Phosphorylation of Recombinant Interferon-gamma by Kinases Released from Various Cells

By B. ROBERT-GALLIOT, M. J. COMMOY-CHEVALIER, P. GEORGES* AND C. CHANY*

Institut National de la Santé et de la Recherche Médicale, U.43 and 1Laboratoire de Biochimie, Hôpital Saint Vincent-de-Paul, 74 avenue Denfert-Rochereau, 75674 Paris Cedex 14, France

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SUMMARY

Recombinant interferon-gamma (IFN-γ) in contact with human embryonic fibroblasts or with a great variety of cells from different animal species was phosphorylated in the presence of [γ-32P]ATP and magnesium ions by a protein kinase released in the culture medium. Using SDS-polyacrylamide gel electrophoresis, we found that both the monomeric (17000 to 18000) and dimeric (34000 to 35000) molecular weight forms of IFN-γ became intensely radioactive. Serine, but not threonine or tyrosine, was phosphorylated. It is of interest that the kinase released from reputedly insensitive cells also phosphorylated IFN-γ. The process did not noticeably degrade the antiviral functions of the molecule nor did it affect, at least in a detectable manner, its anti-proliferative effect on WISH or Daudi cells. Furthermore, the antigenic structure and its capacity to react with monoclonal antibodies were also unaltered. It is presently not known which biological function is regulated by the phosphorylating process.

INTRODUCTION

Phosphorylation probably plays an important role in the transmission of intercellular signals and in the response of individual cells to hormones or other mediators. Protein kinase A is cAMP- and cGMP-dependent. Protein kinase C is activated by diacyl glycerol (an intermediate of the phosphatidylinositol metabolic cycle) in the presence of calcium ions and seems to be involved in the regulation of cell proliferation (Marx, 1984; Nishizuka, 1984). Furthermore, a protein kinase has been reported which, when released from the outer cell membrane, can phosphorylate various substrates, such as phosvitin (Kübler et al., 1983), and for which presently no cellular function has been assigned.

We report here a new observation on the effect of such an apparently constitutively released protein kinase which, in the presence of [γ-32P]ATP and magnesium ions, rapidly phosphorylates human recombinant interferon-gamma (IFN-γ) (Seahill et al., 1983) in its monomeric and dimeric forms. The phosphorylation procedure does not result in a detectable inactivation of the molecule, as expressed in terms of antiviral protection and anti-proliferative effect. Binding to monoclonal antibodies is also not impaired, as estimated by Western blot analysis.

METHODS

Reagents. Recombinant human IFN-γ with a specific activity (sp. act.) of 3 × 10^7 units (U)/mg protein (780 μg/ml) or 7 × 10^7 U/mg protein (185 μg/ml), with a purity of >96% and stabilized with human serum albumin, was provided by Biogen. Monoclonal antibody against human IFN-γ was employed at a dilution of 1/2000 as indicated by Biogen for use in Western blot analysis (sp. act. in solid phase radioimmunoassay 100000 to 300000) and affinity chromatography. IFN-β [sp. act. 2 × 10^7 international units (IU)/mg protein] was purchased from Cytotech and Upsa Laboratories. Lymphoblastoid IFN-α (sp. act. 2 × 10^6 IU/mg protein) was provided by Hayashibara Biochemical Laboratories.

Mouse IFN (sp. act. 4 × 10^6 IU/mg protein) was prepared in Ehrlich ascites tumour cells according to a previously described method (Dussaix et al., 1983).
Phosvitin and human serum albumin were from Sigma. Medium and serum were from Gibco. Activated CH-Sepharose 4B was purchased from Pharmacia.

The protein markers (electrophoresis calibration kit, Pharmacia) were: phosphorylase b (94000 mol. wt.), bovine albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100) and α-lactalbumin (14400).

Horseradish peroxidase-conjugated anti-mouse IgG was prepared in rabbit (Miles Yeda, Rehovot, Israel). [3H]Thymidine was purchased from the Commissariat à l’Energie Atomique (CEA), Gif-Sur-Yvette, France.

Cell culture and IFN assay. Human WISH, HeLa, Hos, mouse L and MDBK (bovine) cells were routinely propagated in Eagle’s MEM supplemented with 10% newborn calf serum. Human embryonic fibroblasts F7000 (Flow Laboratories) were grown in Eagle’s basal medium plus 10% foetal calf serum. Carp cells were received from Dr P. de Kinkelin. Aedes albopictus mosquito cells were provided by Dr L. Rosen.

The different IFNs were assayed by microtitration using the 50% inhibition of vesicular stomatitis virus-induced cytopathic effect in three human cell lines, WISH, HeLa or F7000, for human IFN and in L cells for mouse IFN (Lebon et al., 1980).

The anti-proliferative properties were estimated using WISH or lymphoblastoid Daudi cells cultured in growth medium supplemented with 5% calf serum (5 × 10⁵ cells/ml). After 24, 48 and 72 h of incubation with IFN, the cells were treated with 10 μCi/ml [3H]thymidine (sp. act. 25 Ci/mmol; CEA). The incorporation of [3H]thymidine was measured in the acid-insoluble fractions at 24, 48 and 72 h. In parallel, the number of cells was counted in a haemocytometer.

Radioactive labelling. Adenosine 5’-[γ-32P]triphosphate, triethylammonium salt, with a sp. act. of 10 Ci/mol (370 GBq/mmol), was purchased from Amersham. Labelling was carried out as described earlier (Kübner et al., 1979) using a monolayer of 10⁶ cells, washed twice with prewarmed assay mixture (70 mM-NaCl, 30 mM-Tris-acetate, 5 mM-magnesium acetate, 5 mM-potassium phosphate, 0.5 mM-EDTA, 75 mM-glucose, pH 7-2).

Labelling of the cell-bound IFN. The cells were incubated for 15 min at 37°C with the assay mixture containing either IFN-α, IFN-β or IFN-γ or phosvitin (at the concentrations indicated in the text) and with 0.5 μM-[γ-32P]ATP, with adequate controls. The phosphorylation of IFN bound to the receptor was measured after eliminating the supernatant, washing the cells twice with ice-cold assay mixture (containing 1 mM of unlabelled ATP) and lysing them with sample buffer.

Phosphorylation of unbound IFN after cell contact. After 15 min of incubation at 37°C, the assay mixtures, containing or not containing IFN or phosvitin, were removed from the monolayers of cells, centrifuged at 2500 g for 10 min. The supernatants (300 μl) were incubated in the presence of 0.5 μM-[γ-32P]ATP for 15 min at 30°C. Two aliquots of 100 μl each were deposited on a Whatman 3MM filter and precipitated by washing three times with 10% TCA at 4°C. The radioactivity of the different preparations was determined in a beta scintillation counter (Intertechnique). Another aliquot (also 100 μl) was mixed with a sample buffer to stop the phosphorylation reaction and used for SDS-gel electrophoresis.

Phosphorylation of IFN by kinases released by the cells. The cells were incubated with assay mixture for 15 min at 37°C. The supernatants were collected by centrifugation at 2500 g for 10 min. The IFNs were mixed 1/2 with the supernatants and incubated with [γ-32P]ATP for 15 min at 30°C. The radioactivity after TCA precipitation was counted as described above. SDS-gel electrophoresis was performed in the same manner as detailed below.

Analysis by SDS-polyacrylamide gel electrophoresis. SDS-PAGE was carried out according to a technique previously described (Laemmli, 1970). All the aliquots were incubated with sample buffer (0.1 M-Tris-Tris-HCl pH 6.8, 2.5% SDS, 10% glycerol, 0.6 m-bromophenol blue, 1.54% dithiothreitol), boiled for 3 min at 100°C, and applied to a 15% SDS-polyacrylamide gel. The gels were then autoradiographed (Films Orwo HS 90).

Amino acid analysis. After electrophoresis on polyacrylamide gel, the samples were eluted and hydrolysed with 6 M-HCl at 110°C for 2 h. The analysis of the amino acids was performed on a Liquimat 3 (Roche-Kontron), according to a previously described technique (Capony & Demaille, 1983), using acrylamide chromatography on columns containing DC6A sulphonated polystyrene resin (Durrum). The fractions were eluted with 1 M-trifluoroacetic acid. The order and height of the eluted peaks were estimated by their fluorescence using o-phthaldialdehyde. Each fraction was tested for radioactivity. O-Phosphoserine, O-phosphothreonine and O-phosphotyrosine, employed as reference, were purchased from Sigma.

Electrophoretic transfer. After acrylamide gel (12.5%) electrophoresis, the proteins were blotted to a nitrocellulose filter, using a Trans-Blot cell apparatus (Bio-Rad) (40 V, 4 h) in Tris buffer pH 8.5 (10 mM-Tris-HCl, 150 mM-glycine, 20% methanol). The filter was incubated with monoclonal antibody (dilution 1/2000) against human IFN-γ overnight at 4°C. The complex was then labelled with a peroxidase-conjugated anti-mouse IgG (Miles Laboratories), incubated for 45 min at room temperature and detected with diaminobenzidine and hydrogen peroxide (Towbin et al., 1979).

Affinity chromatography. The same monoclonal antibody as the one used in the electrophoretic transfer experiments was bound to activated CH-Sepharose 4B (Pharmacia). IFN, phosphate-labelled or not, diluted in the assay mixture indicated for phosphorylation (pH 7.2) was added to the affinity column and incubated for 20 h at 4°C. Thereafter, the column was washed, the bound IFN eluted with McIlvaine’s citrate–phosphate buffer pH 2.2, and the fractions were tested for radioactivity.
RESULTS
Phosphorylation of IFN in contact with sensitive cells

To study the phosphorylation of IFN-γ, we used a human transformed cell line (HeLa) and normal human embryonic fibroblasts (F7000). The cells were washed twice with the assay mixture (see Methods) and then treated for 15 min at 37 °C in the presence of 1 ml IFN-γ (3 × 10⁶ U/ml) and 0.5 μM-[γ-³²P]ATP. The cellular phase was solubilized with the sample buffer and then analysed by SDS–PAGE.

The results of the assay in normal fibroblasts (control, lane 1; IFN-treated, lane 2) and in HeLa cells (control, lane 3; IFN-treated, lane 4) are shown in Fig. 1. It is of interest that the number of phosphorylating bands is significantly higher in the transformed HeLa cells when compared to normal fibroblasts. However, in both cases, an intense labelling was seen in the 17000 mol. wt. region and a less reactive but distinct double band in the 35000 mol. wt. area. These are generally attributed to the monomeric and dimeric forms of recombinant IFN-γ and will be proven clearly further on in the text.

Lack of phosphorylation of human IFN-α, IFN-β and mouse IFN

The effect of such cellular protein kinases on other IFN species of human or animal origin was compared (Fig. 2). HeLa cells were treated by human IFN-α (lane 4), IFN-β (lane 2) and IFN-γ (lane 3) and by mouse IFN-α/β (lane 6) in the same manner as described above. An additional control (lane 5) was performed using assay buffer supplemented with human serum albumin at the same concentration (2 mg/ml) as IFN-γ. All IFNs were used at comparable concentrations based on their antiviral effect on HeLa cells (10⁷ reference units in 1 ml). Mouse IFN was used at the concentration of 2 × 10⁶ reference units/ml.

Only lane 3 corresponding to IFN-γ showed labelling in the 17000 and 35000 mol. wt. areas. It is noteworthy that cloned human IFN-α or beta interferons at similar concentrations were also not phosphorylated (data not shown).

Release of protein kinase in the supernatant from human IFN-γ-treated or untreated cells

To explore whether the enzymic activity was bound to the cell membrane, present in the cytosol or, on the contrary, released in the supernatant as known for phosvitin (Kübler et al., 1983), the cells were treated with either 1 ml phosvitin (1 mg/ml) or 1 ml IFN-γ (10⁷ units/ml) for 15 min at 37 °C. The supernatant was then removed and centrifuged and [γ-³²P]ATP and magnesium ions were added. The mixture was incubated for 15 min at 30 °C. The preparation was diluted 1:2 with sample buffer and analysed by SDS–PAGE.

The results obtained after autoradiography are presented in Fig. 3. The zone corresponding to either monomeric or dimeric IFN-γ was strongly labelled by [γ-³²P]ATP, while with phosvitin the phosphorylated bands were found in the 43000 mol. wt. area (Fig. 3a), in agreement with Kübler et al. (1983). When HeLa cells were incubated for 15 min with assay mixture and the supernatant mixed 1:2 with IFN-γ, a similar pattern of phosphorylation was seen with a somewhat lower intensity (Fig. 3b). If IFN was incubated only with [γ-³²P]ATP and buffer, no reaction was detected (data not shown). Thus, protein kinase was released from the cells in the supernatant whether or not they were treated with IFN-γ.

Dose–response relationship

The dose–response effect was studied by measuring phosphorylation using the supernatant from HeLa cells incubated for 15 min at 37 °C with the assay mixture or with a range of increasing concentrations of IFN-γ from 10⁵ to 3 × 10⁷ as shown in Fig. 4. To each aliquot, [γ-³²P]ATP was added for 15 min at 30 °C.

The results show a linear dose–response relationship between the amount of IFN used and the count. This linearity is also illustrated in the insert which represents the SDS-gel electrophoresis of each subsequent IFN aliquot when the reaction was completed.
Fig. 1. Phosphorylation of IFN in contact with F7000 (lanes 1 and 2) or HeLa cells (lanes 3 and 4). Cells were treated for 15 min at 37 °C with [γ-32P]ATP (0.5 μM) in assay mixture alone (lanes 1 and 3) or with the addition of IFN-γ (lanes 2 and 4). The cell phase was analysed on a 15% SDS–polyacrylamide gel and subjected to autoradiography. The positions of the monomeric (17000 mol. wt.) and dimeric (35000 mol. wt.) forms of IFN-γ are indicated.

Fig. 2. Lack of phosphorylation of human IFN-α and IFN-β and mouse IFN-α/β. HeLa cells were treated for 15 min at 37 °C with assay mixture (lane 1), IFN-β (lane 2), IFN-γ (lane 3), IFN-α (lane 4), assay mixture containing 2 mg/ml human serum albumin (lane 5) and mouse IFN-α/β (lane 6). [γ-32P]ATP (0.5 μM) was added at the same time as IFN. The cell phase was analysed on a 15% SDS–polyacrylamide gel and subjected to autoradiography. The positions of the monomeric and dimeric mol. wt. forms of IFN-γ are indicated.
Phosphorylation of recombinant interferon-gamma

Fig. 3. Release of protein kinase in the supernatant. (a) HeLa cells were treated for 15 min at 37°C with 1 ml IFN-γ (3 × 10^7 U) (lane 1) and 1 ml phosvitin (1 mg/ml) (lane 2). The supernatants were centrifuged at 2500 g for 10 min. Phosphorylation was performed at 30°C for 15 min with 0.5 μM[γ-32P]ATP. (b) HeLa cells were incubated with assay mixture for 15 min. The supernatants were centrifuged and diluted 1:2 with IFN-γ (3 × 10^7 U) (lane 1) or with phosvitin (2 mg/ml) (lane 2). Phosphorylation was performed at 30°C for 15 min with 0.5 μM[γ-32P]ATP. The samples were analysed on a 15% SDS-polyacrylamide gel and subjected to autoradiography. The positions of the monomeric and dimeric mol. wt. forms of IFN-γ and the marker protein ovalbumin (43000 mol. wt.) are indicated.

Release of protein kinase from different cell species

It was of interest to investigate to what extent the protein kinase, released from different cell species, phosphorylated human IFN-γ. For reference, phosvitin was employed. The results, summarized in Table 1, clearly show that all the cells tested released protein kinase, even phylogenetically remote species such as fish and mosquito cells. The level of phosphorylation was, however, not parallel when comparing phosvitin and IFN-γ. Furthermore, when controlled in SDS-PAGE, as already indicated previously, both IFN-γ molecular species were labelled, whatever the cell type employed, while in the presence of phosvitin, a 43000 mol. wt. band was seen (data not shown).

Phosphorylation of serine residues

The amino acid residues of IFN-γ, which bind the phosphoryl group from [γ-32P]ATP, were analysed. The major 17000 mol. wt. component eluted from SDS-PAGE was employed. The hydrolysed molecule was applied on a column containing DC6A sulphonated polystyrene resin, as described in Methods (Capony & Demaille, 1983). As reference, phosphoserine, phosphothreonine and phosphotyrosine were used. In Fig. 5, we show the fluorescence diagram obtained. The first important peak represents the unbound material after inoculation. The different fractions were tested for radioactivity. The only phospho-labelled peak was found at the retention time corresponding to phosphoserine, while phosphothreonine and phosphotyrosine were not radioactive. Similar results (not shown here) were obtained using thin-layer electrophoresis (Hunter & Sefton, 1980).
Fig. 4. Dose-response relationship. HeLa cells were treated with the different IFN concentrations for 15 min at 37 °C and phosphorylation was measured in the supernatant incubated in the presence of [γ-32P]ATP for 15 min at 30 °C. Samples were preincubated with 10% TCA and counted. In parallel, at each point, the aliquots were analysed on an SDS–polyacrylamide gel (inset) and subjected to autoradiography.

| Table 1. Phosphorylation of IFN-γ supernatant from different cells* |
|---|---|---|---|
| Cell line | Control (c.p.m.) | IFN-γ (c.p.m.) | Phosvitin (c.p.m.) |
| Human F7000 | 707 | 31957 | 2843 |
| WISH | 870 | 4603 | 6608 |
| HeLa | 796 | 9432 | 10694 |
| Hos | 1089 | 5719 | 9316 |
| Mouse L | 1150 | 13501 | 9730 |
| MDBK | 721 | 11570 | 8664 |
| Carp | 781 | 6211 | 5352 |
| Mosquito | 2424 | 15523 | NT† |

* Cells were incubated with assay mixture for 15 min at 37 °C. The supernatants were collected and diluted 1:2 with either IFN-γ or phosvitin. Phosphorylation was performed as described in Methods.
† NT, Not tested.

Effect of phosphorylation on antiviral functions and binding to monoclonal antibodies

It was of interest to explore whether or not phosphorylation modified the biological properties and the antigenic responsiveness of the recombinant molecule. In six successive experiments we could not detect any change in the capacity to protect WISH cells whether or not the molecule was phosphorylated (data not shown). Furthermore, monospecific antiserum neutralized similarly the antiviral effect of phosphorylated or control IFN.

The effect of phosphorylation on the binding to monoclonal antibodies was studied using a Western blot transfer, as described in Methods. As shown in Fig. 6, the binding capacity of the peroxidase-labelled monoclonal antibody did not seem to be significantly modified in the
Fig. 5. Phosphorylation of serine residues. After hydrolysis, samples were analysed on a column containing DC6A sulphonated polystyrene resin (Durrum). The amino acids were detected by their fluorescence using o-phthalaldehyde. Phosphoserine (P-SER), phosphothreonine (P-THR) and phosphotyrosine (P-TYR) were employed as reference. (a) Elution profile of phosphorylated reference amino acids as measured by fluorescence. The column was inactivated with 1-25 nmol phospho-amino acid standard markers. (b) The only peak containing the radiolabel is that of P-serine (P-SER) (arrow).

Fig. 6. Analysis of phosphorylated IFN-γ by electrophoresis and Western blot transfer. Indirect peroxidase-labelled monoclonal antibody binding of IFN-γ (lane 1) on phosphorylated IFN-γ (lane 2) is shown. Lane 3, autoradiography of the same sample as in lane 2.
Table 2. *Anti-proliferative properties of IFN-γ*

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<th>IFN-γ (10^2 U/ml)</th>
<th>IFN-γ (10^3 U/ml)</th>
<th>IFN-γ (10^4 U/ml)</th>
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<td></td>
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<td>Acid-insoluble</td>
<td>Acid-soluble</td>
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<td>Phosphorylated IFN-γ</td>
<td>471 ± 46</td>
<td>358128 ± 25235</td>
<td>184 ± 56</td>
<td>169123 ± 16076</td>
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<tr>
<td>IFN-γ</td>
<td>471 ± 46</td>
<td>358128 ± 25235</td>
<td>177 ± 48</td>
<td>161598 ± 11168</td>
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* The incorporation of [3H]thymidine in acid-insoluble and soluble fractions was estimated in WISH cells treated for 48 h by phosphorylated or unphosphorylated IFN-γ. Each count represents the mean value and standard variation for four plates.
phosphorylated specimen (lane 2) when compared to the control (lane 1). The small difference seen in the experiment did not show up consistently and cannot be considered as significant. In lane 3, we show the radiolabelled and phosphorylated IFN sample after autoradiography. The preparations in lanes 2 and 3 are identical, the first representing the monoclonal peroxidase-labelled bands and the other showing their radioactivity. Further control experiments were performed in which labelled or unlabelled IFN-γ was bound to the monoclonal IFN-γ antibody column, as indicated in Methods. The analysis of the eluted fractions further confirmed that radioactivity and antiviral protection eluted simultaneously from the column. When submitted to SDS–PAGE, the purified IFN was again localized in the same molecular weight areas (data not shown).

Lack of detectable modification of IFN-γ anti-proliferative properties after phosphorylation

It is well-known that IFN-γ, like the other IFNs, inhibits cell proliferation (Rubin & Gupta, 1980). We have tested human amniotic WISH and lymphoblastoid Daudi cells, studying in parallel DNA synthesis and cell count, as indicated in Methods.

Both the 17000 and 35000 mol. wt. fractions of IFN-γ were incubated on cells and phosphorylated. Then the phosphorylated or unphosphorylated IFN-γ was analysed for its anti-proliferative capacities. In WISH cells, the inhibition of cellular DNA synthesis was not modified by phosphorylation, as shown in Table 2. Similarly, the IFN-induced decrease of the thymidine pool size was not altered. Daudi cells, which are completely insensitive to IFN-α, while highly responsive to IFN-γ while highly responsive to IFN-α, were also unaffected by the phosphorylated or unphosphorylated IFN molecules (data not shown).

DISCUSSION

The data presented here show that recombinant IFN-γ is phosphorylated in serine residues by protein kinases released from the cells. The immune electrophoretic transfer and the affinity chromatographical identification show unequivocally that the phosphorylated proteins are indeed two molecular forms of IFN-γ. Alpha or beta interferons incubated in parallel with the same kinase preparation are not phosphorylated. There are considerable differences in the structure of the IFNα/β and IFN-γ molecules, and this observation represents thus an additional distinction between the two IFN groups.

The dose–response relationship between the concentration of IFN-γ and the amount of IFN molecules phosphorylated is linear.

It is of interest that a number of cell species even located far apart in the phylogenetic scale can release such kinase activity, phosphorylating the IFN-γ. It is thus evident that this procedure is not involved in antiviral activity since such cells are unresponsive to the human IFN species. There is no information as to whether a single kinase is involved or whether different enzymes of this type are able to phosphorylate IFN-γ.

It is also presently not known whether the molecule released from the cells is comparable or not to protein kinase C (Nishizuka, 1984) or the one which activates the phosvitin molecules after being released from the cells. Antigenicity and antiviral and anti-proliferative properties of IFN-γ are seemingly unaffected by phosphorylation, which does not exclude, however, possible changes in other presently unknown biological functions it possesses. Besides its theoretical interest, it is noteworthy that the phosphorylation of IFN-γ is easily obtained and can be applied to biological tests, including radioimmunoassays.

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Note added in proof. After submission of this manuscript, we received information from A. Rashidbaigi, H.-F. Kung and S. Pestka, following our abstract presentation at the Third TNO/ISIR Congress on the Biology of the Interferon System [Chany, C., Robert-Galliot, B., Commoy-Chevalier, M. J. & Georges, P. (1984). Cell-released
protein kinases phosphorylate recombinant interferon-gamma. *Antiviral Research*, September, Abstract 1, p. 37]. These authors have also observed, using a commercial bovine protein kinase, that interferon can be phosphorylated with efficacy and that such labelled material can be used for the study of receptors in sensitive cells [Rashidbaigi, A., Kung, H.-F. & Pestka, S. (1985). Study of the receptor for immune interferon in human histiocytic lymphoma, U937, cells with a $^{32}$P-labelled human recombinant immune interferon. Abstract. *Federation Proceedings* (March), in press.

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