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SPERMINE SPECIFICALLY INHIBITS THE PHOSPHORYLATION OF AN 11,000-DALTON NUCLEAR PROTEIN IN VARIOUS CULTURED MAMMALIAN CELL LINES

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SUMMARY: The effect of polyamines (putrescine, spermidine and spermine) on endogenous protein phosphorylation in mouse neuroblastoma cells was investigated by using techniques of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The results indicated that spermine at 1mM completely inhibited the phosphorylation of the 11,000-dalton and 120,000-dalton proteins in nuclear fractions. The inhibition of the phosphorylation of the 11,000-dalton but not the 120,000-dalton protein by spermine was also observed in five other cell lines examined and appeared to be a general phenomenon. The inhibitory effect of spermine on the phosphorylation of the 11,000-dalton protein was specific, other cations such as ammonium chloride, arginine, putrescine, cyclen and trien were ineffective at equal molar or much higher concentrations.

Mouse neuroblastoma cells grown in tissue culture can be induced to differentiate by cAMP analogs and agents which increase intracellular cAMP content [1,2]. The differentiation of mouse neuroblastoma cells is accompanied by large decreases of ornithine decarboxylase (ODC[1], E.C. 4.1.1.17) activity and polyamine content [3]. Recently we have found that α-fluoromethylornithine, a suicidal enzyme inhibitor of ODC, can potentiate the effect of suboptimal concentrations of dibutyryl cAMP in eliciting maximal differentiation of mouse neuroblastoma cells [4,5]. These results suggest that modulation of polyamine metabolism may be important in the differentiation of mouse neuroblastoma cells. As a step to define the action of polyamines in the differentiation of neuroblastoma cells, we have taken the approach of identifying specific biochemical events mediated by polyamines and to

1. Abbreviations used: ODC, ornithine decarboxylase; PMSF, phenylmethyl sulfonyl fluoride; trien, 1,4,7,10, tetraazadecane; cyclen, 1,4,7,10-tetraazacyclodecane.

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correlate the occurrence of such events with the expression of differentiated phenotypes in the mouse neuroblastoma cells.

Abundant literature evidence has indicated the importance of post-translational modification of proteins via phosphorylation-dephosphorylation as a biological control mechanism [6-8]. Physiological concentrations of polyamines have been shown to enhance activities of certain nuclear kinases from rat liver and hepatoma cells [9-12] and Physarum polycephalum [13].

The purpose of the present study is to examine whether polyamines can specifically affect protein phosphorylation reactions in mouse neuroblastoma cells. During the course of this study we have found that spermine not only stimulated phosphorylation of certain nuclear proteins but also specifically inhibited the phosphorylation of an 11,000-dalton polypeptide. Furthermore, this inhibition appeared to be a general phenomenon which occurred in five other cell lines examined.

MATERIAL AND METHODS

Chemicals [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham, Arlington, IL. Putrescine, spermidine, spermine, chymostatin, pepstatin, phenylmethylsulfonyl fluoride (PMSF) and sodium molybdate were from Sigma Chem. Co. St. Louis, MO. Tissue culture media and sera were from Gibco, Grand Island, N.Y. All other chemicals were of standard reagent grade. Trien (1,4,7,10 tetraazadecane) and cyclen (1,4,7,10 tetraazacyclododecane) were kindly provided by Dr. H. Schugar, Rutgers University.

Cell Culture The N-18 or NB-15 mouse neuroblastoma cell were grown as monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum under conditions previously reported [14]. Cells at an early stationary phase of growth were used for all studies described below unless otherwise indicated. PC12 rat pheochromocytoma cells (a gift of Dr. L.A. Greene, NYU Medical School), S91 mouse melanoma (gift of Dr. J.M. Pawelek, Yale Medical School), C6 mouse glioma cells, L5 rat myoblast (a gift of Dr. A.Y.-C. Liu, Harvard Medical School) and IMR-90 human fibroblasts were grown as previously described [14].

Isolation of Nuclei The monolayer cell cultures were scraped into a homogenization buffer containing 0.05 M Tris-HCl, pH 7.4 and 0.25 M sucrose. Cells were then homogenized by repeatedly forcing the cell suspension through a 25G/5/8 inch needle. The efficacy of the homogenization procedure was evaluated by examining the suspension under phase contrast microscopy. More than 90% of the nuclei remained intact. The crude nuclear pellet was collected by centrifugation at 2000 rpm for 5 min at 4°C in a Sorvall Centrifuge. The crude nuclear fraction was washed twice with the homogenization buffer supplemented with 1mM PMSF and 0.25% Triton X-100. The washed nucelar pellet was resuspended in the same buffer, mixed with 2.4 M sucrose in 0.05 M Tris HCl, pH 8.0 and centrifuged at 20,000 rpm in a SW 25.1 Beckman rotor for 90 min at 4°C. The pellet was termed purified nuclei [15]. Similar results were obtained using nuclear fraction prepared according to another method as described by Rapaport et. al. [16].

Endogenous Protein Phosphorylation The assay mixture contained 1mM EGTA, 20 mM potassium fluoride, 10 mM magnesium acetate, 1 mM sodium molybdate, 5 μM [γ-^{32}P]ATP (3.1 x 10^7 pm per nmol), 1 μg each of chymostatin and pepstatin and purified nuclei equivalent to 150-200 μg proteins in a 0.15 ml of TES buffer (N-tris[hydroxymethyl]-methyl-2-aminoethane sulfonic acid, 60 mM, pH 7.0). Amines were added as hydrochloride salt to the indicated concentrations. The reaction mixture was incubated for 30 min at room temperature. Protein kinase activity was estimated by measuring the amount of [^{32}P]phosphate incorporated into acid-insoluble protein fraction. The endogenous phosphorylation pattern was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradio-graphy. Briefly, at the end of incubation, the phosphorylation reaction mixture was mixed with a one-fifths volume of an SDS-stop solution containing 12% SDS, 0.5 M Tris-HCl (pH 9.0), 10% β-mercaptoethanol, 5mM EDTA, 25% sucrose and 0.005% of the tracking dye, Pyronin Y. Samples were heated at 100°C for 3 min. Aliquots of the samples containing 50 μg of protein were applied on a 7.5-15% acrylamide gradient slab gel. Upon completion of the electrophoresis, the gel was stained, destained and vacuum dried on a Whatman filter paper. Autoradiograph was made by exposing the dried gel on a Kodak X-ray film for an appropriate period of time.

RESULTS AND DISCUSSION

The phosphorylation pattern of purified nuclear fraction from NB-15 mouse neuroblastoma cells is shown in Fig. 1 (Lane A). The pattern was reproducible from experiment to experiment and was independent of the method used to prepare the nuclear fraction. Exogeneously added spermidine and spermine, but not putrescine, stimulated the phosphorylation of many proteins present in the

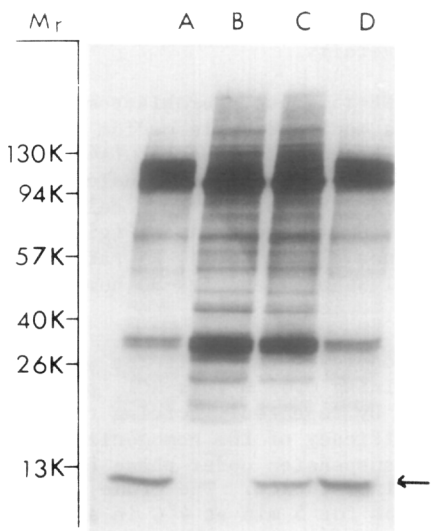

Fig. 1 Effect of polyamines on the endogenous protein phosphorylation pattern in purified nuclear fraction prepared from NB-15 mouse neuroblastoma cells. Lane A, no addition; Lane B, 1mM spermine added; Lane C, 1mM spermidine added; Lane D, 1mM putrescine added. The phosphorylation condition, gel electrophoresis and autoradiographic procedure were described in METHODS. Arrow indicates the 11,000-dalton protein.

nuclear fraction, most notably, the 100,000-, and the 32,000-dalton proteins (Fig. 1, Lanes B and C vs Lane A). In addition to the stimulation, 1 mM spermine almost completely inhibited the phosphorylation of a 120,000-dalton and an 11,000-dalton protein (Fig. 1 Lane B). Spermidine also inhibited the phosphorylation of the 120,000-dalton. However, its inhibitory effect on the phosphorylation of the 11,000-dalton protein was less pronounced. Both the 11,000-dalton and the 120,000-dalton protein represented major phosphorylated proteins in the nuclear fraction. In Fig. 1, Lane A, 11% and 15% of the total incorporated radioactivity were associated with the 11,000-dalton and the 120,000-dalton protein respectively.

To evaluate the specificity of effects of polaymines, we examined and compared the effects of two amines, arginine and ammonium chloride, two synthetic spermine analogs, trien and cyclen, with that of the three naturally occurring polyamines, putrescine, spermidine and spermine (Fig. 2). In this particular experiment a crude nuclear fraction of the NB-15 mouse neuroblastoma cells rather than the purified nuclear fraction was used. A comparison of the results illustrated in Fig. 1 (Lane A) and Fig. 2 (Lane A) indicated that there was little or no difference in the overall phosphorylation pattern of the crude and purified nuclear fraction. In agreement with the results obtained with purified nuclear fraction, 1mM spermine inhibited the phosphorylation of the 120,000- and 11,000-dalton proteins (Fig. 2 Lane C); 1 mM spermidine inhibited the phosphorylation of the 120,000-dalton protein but was less effective in inhibiting the phosphorylation of the 11,000-dalton protein (Fig. 2, Lane B). The effects of spermine and spermidine appeared to be specific; putrescine, arginine and ammonium chloride had no significant effect on the phosphorylation pattern even at concentrations as high as 5mM (Fig. 2 Lanes D-F). Cyclen, a cyclic analog of spermine, neither stimulated nor inhibited phosphorylation of any individual proteins present in the nuclear fraction (Fig. 2 Lane G). Trien, a spermine analog with similar charge property but with a shorter hydrocarbon chain length, mimicked the spermidine in inhibiting the phosphorylation of the 120,000-dalton but not the 11,000-dalton protein (Fig. 2, Lane H). Taken

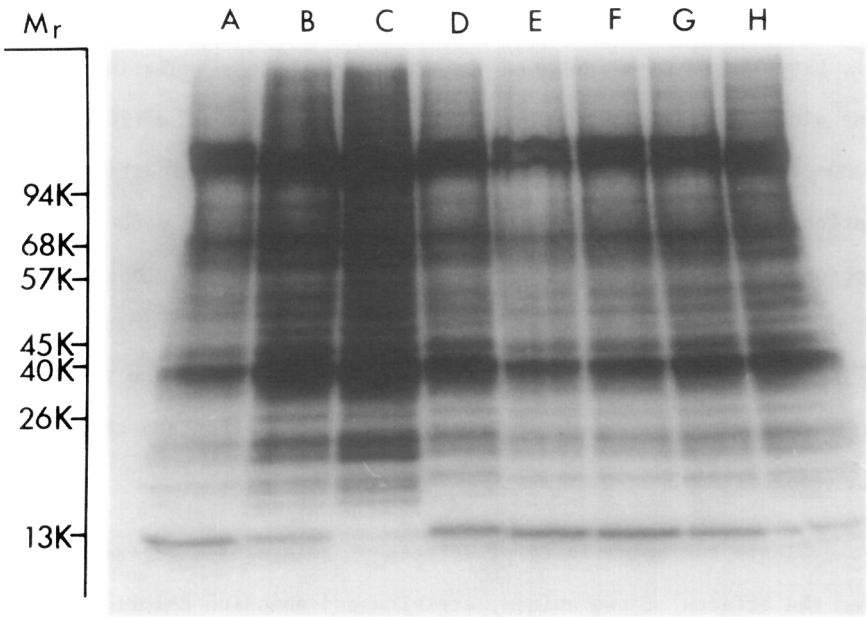

Fig. 2 Specificity of the effect of polyamines on the endogenous protein phosphorylation in crude nuclear fraction of NB-15 mouse neuroblastoma cells. Lane A, no addition; Lane B, 1mM spermidine; Lane C, 1mM spermine; Lane D, 5mM putrescine; Lane E, 5mM ammonium chloride; Lane F, 5mM arginine; Lane G, 1mM cyclen; Lane H, 1mM trien. Arrow indicates the 11,000-dalton protein.

together, these results indicated that: (a) The structural and charge requirements for the inhibition of the 11,000-dalton protein was more stringent than that for the 120,000-dalton protein. Thus at 1mM, spermine, spermidine and trien all inhibited the phosphorylation of the 120,000-dalton protein but only spermine was effective in inhibiting the phosphorylation of the 11,000-dalton protein. (b) The inhibition of the phosphorylation of the 11,000-dalton and the 120,000-dalton proteins and the stimulation of the phosphorylation of all other nuclear proteins by spermine appeared to occur concomitantly. Amines which are ineffective in inhibiting both 11,000-dalton and 120,000-dalton proteins were also ineffective in stimulating phosphorylation of other nuclear proteins. In this regard it may be of interest to note that Hara and Endo [17] reported that spermine and spermidine stimulate casein phosphorylation but inhibit phosvitin phosphorylation in a reaction catalyzed by nuclear protein kinase NII.

Polyamines are ubiquitous organic cations found in both eukaryotes and prokaryotes. Cultured animal cells have been shown to contain putrescine at 0.25-2.5 mM, and spermidine and spermine at the level of 1-2 mM [18]. In view of this consideration and to evaluate the possible generality of the specific effects of spermine at physiological concentration on the phosphorylation of nuclear proteins, we examined the effects of spermine on the endogenous phosphorylation of crude nuclear fractions obtained from various cell lines including C6 glioma cells, L5 myoblasts, S91 melanoma cells, PC12 cells and IMR-90 human fibroblasts (Fig. 3). While there were significant differences in the overall pattern of phosphorylation of nuclear proteins from different cell lines, it is noteworthy that the spermine-mediated inhibition of the phosphorylation of the 11,000-dalton protein occurred in all cell lines studied. The phosphorylation of the 120,000-dalton protein and the inhibition of this phosphorylation by spermine that we observed in mouse neuroblastoma cells (Fig. 1, Lane A and B) were not obvious in these cell lines, suggesting that the phosphorylation of the 120,000-dalton protein may be a neuroblastoma cell-specific event.

Hara et.al. [19] studied the effect of polaymines on the endogenous protein phosphorylation of chromatin fraction isolated from rat liver. These authors

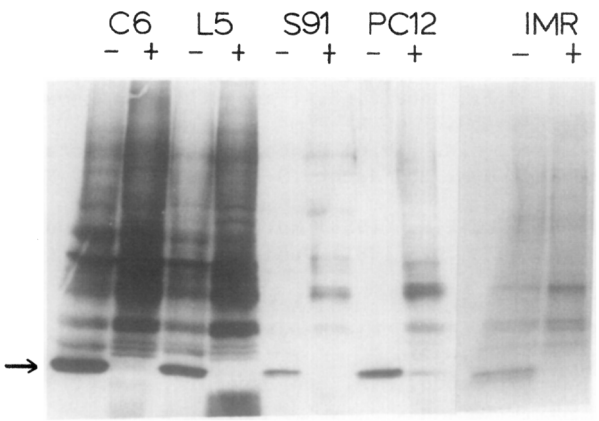

Fig. 3 Effect of spermine on the endogenous phosphorylation of nuclear proteins of various mammalian cell lines. (-), no addition; (+), 1mM spermine added. C6, mouse glioma cell line; L5, rat myoblast cell line; S91, Cloudman S91 mouse melanoma cell line; PC12, rat pheochromocytoma cell line; IMR, IMR-90 human skin fibroblast. Arrow indicates the 11,000-dalton protein.

reported that spermine specifically inhibited the phosphorylation of a 43,000-dalton, a 11,000-dalton and a 10,000-dalton protein. Qi et.al. [20] recently reported that polaymines inhibit phospholipid-sensitive and calmodulin-sensitive Ca^{2+}-dependent protein kinases. These studies together with the data presented above suggest that the action of polyamines, particularly spermine, can be mediated not only by the stimulation of protein phosphorylation but also by the inhibition of protein phosphorylation. Although the function of the 11,000-dalton protein is not known at present, the facts that it is one of the mostly prominently phosphorylated proteins in various mammalian cell lines examined (Fig. 3) and that spermine at physiological concentration specifically inhibits its phosphorylation (Fig. 1, Lane B) suggest that this protein may play an important role in the biochemical processes in which spermine specifically affects cellular function.

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