Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 254-260

TRANSPORT OF γ-INTERFERON INTO THE CELL NUCLEUS MAY BE MEDIATED BY NUCLEAR MEMBRANE RECEPTORS

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Received May 30, 1986

SUMMARY: Purified mouse interferon gamma (MuIFN-γ), a lymphokine having potent antiviral, immunomodulatory, and growth inhibitory activities, is internalized ($t_{\frac{1}{2}}$ <1.0 min) by mouse L929 fibroblasts via receptor-mediated endocytosis. Individual MuIFN-γ molecules, identified by a postembedding immuno-gold technique, are then transported to the cell nucleus, perhaps through nuclear pores, into areas of dense chromatin. Purified, isolated nuclei of L929 cells bind radiolabeled MuIFN-γ specifically and with high affinity (Kd = 2×10^{-10} M). These nuclear membrane receptors, distinct from those for MuIFN-β, number about 24,000/nucleus. Treatment of nuclei with trypsin prevents binding of MuIFN-γ. The demonstration of rapid cellular uptake and transport of MuIFN-γ into the dense chromatin, perhaps facilitated by nuclear receptors, suggests that IFN-γ molecules, alone or bound to receptor, may directly affect genome regulation.

Of the three types of interferon (IFN), the antiviral lymphokine IFN-γ has appreciably greater immunomodulatory and growth-inhibitory activity than either IFN-α or IFN-β. IFN-γ differs from IFN-α and -β in amino acid sequence, antigenicity, receptor specificity (1), and induces a greater number of proteins in cells (2). Binding of IFNs to plasma membrane receptors (3) has been thought to be sufficient to trigger intracellular activities by yet unidentified transmembrane signals or messengers. The fate or locus of action of receptor-bound IFN is not certain. IFN molecules appear to be internalized by receptor-mediated endocytosis (4-8), a specific, selective, and rapid process for internalizing several other cell regulators, such as growth factors and hormones (9,10). Previous internalization studies tracing IFN by electron microscopy relied on complexing and thus modifying IFN molecules with an electron-dense marker

0006-291X/86 $1.50

prior to their uptake (4,6). By immunologically identifying murine IFN-γ molecules on the surface of ultrathin sections of cell embedded in a hydrophilic resin Lowicryl K4M, we show here that natural, unmodified molecules of IFN-γ are very rapidly internalized via receptor-mediated endocytosis and enter the nucleus as has previously been shown for MuIFN-β (5,7,). Specific, high-affinity, nuclear membrane receptors for IFN-γ, also described here, may facilitate nuclear entry and accumulation of IFN-γ. This localization suggests a possibly direct nuclear role for IFN-γ molecules in genome regulation.

MATERIALS AND METHODS

Mouse L929 fibroblasts were grown in suspension as described (5,7). After 3 days at 36°C, the cells were centrifuged, washed three times with Eagle's minimal essential medium (EMEM) without serum, and utilized immediately.

Mouse IFN-γ (MuIFN-γ) was induced in murine T lymphocytes and purified by RNA-affinity chromatography to a specific activity of 1.4×10^7 laboratory units (LU)/mg (11). Antiviral activity of IFNs was titrated by a hemagglutination yield-reduction bioassay using GDVII virus in L cells (12). MuIFN-γ activity is expressed in LU since no homologous international standard is yet available. Purified MuIFN-γ was labeled with [^{125}]I-Bolton-Hunter reagent to a specific activity of 50 μCi/μg (7). Two-thirds of the original antiviral activity was retained; on SDS-PAGE 85% of this activity co-migrated with a 40Kd protein and 15% with a 20Kd protein; both the dimer and the monomer had antiviral activity.

L929 fibroblast nuclei were prepared by a modification of the method of Seale as previously described (7,13). Briefly, thoroughly washed samples of $1-5 \times 10^8$ cells were subjected to hypotonic lysis, homogenization by 35 strokes of a type-B tight-fitting Dounce pestle, and centrifugation for 4 min at 800 x g; the homogenate was resuspended in lysis buffer and sedimented at 1,000 x g for 10 min on a 15% Ficoll cushion. The nuclei were washed in lysis buffer, resuspended in EMEM, and counted. More than 65% of the nuclei appeared to be free from cytoplasmic residue and cytoplasmic and plasma membrane enzyme markers. The degree of purity was monitored both by measuring the contamination with marker enzymes from plasma membrane (4.7% of total alkaline phosphodiesterase) or from endoplasmic reticulum (7% of total NDAPH cytochrome (P-450) reductase) (13) and by electron microscopy (≥65% free of cytoplasmic membranes) (7). Suspensions of nuclei were capable of incorporating significant amounts of [^{32}P]dCTP (up to 100 pmol/40 min) in an *in vitro* DNA replication system (13), confirming their functional activity. Increasing amounts of MuIFN-γ were applied to triplicate samples of 5×10^5 nuclei, kept on ice for 15 min and processed as previously described for MuIFN-β (7,13).

For electron microscopy mouse L929 fibroblasts grown in suspension were washed three times with cold (4°C) Eagle's minimal essential medium (EMEM) without serum, and incubated at 4°C for 30 min with 10,000 LU/ml highly purified MuIFN-γ. After the cells were washed with cold EMEM, they were injected as a thick suspension (10^8 cells/0.5 ml EMEM) into 10 ml of EMEM prewarmed to 37°C, and aliquots (~10^7 cells) transferred at intervals

(0-15 min) into cold (4°C) fixative (0.5% glutaraldehyde, 4% paraformaldehyde in EMEM) for 30 min.

Fixed cells were washed for 1 hr with 0.1 M lysine to block free aldehyde groups, dehydrated in dimethylformamide, and embedded in the Lowicryl K4M (14). Ultrathin sections were cut and collected on 300-mesh copper grids covered with Formvar film. The sections were first floated on a drop of 0.4% bovine serum albumin for 1 hr, rinsed briefly, and transferred onto a drop of anti-MuIFN-γ rabbit IgG (1:900) (provided by Dr. Havell, Saranak Lake, NY). This IgG preparation was specific for MuIFN-γ and did not neutralize MuIFN-β; extensive absorbtion with L929 cells before use did not cause any loss of potency (1:70,000 against 10 LU MuIFN-γ). After 1 hr incubation at room temperature, the sections were thoroughly washed with warm water (~60°C) and exposed to 1:500 dilution of colloidal gold-protein A conjugate (CGPA) (E-Y Laboratories, San Mateo, CA) to allow for binding of the protein A to the Fc portion of the IgG. This dilution of CGPA gave a nonspecific CGPA adherence to Lowicryl K4M of 0.008 ± 0.002 ($p<0.05$) CG particles per 1 μm^2 of the plastic outside the cell sections (5). Sections of cells not treated with IFN and processed as described served as one set of controls; another control consisted of sections of cells treated with IFN but not incubated with anti-IFN antibody. Electron microscopy was performed with a Philips EM400 electron microscope at magnifications x3,600-x28,000.

RESULTS AND DISCUSSION

The method of following the uptake of [^{125}I]-MuIFN-γ into a mass population of mouse L929 cells revealed rapid internalization of the [^{125}I] label ($t_{1/2}$ < 1.0 min) (data not shown), similar to results obtained with human IFN-γ (8) in human cells and murine IFN-β in L929 cells (5,7); this method, however, cannot unequivocally identify where IFN-γ goes in the cell.

Fig. 1 shows electron micrographs of ultrathin sections of L929 cells incubated at 37°C for different lengths of time with natural MuIFN-γ. In the first 60 sec, IFN molecules were detected on coated areas of the plasma membrane and in coated pits (1a,b). Coated pits containing IFN were seen to invaginate forming coated vesicles (1c) and receptosomes (1d). This process, characteristic of RME, took place within 1-2 min of incubation at 37°C. The label was located very close to the membrane of these organelles, implying that IFN was not detached from the receptor. Within 2 min of incubation the majority of MuIFN molecules were detected on and in the cell nucleus (1e,f), although some IFN could be found in the nucleus even within 60 sec. On many micrographs IFN was shown to be lcoated in or near nuclear pores (1e).

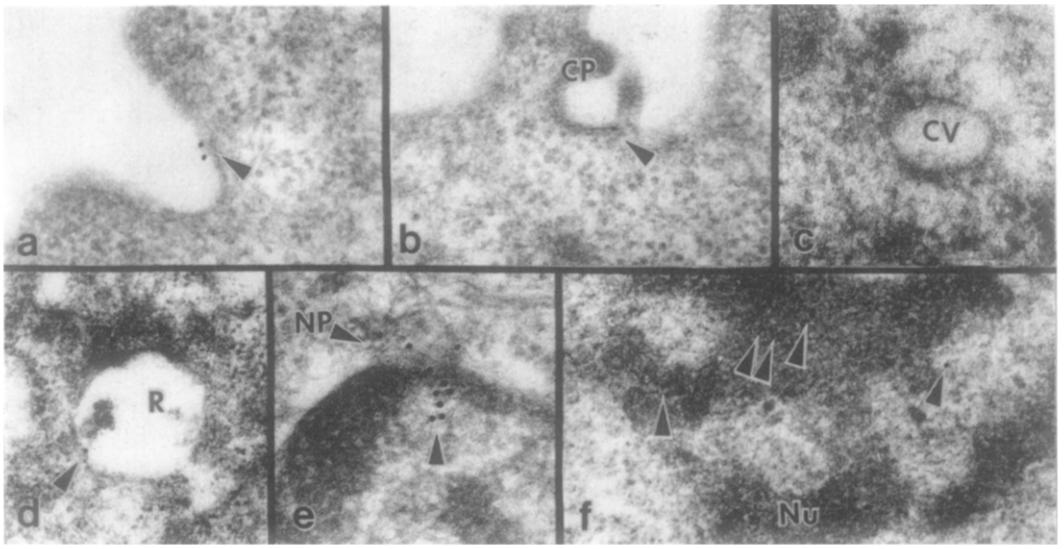

Fig. 1. Electron photomicrographs showing rapid receptor-mediated endocytotic movement of MuIFN-γ through the cytoplasm and into the nucleus of L929 mouse fibroblasts. After treatment at 4°C with MuIFN-γ, cells were incubated at 37°C for different periods (1-15 min), fixed, and embedded in the hydrophilic resin Lowicryl K4M. Ultrathin sections were treated with anti-MuIFN-γ IgG (1:900) and then CGPA (1:500). (a and b) - 60 sec at 37°C after treatment with IFN; MuIFN-β located on coated areas of plasma membrane (CM) and in coated pits (CP). (c) - 1-2 min; MuIFN-γ located in a coated vesicle (CV); (e and d) - 2 min; MuIFN-γ located in receptosome (R); and on the nuclear (Nu) envelope; (e) - in a nuclear pore (NP). In 2 min the majority of the label was located inside the nucleus (Nu).

These results provide direct, unambiguous evidence that naturally internalized unmodified MuIFN-γ molecules are transported by RME to the L929 nucleus where they reside mainly in areas of dense chromatin. Isolated L929 cell nuclei also bind [^{125}I]-MuIFN-γ (Fig. 2). The nuclear binding is specific, since an excess (>30-fold) of unlabeled MuIFN-γ competes with the [^{125}I]-MuIFN-γ for nuclear membrane binding sites (Fig. 2a). By Scatchard analyses there are 24,000 high-affinity binding sites/nucleus, with a Kd of 2 x 10^{-10} M, as well as some low-affinity sites as indicated by the bi-phasic curve (Fig. 2b). High- and low-affinity nuclear binding sites have also been observed with other ligands (15,16,17). Brief treatment of isolated nuclei with trypsin (3 mg/ml) reduced binding of [^{125}I]-MuIFN-γ by 88%, suggesting that the nuclear receptor has a protein moiety. High-molarity salt (1 M NaCl), known to elute IFNs from nucleic acid affinity columns (18), reduced binding by

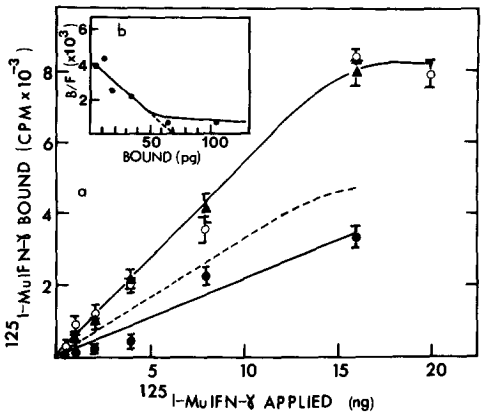

Fig. 2a. Binding of [^{125}I]-MuIFN-γ to isolated L929 cell nuclei. Increasing amounts of [^{125}I]-MuIFN-γ were added to 5 x 10^5 L929 nuclei incubated at 4°C for 15 min, after which the amount of nucleus-bound [^{125}I] was determined (7). O, [^{125}I]-MuIFN-γ alone; ●, binding of [^{125}I]-MuIFN-γ in the presence of 500 ng of cold MuIFN-γ; ▲, binding of [^{125}I]-MuIFN-γ in the presence of 800 ng of cold MuIFN-β (1.3 x 10^8 IU/mg, Lee Biomolecular). The vertical bars indicate the standard error of the mean. The dashed line represents the specific binding calculated by subtracting the cpm of [^{125}I] bound in the presence of cold MuIFN-γ from the cpm of total [^{125}I] bound.

Fig. 2b. The inset figure is a Scatchard plot of the data for specific binding of [^{125}I]-MuIFN-γ (as the dimer).

only 12%. Lack of competition for nuclear binding of [^{125}I]-MuIFN-γ by excess unlabeled MuIFN-β indicates that β and γ IFNs have different nuclear receptors (5), a distinction that has also been noted for the plasma membrane receptors for these IFNs (3). Trapping of [^{125}I]-MuIFN-γ by chromatin as illustrated by electron microscopy may possibly account for the values observed at low bound/free ratios shown in the Scatchard plot (Fig. 2b, inset).

The physiological significance of the very rapid transport of IFN-γ into the nucleus, which may be facilitated by nuclear membrane receptors has still to be determined. Recent evidence suggests a possible role for early, direct nuclear IFN effects. Human IFN-α and -β induce new synthesis of several mRNAs within 5-10 min, and recombinant human IFN-γ within 30 min after application to the cells (19-22). Various biologically active

ligands, such as insulin, nerve and epidermal growth factors, and steroid hormones, bind to the nuclear membrane or the nuclear matrix (15,16,17). Indeed, the treatment of isolated nuclei with insulin increases nuclear mRNA efflux, nucleotide triphosphatase activity, and protein phosphorylation (15). Brief treatment of isolated nuclei of mouse L cells with murine IFN-β rapidly decreases RNA efflux from the nuclei in dose-dependent manner (13). The very rapid uptake and transport of immunologically recognizable IFN-γ molecules through the cytoplasm and into the dense chromatin areas of the nucleus suggests that IFN molecules may act as their own messengers, either alone or bound to receptor, and may be directly involved in genome regulation.

ACKNOWLEDGMENTS

We thank Dr. Edward Havell for his gift of anti-MuIFN-γ antibody. We also thank Janet DeBruin for technical assistance. This work was supported in part by grants from the Walter Schroeder Foundation, the Stackner Family Foundation, and the NIH.

REFERENCES

1. Epstein, L.B. (1984) in Interferon, Volume 2: Interferons and the Immune System, J. Vilcek and E. DeMaeyer, eds., pp. 185-220, Elsevier, Amsterdam.
2. Weil, J., Epstein, C.J., Epstein, L.B., Sedmak, J.J., Sabran, J.L., and Grossberg, S.E. (1983) Nature (London) 301, 437-439.
3. Zoon, K. and Arnheiter, H. (1984) Pharmac Ther 24, 259-278.
4. Zoon, K.C., Arnheiter, H., Zur Nedden, D., Fitzgerald, D.J.P., and Willingham, M.C. (1983) Virology 130, 195-203.
5. Kushnaryov, V.M., DeBruin, J., LeMense, G., MacDonald, H.S., Sedmak, J.J., and Grossberg, S.E. (1986) J Interferon Res (in press).
6. Kushnaryov, V.M., MacDonald, H.S., Sedmak, J.J., Grossberg, S.E. (1983) Infect Immun 40, 320-329.
7. Kushnaryov, V.M., MacDonald, H.S., Sedmak, J.J., Grossberg, S.E. (1985) Proc Natl Acad Sci USA 82, 3281-3285.
8. Anderson, P., Yip, Y.K., Vilcek, J. (1983) J Biol Chem 258, 6497-6502.
9. Wileman, T., Harding, C., Stahl, P.L. (1985) Biochem J 232, 1-14.
10. Anderson, R.G.W., Kaplan, J. (1983) Modern Cell Biol 1, 1-52.
11. Taylor, J.L., Sedmak, J.J., Jameson, P., Lin, Y.-G., Grossberg, S.E. (1984) J Interferon Res 4, 315-327.
12. Jameson, P. and Grossberg, S.E. (1984) In Interferons, Methods in Enzymology, S. Pestka, ed., pp. 312-315, Academic Press, New York.
13. MacDonald, H.S., Kushnaryov, V.M., Hevner, R., Sedmak, J.J., and Grossberg, S.E. (1986) J Interferon Res (in press).
14. Altman, L.G., Schneider, B.G., and Papermaster, D. (1984) J Histochem Cytochem 32, 1217-1223.
15. Goldfine, I.D., Kriz, R.M., and Wong, K.Y. (1981) In Receptor-mediated Binding and Internalization of Toxins and Hormones, pp. 233-250, Middlebrook, J.L., Kohn, L.D., eds., Academic Press, New York.

16. Yanker, B., and Shooter, E. (1980) Proc Natl Acad Sci USA 76, 1269-1273.
17. Johnson, L.K., Vlodavsky, I., Baxter, J.D., Gospodarowicz, D. (1980) Nature (London) 287, 340-343.
18. Tatchell, K. and Van Holde, K.E. (1977) Biochemistry 16, 5295-5303.
19. Friedman, R.L., Manly, S.P., McMahon, M., Kerr, I.M., and Stark, G.R. (1984) Cell 38, 745-755.
20. Friedman, R.L., and Stark, G.R. (1985) Nature (London) 314, 637-639.
21. Larner, A.C., Jonak, G., Cheng, Y.-S.E., Korant, B., Knight, E., and Darnell, J., Jr. (1984) Proc Natl Acad Sci USA 81, 6733-6737.
22. Luster, A.D., Unkeless, J.C., and Ravetch, J.V. (1985) Nature (London) 315, 672-676.