Vol. 106, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1982 Pages 305-311

# INTERFERON INDUCTION OF POLYAMINE-DEPENDENT PROTEIN KINASE ACTIVITY IN EHRLICH ASCITES TUMOR CELLS

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Received April 1, 1982

SUMMARY: Treatment of Ehrlich ascites tumor cell cultures in vitro with interferon induces a protein kinase activity that is activated by the polyamines, spermidine and spermine. Putrescine antagonizes the activation. The protein kinase yields a phosphorylated endogenous polypeptide of $M_r$ 68,000-70,000. The polyamine-dependent protein kinase activity cofractionates with a double-stranded RNA-dependent protein kinase activity during affinity chromatography on poly (I)·poly (C) - agarose or by chromatography on phosphocellulose. The double-stranded RNA-dependent protein kinase also phosphorylates an endogenous polypeptide of $M_r$ 68,000-70,000. Unsuccessful attempts to discriminate between these two protein kinase activities on the bases of their respective capacities to be activated by either double-stranded RNA or spermidine/spermine, suggest that a single protein kinase enzyme may be activated by these strikingly dissimilar modifiers.

Recent studies directed towards understanding the molecular mechanisms of interferon action have focused on the central role of interferon induction of unique enzymes assumed to have a function in establishment of the antiviral state. Thus, pretreatment of mammalian cells with interferon results in the appearance of a new protein kinase activity. The protein kinase activity requires double-stranded RNA (dsRNA) for maximum catalytic activity. This enzyme phosphorylates two endogenous polypeptides of apparent molecular weights ($M_r$), 35,000 and 67,000 (1). The $M_r$ 35,000 polypeptide is proposed to be the smallest of the subunits of one of the initiation factors, eIF-2, involved in protein synthesis (2). The identity of the $M_r$ 67,000 phosphopolypeptide is unknown, although its phosphorylation can be used as a marker for interferon action (3).

Recently, a protein kinase was discovered in nuclei of the slime mold, Physarum polycephalum (4) and in bovine spermatozoa (5), which phosphorylates a $M_r$ 68,000-70,000 polypeptide in a reaction that is dependent on the polyamines, spermidine and spermine. Putrescine antagonized the activation by spermidine and spermine. In the case of P. polycephalum, the $M_r$ 70,000 phosphate-acceptor protein was identified to be the enzyme, ornithine decarboxylase (OrnDCase) (6).

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0006-291X/82/100305-07$01.00/0

Phosphorylation of OrnDCase by the polyamine-dependent protein kinase reaction inhibited its catalytic capacity to decarboxylate ornithine. The similarities of $M_r$ of the protein substrates for the polyamine-dependent and the dsRNA-dependent protein kinases, coupled with the recent finding that interferon treatment of mammalian cells results in a rapid loss of OrnDCase activity (7-10), prompted us to investigate the relationship of interferon treatment to the expression of these two protein kinase activities.

This communication reports the finding of an interferon-induced protein kinase activity in Ehrlich ascites tumor cells which is activated by the polyamines, spermidine and spermine. The endogenous substrate which is phosphorylated is a $M_r$ 68,000-70,000 polypeptide. The polyamine-dependent protein kinase activity could not be differentiated from the dsRNA-dependent protein kinase activity in crude cell extracts, or in preparations partially purified by affinity chromatography on poly (I)·poly (C) - agarose (1), or in preparations fractionated by phosphocellulose chromatography (4).

## MATERIALS AND METHODS

Ehrlich ascites tumor cells (ATCC# CCL-77) were grown in Eagles minimal essential medium supplemented with 10% fetal calf serum (Irvine Scientific Co.). Cells were grown in shake-cultures until the cell density reached 2 x $10^6$ cells/ml. Cultures were then treated with 400 units/ml of mouse fibroblast interferon (specific activity: 3 x $10^5$ units/ml) for 21 hr (11) before isolation of cytoplasmic and nuclear protein fractions (12). Polyamine-dependent protein kinase was isolated by chromatography on phosphocellulose (4). Interferon-induced, dsRNA-activated protein kinase was isolated by affinity chromatography on poly (I)·poly (C) - agarose (P·L Biochemicals, Inc.) (1). Discontinuous gel electrophoreses were conducted using 15% sodium dodecylsulfate (SDS)-polyacrylamide gels (15,16). Protein concentration was determined using Folin's reagent (4).

## RESULTS AND DISCUSSION

Polyamine-dependent protein kinase from nuclei of P. polycephalum (4), bovine spermatozoa (5), and rat liver (unpublished), can be successfully purified on phosphocellulose chromatography. A portion of the protein kinase and its $M_r$ 70,000, phosphate-acceptor substrate protein copurify as a complex by this procedure. Thus, the protein kinase can be detected by measuring its capacity to phosphorylate the endogenous substrate protein.

Phosphocellulose chromatography. In order to determine whether a similar complex could be isolated from ascites tumor cells, control cells which had not been exposed to interferon were fractionated into separate cytoplasmic and nuclear protein preparations. Each control preparation was subsequently fractionated further by chromatography on Bio-Rex 70 and then on phosphocellulose (4). Table 1 (column (d)) shows the results. The control cytoplasmic fraction (preparation #1) demonstrated a protein kinase activity that was stimulated over 4-fold by a combination of 0.5 mM each of spermidine and spermine. dsRNA, supplied as poly

Table 1. Effect of Polyamines and Poly (I) · Poly (C) on Protein Kinase, Fractionated by Phosphocellulose Chromatography and by Affinity Chromatography on Poly (I) · Poly (C) - Agarose, From Control and Interferon-Treated Ascites Tumor Cells.

| Preparation No. | Protein Kinase Preparation | Addition[a] | Specific Activity of Protein Kinase | |
|---|---|---|---|---|
| | | | from phosphocellulose chromatography | from poly (I) · poly (C) affinity chromatography |
| | | | (pmoles/min/mg enzyme)[b] | |
| (a) | (b) | (c) | (d) | (e) |
| 1 | Control cytoplasmic | None | 85 | 32 |
| | | Polyamines | 374 | 109 |
| | | dsRNA | 359 | 115 |
| | | Polyamines + dsRNA | 371 | 152 |
| 2 | Control nuclear | None | 28 | 19 |
| | | Polyamines | 29 | 27 |
| | | dsRNA | 43 | 38 |
| | | Polyamines + dsRNA | 44 | 43 |
| 3 | Interferon-treated cytoplasmic | None | 490 | 475 |
| | | Polyamines | 1401 | 2418 |
| | | dsRNA | 1436 | 2030 |
| | | Polyamines + dsRNA | 1997 | 2556 |
| 4 | Interferon-treated nuclear | None | 355 | 809 |
| | | Polyamines | 977 | 3429 |
| | | dsRNA | 1015 | 3909 |
| | | Polyamines + dsRNA | 1376 | 4008 |

[a]Polyamines were a mixture of spermidine plus spermine, each 0.5 mM. Double-stranded RNA (dsRNA) was poly (I) · poly (C), potassium, salt, and was added to a final concentration of 1 μgm/ml.

[b]The specific radioactivity of [$\gamma$-$^{32}$P]ATP was 3.49 x $10^8$ cpm/μmol for assays given in column (D) and 2.85 x $10^8$ cpm/μmol for assays given in column (E). Each assay contained 34 μg of enzyme preparation. Other details were as those previously described (6).

(I)·poly (C) (P·L Biochemicals, Inc.) at 1 μgm/ml, stimulated protein kinase activity to a similar extent in the same preparation. Simultaneous additions of polyamines and dsRNA produced a comparable enhancement. The control nuclear fraction (preparation #2, column (d)) demonstrated little protein kinase activity which was essentially unaffected by polyamines, dsRNA, or a combination of the two.

Ascites tumor cells were next treated with mouse fibroblast interferon for 2 hr prior to fractionation into separate cytoplasmic and nuclear protein preparations. Each interferon-treated preparation was also fractionated further by chromatography on Bio-Rex 70 and then on phosphocellulose. Table 1 (column (d)) shows that the interferon-treated, cytoplasmic fraction (preparation #3)

demonstrated an enhanced level of protein kinase activity compared to the control cytoplasmic preparation (preparation #1), and the activity was enhanced 2.8-fold by the polyamines or by poly (I)·poly (C). Simultaneous additions of the polyamines and dsRNA produced a 4-fold stimulation compared to assays that were conducted without addition of a modifier. The interferon-treated nuclear fraction (preparation #4, column (d)) demonstrated markedly enhanced protein kinase activity compared to the control (preparation #2). The level of enhanced protein kinase activity elicited by the polyamines (2.7-fold), dsRNA (2.8-fold) or a combination of the polyamines plus dsRNA (3.9-fold) were the same as the levels of enhancement observed under similar conditions in the interferon-treated cytoplasmic preparation.

A comparison of the protein kinase specific activities (column (d)) in the control nuclear preparation (preparation #2) and the interferon-treated nuclear preparation (preparation #4) was most striking. Interferon elicited an apparent 33-fold induction of polyamine-dependent protein kinase activity (cf. 43 vs 1015) in the nucleus. Combined additions of the polyamines and of dsRNA evoked a 31-fold increase (cf. 44 vs 1376). Since the combined additions evoked an increase which was not significantly greater than that observed with single additions of the polyamines or dsRNA, these results suggest that a single protein kinase enzyme may be activated both by a spermidine/spermine mixture and by dsRNA. This proposal was supported further by exhaustive phosphorylation experiments (data not shown). Protein kinase reactions were activated by polyamines and were allowed to continue for 60 min. The phosphate-acceptor substrate protein available to the polyamine-dependent reaction was depleted after 60 min. Addition of dsRNA to these reaction mixtures evoked no additional phosphorylation. The converse of this reaction, in which exhaustive phosphorylation was activated by dsRNA, then followed after 60 min by addition of polyamines, also yielded no additional phosphorylation. These experiments indicated that the polyamine-dependent protein kinase and the dsRNA-dependent protein kinase may phosphorylate the same endogeneous substrate protein (see below).

Poly (I) · poly (C) - agarose chromatography. Interferon-induced, dsRNA-dependent protein kinase can be fractionated by affinity chromatography on poly (I) · poly (C) - agarose (1). Thus, it was important to determine whether dsRNA-dependent protein kinase activity retained on poly (I) · poly (C) - agarose responded similarly to the polyamines and dsRNA as the polyamine-dependent protein kinase which was isolated on phosphocellulose as described in the previous section. Control and interferon-treated ascites tumor cells were first fractionated into cytoplasmic and nuclear preparations. Each respective preparation was subsequently subjected to affinity chromatography on poly (I) · poly (C) - agarose (1). The independently isolated protein kinase preparations were then tested for responses to the modifiers, polyamines or dsRNA. Table 1 (column

(e)) shows the results. The control cytoplasmic preparation (preparation #1) and the control nuclear preparation (preparation #2) both contained low levels of protein kinase activity. The nuclear fraction demonstrated little protein kinase activity that could be activated by the polyamines, dsRNA, or a combination of both. In contrast, the corresponding interferon-treated fractions were markedly elevated in protein kinase activity which was strongly responsive to stimulation by either the polyamines or dsRNA. The protein kinase specific activity in the interferon-treated nuclear preparation was stimulated 127-fold over the control preparation by the polyamines (cf. 27 vs 3429). dsRNA stimulated the same protein kinase activity 102-fold over the control (cf. 38 vs 3909). A combination of the polyamines and dsRNA activated the interferon-treated nuclear protein kinase 93-fold over the control (cf. 43 vs 4008). Again, the fact that the combined modifiers enhanced the protein kinase activity to an extent comparable to that of each individual modifier (cf. 4008 vs 3909 or 3429), suggested that one protein kinase was responsible for the phosphorylation reaction.

Endogenous phosphate-acceptor proteins. The proposal in the previous sentence above gained support from analyses of the products of the phosphorylation reactions as shown in representative profiles in Fig. 1. The phosphorylated product in all reactions containing samples from the interferon-treated nuclear preparation, plus polyamines and/or dsRNA, was a single [$^{32}$P]phosphopeptide of $M_r$ 68,000 - 70,000 (Fig. 1, panel A). Thus, a spermidine/spermine mixture, dsRNA, or a combination of the two polyamines plus dsRNA, activated a protein kinase reaction to phosphorylate a unique substrate protein of $M_r$ 68,000 - 70,000. Whether the polyamine-dependent protein kinase and the dsRNA-dependent protein kinase catalyze phosphorylation of the same identical polypeptide is unknown at this time. It can only be concluded that the reaction product in either case was a phosphoprotein of $M_r$ 68,000 - 70,000.

The interferon-treated cytoplasmic preparation catalyzed phosphorylation of two polypeptides of similar $M_r$ in the presence of polyamines, dsRNA, or a combination of the two modifiers (Fig. 1, panel B). The [$^{32}$P]phosphopeptides had $M_r$ values of 70,000 and 135,000, respectively. In contrast, those labeled phosphopeptides were not observed among the products of any reactions conducted in the absence of the polyamines and/or dsRNA. Also, they were not produced by the control cytoplasmic or control nuclear enzyme preparations in the absence or the presence of the polyamines and/or dsRNA. These results showed that the phosphorylation reactions which yielded the $M_r$ 70,000 and 135,000 phosphopeptides were critically dependent on the modifiers, polyamines or dsRNA, for activation. The small amount of phosphorylation observed by the control cytoplasmic or the control nuclear preparations in the absence of the modifiers represented phosphorylated products which could not be detected on the polyacrylamide gel system used.

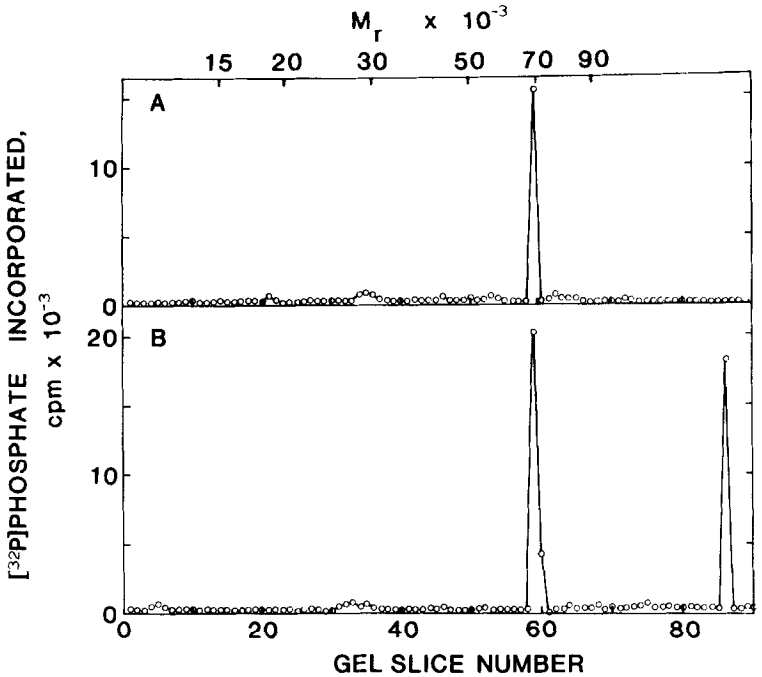


Fig. 1. Content of $^{32}P_t$ in SDS/polyacrylamide rod gels following labeling of endogeneous peptides with [$\gamma$-$^{32}$P]ATP by protein kinase preparations fractionated on poly (I) · poly (C) - agarose. Labeling was conducted in an in vitro protein kinase assay described in Fig. 2 of reference (6). (A) The protein kinase preparation (58 µgm) was derived from the soluble nuclear proteins of ascites tumor cells pretreated with interferon as described in Materials and Methods. The protein kinase was activated with a combination 0.5 mM spermidine, 0.5 mM spermine, and 1 µgm / ml of poly (I) · poly (C). (B) The protein kinase preparation (39 µgm) was derived from the soluble cytoplasmic proteins of ascites tumor cells pretreated with interferon. The protein kinase was similarly activated by polyamines and poly (I) · poly (C) as in (A). The specific radioactivity of [$\gamma$-$^{32}$P]ATP in all cases was 6.8 x $10^{10}$ cpm/µmol.

All activations by spermidine and spermine, that are summarized in Table 1, were completely antagonized by 0.5 mM putrescine (data not shown).

The polyamines, spermidine and spermine, are highly cationic at pH 6.5, the pH of the enzymatic assays described herein. dsRNA is highly anionic at the same pH. Several findings reported here suggest that a single protein kinase enzyme is capable of activation by these strikingly dissimilar modifiers. The polyamine-dependent protein kinase and the dsRNA-dependent protein kinase activities cofractionated on phosphocellulose or on poly (I) poly (C) - agarose. Both activities yielded endogenous, phosphorylated products of the same $M_r$. Lastly, the two enzyme activities could not be differentiated on the bases of their respective activations by the polyamines or dsRNA in a common preparation.

The $M_r$ 68,000 - 70,000 phosphate-acceptor protein for both polyamine- and dsRNA-dependent protein kinases in Ehrlich ascites tumor cells has not yet been

rigorously identified. However, nuclear and cytoplasmic preparations which contain the phosphate-acceptor protein, also demonstrate low OrnDCase activity. Phosphorylation of the phosphate-acceptor protein correlates with the loss of OrnDCase activity (unpublished data). There are strong parallels between the properties reported here for phosphorylation of the $M_r$ 68,000 - 70,000 polypeptide in Ehrlich ascites tumor cells and those that we have discovered in three other eukaryotic sources (4-6). The parallels are sufficiently strong to pursue the question of whether phosphorylation of OrnDCase with concomitant suppression of polyamine synthesis may be another target for interferon induction of protein kinase.

Acknowledgements: This investigation was supported by research funds from Public Health Service Grant 18538 from the National Institute of General Medical Sciences.

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