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CHARACTERISATION OF THE INTERFERON-MEDIATED PROTEIN KINASE BY POLYCLONAL ANTIBODIES

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SUMMARY - Interferon-treated human cells show an enhanced level of a double-stranded (ds) RNA-dependent protein kinase activity which is manifested by the phosphorylation of an endogenous 72,000 molecular weight protein (p72K kinase). By the use of murine polyclonal antibodies against this p72K kinase, here we have characterized the protein kinase activity associated with immune complexes precipitated from extracts of interferon-treated cells. Precipitation of the p72K kinase by the polyclonal antibodies results in the formation of a complex in which the kinase activity is manifested by phosphorylation of the 72K protein. This phosphorylation, however, is independent of dsRNA. Such immunoprecipitates can also phosphorylate exogenous substrates, calf thymus histones and the α subunit of protein initiation factor eIF2.

Interferon-treated cells show an enhanced level of a protein kinase activity which is manifested by the phosphorylation of (a) an endogenous 67K protein (p67K kinase) in mouse cells or a 72K protein (p72K kinase) in human cells ; (b) endogenous or exogenous 35K protein which is the α subunit of protein initiation factor eIF2 ; and (c) added histones (see reference 1 for details). In crude cell extracts or in partially purified kinase fractions, the phophorylation of these three proteins (67K or 72K, eIF2 and histones) is enhanced considerably by the presence of double-stranded (ds)RNA (2-5). This kinase activity is independent of cyclic AMP or cyclic GMP, is markedly stimulated by Mn^{2+} and is not inhibited by heparin (6). It phosphorylates the 67K and the 72K proteins by their serine and threonine residues (7,8). In cell-free systems, the role of such dsRNA-dependent protein kinase activity is considered to be the phosphorylation of eIF2 (9,10). The phosphorylation of the 67K and 72K proteins have been shown in

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interferon-treated cells during virus infection (11,12), but the significance of this and its correlation with the phosphorylation of eIF2 remains to be clarified.

MATERIALS AND METHODS

γ-^{32}P-ATP was supplied by Amersham International. Poly(I).poly(C) and poly(A).poly(U) were from P.L. Biochemicals.Sepharose 4B and protein A-Sepharose Cl-4B were from Pharmacia. Poly(I).poly(C)-Sepharose and poly(A).poly(U)-Sepharose were prepared as previously described (6). Calf thymus histones HIIA and milk casein were from Sigma. Rabbit anti-human fibrinogen and factor XIII sera were from Institut Behring. Partially purifed preparation of eIF2 was the generous gift of Dr. M.J. Clemens (13).

Mouse L-929 and human Hela cells were grown in basal medium (Eagle's) with Earle's salts containing 10% newborn calf serum. Cell extracts (1500g supernatant fluids) were prepared by incubation of cell monolayers in lysis buffer containing 10 mM 4-(2-Hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.6, 10 mM KCl, 2mM magnesium acetate, 7mM 2-mercaptoethanol, 0.5% nonionic detergent NP-40 (BDH Chemicals Ltd, Poole, England), and aprotinin at 100 unit/ml (Zymofren, Specia). Human Daudi cells were grown in suspension in RPMI-1640 medium (Grand Island Biological Company, U.S.A.) containing 10^{-5}M 2-mercaptoethanol and 10% fetal calf serum. For the preparation of cell extracts, Daudi cells were first washed with phosphate buffered saline before addition of lysis buffer. Cell extracts were centrifuged at 12,000g and the supernatants (S 12) stored at -80°C. In all the experiments described here, S 12 fractions were used. Treatment of cells with interferon was at 500-1000 NIH units/ml for 18-22 hours. Human leukocyte (α) interferon (10^8 units/mg of protein) and mouse (α + β) interferon ($5x10^7$ units/mg of protein) were purified in the laboratory (8,14).

The p72K kinase from interferon treated Daudi Cells was purified on poly(I).poly(C)-Sepharose in the presence of heparin (6). The kinase was eluted by Hepes buffer containing 1M KCl and mouse albumin at 0.5mg/ml (2) and dialyzed extensively against phosphate-buffered saline. Five Balb/c female mice (6 weeks old) were injected intraperitoneally, 6 times at 2 weeks interval, with the purified preparation of p72K kinase (material equivalent to that from 10^8 cells) supplemented with Freund's Adjuvant. These mice received 3 further injections of p72K kinase stabilized on poly(A).poly(U)-Sepharose (200μl) without Freund's Adjuvant. One day after the last injection, these immunized mice as well as normal mice received intraperitoneally a suspension of 10^6 sarcoma 180/TG cells in order to prepare hyperimmune ascitic fluid (15). Ten days later, the ascitic cells were removed, centrifuged (200g, 5 min) and the peritoneal fluid was collected. The immunoglobulins in this fluid were concentrated at 50% $(NH_4)_2SO_4$, dialyzed against 10mM phosphate buffer pH8.0 and further purified on a DEAE-cellulose column (DE-52, Whatman) by elution at 40 mM phosphate buffer pH8.0.

The protein kinase activity was assayed in the immune complexes by phosphorylation of the 72K protein. Enzyme preparations were first incubated (60 min, 4°C) with the mouse anti-p72K kinase antibodies before further incubation (3hr, 4°C) with protein A-Sepharose (100μl) in buffer A:Tris-HCl pH 7.6, 100mM KCl, 2.5mM Mg $(OAc)_2$, aprotinin (10 units/ml), heparin 5 units/ml and 20% glycerol, v/v. The immune complexes bound to protein-A Sepharose were washed batchwise and consecutively with twice 4ml of buffer B (as buffer A but containing

0.5% NP40), twice 4ml of buffer A and once with buffer C (as buffer A but containing 10 mM $MnCl_2$ and 1mM dithiotrietol). Finally, the immune complexes bound to protein A-Sepharose were incubated (45min, 30°C) in 50µl of buffer C containing 1µM γ-^{32}P-ATP (50-100 Ci/mmol). The phosphorylation reaction was stopped by the addition of 50µl of 2 fold concentrated electrophoresis sample buffer : 130 mM Tris-HCl pH 6.8, 2 M urea, 8.5% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 1.5M 2-mercaptoethanol and 35 % glycerol, v/v. The different samples were analyzed by polyacrylamide slab gel electrophoresis in SDS (6).

RESULTS AND DISCUSSION

It is still not clear whether phosphorylation of the 72K protein in kinase preparations from interferon treated human cells is due to autophosphorylation of the protein kinase or it reflects the phosphorylation of a substrate by a protein kinase enhanced by interferon. In order to rule out any misunderstanding we propose to refer to such a system as p72K kinase, i.e., a protein kinase activity which is manifested by the phosphorylation of a defined 72K protein.

The presence of antibodies against the p72K kinase were detected by immunoprecipitation of the kinase with serum from the immunized mice. The immune complexes immobilized on protein A-Sepharose were washed four times with buffer before incubation with γ-^{32}P-ATP (Materials and Methods). The protein kinase activity was then revealed by phosphorylation of the endogenous 72K protein. Figure 1 shows the proteins phosphorylated in the immune complexes prepared by the use of serum containing antibodies and extracts from interferon treated human Daudi and HeLa and mouse L-929 cells. The phosphorylation of the 72K protein was observed in the immune complexes from extracts of Daudi and Hela cells but not from extracts of L-929 cells (Fig.1; lanes Ig+). These polyclonal antibodies did not precipitate a similar protein kinase activity that we have previously described in human plasma (16, 17 ; data not shown).Thus they were specific of the p 72K kinase from human cells. The phosphorylation of the 72K protein was not observed in immunoprecipitates using serum from normal (unimmunized) mice (Fig. 1; lanes Ig-). Other proteins were also phosphorylated in immunoprecipitates from extracts of Daudi cells (Fig 1, section Daudi).

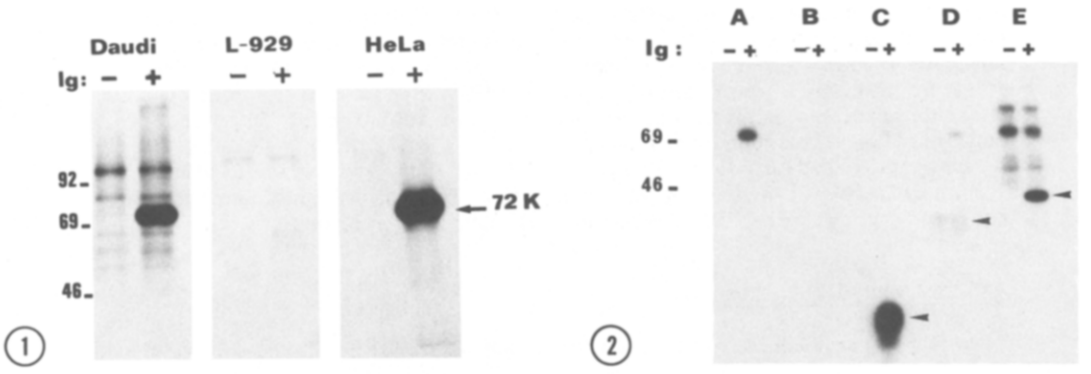

Fig. 1 - Phosphorylation of the 72K protein in the immune complex. Extracts (1mg of protein) from interferon-treated Daudi, L-929 and HeLa cells were incubated with 5 µl of serum from control (unimmunized) mouse (lanes Ig-) or from the mouse producing anti-p72K kinase antibodies (lanes Ig+). The immunoprecpitates recovered by protein A-Sepharose, were washed with buffer (A, B and C), assayed for protein kinase activity ("Materials and Methods") and the phosphorylated proteins were analyzed by polyacrylamide gel (8,5%) electrophoresis. An autoradiograph of a stained and dried gel is shown. The numbers on the left give the molecular weight of protein markers in thousands : phosphorylase B : 92 ; bovine plasma albumin : 69 ; ovalbumin : 46. The position of the ^{32}P-labelled 72K protein is indicated by the arrow on the right.

Fig. 2 - Phosphorylation of exogenous substrates by the protein kinase in the immune complex. Extracts (1mg) from interferon-treated Daudi cells were incubated in the presence of control (normal mouse) immunoglobulins (10µg) (lanes Ig - in sections A to E) or purified immunoglobulins (10 µg) containing anti-p72K kinase antibodies (lanes Ig+ in sections A to E) and the immune complexes were recovered by protein A-Sepharose. In section A, the protein kinase activity was assayed as such as described in the legend of Fig. 1. In sections B to E, the immune complexes bound to protein A-Sepharose were first incubated (30°C, 30 min) with cold ATP (10 µM) to phosphorylate the 72K protein and then were washed once in buffer C before incubation (30°C, 30 min) with γ-^{32}P-ATP and the different substrates - B: no substrate ; C : 10µg of calf thymus histone HIIA ; D : 10 µg of casein ; E : 10 µl of partially purified preparation of eIF2 (10 mg/ml). The different reactions were stopped by electrophoresis sample buffer and the ^{32}P-labelled proteins analyzed by polyacrylamide gel (12.5 %) electrophoresis. An autoradiograph of a stained, dried gel is shown. The numbers on the left give the molecular weight of protein markers. The arrow heads show the position of histone, casein and the smallest subunit(35K) of eIF2 in C, D and E, respectively. The 72K protein in sections B to E (lanes Ig +) is not radioactively ($^{32}PO_4$) labelled because it was first phosphorylated with cold ATP.

The phosphorylation of these however, were not specific since they were observed with serum from both normal and unimmunized mice. When purified immunoglobulins ("Materials and Methods") were used in the immunoprecipitation then only phosphorylation of the 72K protein was observed (Fig. 2 ;section A, Ig +).

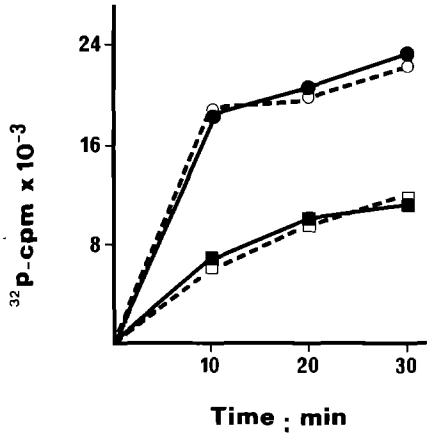

Fig. 3 - Kinetics of phosphorylation of the 72K protein in immune complexes from extracts of control and interferon-treated Daudi cells. The p72K kinase in extracts (1mg of protein) from control (□ ; ■) and interferon-treated (○ ; ●) Daudi cells was immunopecipitated with purified total immunoglobulins (10 µg) and the immune complex bound to protein A-Sepharose was assayed for protein kinase activity in the absence (□ ; ○) or in the presence of 1µg/ml of poly(I).poly(C) (■ ; ●). At different times as indicated on the abscissa, the phosphorylation reaction was stopped by the addition of electrophoresis sample buffer and heating (95°C for 3 min). All the samples supplemented with bovine plasma albumin (5 µg) were then analyzed by polyacrylamide slab gels. The ^{32}P-labelled 72K proteins were localized in the stained gels according to the mobility of albumin (69K) and pieces of gel (0.5 x 1cm) were cut and the radioactivity measured by liquid scintillation (ordinate).

Figure 3 shows the kinetics of phosphorylation of the 72K protein in immune complexes prepared from extracts of control and interferon-treated Daudi cells, in the absence or presence of poly(I).poly(C). The kinetics and the level of phosphorylation of the 72K protein in such immune complexes are identical with or without dsRNA. In contrast to crude cell extracts therefore, the 72K kinase becomes independent of dsRNA in immune precipitates. The loss of dependance on dsRNA for activity of the kinase in the immune complexes might be due to a modification in the conformation of the 72K kinase during immunoprecipitation. It might also be due to elimination of a regulatory protein which in crude extracts renders the kinase dependent to dsRNA. In accord with the latter, there has been a report for the presence of a phosphatase in cell extracts which is inhibited by dsRNA (18). Furthermore, we have previously shown that the activity of the

p67K kinase in a highly purified enzyme fraction from mouse L-929 cells is independent of dsRNA(2).

The level of phosphorylation of the 72K protein is much higher in the immune complexes from extracts of interferon-treated than control cells (Fig.3), thus indicating that this protein kinase is the one associated with the action of interferon (1). Further evidence for this was provided by characterising the ^{32}P-labelled 72K protein. This protein is composed of several subspecies with isoelectric points in the range of 7.2 to 8.2 and on partial proteolysis by Staphyloccocus aureus V8 protease, it yields several phosphopolypeptides of molecular weights between 56 K to 23 K (data not presented). These results are typical of the 72K protein, the phosphorylation of which is enhanced in extracts of human cells treated with interferon (8). In addition to the phosphorylation of the endogenous 72K protein, the protein kinase activity in the immune complex has the capacity to phosphorylate exogenous substrates such as calf thymus histones (Fig. 2 ; section C, Ig +) and the subunit of eIF2 (Fig. 2 ; section E, Ig+). Similar to the phosphorylation of endogenous 72K protein, the phosphorylation of these exogenous substrates is independent of dsRNA (data not presented). For these experiments, immune complexes were first incubated with cold ATP before further incubation with exogenous substrates and γ-^{32}P-ATP (see the legend of Fig.2). For this reason ^{32}P-labelling of the 72K protein is not observed (Fig. 2; sections B,C,D and E). Casein was not phosphorylated by the protein kinase activity in the immune complex (Fig.2 ; section D, Ig +). The capacity to phosphorylate basic proteins (histones) but not acidic ones (casein) is a characteristic of the interferon-mediated protein kinase.

These murine polyclonal antibodies directed against human p72K kinase can be used for characterisation of such activity in relation to the mechanism of action of interferon. Furthermore, detection of anti - p72K kinase antibodies by the immunoprecipitation technique, provides

a convenient method which could be used efficiently for screening monoclonal antibodies. These latter are essential for further characterisation of the p72K kinase and are necessary to find out whether phosphorylation of the 72K protein is due to an autophosphorylation of the kinase or it reflects phosphorylation of a substrate by a protein kinase.

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REFERENCES

1. Hovanessian, A.G. (1979) Differentiation. 15, 139-151.
2. Hovanessian, A.G. and Kerr, I.M. (1979) Eur. J. Biochem. 93, 515-526.
3. Sen, G.C., Taira, H. and Lengyel, P. (1978) J. Biol. Chem. 253, 5915-5921.
4. Kimchi, A., Zilberstein, A., Schmidt, A., Shulman, L. and Revel, M. (1979) J. Biol. Chem. 254, 9846-9853.
5. Lasky, S.R., Jacobs, B.L. and Samuel, C.E. (1982) J. Biol. Chem. 257, 11087-11093.
6. Hovanessian, A.G., Rivière, Y. and Krust, B. (1983) Anal. Biochem. 129, 349-356.
7. Samuel, C. (1979) Proc. Natl. Acad. Sci. USA, 76, 515-526.
8. Krust, B., Galabru, J. and Hovanessian, A.G. (1984) J. Biol. Chem. 259,8494-8498.
9. Farrel, P.J., Sen, G.C., Dubois, M.F., Ratner, L., Slattery, E. and Lengyel, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5893-5896.
10. Zilberstein, A., Kimchi, A., Schmidt, A. and Revel, M. (1978) Proc. Natl. Acad. Sci. USA 75, 4734-4738.
11. Gupta, S.L., Holmes, S.L. and Mehra, L.L. (1982) Virology 120, 495-499.
12. Nilsen, T.W., Maroney, P.A. an Baglioni, C. (1982) J. Biol. Chem. 257, 14593-14596.
13. Clemens, M.J., Pain, V.M., Wong, S.T. and Henshaw, E.C. (1982) Nature (Lond.) 296, 93-95.
14. Meurs, E., Rougeot, C. Svab, J., Laurent, A.G., Hovanessian, A.G., Robert, N., Gruest, J., Montagnier L. and Dray, F. (1982) Infect. Immun. 47, 919-926.
15. Sartorelli, A.C., Fischer, D.S. and Downs, W.G. (1966) J. Immunol. 96, 676-684.
16. Hovanessian, A.G., Rollin, P., Rivière, Y., Pouillard, P., Sureau, P. and Montagnier, L. (1981) Biochem. Biophys. Res. Commun. 103, 1371-1377.
17. Krust, B., Galabru, J. and Hovanessian, A.G. (1983) Biochem. Biophys. Res. Comm. 117, 350-357.
18. Epstein, D.A., Torrence, P.F and Friedman, R.M. (1980) Proc. Natl. Acad. Sci. USA 77, 107-111.