Interferon alters the pattern of secreted proteins from Ehrlich ascites-tumour cells

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Treatment of Ehrlich ascites-tumour (EAT) cells with interferon (IFN) abolished their ability to secrete a 32 kDa protein that was secreted by growing EAT cells. These IFN-treated cells secreted two proteins (molecular masses 100 and 89 kDa as estimated by SDS/polyacrylamide-gel electrophoresis) that were not detected in two-dimensional gel electrophoresis of the culture fluid of untreated EAT cells. The sequence of 20 amino acids from the *N*-terminal end of the 32 kDa protein was very similar to portions of sequences of mouse proviral *gag* proteins.

INTRODUCTION

IFN (interferon)- α and - β are proteins with antiviral, immunomodulatory and cell-growth-regulatory activities [1]. They were shown to induce the synthesis of several cell-associated proteins [2–15]. Furthermore, as we previously reported, treatment with IFN- β also alters the pattern of secreted proteins from quiescent and platelet-derived-growth-factor-treated BALB/c-3T3 cells [16]. BALB/c-3T3 cells secrete a 89 kDa protein in response to IFN treatment [16,17]. Recently, other cell lines (L929, WDFN, CAK-TK⁻) were also shown to secrete a similar protein [18]. To clarify the biological function(s) of the 89 kDa protein, a large-scale purification is required. Therefore we started to analyse the effect of IFN on Ehrlich ascites-tumour (EAT) cells.

Here we present further observations on the effects of mouse IFN- α and - β on protein secretion by EAT cells.

EXPERIMENTAL

Materials

The monolayer-type EAT cell line was kindly given to us by Dr. Peter Lengyel (Yale University). MEM and FBS were obtained from Gibco. Partially purified recombinant mouse IFN- α (specific activity 1×10^6 i.u./mg) [19] and native IFN produced in Newcastle-disease-virus-infected mouse L-cells, purified through controlled-pore-glass (specific activity of 1×10^7 i.u./mg) were gifts from Dr. Hideharu Taira (Tokyo Metropolitan Institute for Medical Research). Purified recombinant mouse IFN- β (99.7% pure by SDS/PAGE; specific activity of 3.0×10^7 i.u./mg) [20] was provided by the Basic Research Laboratories, Toray Industries. Phenyl-Sepharose CL-4B, DEAE-Sepharcel, and CM-Sepharose were obtained from Pharmacia Fine Chemicals, and phosphocellulose (P11) was obtained from Whatman. Phenyl-Sepharose-treated calf serum (PS-CS) was prepared as described in [17]. [³⁵S]Methionine (sp. radioactivity 1150 Ci/mmol) was from Amersham. The h.p.l.c. system consisted of an HLC-803D chromatograph, GE4 gradient former, and UV-8000 monitor from Toyo Soda

Co. The C-4 reverse-phase column (10 mm $\times$ 250 mm) for h.p.l.c. was a product of Senshu Science Co. Acetonitrile and trifluoroacetic acid were high-grade reagents for amino-acid-sequence analysis. Other chemicals were of reagent grades. Buffer A consisted of 20 mM-Tris/HCl (pH 7.5 at 10 °C), 10% (v/v) glycerol, 0.02% NaN₃ and 10 μ M-PMSF.

Cell culture

Mouse EAT cells were cultured, in MEM + 10% FBS at 36 °C in CO₂/air (1:19), as a monolayer in a plastic culture flask.

Labelling of the culture fluid from EAT cells for fluorographic analysis

Each 75 cm² tissue-culture flask was washed twice with 50 ml of MEM (–methionine), supplemented with 5 ml of MEM (–methionine) containing 1% PS-CS and 30 μ Ci of [³⁵S]methionine/ml, and, in the case of the cells which had been treated with IFN, also supplemented with 1000 i.u. of mouse IFN- α or - β /ml. After incubation at 36 °C for 6 h, the culture fluid was collected.

Two-dimensional gel electrophoresis

The original method of O'Farrell [21] was employed. The first dimension was isoelectric focusing, using mixtures of 1.6% Ampholine pH 5–7 and 0.4% Ampholine pH 3.5–10, both obtained from LKB. The second dimension was SDS/10% PAGE [22]. By the use of pI calibration kits ranging from pH 4.7 to 10.6 (BDH), the pH gradient was linear from pH 5.65 to 8.3 in our hands, but non-linear beyond this region.

Production and purification of 100 and 89 kDa proteins

For efficient production of the 100 and 89 kDa proteins, EAT cells were split in a 1:20 ratio into 60 150 cm² tissue-culture flasks, each containing 50 ml of MEM + 10% FBS, and cultured for 2 days. To each flask was added 1000 i.u. of native mouse IFN/ml, which had been produced from L-cells and partially purified, and the cells were cultured for a further 24 h. Each of the

Abbreviations used: EAT, Ehrlich ascites tumour; FBS, fetal bovine serum; IFN, interferon; MEM, minimum Eagle's medium; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; PS-CS, phenyl-Sepharose-treated calf serum; PTH, phenylthiohydantoin; AKV, a murine-leukaemia virus residing in the germ line of AKR mice.

58 flasks was washed twice with 50 ml of MEM, to which were then added 15 ml of MEM containing 1% PS-CS and 1000 i.u. of IFN/ml, and the cells incubated for 6 h. The culture fluid was centrifuged at 1000 *g* for 5 min, and the supernatant fraction was collected. To obtain radiolabelled proteins to serve as markers during the purification, each of the two flasks was washed twice with 50 ml of MEM (–methionine); 15 ml of MEM (–methionine) containing 1% PS-CS, 1000 i.u. of IFN/ml and 20 μ Ci of [³⁵S]methionine/ml were then added and the cells were incubated for 6 h at 36 °C. Thereafter, the radioactive culture fluid was centrifuged at 1000 *g* for 5 min. The supernatant fraction was collected and then mixed with the pooled unlabelled supernatant fraction from the 58 flasks.

A 900 ml portion of culture fluid from 60 150 cm² tissue-culture flasks was applied at a flow rate of 5 ml/h to a 10 ml phenyl-Sepharose CL-4B column which had been equilibrated with MEM. The column was washed with 30 ml of MEM and then with 200 ml of buffer A containing 50 mM-NaCl. The column was developed with buffer A containing 50 mM-NaCl and 0.5% (w/v) Triton X-100, and the radioactivity-containing fractions were pooled.

The phenyl-Sepharose fraction (35 ml) was applied with a flow rate of 1.5 ml/h to a 10 ml phosphocellulose column which had been equilibrated with buffer A containing 50 mM-NaCl and 0.1% (w/v) Triton X-100. The column was developed with the same buffer, and the flow-through fraction, which contained radioactivity, was pooled.

The phosphocellulose flow-through fraction (52 ml) was applied with a flow rate of 2.4 ml/h to a 10 ml DEAE-Sephacel column which had been equilibrated with buffer A containing 50 mM-NaCl and 0.1% (w/v) Triton X-100. After washing with 50 ml of the same buffer, with 50 ml of buffer A containing 0.1 M-NaCl and 0.1% (w/v) Triton X-100, and with 50 ml of buffer A containing 0.2 M-NaCl and 0.1% (w/v) Triton X-100, sequentially, the column was developed with 50 ml of buffer A containing 0.3 M-NaCl and 0.1% (w/v) Triton X-100. A single radioactive peak was eluted in this fraction.

Production and purification of the 32 kDa protein

For production of the 32 kDa protein, EAT cells were split in a 1:20 ratio into 66 150 cm² tissue-culture flasks, each containing 50 ml of MEM + 10% FBS, and cultured for 3 days. Each of the 62 flasks was washed twice with 50 ml of MEM, then 15 ml of MEM + 1% PS-CS were added and the cells were incubated for 6 h. The culture fluid was centrifuged at 1000 *g* for 5 min, and the supernatant fraction was collected. To obtain radiolabelled marker proteins for the purification, each of the four flasks was washed twice with 50 ml of MEM (–methionine); 15 ml of MEM (–methionine) containing 1% PS-CS and 20 μ Ci of [³⁵S]methionine/ml were then added and the cells were incubated for 6 h at 36 °C. Thereafter, the radioactive culture fluid was centrifuged at 1000 *g* for 5 min. The supernatant fraction was collected and mixed with the pooled unlabelled supernatant fraction from the 62 flasks.

The culture fluid (990 ml) from 60 150 cm² tissue-culture flasks was applied with a flow rate of 5 ml/h to a 10 ml phenyl-Sepharose CL-4B column that had been equilibrated with MEM. The column was washed with

30 ml of MEM and then with 300 ml of buffer A containing 50 mM-NaCl. The column was developed with buffer A containing 50 mM-NaCl and 0.5% (w/v) Triton X-100, and the radioactivity-containing fractions were pooled.

The phenyl-Sepharose fraction (67 ml) was applied at a flow rate of 1.3 ml/h to a 10 ml DEAE-Sephacel column that had been equilibrated with buffer A containing 50 mM-NaCl and 0.1% (w/v) Triton X-100. The column was developed with the same buffer, and the flow-through fraction, which contained radioactivity, was pooled.

The DEAE-Sephacel flow-through fraction (70 ml) was applied at a flow rate of 2.7 ml/h to a 10 ml CM-Sepharose column that had been equilibrated with buffer A containing 50 mM-NaCl and 0.1% (w/v) Triton X-100. The column was developed with the same buffer and the flow-through fraction, which contained radioactivity, was pooled.

An 18 ml portion of the CM-Sepharose flow-through fraction (total vol. 70 ml) was applied at a flow rate of 3 ml/min to a preparative reverse-phase h.p.l.c. column (C-4; 10 mm $\times$ 250 mm) that had been equilibrated with 0.1% (v/v) trifluoroacetic acid, using an HLC-803D chromatograph interfaced with a UV-8000 monitor (Toyo Soda). The column was developed with an 120 min linear gradient of acetonitrile (0–70%) in the presence of 0.1% (v/v) trifluoroacetic acid. A single radioactive peak was eluted at about 77 min retention time.

Amino-acid-sequence determination

The sample was processed for Edman degradation by using a gas phase sequencer (Applied Biosystems; type 470 A), and the resultant amino acid PTH derivatives were analysed with a PTH analyser (Applied Biosystems; type 120 A).

RESULTS AND DISCUSSION

Alteration of the pattern of secreted protein(s) in the culture fluid of IFN-treated EAT cells

We analysed the labelled culture fluid from untreated EAT cells and that from IFN-treated EAT cells after 24 h of IFN treatment. A 32 kDa protein that was secreted continuously by the untreated cells (indicated by the arrow in Fig. 1) disappeared completely after the treatment with IFN (1000 i.u./ml) (see also arrow in Fig. 2). On the other hand, 100 and 89 kDa doublet protein bands appeared when EAT cells were treated with IFN (1000 i.u./ml) (indicated by the arrowheads in Figs. 1 and 2). Furthermore, a protein band of 10.5 kDa ($\triangleright$, Fig. 1), which was immunoprecipitated with an antiserum to β_2 -microglobulin, was also increased by IFN treatment, as reported previously [16]. The increase in the number of EAT cells was diminished by the treatment with mouse IFN- α and IFN- β , and this effect was pronounced after 2 days of IFN treatment [23]. Therefore we also studied the culture fluid at 2, 3 and 4 days of IFN treatment. The results (not shown) were essentially the same as those illustrated in Fig. 1. The 32 kDa protein was very basic, and the 100 and 89 kDa doublet protein bands were very acidic. Because they were in the non-linear range of our two-dimensional gel-electrophoresis system, the exact isoelectric points of these proteins could not be measured. However, these extreme iso-

Fig. 1. Alteration of the pattern of secreted proteins in the culture fluid of EAT cells after treatment with mouse IFN

(a) EAT cells were split in a 1:20 ratio in 75 cm² tissue-culture flasks. After being cultured for 48 h, the cells were treated with partially purified mouse native IFN (1000 i.u./ml) (lane 2) or left untreated (lane 1). At 24 h after the addition of IFN, each flask was washed with MEM (–methionine) and labelled with [³⁵S]methionine for 6 h as described in the Materials and methods section. The untreated cells were also labelled by the same procedure. An amount of culture fluid corresponding to 50000 c.p.m. of trichloroacetic acid-precipitable radioactivity was fractionated by gel electrophoresis in a 10–20% linear-gradient polyacrylamide gel in the presence of SDS and revealed by fluorography as described previously [16]. A [¹⁴C]methylated protein mixture from Amersham was used as a source of molecular-mass (*M*) markers. (b) The experiment was the same as in (a). The cells were treated with recombinant mouse IFN-α (1000 i.u./ml) (lane 2) or recombinant mouse IFN-β (lane 4) or they remained untreated (lanes 1 and 3). The arrowhead (➤), arrow (→), and open triangle (▷) indicate the positions of the 100, 32 and 10.5 kDa proteins respectively. Abbreviation: *M*, molecular mass.

electric points were advantageous for the purification of these proteins.

The purifications of the IFN-induced 100 and 89 kDa secreted proteins and the IFN-suppressed 32 kDa secreted protein were performed as described in the Materials and methods section. The silver-staining patterns of the final purified samples are shown in Fig. 3. Both the 100 and 89 kDa IFN-induced secreted proteins were eluted in exactly the same fraction through three column chromatographic procedures, suggesting that

Fig. 2. Analysis of culture fluid from IFN-treated or control EAT cells by two-dimensional gel electrophoresis

An amount corresponding to 50000 c.p.m. of trichloroacetic acid-precipitable radioactivity of the labelled culture fluid from EAT cells, which had been treated with mouse IFN-β for 24 h (b) or which had not been treated with IFN (a), was analysed by two-dimensional gel electrophoresis as described in the Materials and methods section. Each gel was processed for fluorography as described in Fig. 1. The arrowhead (➤) in (b) indicates the position of the 100/89 kDa protein doublet, and the arrow (→) in (a) indicates the position of the 32 kDa protein. The arrowhead in (a) and the arrow in (b) indicate the absence of these corresponding proteins.

these were of very similar nature. This similarity was also suggested by the similar mobility of these two proteins in two-dimensional gel electrophoresis [arrowhead (➤) in Fig. 2]. The *N*-terminal sequence analysis of the 100 and 89 kDa proteins had been performed after electroblotting on to a poly(vinylidene difluoride) membrane as described in [24], since the conventional chromatographic procedure could not separate the 100 and 89 kDa proteins. Although ~60 pmol each of the 100 and 89 kDa proteins had been used, the level of *N*-terminal amino acids was under that detectable, indicating that the *N*-termini of these proteins were probably blocked.

The 100 and 89 kDa protein doublet, secreted from the IFN-treated EAT cells, is very similar to the 89 kDa protein from BALB/c-3T3 cells; the purification procedure was the same, and they were eluted in the same

Fig. 3. Silver staining of the 100 and 89 kDa protein doublet and the 32 kDa protein preparations purified from the culture fluid of EAT cells

The DEAE-Sephacel fraction of the 100 and 89 kDa protein preparation from the culture fluid of IFN- β -treated EAT cells (lane 1) and the final fraction of the 32 kDa protein (C-4 reverse-phase column chromatography) purified from the culture fluid of EAT cells not treated with IFN (lane 2) were analysed by SDS/PAGE using 12.5 % gels, and proteins were revealed by silver staining [31].

	5	10
32 kDa	Pro-Leu-Arg-Leu-Gly-Gly-Asn-(Gly)-Gln-Phe-	
p30(AKV)	Pro-Leu-Arg-Leu-Gly-Gly-Asn-Gly-Gln-Leu-	
	15	20
	Gln-Tyr-Trp-Pro-Phe-Ser-Ser-(Leu)-Asp-Leu	
	Gln-Tyr-Trp-Pro-Phe-Ser-Ser-Ser-Asp-Leu	

Fig. 4. *N*-Terminal-20-amino-acid sequence of the 32 kDa protein

The 32 kDa protein ('32 kDa') was analysed for its *N*-terminal sequence as described in the Materials and methods section. Amino acid in parentheses are uncertain due to limitations of the procedure used. The *N*-terminal sequence of the p30 *gag* protein of AKV ['p30(AKV)'] is shown for comparison [25].

fraction through three column procedures. Preliminary experiments showed that the 100 and 89 kDa protein doublet did not bind to a poly(I)·poly(C)-agarose column, unlike the 89 kDa protein from BALB/c-3T3 cells. A larger-scale purification of these proteins is needed to clarify their biological function(s).

N-Terminal sequence analysis of the 32 kDa protein

The purified 32 kDa protein fraction (lane 2 in Fig. 3) was analysed for its *N*-terminal sequence, and the results are shown in Fig. 4. The sequence showed 90 % identity

with that of residue nos. 215–234 of *gag* polyprotein of AKV [25] and 85 % identity with that of *gag* polyproteins from Abelson murine-leukaemia virus [26], Moloney murine-leukaemia virus [27], and Moloney murine-sarcoma virus [28]. This results raises the possibility that this EAT monolayer-cell line is a spontaneous producer of a provirus. The relation between cancer phenotype and expression of a provirus is unclear. From the viewpoint of the antiviral activity of IFN [29], the mode of inhibition of the secretion of this protein is intriguing. Perhaps this might be explained by the impairment of virus production described by Friedman & Ramseur [30].

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REFERENCES

- Lengyel, P. (1982) *Annu. Rev. Biochem.* **51**, 251–282
- Burrone, O. R. & Milstein, C. (1982) *EMBO J.* **1**, 345–349
- Colonna, R. J. & Pang, R. H. L. (1982) *J. Biol. Chem.* **257**, 9234–9237
- Fellous, M., Nir, U., Wallach, D., Merlin, G., Rubinstein, M. & Revel, M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3082–3086
- Samanta, H., Dougherty, J. P., Brawner, M. E., Schmidt, H. & Lengyel, P. (1982) *UCLA Symp. Mol. Cell. Biol.* **25**, 59–72
- Wallach, D., Fellous, M. & Revel, M. (1982) *Nature (London)* **299**, 833–836
- Yoshie, O., Schmidt, H., Reddy, E. S. P., Weissman, S. & Lengyel, P. (1982) *J. Biol. Chem.* **257**, 13169–13172
- Chebath, J., Merlin, G., Metz, R., Benesch, P. & Revel, M. (1983) *Nucleic Acids Res.* **11**, 1213–1226
- Cheng, Y.-S. E., Colonna, R. J. & Yin, F. H. (1983) *J. Biol. Chem.* **257**, 9234–9237
- St. Laurent, G., Yoshie, O., Floyd-Smith, G., Samanta, H., Sehgal, P. B. & Lengyel, P. (1983) *Cell (Cambridge, Mass.)* **33**, 95–102
- Friedman, R. L., Manly, S. P., McMahon, M., Kerr, I. M. & Stark, G. R. (1984) *Cell (Cambridge, Mass.)* **38**, 745–755
- Larner, A. C., Jonak, G., Cheng, Y.-S. E., Korant, B., Knight, E. & Darnell, J. E., Jr. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6733–6737
- Staeheli, P., Prochazka, M., Steigmeier, P. A. & Haller, O. (1984) *Virology* **137**, 135–142
- Yasui, H., Takai, K., Yoshida, R. & Hayaishi, O. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6622–6626
- Hovanessian, A. G., Laurent, A. G., Chebath, J., Galabru, J., Robert, M. & Svab, J. (1987) *EMBO J.* **6**, 1273–1280
- Tominaga, S. & Lengyel, P. (1985) *J. Biol. Chem.* **260**, 1975–1978
- Tominaga, S., Tominaga, K. & Lengyel, P. (1985) *J. Biol. Chem.* **260**, 16406–16410
- Veomett, G. E. (1988) *J. Interferon Res.* **8**, 217–226
- Shaw, G. D., Boll, W., Taira, H., Mantel, N., Lengyel, P. & Weissmann, C. (1983) *Nucleic Acids Res.* **11**, 555–573

20. Matsuda, S., Utsumi, J. & Kawano, G. (1986) *J. Interferon Res.* **6**, 519–526
21. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
23. Tominaga, S. (1988) *J. Cell. Physiol.* **135**, 350–354
24. Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038
25. Herr, W. (1984) *J. Virol.* **49**, 471–478
26. Reddy, E. P., Smith, M. J. & Srinivasan, A. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3623–3627
27. Shinnick, T. M., Lerner, R. A. & Sutcliffe, J. G. (1981) *Nature (London)* **293**, 543–548
28. Reddy, E. P., Smith, M. J. & Aaronson, S. A. (1981) *Science* **214**, 445–450
29. Friedman, R. M. (1977) *Bacteriol. Rev.* **41**, 543–567
30. Friedman, R. M. & Ramseur, J. M. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3542–3544
31. Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363

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