 000 cpm IF-treated ≡ 100). Arrows indicate elution positions of: BD, blue dextran; BSA, (68,000); PX, horseradish peroxidase (40,000); CA, carbonic anhydrase (31,000); CT, α-chymotrypsinogen (25,000); MG, myoglobin (17,200); CC, cytochrome c (12,400); and L, [^{3}H]leucine.

factors (Fig. 1). Based on the level of inhibition of reovirus mRNA translation per amount of [^{14}C]valine-labeled ribosomal salt-wash

Table 1

Recovery of inhibitory and stimulatory activities present in 0.5 M KCl ribosomal salt-washes from interferon-treated and untreated L_{929} cells after Bio-Gel P150 column chromatography

Ribosomal	Salt-wash Protein			Inhibitor		Stimulator	
Salt-wash	Applied	Recovered		Applied	Recovered	Applied	Recovered
	cpm X 10^{-5}		%	*units*[a]		*units*[a]	
Interferon-treated	13.8	11.0	80	6570	12,930[b]	-	6300
Untreated	8.4	6.5	77	-	4700[b]	4200	8450

[a]One inhibitory or stimulatory unit inhibits or stimulates, respectively, the rate of [^{3}H]leucine incorporation in response to reovirus mRNA by one percent catalyzed by the ascites protein synthesizing system.

[b]Inhibitor units were calculated from fractions 22 through 46; column internal volume peak, fractions 47-55, was not included in calculation.

protein, the interferon-mediated inhibitor was purified >100-fold by Bio-Gel P150 chromatography from the 0.5 M KCl ribosomal salt-wash fraction.

Protein nature of the purified interferon-mediated inhibitor. SDS-Polyacrylamide gel electrophoresis of the Bio-Gel column fractions revealed that the fractions containing the interferon-mediated translational inhibitor (Fig. 1, fractions 32-43) contained a [^{14}C]valine-labeled low-molecular-weight component (Fig. 2) that stained with Coomassie brilliant blue. The apparent molecular weight on 15% polyacrylamide gels was $\leq$10,000 daltons. The incorporation of radioactive amino acid, present as [^{14}C]valine in the culture medium during interferon treatment, into the low-molecular-weight component present in the interferon-mediated inhibitor fraction indicates that the $\leq$10K component is, at least in part, protein in nature. Furthermore, the activity of the inhibitor was reduced by treatment with trypsin. In addition to the ^{14}C-labeled low-molecular weight component, various

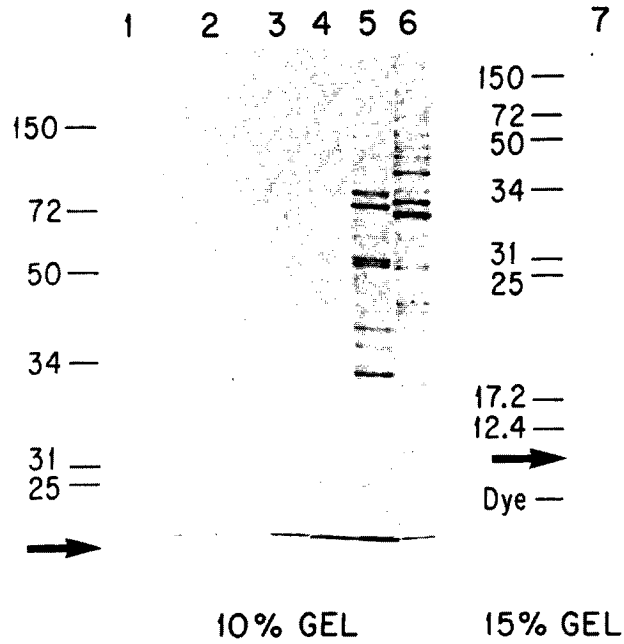
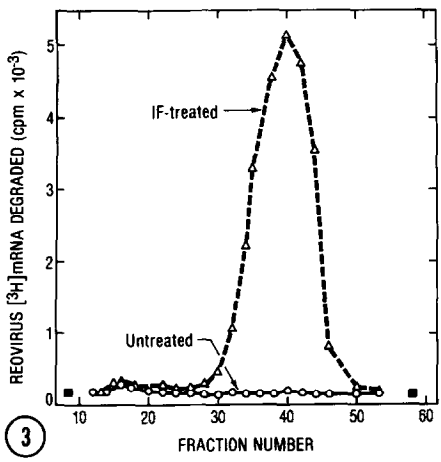


Fig. 2. Autoradiograms of Bio-Gel P150 column fractions obtained by chromatography (Fig. 1) of the 0.5 M KCl ribosomal salt-wash prepared from [^{14}C]valine-labeled interferon-treated cells. Polypeptides were separated by SDS-polyacrylamide slab gel electrophoresis (18). Fractions 46-55 (#1) and 35-43 (#2 and #7) were concentrated ca. 40-fold; 40 µl was applied to the gel. The remaining fractions were concentrated ca. 20-fold, and 40, 20, 40 and 5 µl were applied for fractions 30-34 (#3), 24-29 (#4), 19-22 (#5), and 14-18 (#6) respectively. Standards: reovirion proteins λ (150,000), μ_2 (72,000), σ_3 (34,000); α-amylase (50,000); CA; CT; MG; and CC.

other ribosome-associated proteins, including two of apparent molecular weight near 50 K, were labeled with [^{14}C]valine during interferon treatment (Fig. 2).

Nuclease activity associated with the purified interferon-mediated inhibitor. The fractions obtained from the Bio-Gel fractionation of the interferon-treated and untreated ribosomal washes (Fig. 1) were analyzed for their ability to catalyze the nucleolytic degradation of reovirus [^{3}H]mRNA. As shown in Fig. 3, P150 fractions corresponding to the interferon-mediated translational inhibitor (Fig. 1) contained significant levels of nuclease activity; the corresponding fractions obtained



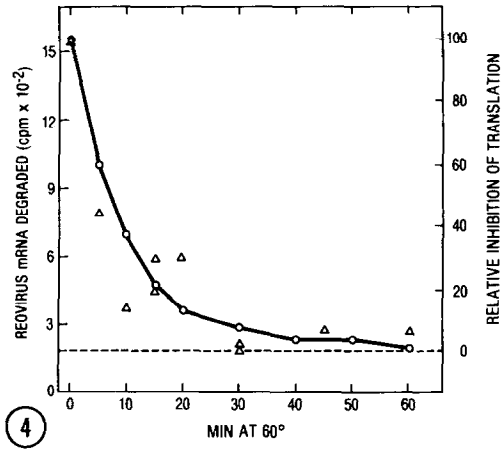


Fig. 3. Bio-Gel P150 elution profile of interferon-mediated nucleolytic activity. The fractions obtained from chromatography (Fig. 1) of the 0.5 M KCl ribosomal salt-wash fraction from interferon-treated (Δ---Δ) and untreated (O——O) cells were assayed for nucleolytic degradation of reovirus [^{3}H]mRNA. The 25 µl reaction mixture contained 0.625 µl column fraction and 1 µg reovirus [^{3}H]mRNA (8400 cpm/µg) in 40 mM MES (2[N-morpholino]ethane sulfonic acid) pH 6.2, 120 mM KCl and 1 mM DTT; after 10 min incubation at 25°, 25 µl of ice-cold 12.5% (w/v) $HClO_4$-0.37% (w/v) uranyl acetate was added, the sample was centrifuged, and the radioactivity of a 35 µl aliquot of the supernatant solution was determined in Aquasol-II. ■ , buffer controls.

Fig. 4. Heat inactivation of interferon-mediated translational inhibitor and nucleolytic activity. Fraction 38 obtained from the chromatography of ribosomal salt-wash from interferon-treated cells (Fig. 1) was incubated at 60° for 0-60 min under argon atmosphere in sealed polypropylene tubes. The tubes were then cooled in ice and centrifuged to collect any condensate before assaying for the ability to: (Δ), inhibit reovirus mRNA translation as measured under Fig. 1 legend; and (O) degrade reovirus [^{3}H]mRNA as measured under Fig. 3 legend with the broken line indicating the assay blank value.

from untreated cells did not contain detectable nuclease activity (Fig. 3). The nucleolytic activity did not require prior activation by dsRNA and ATP as did the nuclease reported by Sen *et al.* (11). It is conceivable that sufficient reovirus genome dsRNA contaminated our [^{3}H]mRNA substrate, or that the nuclease was already "activated". The P150 eluate peak of nuclease activity tailed towards the higher molecular weight region of the column, indicating that it may have been interacting

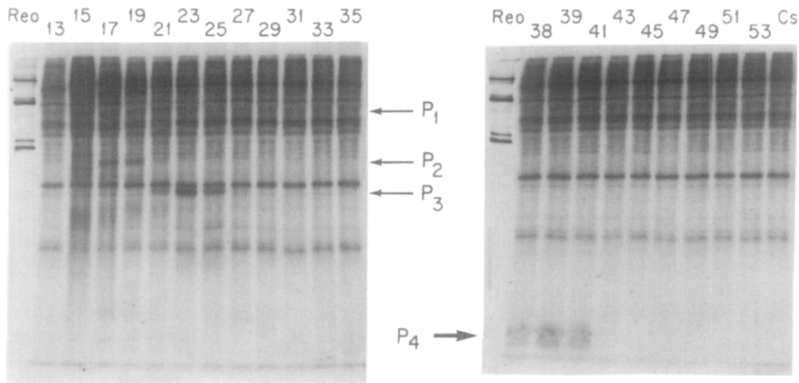


Fig. 5. Autoradiogram of 15% SDS-polyacrylamide gel showing [γ-^{32}P]ATP-mediated phosphorylation of Bio-Gel P150 fractionated ribosomal salt-wash proteins obtained from interferon-treated cells (Fig. 1). The phosphorylations were performed *in vitro* as previously described (15); 10 µl of each column fraction was assayed in the presence of 50 µg cell-sap protein and 1 µg/ml reovirus genome dsRNA. 15 µl was applied to the gel. Reo, [^{14}C]valine-labeled reovirion protein standard; 13, 15, 17,..., column fractions assayed for phosphorylation; Cs, phosphorylation of cell-sap proteins in the absence of added column fraction. ' Phosphorylated proteins enhanced in interferon-treated-cell fractions are indicated by P_1, P_2 and P_3(15), and P_4.

with larger molecular weight species (16).

Effect of heat on the interferon-mediated translational inhibitor and nucleolytic activity. The nuclease activity of the chromatographically-purified interferon-mediated inhibitor was progressively reduced by heating at 60°; a concomitant decrease in the activity of the inhibitor of reovirus mRNA translation was also observed following incubation at 60° (Fig. 4). The extent of inactivation of the translational inhibitor activity and the nuclease activity was dependent, in similar manner, upon both the temperature and the length of time heated.

Phosphorylation of the purified interferon-mediated inhibitor. The fractions obtained from the Bio-Gel P150 fractionation of the ribosomal salt-washes from interferon-treated and untreated cells were analyzed for [γ-^{32}P]ATP-mediated phosphorylation in the presence and absence of cell-sap and dsRNA. Specific phosphorylation was observed of a low-

molecular-weight component ($\leq$10K), designated P_4, which was present in column fractions 38-41 obtained by chromatography of ribosomal wash from interferon-treated cells (Fig. 5); these fractions contained the interferon-mediated translational inhibitor (Fig. 1) and the interferon-mediated nuclease activity (Fig. 3). The phosphorylation of the low-molecular weight component, which was independent of dsRNA but dependent upon cell-sap, was not detectable in the corresponding fractions obtained from untreated control cells. The Bio-Gel P150 column also resolved three additional proteins which were phosphorylatable *in vitro*, and which were previously designated (15) as P_1, $\sim$50K; P_2, $\sim$30K; and P_3, $\sim$20K (Fig. 5). The P_3 component was present in the column fractions that contained the common inhibitor of translation (Fig. 1 & Fig. 5). Roberts *et al.* (12) recently described a dsRNA-dependent phosphorylation of a low-molecular-weight "component Z" present in the cell-sap fraction from interferon-treated mouse L cells. However, component P_4 appears to differ from the inhibitor described by Kerr and colleagues (12,19) in a number of parameters including apparent dsRNA-independent phosphorylation and thermolability (Fig. 4) of the inhibitory activity associated with the P_4-containing fraction.

In summary, we have isolated an interferon-mediated inhibitor of viral mRNA translation associated with mouse L_{929} ribosomes. The fraction which contained this translational inhibitor also possessed interferon-mediated nuclease activity, a low-molecular-weight [^{14}C]valine-labeled component, and a smaller component which was phosphorylated *in vitro* with [γ-^{32}P]ATP. These $\leq$10K components were resolved from previously described interferon-mediated (phosphorylatable) components of $\sim$50K, $\sim$30K and $\sim$20K (7,15). The biochemical relationship, if any, between the multiple interferon-mediated molecular changes observed in murine cells, and the specific molecular mechanism by which interferon-mediated components function in the antiviral state remain open questions.

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