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CHARACTERIZATION OF IMMUNE TYPE INTERFERON (IFNγ)-INDUCED CYTOPLASMIC PROTEIN KINASE ACTIVITY IN MOUSE L CELLS

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SUMMARY: Mouse immune interferon (IFNγ) induced double-stranded RNA-independent protein kinase activity in the cytoplasmic fraction of mouse L cells as measured against a histone substrate. Chromatographic purification separated the activity into three distinct kinases of molecular weights of approximately 100K (kinase I), 70K (kinase II), and 70K (kinase III). Partially purified IFNγ was as effective as crude in inducing protein kinase activity. Induction was blocked by treatment of IFNγ with specific anti-IFNγ antibody or by treatment of the L cells with actinomycin D. Kinase II activity was different from that of kinases I and III in that it showed Ca-dependence in the absence of Mg^{2+}, was inhibited in activity by the SH binding agent N-ethylmaleimide, and could use the cellular enzymes RNase A and hexokinase as substrates. The described protein kinases could play an important role in mediation of IFNγ effects, particularly where phosphorylated enzyme substrates were shown to have altered activity.

It has been demonstrated by several laboratories that treatment of cells with interferon (IFN) results in increased total protein kinase activity and increased protein phosphorylation of cellular and ribosomal proteins (1-7). We and others have partially purified and characterized a ribosomal-associated IFN-induced dsRNA-dependent protein kinase, which phosphorylates the small subunit (37K) of eIF-2, resulting in the inhibition of initiation of viral protein synthesis (4,7,8). Recently, we reported that IFNγ also induces dsRNA-dependent protein kinase activity in mouse L cells (9). The kinetics of induction of IFNγ-induced ribosomal kinase was different from that observed for virus-induced IFNs as was also shown for induction of 2',5'-oligo A synthetase (10-12).

The present study was undertaken to further determine and characterize the biological properties of IFNγ-induced kinase activity in the cytoplasmic fraction of mouse L cells. We found that IFNγ induces an increase in at least three distinct cytoplasmic kinases as measured against a histone substrate. A possible

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biological role of the IFNγ-induced kinases in the IFN-induced biochemical events is discussed.

MATERIALS AND METHODS

Chemicals - [γ-^{32}P]ATP (19.0 Ci/mol) was obtained from Amersham Corp. Casein, phosvitin, pancreatic ribonuclease A (RNase A), dithiothreitol (DTT), calf thymus histone (type II-a) and cAMP were obtained from Sigma Chemicals Corp. DEAE-cellulose (DE-52) was obtained from Whatman. Concanavalin A-Sepharose and Sephacryl S-200 were obtained from Pharmacia.

Mouse IFNγ preparation - IFNγ was produced in C57Bl/6 mouse spleen cell cultures using staphylococcal enterotoxin A as an inducer as previously described (13). The produced IFNγ was partially purified by adsorption to controlled Pore-glass beads and elution with 20% ethylene glycol and 1 M NaCl in phosphate buffered saline (PBS) (14). Following dialysis against PBS, the IFNγ fraction was applied on a concanavalin A-Sepharose column and eluted with 0.25 M α-methyl-D-mannoside in PBS. The final product had a specific activity of approximately 10^5 units/mg protein.

Treatment of the cells with IFNs - Confluent monolayers of mouse L cells (about 10^8 cells) in 150 cm^2 flasks were treated with 300 reference units/ml of IFN for 12 hrs at 37°C. The cells were harvested by scraping with a rubber policeman, centrifuged, washed twice with PBS, and cytoplasmic fractions were prepared as described below for testing protein kinase activity.

Cytoplasmic fraction (crude kinase fraction) - The cytoplasmic fractions from untreated and IFN -treated cells were prepared as follows. The cells (about 10^8 cells) were gently homogenated with a glass homogenizer in 3 ml of 10 mM Tris-HCl (pH 7.5) containing 2 mM $CaCl_2$, 2 mM dithiothreitol (DTT) and 10 mM KCl, and then centrifuged for 20 min at 12,000 rpm. The supernatant was recentrifuged at high speed (105,000 Xg for 45 min) to remove ribosomes from the fraction. The obtained supernatant was concentrated to 1.0 ml using polyethylene glycol 20000 powder. The concentrated solutions were dialyzed overnight at 4°C against 10 mM Tris-HCl (pH 7.5) containing 0.5 M KCl, 2 mM DTT and 10% glycerol (Buffer A). The dialyzed fractions were used as crude kinase preparations (cytoplasmic fractions) for the purification and kinase assay in this study.

Assay of protein kinase activity - The complete reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 4 mM DTT, 10 µg of bovine serum albumin (BSA), 100 µM [γ-^{32}P]ATP (2,000 cpm/pmol), the indicated amounts of substrate (histone or other proteins), the indicated amounts of either Mg^{2+} or Ca^{2+} and the indicated amounts of protein kinase purified or unpurified from the cytoplasmic fraction of mouse L cells. After incubation for the periods indicated at 37°C, the enzyme reaction was terminated by the addition of 0.5 ml of 0.2 M sodium pyrophosphate containing BSA (1 mg/ml) and 0.5 ml of 20% trichloroacetic acid. The precipitate was collected by filtration through a Whatman glass filter (type GF/F) and the [^{32}P]radioactivity of the filter was determined by a liquid scintillation spectrometer after drying.

Protein measurement - Protein concentration of the enzyme preparations was determined by the method of Lowry et al. (15).

RESULTS

IFNγ-induced cytoplasmic protein kinase activity in mouse L cells

When mouse L cells (about 10^8 cells) were exposed to crude mouse IFNγ (300 reference units/ml) for 12 hrs at 37°C, the activity of cytoplasmic protein kinase (histone phosphorylation with [γ-^{32}P]ATP) from such cells was significantly increased as compared with that of untreated cells (Table 1). This increased protein kinase

Table 1. Characterization of interferons as stimulators of cytoplasmic protein kinase activity in mouse L cells.

Treatments	Protein kinase activity (pmol [γ-^{32}P]ATP/µg histone)
None	18.5
Mouse IFNγ	63.8
Mouse IFNγ + actinomycin D	22.8
Mouse IFNα/β	40.2
Human IFNα	19.1
Mouse IFNγ + anti-mouse IFNγ antiserum	23.0

Crude kinase fractions (cytoplasmic fractions) were prepared from untreated mouse L cells or cells treated with 300 units/ml of mouse IFNγ (crude), or cells treated with 300 units/ml of human IFNα (specific activity 10^5 units/mg) for 6 hrs at 37°C. Actinomycin D (1 µg/ml) was added to the cell cultures at the same time as IFNγ. Protein kinase activity of the fractions was assayed using either histone (type II-A) in the presence of Mg^{2+} (3 mM) or phosvitin in the presence of Ca^{2+} (3 mM) as described in Materials and Methods. Anti-mouse IFNγ antiserum was preincubated with mouse IFNγ (10^3 units/ml) for 30 min at 37°C before exposure to the cells, at a concentration that completely neutralized the antiviral activity.

activity was also obtained when partially purified IFNγ (10^5 units/mg) was used. However, no increase of protein kinase activity was observed in cells treated with human leukocyte IFN (IFNα) which does not induce appreciable antiviral resistance in mouse L cells, or from cells treated with mouse IFNγ together with actinomycin D (1 µg/ml), which inhibits the development of antiviral state induced by IFNs. Also, anti-IFNγ antiserum neutralized the ability of IFNγ to induce protein kinase activity in the cells. This antiserum is not reactive against the lymphokines, interleukins 1 and 2 or colony stimulating factor (unpublished data). These data strongly suggest that mouse IFNγ is responsible for the increase of total protein kinase activity in the cytoplasmic fraction of mouse L cells.

Partial purification of IFNγ-induced kinases

The cytoplasmic fractions from untreated and IFNγ-treated cells were passed through a Sephacryl S-200 column (1.5 X 50 cm) previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 0.5 M KCl, 2 mM DTT and 10% glycerol to separate kinase activities by their molecular size differences. As shown in Fig. 1, two distinct kinases (P-I and P-II) were detected using histone (or casein) as a phosphate acceptor. The kinase activity of the first peak (P-I) was significantly stimulated by the addition of 10 µM cAMP, whereas the activity of the second peak (P-II) was

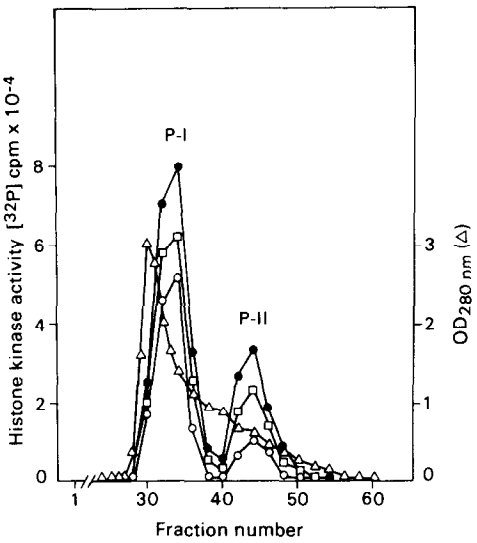

Fig. 1. Separation of protein kinase activities by Sephacryl S-200 gel filtration. The cytoplasmic fractions were prepared from untreated cells (○) and cells treated with mouse IFNγ (300 units/ml) for 2 hrs (△) and 6 hrs (●), respectively, at 37°C. Aliquots (5 µl) were assayed against a histone substrate as described in the Materials and Methods. Absorbance at 280 nm (X).

slightly inhibited by cAMP. Total protein kinase activities of both fractions (P-I and P-II) from IFNγ-treated cells increased with increased time of exposure of the cells to IFN. The specific activity (histone phosphorylation/µg protein/min at 37°C) of the second peak (P-II kinase) was increased approximately 3-fold 6 hr after treatment of the cells with IFNγ (300 units/ml) as compared with that of untreated cells.

In order to further characterize the two distinct kinases (P-I and P-II) from IFNγ-treated cells, the fractions were separately applied on a DEAE-cellulose column. The P-I kinase eluted as a single peak between 0.15 M and 0.2 M KCl in the buffer (Fig. 2). The active fractions were pooled and designated as kinase I. On tne other hand, the P-II kinase was separated into two kinases: the first peak (kinase II) was eluted between 0.05 M and 0.1 M KCl and the other (kinase III) was eluted between 0.2 M and 0.3 M KCl (Fig. 3). Only kinase II phosphorylated RNase A. The active fractions in the DEAE-cellulose column chromatography were pooled, respectively, and immediately concentrated by polyethylene glycol 20000. The molecular weight of kinases I, II and III as determined on a Sephacryl S-200 column

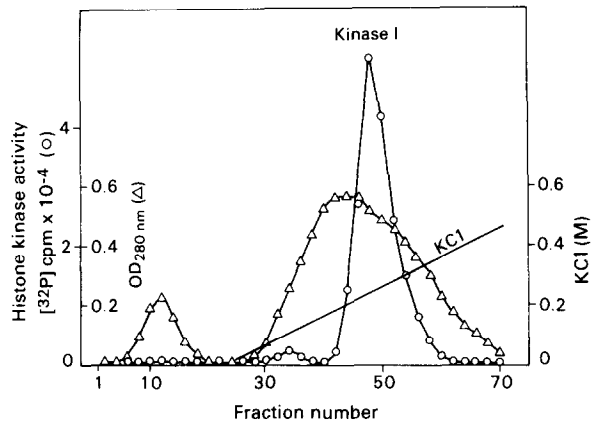

Fig. 2. DEAE-cellulose column chromatography of P-I fraction from the cytoplasmic fraction of IFNγ-treated cells. The P-I kinase fraction was applied to a column (1.5 X 8 cm) of DEAE-cellulose previously equilibrated with 10 mM Tris-HCl (pH 7.5) containing 2 mM DTT and 10% glycerol. The column was washed with 10 ml of the same buffer and fractions (0.5 ml) were collected. Elutions were carried out with a linear gradient (0 M to 0.5 M KCl) in the buffer. Aliquots (5 µl) were assayed as described in the Materials and Methods. Histone phosphorylating activity (○) and absorbance at 280 nm (X).

were approximately 100K, 70K and 70K, respectively, as measured by comparison with marker proteins. Kinases I, II and III were purified about 250-fold, 350-fold and 318-fold with activity yields of 45%, 25% and 38%, respectively.

Requirements for IFNγ-induced kinase activities

The optimal conditions for activity of the kinases are summarized in Table 2. Either histone or casein were suitable phosphate acceptors. The optimal

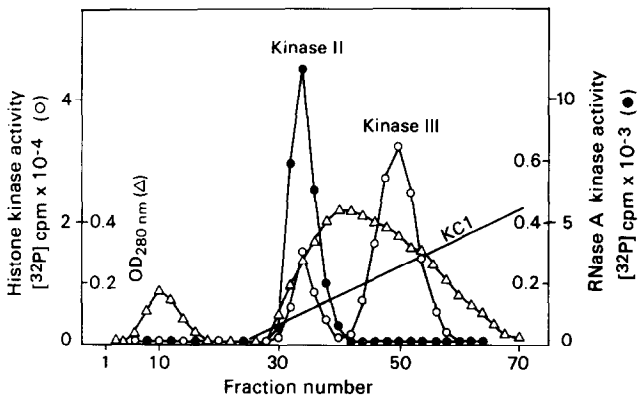

Fig. 3. DEAE-cellulose column chromatography of P-II fraction from the cytoplasmic fraction of IFNγ-treated cells. The DEAE-cellulose column chromatography of P-II kinase was performed as described in Fig. 2. Histone phosphorylating activity was in the presence of Mg^{2+} (5 mM) (○). RNase phosphorylating activity was in the presence of Ca^{2+} (3 mM) (●). Absorbance at 280 nm (X).

Table 2. Requirements for activity of the kinases from IFNγ-treated cells

Reaction mixtures	Protein kinase activity (pmol [γ-^{32}P]ATP/μg histone) Kinase I	Kinase II	Kinase III
Complete	36.6	4.9	15.4
Complete - Mg^{2+}	0.2	0.1	0.1
Complete - Mg^{2+} + Ca^{2+}	0.3	6.4	0.1
Complete - Histone	0.1	0.1	0.1
Complete - DTT + NEM	32.9	0.1	13.1
Complete + cAMP	58.5	2.8	14.3
Complete + dsRNA	32.6	5.0	14.6

The complete reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$ or 3 mM $CaCl_2$, 4 mM DTT, 10 μg of BSA, 20 μg of histone (type II-A), 100 μM [γ-^{32}P]ATP and either kinase I, kinase II or kinase III at 0.5 μg each. The reaction mixture was incubated for 10 min at 37°C. The concentration of cAMP, N-ethylmaleimide (NEM) and dsRNA (poly rI:poly rC) was 3 μM, 1 mM and 100 ng/ml, respectively.

concentration of Mg^{2+} for the three kinases was 3-5 mM. Kinases I and III had an absolute requirement for Mg^{2+} for activity, and were not activated by Ca^{2+}. In contrast to this, kinase II was activated by Ca^{2+} in absence of Mg^{2+} for protein phosphorylation. cAMP (3 μM) enhanced kinase I activity about 2.6-fold. N-ethylmaleimide (NEM), an inhibitor of SH enzymes, strongly inhibited the activity of kinase II but had no effect on the other kinases. No stimulating effect of dsRNA (poly rI: poly rC) on the three kinase activities at any concentration (10 ng/ml to 1 mg/ml) was observed.

Substrate requirements

The substrate requirements of the partially purified kinases are summarized in Table 3. Kinases I and III were similar in phosphorylation of histone, phosvitin and casein in the presence of Mg^{2+} (5 mM), while hexokinase and RNase A were poor phosphate acceptors. In contrast to this, kinase II phosphorylated both RNase A and hexokinase in the presence of Ca^{2+} (3 mM). These results show that kinase II is a Ca-dependent protein kinase (in the absence of Mg^{2+}). The data show that the substrate requirements of kinase II are different from those of kinases I and III.

DISCUSSION

We have previously shown that mouse IFNγ can induce ribosome-associated dsRNA-dependent protein kinase activity in mouse L cells that phosphorylates the α subunit

Table 3. Substrate requirement of the three distinct kinases purified from the cytoplasmic fraction of IFNγ-treated cells

Substrate	Protein kinase activity (pmol [γ-^{32}P]ATP/μg histone)					
	Kinase I		Kinase II		Kinase III	
	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}	Ca^{2+}
Histone	36.5	N.D.	4.4	6.3	14.5	N.D.
Casein	1.4	N.D.	2.8	2.2	8.1	N.D.
Mouse IgG	2.9	N.D.	3.1	4.8	1.8	N.D.
RNase A	1.7	N.D.	3.7	5.7	1.5	N.D.
Hexokinase	N.D.	N.D.	1.9	2.8	N.D.	N.D.
Phosvitin	1.8	N.D.	5.2	9.1	13.1	N.D.
BSA	0.1	N.D.	N.D.	N.D.	N.D.	N.D.

The protein kinase activity of kinase I, kinase II and kinase III (all at 0.5 μg) was incubated, respectively, for 10 min at 37°C with various substrates (20 μg each) in the presence of either Mg^{2+} (5 mM) or Ca^{2+} (3 mM). The activity of kinase I was assayed in the presence of 3 μM cAMP. N.D. = not detected.

of eukaryotic peptide initiation factor eIF-2 (9). We have shown here that IFNγ can also induce cytoplasmic dsRNA-independent protein kinase activity in L cells as measured against a histone (or casein) substrate. Kinase purification, using Sephacryl S-200 gel filtration and DEAE-cellulose ion exchange chromatography, separated the activity into three distinct kinases of molecular weights of approximately 100K (kinase I), 70K (kinase II), and 40K (kinase III). IFNγ is responsible for the induction of the three cytoplasmic kinases as evidenced by the observation that: (a) partially purified mouse IFNγ (10^5 units/mg protein) induced the kinase activity to about the same level as that observed with crude mouse IFNγ, (b) specific anti-IFNγ antiserum neutralized the ability of IFNγ to induce activity, (c) actinomycin D blocked induction, (d) and mouse IFNα/β, but not human IFNα, induced kinase activity, thus demonstrating species specificity in induction.

The three kinases are similar in that they are all dsRNA-independent, which differentiates them from the well known ribosome-associated, dsRNA-dependent protein kinase (7-11). They are, however, functionally distinct enzymes. Kinase I exhibited cAMP-dependent activity, while kinases II and III did not. All three kinases required Mg^{2+} for activation, but only kinase II was activated in the presence of Ca^{2+} (absence of Mg^{2+}). Only kinase II was sensitive to NEM, an

inhibitor of SH enzymes. Substrate requirement for activity also differentiated kinase II from kinases I and III, since it was active against RNase and hexokinase while the others were inactive. Preliminary data suggest that kinase II phosphorylation of RNase A significantly enhances enzymatic activity.

The demonstration that IFNγ can induce several cytoplasmic protein kinase activities provides additional possible mechanisms by which IFNγ can exert a pleiotropic effect in regulation of cellular function. Studies are in progress to further determine possible cellular enzyme substrates for the cytoplasmic kinase activities, and the possible alteration of enzyme function as a result of the phosphorylation.

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