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DISSOCIATION OF PROTEIN KINASE ACTIVITY AND THE INDUCTION OF THE ANTIVIRAL STATE IN A CELL LINE RESPONSIVE TO THE ANTIVIRAL EFFECTS OF INTERFERON

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SUMMARY: Interferons or oxidized glutathione were found to induce double-stranded RNA-dependent protein kinase activity in mouse L cells that phosphorylates the α subunit of eukaryotic peptide initiation factor 2. A mixture of leukocyte/fibroblast interferons as well as immune interferon induced the protein kinase and also suppressed virus replication in the L cells. Oxidized glutathione was equally effective in inducing protein kinase activity, but it did not induce an antiviral state in these cells. The data suggest that a simple cause and effect relationship does not exist between protein kinase induction and the establishment of the antiviral state in a cell that is responsive to the antiviral effects of interferon.

Several interferon (IFN)[1]-induced molecular events have recently been described which may play a role in expression of IFN-mediated biological activities, such as establishment of an antiviral state and immunosuppression (reviewed in 1). Suppression of viral and cellular protein synthesis is clearly a major consequence of IFN activity. Translational inhibition induced by IFN treatment of susceptible cells can be demonstrated in cell-free extracts. Two well-established enzymatic activities appear as a consequence of exposing susceptible cells to IFN. Both are dependent on preactivation by double-stranded RNA (dsRNA) after induction by IFN. One is a 2', 5'-oligoisoadenylate synthetase (2-4); the other is a cAMP-independent protein kinase that phosphorylates the small (α) subunit of peptide initiation factor2(eIF-2α; 2, 4-6). The latter enzyme is found in ribosomal extracts of IFN-treated cells. Phos-

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phorylation of eIF-2α has been correlated with inhibition of protein synthesis, or more specifically peptide initiation, as reviewed recently (7).

We have shown previously that IFN and sulfydryl (SH)-reactive agents such as oxidized glutathione (GSSG) act in a similar manner to suppress the *in vitro* antibody-production response of mouse spleen cells (3). In the study reported here, mouse L-cells were treated with L-cell IFN (IFN-α/β), immune IFN (IFN-γ), or GSSG. Induction of the antiviral state in L cells was tested and compared with the appearance of the eIF-2α protein kinase activity in ribosomal extracts.

MATERIALS AND METHODS

Cell Culture. The mouse L-cell fibroblast line (L-Galveston) was grown in 2000 ml roller bottles at 37°C to a density of 4 x 10^8 cell/bottle. This L-cell line is routinely used in mouse IFN assays. Growth medium consisted of RPMI-1640 and 5% fetal calf serum (GIBCO). One day after seeding, fresh medium containing the substance to be tested was added. Partially purified mouse IFN-α/β (L-cell IFN) and staphylococcal enterotoxin A-induced mouse IFN γ were used at 300 units/ml. The IFNs were prepared as described below. GSSG (Sigma) was used at 1 mg/ml. The cultures were incubated for an additional 24 hours at 37°C. The cells were harvested with a rubber policeman.

Preparation of Interferons. L cell IFN is a mixture of α (leukocyte) and β (fibroblast) IFNs and is designated as IFN-α/β here (9). It was produced by stimulation of L cells with Newcastle disease virus and partially purified by adsorption to Controlled Pore-Glass (CPG) beads, followed by chromatography on an Ultragel AcA54 column (10). The final product has a specific activity of about 10^7 units/mg protein. IFN-γ was produced in mouse spleen cells as previously described (11) and purified on CPG beads and by Ultragel AcA chromatography as described above. The final product had a specific activity of approximately $10^{4.5}$ to $10^{5.0}$ units/mg protein. Interferon activity was assayed in microliter plates by plaque reduction as previously described (11).

Preparation of the Ribosomal Salt Wash Fraction. The procedure described by Pinphanichakarn *et al.* (12) was followed to lyse the cells. The postmitochondrial supernatant was then centrifuged for 4 hr at 45,000 rpm (Ti50 rotor). The supernatant was discarded. The translucent ribosomal pellet was rinsed, then suspended in a solution containing 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM dithiothreitol and 0.1 mM EDTA. KCl, 4 M, was added to the ribosome suspension to give a final concentration of 0.5 M and the ribosomes were stirred on ice for 30 min. The ribosomes were removed by ultracentrifugation as described above. The supernatant comprised the ribosomal salt wash fraction and represents the protein kinase fraction used in the studies described below. Its protein concentration was determined from absorbance at 260 nm and 280 nm (13). The ribosomal salt wash fraction was dialyzed overnight against 10 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM dithioerythritol, 0.1 mM EDTA, then stored in small aliquots at -80°C.

Assay of Protein Kinase Activity. Activation of protein kinase activity was carried out with unlabeled ATP as described by Petryshyn *et al.* (14). The reaction mixture used for kinase activation contained in a final volume of 50 µl, 25 mM Tris-HCl (pH 7.7), 2 mM $mg(OAc)_2$, 1 mM dithiothreitol, 80 mM KCl, 5 mM creatine phosphate (Sigma), 2.5 µg creatine phosphate kinase (Sigma) and

0.1 mM ATP, 40 μg of salt wash fraction protein from treated or untreated cells, and as indicated 1.0 ng (20 ng/ml final concentration) of reovirus dsRNA (a gift from Dr. A.J. Shatkin, Roche Institute for Molecular Biology, Nutley, NJ). Incubation was for 20 min. at 30°C. Phosphorylation of eIF-2 with the pre-activated protein kinase was carried out essentially as described previously (15,16). A 20 μl aliquot of the reaction mixture described above containing the activated kinase was enlarged to a total volume of 30 μl containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 3 μg of purified eIF-2 prepared from rabbit reticulocytes as previously described (17) and 0.3 mM [γ-^{32}P]ATP (1-2 Ci/mmol; New England Nuclear, Boston). The mixture was incubated for 10 min at 37°. The reaction was terminated by the addition of 30 μl of "electrophoresis sample buffer". Proteins were analyzed on 15% polyacrylamide gels in SDS as described previously (15,16). Autoradiograms were prepared and scanned with a densitometer to determine the integrated area under the 38,000 dalton eIF-2α band. Under the assay conditions used, phosphorylation of eIF-2α was proportional to the amount of the kinase activity present in the reaction mixture, as judged from the densitometer scans of the autoradiograms

RESULTS AND DISCUSSION

It is well established that interferon induces protein kinase activity in mouse L cells that will phosphorylate the α subunit of eIF-2 (18). This protein kinase activity is dependent on dsRNA, has high specificity for eIF-2α or histones, and is associated with the ribosomes. It can be washed off the ribosomes with 0.5 M KCl in a fraction called ribosomal salt wash. Treatment of L-cells with GSSG also appears to induce a ribosome-bound, dsRNA-dependent eIF-2α kinase that may be identical to the enzyme induced by interferon. Purified eIF-2 from rabbit reticulocytes was phosphorylated with the salt wash fraction of ribosomes isolated from treated and untreated cells as described in Methods. Peptides present in the resulting reaction mixture were separated by electrophoresis in sodium dodecyl sulfate in polyacrylamide gels from which autoradiograms were made. Densitometer tracings of the eIF-2α region of these radioautographs are presented in Fig. 1. The results indicate increased eIF-2α protein kinase activity in the salt wash fraction from cells that had been treated with either IFN-γ or GSSG compared to the salt wash fraction from untreated cells. Surprisingly, GSSG appears to induce a higher level of kinase activity than IFN-γ. The eIF-2α protein kinase activity from GSSG-treated cells is decreased appreciably if dsRNA is not added to the reaction mixture in which the enzyme is activated. A similar dependence on dsRNA has been demonstrated

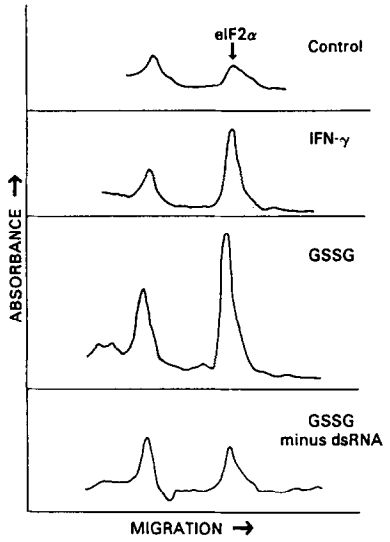

Fig. 1. Scan of autoradiograms of protein kinase activity induced in L cells by IFN-γ or GSSG. Kinase activity was determined with L cell ribosome extracts, purified reticulocyte eIF-2, dsRNA, and [γ^{32}P]ATP as described in Materials and Methods.

with the eIF-2α protein kinase from cells treated with IFN-γ (data not shown). These results indicate that IFN-γ, like IFN-α/β, is capable of inducing dsRNA-dependent protein kinase activity in mouse L cells. Dependence on dsRNA distinguishes this protein kinase from the eIF-2α kinase that is activated in lysates of rabbit reticulocytes by GSSG (20,21). IFN-γ used in these experiments was partially purified ($10^{4.5}$ to $10^{5.0}$ units/mg protein) and separated from lymphotoxin activity (H.M. Johnson and C. Robbins, in preparation).

Falcoff and his coworkers (19) have shown induction by IFN-γ in L-cells of what may be the same protein kinase. This IFN preparation was isolated from spleen cells of nude mice that had been grown in tissue culture with phytohemagglutinin. Increased dsRNA-dependent phosphorylation of a 67,000 dalton peptide was observed in extracts from treated cells. A 67,000 dalton phosphopeptide has been associated with eIF-2α protein kinase activity induced by interferons (2,4) and has been suggested to be a component of the activated dsRNA-dependent kinase.

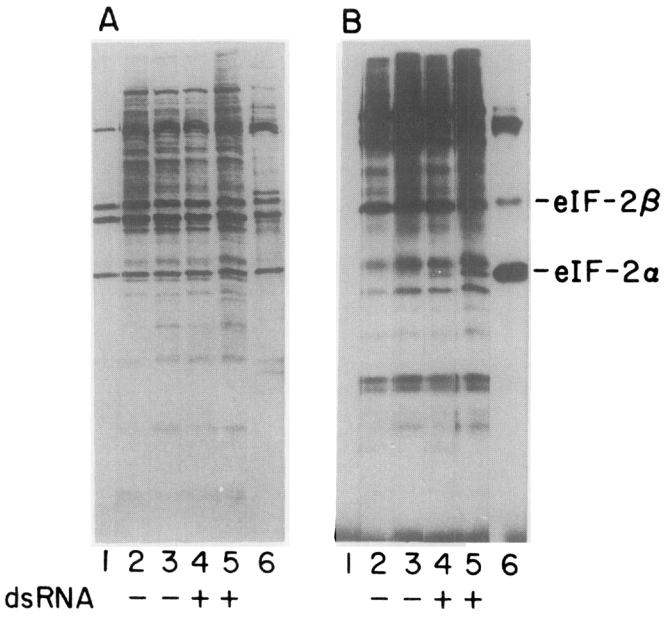

Fig. 2. Analysis by gel electrophoresis of the GSSG-induced protein kinase. The salt wash fraction was prepared from GSSG-treated and control L-cells and incubated in the absence or presence of dsRNA to activate the protein kinase as described under Methods. Then all samples were incubated with 3 μg rabbit reticulocyte eIF-2 and [γ-^{32}P]ATP (1.45 Ci/mol). Track 1: eIF-2 alone, tracks 2 and 4: salt wash fraction from control cells, tracks 3 and 5: salt wash fraction from GSSG-treated cells. DsRNA was added during preactivation where indicated. Track 6: 2.4 μg of the heme-controlled rabbit reticulocyte eIF-2α protein kinase (prepared as outlined in ref. 23) was added. A. Stained gel. B. Autoradiogram.

A representative stained gel and the autoradiogram derived from it showing the protein kinase activity induced by GSSG is given in Fig. 2. A number of peptides are phosphorylated in the relatively crude extracts including the β subunit of eIF-2. In reticulocytes the β subunit of eIF-2 is phosphorylated by a cAMP-independent kinase that is distinct from any of the eIF-2α kinases that have been characterized (22,23). The activity of this kinase is not affected by low concentrations of dsRNA. It is useful to compare phosphorylation of eIF-2α and eIF-2β in the presence and absence of dsRNA as an indication of dsRNA-dependent kinase activity. Increased phosphorylation of eIF-2α in

Table I

Effect of IFN and GSSG on Protein Kinase and Antiviral Activity of L Cells

Experiment	Treatment[a]	Fold increase in protein kinase activity	Antiviral activity[b]
1	IFN-α/β	3.8	300
	GSSG	2.0	0
	Control	---	0
2	IFN-γ	2.8	300
	GSSG	3.5	0
	Control	---	0
3	IFN-α/β	3.5	300
	IFN-γ	1.2	300
	Control	---	0

[a] IFN-α/β= L-cell, virus-induced interferon, 300 U/ml; GSSG=oxidized glutathione, 1 mg/ml; IFN-γ=mouse spleen cell, staphylococcal enterotoxin A-induced interferon, 300 U/ml. dsRNA was present in all assays.

[b] Antiviral activity expressed as units IFN/ml, as measured in viral plaque-reduction assay (11).

the presence of dsRNA is observed with the salt wash fraction from cells treated with GSSG but there is little or no difference in phosphorylation of eIF-2β. For comparison, eIF-2 that has been phosphorylated with the heme-controlled eIF-2α protein kinase from rabbit reticulocytes (cf. 7) is included in Fig. 2.

Even though GSSG was at least as effective as IFN-γ for induction of the eIF-2α kinase activity, the data of Table I appear to indicate that a concomitant induction of the antiviral state did not occur in cells treated with GSSG.

Antiviral activity was measured in the viral plaque-reduction assay (11) as briefly outlined under Materials and Methods. In this assay, replication of vesicular stomatitis virus was tested with cells pretreated as described in the table. GSSG did not induce activity that would suppress virus replication. Similar results were obtained when replication of Mengo virus was tested in L cells pretreated as given in Table I (unpublished data).

Induction of protein kinase activity by IFN has been suggested as a possible mechanism for induction of the antiviral state in cells (reviewed in 1). The data with GSSG suggest that a simple cause and effect relationship does not exist between protein kinase induction and establishment of the antiviral state in a cell responsive to the antiviral effects of IFN. Efficient virus replication was observed in human HEC-1 cells that have a high level of dsRNA-dependent eIF-2α kinase activity without induction (24). The antiviral state was not induced by treatment of the cells with interferon. No correlation was found between the IFN-mediated antiviral state and protein kinase activity in three murine cell lines (25). It is also possible that the protein kinase activities induced in L cells by IFN and GSSG represent different enzymes, and that these enzymes affect the induction of the antiviral state differently. However, IFN and GSSG have several common biological and biochemical properties in intact cells. Both molecules suppress the in vitro antibody response with similar kinetics (8). The suppressions are reversed by adding reducing agents, such as 2-mercaptoethanol, after the immunosuppressive state has been induced. IFN and GSSG also inhibit the hexose monophosphate shunt pathway (26), which may cause increased GSSG levels in the cell. It is possible that the above events may be related to IFN and GSSG induction of protein kinase activity. GSSG may be quite useful, then, in elucidating non-antiviral effects of IFN such as immune regulation and anti-cellular activity. Further, increased protein kinase activity may be important in the expression of these non-antiviral IFN-induced biological events.

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REFERENCES

1. Vilcek, J., Gresser, I., and Merigan, T.C. (eds). 1980. Ann. N.Y. Acad. Sci. 350.

2. Farrell, P.J., Sen, G.C., Dubois, M.F., Ratner, L., Slattery, E., and Lengyel, P. 1978, Proc. Natl. Acad. Sci. USA 75: 5893-5897.
3. Kerr, I.M., Brown, R.E. 1978. Proc. Natl. Acad. Sci. USA 75: 256-260.
4. Zilberstein, A., Kimchi, A., Schmidt, A., and Revel, M. 1978. Proc. Natl. Acad. Sci. USA 75: 4734-4738.
5. Roberts, W.K., Hovanessian, A., Brown, R.E., Clemens, M., and Kerr, I.M. 1976. Nature 264: 477-480.
6. Samuel, C.E. 1979. Proc. Natl. Acad. Sci. USA 76: 600-604.
7. Kramer, G., and Hardesty, B. 1980. Cell Biology, D.M. Prescott and L. Goldstein, eds. 4: 69-105.
8. Johnson, H.M. 1980. Proc. Soc. Exp. Biol. Med. 164: 380-385.
9. Stewart, W.E., Blalock, J.E., Burke, D.C., Chany, C., Dunnick, J.K., Falcoff, E., Friedman, R.M., Galasso, G.J., Joklik, W.K., Vilcek, J.T., Youngner, J.S., and Zoon, K.C. 1980. J. Immunol. 125: 2353.
10. Georgiades, J.A., Langford, M.P., Stanton, G.J., and Johnson, H.M. 1979. IRCS Medical Science 7: 559.
11. Osborne, L.C., Georgiades, J.A., and Johnson, H.M. 1979. Infect. Immun. 23: 80-86.
12. Pinphanichakarn, P., Kramer, G., and Hardesty, B. 1977. J. Biol. Chem. 252: 2106-2112.
13. Warburg, O., and Christian, W. 1942. Biochem. 310, 384-421.
14. Petryshyn, R., Levin, D.H., and London, I.M. 1980. Biochem. Biophys. Research Comm. 94: 1190-1198.
15. Kramer, G., Cimadevilla, J.M., and Hardesty, B. 1976. Proc. Natl. Acad. Sci. USA 73: 3078-3082.
16. Kramer, G., Henderson, A.B., Pinphanichakarn, P., Wallis, M.H., and Hardesty, B. 1977. Proc. Natl. Acad. Sci. USA 74: 1445-1449.
17. Odom, O.W., Kramer, G., Henderson, A.B., Pinphanichakarn, P., and Hardesty, B. 1978. J. Biol Chem. 253: 1807-1816.
18. Kerr, I.M., Williams, B.R.G., Hovanessian, A.G., Brown, R.E., Martin, E.M., Gilbert, C.S. and Birdsall, N.J.M. 1979 in "Modern Trends in Human Leukemia III" (R. Neth, R.C. Gallo, P.-H. Hofschneider, K. Mannweiler, eds.) Springer Verlag, Berlin, Heidelberg, New York pp. 291-294.
19. Wietzerbin, J., Stefanos, S., Falcoff, R., Lucero, M., Catinot, L., and Falcoff, E. 1978. Infect. Immun. 21: 966-972.
20. Legon, S., Brayley, A., Hunt, T., and Jackson, R.J. 1974. Biochem. Biophys. Res. Commun. 56: 745-752.
21. Ernst, V., Levin, D. H. and London, I.M. 1978. Proc. Natl. Acad. Sci. USA 75: 4110-4114.
22. Hathaway, G.M., Lundak, T.S., Tahara, S.M., and Trough, J.A. 1979. Meth. Enzym. 60: 495-511.
23. Kramer, G., and Hardesty, B. (1981) Curr. Topics in Cell. Regul. 20, in press.
24. Verhaegen, M., Divizia, M., Vandenbussche, P., Kuwata, T., and Content, J. 1980. Proc. Natl. Acad. Sci. USA 77: 4479-4483.
25. Hovanessian, A.G., Meurs, E., and Montagnier, L. 1981. J. of Interferon Research 1: 179-190.
26. Saneto, R., Holstun, S., and Johnson, H.M. 1980. IRCS Medical Science 8: 921-922.