Rapid induction of nucleoside-diphosphate kinase in HeLa S3 cells by human-type interferons

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Nucleoside-diphosphate (NDP)-kinase can be considered to be induced by human-type interferons (HuIFNs) rapidly, since an enzyme increase was detected within 2 h of incubation of HeLa S3 cells with HuIFNs, while incubation with heterologous mouse IFNs had no such effect. The enzyme increase induced by HuIFNs reached a plateau at 6 h after treatment. Actinomycin D (0.5 μg/ml) significantly blocked the enzyme increase induced by HuIFNs in the cells. A possible biological role of the enzyme in the IFN-induced biochemical events is discussed.

Human type interferon *Recombinant interferon* *Nucleoside-diphosphate kinase* *Enzyme induction (HeLa S3 cell)*

1. INTRODUCTION

It has been clearly shown that treatment of sensitive cells with interferons (IFNs) results in the induction of a wide variety of new polypeptides [1–3]. Although the biological significance of 2′,5′-oligo(A)synthetase [4,5] and of dsRNA-dependent protein kinase [6–8] in the development of an antiviral state has been studied in detail [9], the location and the physiological activity of most IFN-induced polypeptides remain to be elucidated. To study the biochemical mechanisms involved in the initiation of IFN-induced cell activation and cell differentiation, we recently established a new analytical method combining SDS-PAGE followed by autoradiography [10–12]. Under our established conditions, NDP-kinase (EC 2.7.4.6), which catalyzes a phosphate transfer in a wide variety of nucleoside 5′-di- and triphosphates [13], can be selectively detected in cell extracts from various mammalian cells with [γ-^{32}P]ATP in the presence of 3 mM Ca^{2+} [10]. It is possible to determine the ability of IFNs to induce NDP-kinase in various cells since the [^{32}P]phosphoenzymes formed can be quantitatively determined by the nitrocellulose membrane method [10,11].

Here, we examined the ability of HuIFNs to induce NDP-kinase in HeLa S3 cells, in order to determine the physiological role of the enzyme in the initiation of IFN-induced cell activation and cell differentiation.

Abbreviations: HuIFN, human-type interferon; NDP-kinase, nucleoside-diphosphate kinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (20.0 Ci/mmol) was obtained from Amersham, nucleoside 5′-diphosphates (ADP, GDP, CDP and UDP), nucleoside 5′-triphosphates (ATP, GTP, CTP and UDP), DTT and PMSF from Sigma, Sephacryl S200 from Pharmacia and nitrocellulose membrane filter (type TM-1, 0.65 μm) from Toyo Roshi (Tokyo).

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2.2. *Interferons*

Human recombinant interferon γ (rHuIFNγ, spec. act. 10^6 units/mg protein), native human IFNγ (HuIFNγ, 10^6 units/mg protein), human IFNα (HuIFNα, 10^6 units/mg protein) and mouse interferons (rMuIFNγ and native MuIFNγ, 10^6 units/mg protein) were kindly supplied by Shionogi Research Laboratories (Osaka). HuIFNβ, produced by the superinduction method on human diploid foreskin fibroblast cells, was prepared in our laboratories. This HuIFNβ preparation had a specific activity of 10^6 units/mg protein. Anti-HuIFNγ antiserum was kindly donated by Dr M.P. Langford (University of Texas Medical Branch, Galveston, TX).

2.3. *Treatment of HeLa S3 cells with IFNs*

Confluent monolayers of HeLa S3 cells in 150 cm^2 flasks were treated at 37°C in a CO_2 incubator with various IFNs at the indicated titers (international unit as an antiviral activity) for the indicated periods. The IFN-treated and untreated cells were separately harvested by scraping with a rubber policeman, centrifuged and then washed twice with phosphate-buffered saline (PBS, pH 7.2). The cytoplasmic fractions from these cells were prepared as described below for determining NDP-kinase.

2.4. *Crude NDP-kinase preparation*

The cytoplasmic fractions from rHuIFNγ-treated and untreated HeLa S3 cells were prepared as follows: these cells (approx. 5×10^8 cells) were gently homogenized with a glass homogenizer in 0.5 ml of 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, 0.5 mM PMSF, 50 mM KCl and 10% glycerol, and then centrifuged for 20 min at 30000 rpm at 4°C. Under these conditions, over 95% of the total NDP-kinase in these cells was extracted. The obtained supernatants were separately dialyzed overnight at 4°C against the enzyme-extracting buffer and the dialysates were used as crude NDP-kinase preparations.

2.5. *Assay for phosphoenzyme formation of NDP-kinase*

As we previously reported [10,11], NDP-kinase in the crude cell extracts from IFN-treated and untreated HeLa S3 cells rapidly formed a phosphoenzyme, an enzyme-bound high-energy phosphate intermediate, when incubated with [γ-^{32}P]ATP in the presence of divalent cations, such as 3 mM Ca^{2+} and 3 mM Mg^{2+}. The [^{32}P]phosphoenzyme formed was quantitatively determined by the nitrocellulose membrane method [10–12].

2.6. *SDS-PAGE and autoradiography*

SDS-PAGE of the crude and partially purified NDP-kinases was performed according to a modification of the method of Laemmli [14]. The ^{32}P-labelled NDP-kinase polypeptides were detected by autoradiography after SDS-PAGE as in [10–12].

3. RESULTS AND DISCUSSION

To detect NDP-kinase polypeptides in the crude preparation from HeLa S3 cells, the preparation

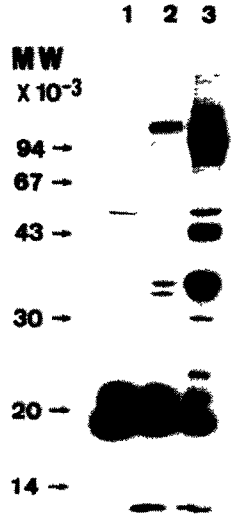

Fig.1. Detection of NDP-kinase subunits as [^{32}P]phosphorylated polypeptides by SDS-PAGE followed by autoradiography. The reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 2 mM DTT, 1 mM EDTA, 20 μM [γ-^{32}P]ATP (5000 cpm/pmol) and the crude cell extract (about 100 μg protein) from HeLa S3 cells. The mixtures were separately incubated for 5 min in an ice bath in the presence of various divalent cations (3 mM Ca^{2+}, 3 mM Mg^{2+} and 3 mM Mn^{2+}) and protein phosphorylation was arrested by the addition of an SDS-PAGE sample buffer. After incubation for 30 min at 50°C, aliquots (about 20 μg protein) were analyzed by SDS-PAGE followed by autoradiography. Lanes: 1, polypeptide phosphorylation in the presence of 3 mM Ca^{2+}; 2, 3 mM Mg^{2+}; 3, 3 mM Mn^{2+}.

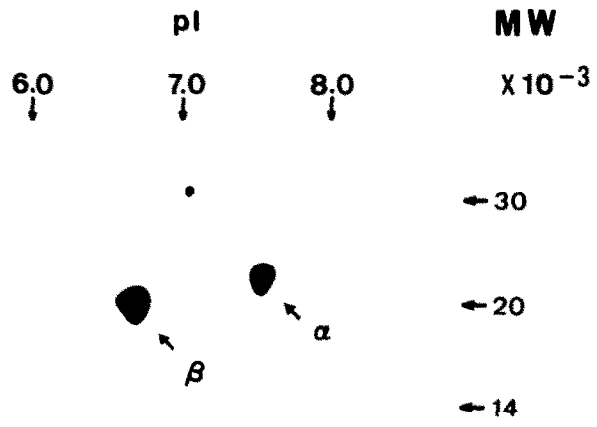

Fig.2. Subunit analysis of the ^{32}P-labelled NDP-kinase by two-dimensional gel electrophoresis. After the crude cell extract (about 50 μg protein) from HeLa S3 cells was incubated for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of 3 mM Ca^{2+}, an aliquot (about 10 μg protein) was directly analyzed by two-dimensional gel electrophoresis according to the modified method of O'Farrell et al. [15]. [^{32}P]Phosphorylated polypeptides were detected by autoradiography after gel electrophoresis.

was incubated for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of either 3 mM Ca^{2+}, 3 mM Mg^{2+} or 3 mM Mn^{2+}. The [^{32}P]phosphorylated polypeptides in the reaction mixtures were analyzed by SDS-PAGE followed by autoradiography. An autoradiogram showed two distinct polypeptides (M_r 21000 and 19000) to be highly phosphorylated in the presence of 3 mM Ca^{2+} or 3 mM Mg^{2+}, whereas several polypeptides including those of M_r 21000 and 19000 were highly phosphorylated in the presence of 3 mM Mn^{2+} (fig.1). The Ca^{2+} concentration was very important in this experiment, because 3 mM Ca^{2+} completely inhibited cellular protein phosphorylation by protein kinases without any reduction in the phosphorylation of these two polypeptides. In addition, two-dimensional gel electrophoresis followed by autoradiography confirmed the existence of two [^{32}P]phosphorylated polypeptides [M_r 21000 (pI 7.5) and M_r 19000 (pI 6.7)] after phosphorylation under defined conditions (incubation for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of 3 mM Ca^{2+}) (fig.2). It was con-

Table 1

Characterization of interferons as inducers of NDP-kinase in HeLa S3 cells

Treatment	NDP-kinase level (%)
	[^{32}P]Phosphate incorporation into the enzyme
Untreated control	100 (4909 cpm)
rHuIFNγ (30 U/ml)	203
(100 U/ml)	352
(300 U/ml)	443
(1000 U/ml)	477
(100 U/ml) + actinomycin D (0.5 μg/ml)	165
HuIFNγ (100 U/ml	437
(100 U/ml) + anti-HuIFNγ antiserum	121
HuIFNα (100 U/ml)	429
HuIFNβ (100 U/ml)	460
rMuIFNγ (100 U/ml)	102
MuIFNγ (100 U/ml)	101

HeLa S3 cells (approx, 10^8 cells) were treated with the indicated IFNs at the indicated titers (IU/ml as antiviral activity) for 6 h at 37°C in a CO_2 incubator. The NDP-kinase level ([^{32}P]phosphate incorporation into the enzyme per μg protein) in the cell extracts from these IFN-treated and untreated cells was determined by the nitorcellulose membrane method [10] after incubation for 5 min in an ice bath with [γ-^{32}P]ATP in the presence of 3 mM Ca^{2+}. Anti-HuIFNγ antiserum was preincubated with HuIFNγ(10^3 U/ml) for 30 min at 37°C before exposure to the cells at a concentration that completely neutralized the antiviral activity

cluded that these two highly phosphorylated polypeptides (M_r 21000 and 19000) in the crude preparation from HeLa S3 cells are identical to NDP-kinase subunits because of the following experimental results: (i) similar phosphorylation of these two polypeptides occurred with the incubation of one of the (γ-^{32}P)-labeled nucleoside 5′-triphosphates in the presence of 3 mM Ca^{2+} without strict base specificity; (ii) [^{32}P]phosphates on the [^{32}P]phosphorylated polypeptides were unstable under acidic conditions (1 N HCl) or on boiling (5 min); (iii) the molecular sizes (M_r 21000 and 19000) of these highly phosphorylated polypeptides in the crude preparation exactly corresponded to the sizes determined for NDP-kinase subunits (M_r 21000 and 19000) [10]; and (iv) the partially purified phosphorylating polypeptide fraction (Sephacryl S200 fraction, fig.3) exhibited NDP-kinase activity (ADP formation from ATP) when the fraction was assayed by the coupled pyruvate kinase-lactate dehydrogenase assay [16] (not shown).

Since (i) NDP-kinase in the crude preparation from HeLa S3 cells preferentially formed a [^{32}P]-phosphoenzyme (reaction intermediate) when incubated with [γ-^{32}P]ATP in the presence of 3 mM Ca^{2+} (figs 1 and 2); and (ii) the [^{32}P]phosphoenzymes formed were quantitatively determined by the nitrocellulose membrane method [10,11], it would be possible to compare the enzyme level between IFN-treated and untreated cells. We found that treatment of HeLa S3 cells with rHuIFNγ (100 units/ml for 2 h at 37°C in a CO_2 incubator) results in a significant increase (about 2.3-times) in NDP-kinase compared with that in untreated cells. This increase was dose-dependent until 300 units/ml rHuIFNγ (table 1). The enzyme level reached a plateau at 6 h after treatment. After 24 h of continuous exposure, treated cells could not be distinguished from untreated control cells on the basis of their NDP-kinase levels. Specific anti-HuIFNγ antiserum neutralized the ability of native HuIFNγ to increase the enzyme level in the cells. This enzyme increase was significantly blocked when actinomycin D (0.5 μg/ml) was added simultaneously with rHuIFNγ (100 units/ml). HuIFNα as well as HuIFNβ and HuIFNγ increased the enzyme to similar levels with the same unitages (100 units/ml) in the cells, whereas mouse recombinant IFNγ (MuIFNγ) did not affect enzyme induction. Taken together, these results show that HuIFNs are responsible for the increase in NDP-kinase level in HeLa S3 cells with cell species specificity, as has been shown in the induction of 2′,5′-oligo(A)synthetase [4], dsRNA-dependent protein kinase [5,6] and Ca^{2+}-dependent protein kinase (C-kinase) [17,18].

To determine the enzymatic properties of NDP-kinases from rHuIFNγ-treated and untreated HeLa S3 cells, the enzymes were partially purified from these cells by Sephacryl S200 gel filtration in the presence of 6 M urea, as previously reported with respect to purification of the enzyme from mouse NK cells [11]. As shown in fig.3, similar elution profiles of the proteins from these cells were observed, and the [^{32}P]phosphate-incorporating activity with a single peak was detected in each fraction. The specific activity ([^{32}P]phosphate incorporation into the enzyme subunits per μg protein) in the main peak fraction (fraction 68) of rHuIFNγ-treated cells was approx. 3-times higher than that determined in the same fraction of untreated cells. There were no significant differences, however, in the enzymes from rHuIFNγ-treated and untreated cells in the following enzymatic properties: (i) requirements for divalent cations, such as Ca^{2+} and Mg^{2+} for their enzyme activity at the optimum pH of 7.5–8.0; (ii) kinetics of phosphoenzyme formation; (iii) inactivation kinetics of the enzymes at 60°C; and (iv) subunit structure [$\alpha_2\beta_2$, α-subunit (M_r 21000) and β-subunit (M_r 19000)] (not shown).

The events leading to the increase in NDP-kinase occurred rapidly with HuIFNs, since it was detected within 2 h of incubation, but not with heterologous mouse IFNs (table 1). A similar enzyme increase was observed when mouse L cells were treated with mouse IFNs under similar conditions. The observations that (i) no significant difference in the enzymes from rHuIFNγ-treated and untreated cells was detected; and (ii) actinomycin D blocked the enzyme increase induced by HuIFNs suggest that IFNs may be responsible for the enhancement of enzyme synthesis at the transcriptional level. A similar IFN-induced membrane-associated polypeptide, which is correlated with the cessation of cell growth, has been recently reported as one of the polypeptides rapidly induced by IFNs [19]. The induction kinetics and the molecular size (M_r 18000–20000) of the IFN-

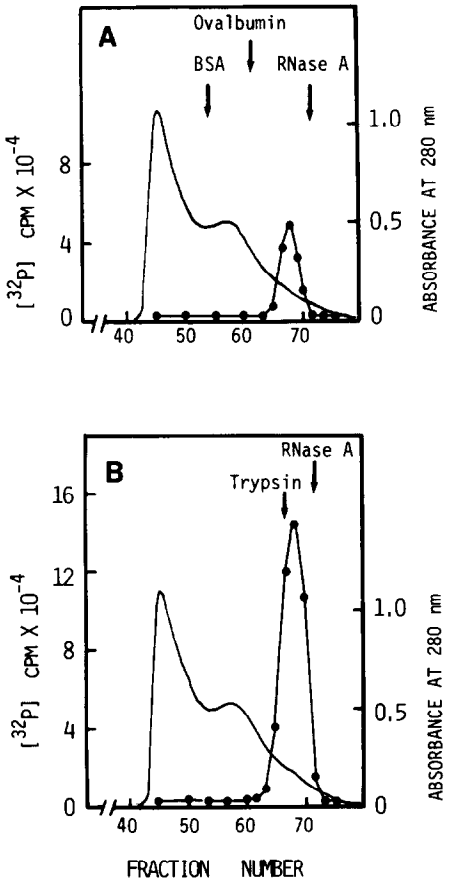

Fig.3. Partial purification of NDP-kinases from rHuIFNγ-treated and untreated HeLa S3 cells by Sephacryl S200 gel filtration in the presence of 6 M urea. HeLa S3 cells (approx. 10^9 cells) were exposed to rHuIFNγ (100 U/ml) for 6 h at 37°C in a CO_2 incubator. The crude NDP-kinase preparations from IFN-treated and untreated cells were prepared as described in section 2. The enzyme preparations (approx. 12 mg protein each) were passed separately through a Sephacryl S200 column (1.2×85 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.5) containing 6 M urea and 2 mM DTT. Elution was carried out with the same buffer containing 6 M urea and 2 mM DTT, and 1.0 ml fractions were collected. The [^{32}P]phosphate-incorporating activity of the indicated fractions was determined by the nitrocellulose membrane method. Arrows indicate the occurrence of bovine serum albumin (BSA, M_r 67 000), ovalbumin (M_r 45 000), trypsin (M_r 24 000) and RNase A (M_r 13 000). [^{32}P]Phosphate-incorporating activity (●) and absorbance at 280 nm (——). [A] Untreated control HeLa S3 cells, [B] rHuIFNγ-treated cells.

induced membrane-associated protein are similar to those shown here in the NDP-kinase subunits. However, the physiological activity of the IFN-induced membrane-associated protein and the mechanism by which IFNs inhibit cell growth are unclear at present.

Earlier reports concerning the physiological role of NDP-kinase indicated that (i) the enzyme acts as a key enzyme in supplying nucleoside 5′-triphosphates (NTPs) from nucleoside 5′-diphosphates (NDPs) for RNA synthesis (transcription) [10,13]; and (ii) the enzyme can initiate RNA synthesis in vitro [20,21]. Because of these physiological activities, it seems that the enzyme may play an important role in polypeptide induction by IFNs. Furthermore, IFN-induced polypeptides could be classified into two types: rapid-type inducing polypeptides (e.g. NDP-kinase, adenylate cyclase and membrane-associated 20 kDa protein), which are induced within 2 h of incubation of the cells with IFNs, and delayed-type inducing polypeptides (e.g. 2′,5′-oligo(A)synthetase, dsRNA-dependent protein kinase and phosphodiesterase), which are detectable after exposure of the cells to IFNs for longer than 3 h at 37°C. This time period for the classification of IFN-induced polypeptides was determined on the basis of the inhibitory effect of actinomycin D on the induction of these polypeptides by IFNs [1–3, 17–19]. The rapid-type polypeptides are incompletely blocked by actinomycin D and are preexistent at low levels in untreated cells, whereas the delayed-type polypeptides are extremely sensitive to actinomycin D and are undetectable in untreated cells [1–3,19].

The observation that the induction kinetics of both NDP-kinase and C-kinase are similar [17,18] is interesting in that it suggests that NDP-kinase may be correlated with the initiation of cellular protein phosphorylation by the protein kinases in IFN-treated cells. This possibility is supported by the following indirect evidence: (i) NDP-kinase supplies phosphate donors (ATP and GTP) for the protein kinases; and (ii) C-kinase [22] is responsible for the metabolic alteration [17,18] and cell activation [23] through cellular protein phosphorylation by the kinases. Experiments to determine (i) the physiological role of NDP-kinase and (ii) the physiological correlation between NDP-kinase and C-kinase in the initiation mechanisms involved in cell activation and cell differentiation induced by IFNs are currently underway.

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