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Polyamine-stimulated phosphorylation of prostatic spermine-binding protein is mediated only by cyclic AMP-independent protein kinases

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Spermine-binding protein (a rat ventral prostatic protein with high affinity for spermine) was phosphorylated *in situ* through the action of intrinsic cellular protein kinase(s), suggesting it to be a phosphoprotein *in vivo*. The purified protein served as a substrate in a number of cyclic AMP-independent protein kinase reactions *in vitro*, but not for cyclic AMP-dependent, Ca^{2+} + calmodulin-dependent or Ca^{2+} + phospholipid-dependent protein kinases. Available data indicate that at least one of the cyclic AMP-independent protein kinases (cytosolic protein kinase C2) may be physiologically relevant in mediating the phosphorylation of this protein. The phosphorylation reaction was stimulated several-fold in the presence of spermine. Spermidine was somewhat less effective, whereas putrescine, cadaverine and 1,6-hexanediamine were minimally active. Phospho amino acid analysis of ^{32}P-labelled spermine-binding protein indicated that phosphoserine was the only labelled phospho amino acid. Spermine-binding protein did not undergo autophosphorylation, or modify the stimulative effect of spermine on the phosphorylation of other substrates such as non-histone proteins. *In situ* the phosphorylation of spermine-binding protein in tissue from castrated rats was markedly diminished as compared with the normal. Since the phosphorylation of spermine-binding protein appears to be mediated by cyclic AMP-independent protein kinase(s) whose activity in the prostate is under androgenic control, it is suggested that androgen-dependent modulation of the protein kinase(s) exerts a regulatory control (via phosphorylation–dephosphorylation) on the spermine-binding activity and stability of this protein *in vivo*. Further, since this protein is a substrate for only the cyclic AMP-independent protein kinases, it could serve as a tool for the investigation of such kinases.

Rat ventral prostate contains an acidic protein (M_r 35000) with a relatively high affinity for the naturally occurring polyamine spermine (Liang *et al.*, 1978). This protein, named as spermine-binding protein, has been purified and characterized. It is present in large amounts in the prostate, and appears to be dependent on the androgenic status of the animal, since spermine-binding activity is diminished on castration and is increased when castrated animals are given a single dose of 5α-dihydrotestosterone (Mezetti *et al.*, 1979). However, the physiological significance of this protein remains obscure. It was thought that it might act as a carrier for spermine (Liang *et al.*, 1978).

Treatment of the spermine-binding protein with a protein phosphatase decreases its ability to bind spermine, suggesting that this protein is a phosphoprotein and that its phosphorylation promotes spermine-binding activity (Liang *et al.*, 1978). In view of this, the present work was undertaken (*a*) to determine if the spermine-binding protein is a phosphoprotein *in situ*, (*b*) to characterize the protein kinase reactions involved in the phosphorylation of spermine-binding protein, and to determine the effects of polyamines on those reactions,

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and (*c*) to determine whether or not the presence of spermine-binding protein along with polyamines influenced the phosphorylation of endogenous chromatin-associated phosphoproteins (i.e. if it acts as a modulator or a ligand in mediating the effects of spermine in this reaction). Stimulation of prostatic chromatin-associated protein kinase reactions by polyamines *in vitro* has been well documented (Ahmed *et al.*, 1978, 1979, 1981, 1983*a*,*b*).

We now report that spermine-binding protein is phosphorylated in intact tissue. The purified protein can serve as a substrate for cyclic AMP-independent protein kinase reactions (such as those associated with cytosolic and nuclear fractions), but not for cytosolic cyclic AMP-dependent or Ca^{2+}+calmodulin-dependent or Ca^{2+}+phospholipid-dependent protein kinases *in vitro*. Spermine, and to a lesser extent other polyamines, markedly enhanced the cyclic AMP-independent protein kinase-mediated phosphorylation of spermine-binding protein. A preliminary report of this work has been presented (Goueli *et al.*, 1983).

Materials and methods

Rats

Male Sprague–Dawley rats weighing 295–325g from Harlan Co. (Indianapolis, IN, U.S.A.) were used as the source of tissues. Orchiectomy was performed under light ether anaesthesia as described previously (Ahmed *et al.*, 1978).

Spermine-binding protein

This was purified from rat ventral prostate by the method described previously (Hiipakka *et al.*, 1984).

Chemicals

Polyamines (spermine hydrochloride and spermidine hydrochloride) and other aliphatic amines were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). They were adjusted to pH 7.4 before use by adding Tris base. All other reagents were of the highest purity available.

Enzyme sources

Cytosolic cyclic AMP-dependent protein kinase (from bovine heart) was purchased from Sigma Chemical Co., or was purified from rat ventral prostate as described previously (Goueli & Ahmed, 1980). Nuclei were prepared from rat liver and ventral prostate as detailed previously (Ahmed, 1971). Chromatin and non-histone protein fraction were isolated exactly as described previously (Ahmed *et al.*, 1978, 1983*b*). Other highly purified protein kinases used were prostatic and liver nuclear cyclic AMP-independent protein kinases (active towards casein, phosvitin and non-histone proteins), and liver cytosolic cyclic AMP-independent protein kinases (active towards casein and phosvitin). These enzymes have been described in a number of tissues and are called N1 and N2 protein kinases to indicate nuclear source (see, e.g., Thornburg *et al.*, 1979; Rose *et al.*, 1981; Erdmann *et al.*, 1982; Goueli & Ahmed, 1984) or casein kinase I and II (kinases C1 and C2 in the present paper) to indicate cytosolic or whole tissue source (see, e.g., Hathaway & Traugh, 1979; Dahmus, 1981; Cochet *et al.*, 1981; Tse *et al.*, 1984). The enzymes from liver and prostatic tissues were purified in this laboratory, and their general characteristics were as follows. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 10% (w/v) gels (Laemmli, 1970) of N1 and C1 enzymes from liver showed a single band of M_r 37000, and of N2 from liver and prostate and C2 from liver cytosol showed three subunits of M_r 42000, 38000 and 26000 for each of the three enzyme preparations. N1- and C1-type enzymes with casein as substrate (5mg/ml) required 150mM-NaCl, 5mM-$MgCl_2$ and 0.1mM-[γ-^{32}P]ATP for maximal activity, which was generally 50000nmol of ^{32}P incorporated into casein/h per mg of enzyme. They were not inhibited by heparin up to a concentration of 10μg/ml in the reaction mixture. N2- and C2-type enzymes required similar reaction conditions, but were inhibited by heparin (50% inhibition in the presence of 2μg of heparin/ml). In addition, these enzymes were able to utilize GTP as well as ATP as the phosphate donor. The activity of the purified C2 and N2 with casein as substrate was generally about 100000nmol of ^{32}P/h per mg of enzyme. Thus the properties of the prostatic and liver nuclear protein kinases (N1 and N2) as well as cytosolic protein kinases (C1 and C2) used by us were comparable with those described by others for similar enzymes from other tissues (references given above).

Phosphorylation of spermine-binding protein in vitro

Phosphorylation of spermine-binding protein was measured in a reaction medium (final volume 0.2ml) consisting of 0.1mM-[γ-^{32}P]ATP (50–100d.p.m./pmol of ATP), 1.0mM-$MgCl_2$, 30mM-Tris/HCl buffer, pH 7.45 (measured at 37°C), 10μg of spermine-binding protein and chromatin (equivalent to 2μg of DNA) or non-histone protein fraction (2μg of protein) as the source of cyclic AMP-independent protein kinase activity. When purified protein kinases were used the amount added per reaction was 1μg of enzyme protein/ml of reaction volume. The reaction was initiated by adding the enzyme preparation and was carried

out at 37°C for a period of up to 10min. It was terminated by the addition of trichloroacetic acid to a final concentration of 15% (w/v). The precipitated protein was washed free of [γ-^{32}P]ATP before measurement of radioactivity incorporated in it, as described previously (Ahmed *et al.*, 1975). Zero-time blanks and appropriate controls were included to take into account the incorporation of ^{32}P into the endogenous proteins of chromatin or non-histone proteins. Activity of cytosolic cyclic AMP-dependent protein kinase was measured as described previously (Ahmed *et al.*, 1983*b*).

Immunoprecipitation of spermine-binding protein from rat ventral prostate tissue incubated with [^{32}P]P_i

Ventral prostates from normal rats (three rats, 1.3g of tissue) and from rats 48h after castration (three rats, 1.1g of tissue) were minced and washed at room temperature with 10ml of incubation medium containing 30mM-Mops/NaOH buffer, pH7.2 (at 37°C), 125mM-NaCl, 7mM-KCl, 3mM-$MgCl_2$, 1mM-$MnCl_2$, 0.5mM-$CaCl_2$, 10μM-$ZnCl_2$, 10mM-glucose and 1mM-spermine. Tissue was collected by centrifugation at 1000*g* for 5min (at $r_{av.}$ 6.9cm) at 4°C and resuspended in 10ml of incubation medium containing 1mCi of carrier-free [^{32}P]P_i (Amersham, Arlington Heights, IL, U.S.A.). The tissue was incubated at 37°C for 2h with constant shaking and then placed on ice. Subsequent steps were performed at 0–4°C. Tissue was collected by centrifugation and washed by resuspension in 10ml of 10mM-Tris/HCl buffer, pH7.5, containing 150mM-NaCl and centrifuging as above. This washing step was repeated two more times. The tissue was resuspended in 3ml of 20mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA, 150mM-NaCl and 1mM-phenylmethanesulphonyl fluoride and homogenized in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 100000*g* for 1h (at $r_{av.}$ 7.8cm) at 4°C, and the supernatant was retained. The supernatant was placed on a DEAE-Sephacel column (1cm × 5cm) equilibrated with homogenization buffer. The column was washed with 30ml of 20mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA and 200mM-NaCl, and the wash was discarded. The column was then eluted with 20ml of 20mM-Tris/HCl buffer, pH7.5, 1mM-EDTA and 300mM-NaCl. The eluate was dialysed against water and then freeze-dried. This fraction is enriched in spermine-binding protein (Liang *et al.*, 1978). The freeze-dried sample was dissolved in 1ml of 20mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA, and spermine-binding protein was immunoprecipitated from 100μl of sample by addition of 100μg of affinity-purified antibodies to spermine-binding protein (Hiipakka *et al.*, 1984). After incubation of the mixture for 5h at 4°C, 300μl of Pansorbin (a 10% suspension of formalin-fixed heat-treated *Staphylococcus aureus*; Calbiochem–Behring, San Diego, CA, U.S.A.) in 50mM-Tris/HCl buffer, pH7.5, containing 5mM-EDTA, 150mM-NaCl, 0.5% (w/v) Triton X-100 and 0.2% (w/v) sodium dodecyl sulphate (buffer A) was added. After incubation for 1h at 4°C, the samples were centrifuged at 10000*g* (at $r_{av.}$ 6.9cm) for 2min at 4°C. The pellet was washed by resuspension in 1ml of buffer A and centrifuged at 10000*g* (at $r_{av.}$ 6.9cm) for 2min at 4°C. The wash was repeated four more times. The pellet was resuspended in 100μl of 62.5mM-Tris/HCl buffer, pH6.8, containing 1% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol and 0.01% Bromophenol Blue and heated at 100°C for 5min. After cooling to room temperature the samples were centrifuged at 10000*g* (at $r_{av.}$ 6.9cm) for 10min at 4°C. Portions were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 12.5% (w/v) polyacrylamide gels (Laemmli, 1970). Radioactivity was located in dried gels by autoradiography at −70°C with intensifying screens. Efficiency of immunoprecipitation of spermine-binding protein was checked by recovery of ^{125}I-labelled spermine-binding protein (Hiipakka *et al.*, 1984) added to parallel incubations. Recovery was greater than 90%.

Other methods

Amino acid analysis of the ^{32}P-labelled spermine-binding protein was carried out by the procedure described by Hunter & Sefton (1980). ^{32}P-labelled spermine-binding protein was subjected to sodium dodecyl sulphate/urea/10%-polyacrylamide-gel electrophoresis by the procedure of Laemmli (1970). The gels were dried, and radioactivity incorporated was detected by autoradiography, with a Kodak No-Screen X-ray film. All other procedures were the same as described previously (Ahmed *et al.*, 1983*a*,*b*).

Results

Phosphorylation of spermine-binding protein in situ

Ventral prostatic tissue from normal and 48h-castrated rats was incubated with [^{32}P]P_i to label the various cellular phosphoproteins. Cytosol was then prepared, and spermine-binding protein was isolated by using specific antibodies against this protein. The gel-electrophoretic profile and the autoradiographic detection of the immunoprecipitated proteins are shown in Fig. 1. It is clear that spermine-binding protein was labelled with ^{32}P *in situ*, indicating that it is a phosphoprotein. The incorporation of ^{32}P *in situ* was markedly de-

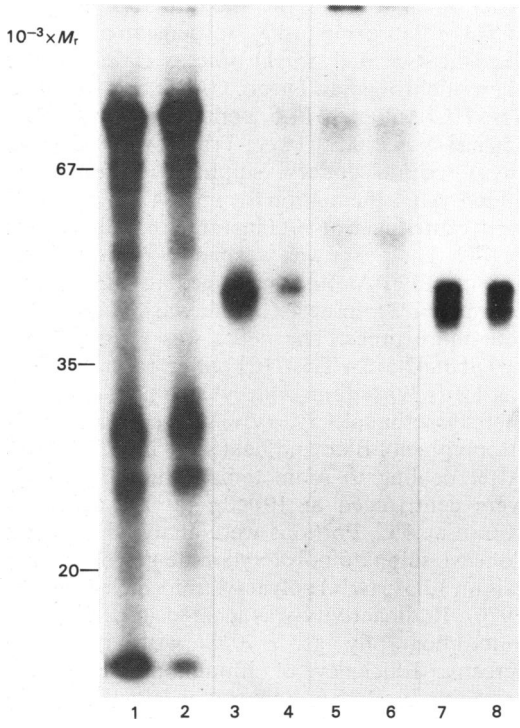

Fig. 1. *Immunoprecipitation of spermine-binding protein from rat ventral-prostate tissue incubated with* $[^{32}P]P_i$
Spermine-binding protein was partially purified from rat ventral-prostate cytosol prepared from tissue incubated *in vitro* with $[^{32}P]P_i$ (see the Materials and methods section). Samples were then immunoprecipitated with antibodies to spermine-binding protein, and the immunoprecipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and autoradiography. Lanes 1 and 2 are the ^{32}P-labelled samples before immunoprecipitation but treated to remove nucleic acids and phospholipids (Bitte & Kabat, 1974). Lanes 3 and 4 are the products of immunoprecipitation with antibodies to spermine-binding protein. Lanes 5 and 6 are the products of immunoprecipitation with control IgG (IgG isolated from rabbits before immunization with spermine-binding protein). Lanes 7 and 8 are the products of immunoprecipitation with antibodies to spermine-binding protein where ^{125}I-labelled spermine-binding protein was added to the samples before immunoprecipitation. Lanes 1, 3, 5 and 7 are the results from normal rats, and lanes 2, 4, 6 and 8 are the results from castrated (48h) rats. The numbers on the left side of the figure indicate the positions of ^{14}C-labelled M_r markers. Exposure times for autoradiography were as follows: lanes 1 and 2, 24h; lanes 3, 4, 5 and 6, 120h; lanes 7 and 8, 5h. The different exposure times were used for ^{125}I and for ^{32}P because of differences in the maximum value of kinetic energy ($E_{max.}$) characteristic of the nuclide in question or to account for the variations in the radioactivity of radiolabelled material in the gels.

creased in tissue from animals that had been castrated 48h previously. Experiments were therefore undertaken to determine the nature of the protein kinase reactions that may be involved in the phosphorylation of spermine-binding protein *in vivo*.

Effects of various effector-dependent protein kinases on the phosphorylation of spermine-binding protein

As shown in Table 1, the purified cyclic AMP-dependent protein kinase from bovine heart cytosol (active towards histone) did not catalyse the phosphorylation of purified spermine-binding protein *in vitro*. This result was also confirmed by utilizing partially purified rat ventral-prostate cytosolic cyclic AMP-dependent protein kinase (result not shown). Addition of 3-isobutyl-1-methylxanthine (at a concentration of 0.5mM), an inhibitor of phosphodiesterase, did not influence these reactions. The result in Table 1 also indicated that spermine-binding protein had no significant influence on the endogenous phosphorylation of this kinase. Both of these enzyme preparations avidly phosphorylated histone and were stimulated by cyclic AMP by 5–7-fold in that reaction. Purified phospholipid-sensitive Ca^{2+}-dependent protein kinase and Ca^{2+}+calmodulin-dependent protein kinase were also unable to phosphorylate spermine-binding protein in the presence or in the absence of spermine (results not included; see also the legend to Fig. 4).

General characteristics of phosphorylation of spermine-binding protein by chromatin-associated cyclic AMP-independent protein kinase reaction in vitro

In view of the above-described results, it was decided to examine phosphorylation of spermine-binding protein in a number of prostatic cyclic AMP-independent protein kinase reactions *in vitro*. As a first step, chromatin and non-histone protein were employed as the source of this kind of kinase activity, which has been shown to be androgen-sensitive in the prostate (Ahmed *et al.*, 1979, 1981). The results shown in Figs. 2 and 3 describe the general characteristics of chromatin-associated protein kinase-mediated ^{32}P incorporation into spermine-binding protein. As shown in Fig. 2(*a*), the optimal requirement for $MgCl_2$ in the reaction was near 1.0mM in the absence of spermine. There was no significant change in the rate of incorporation when the $MgCl_2$ concentration was varied from 1mM to 3mM. However, when 1.0mM-spermine was present, the activity declined sharply at 2.0mM- and 3.0mM-$MgCl_2$. There was no indication of a substitution of spermine for $MgCl_2$ in these reactions. Univalent salt such as NaCl added up to a concentration of 200mM did not influence the phosphorylation of spermine-

Table 1. *Test of activity of bovine heart cytosolic cyclic AMP-dependent protein kinase with spermine-binding protein as substrate*

Where indicated, spermine was present at a concentration of 1 mM, and cyclic AMP at 2 μM. All other experimental details were the same as given in the Materials and methods section. The activity of this enzyme towards mixed histones as substrate in the presence of 2 μM-cyclic AMP was 7920 nmol of ^{32}P/h per mg of enzyme.

System	Protein kinase activity (nmol of ^{32}P incorporated/h per mg of enzyme protein)
Kinase alone	5.7
Kinase + cyclic AMP	7.2
Kinase + cyclic AMP + spermine	4.2
Kinase + cyclic AMP + spermine-binding protein	7.3
Kinase + spermine + spermine-binding protein	3.3
Kinase + cyclic AMP + spermine + spermine-binding protein	3.4

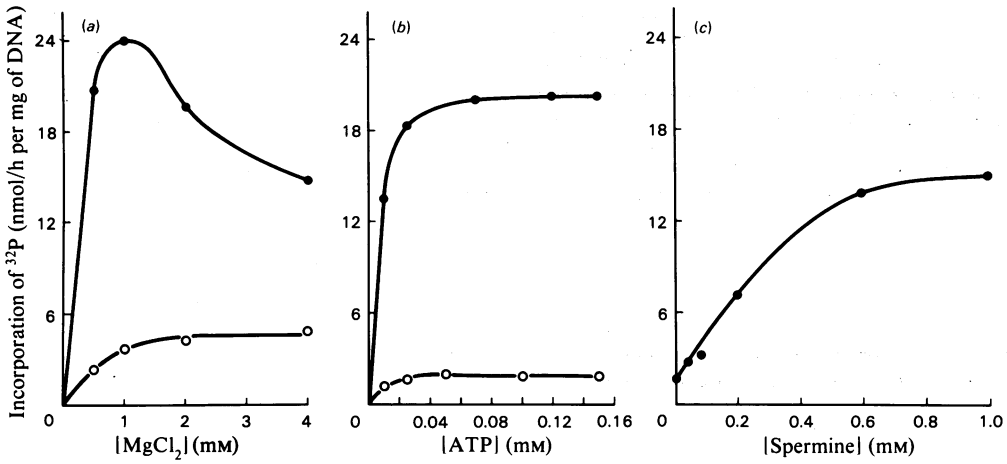

Fig. 2. *Phosphorylation of spermine-binding protein by rat ventral-prostate chromatin-associated protein kinase activity: (a) effect of $MgCl_2$ concentration, (b) effect of ATP concentration, and (c) effect of spermine concentration*

The experimental details were the same as described in the Materials and methods section. ○, No spermine; ●, 1 mM-spermine.

binding protein; however, at a concentration of 300 mM it become somewhat inhibitory. Addition of 1.0 mM-dithiothreitol to the reaction had no effect on the phosphorylation of spermine-binding protein in the presence of spermine (results for the latter two observations are not given).

Fig. 2(*b*) shows the effect of varying the ATP concentration on the phosphorylation of spermine-binding protein. The maximum activity was observed when the ATP concentration was 30 μM or higher; the apparent K_m for ATP was 13 μM with a $V_{max.}$ of 32.7 nmol of ^{32}P incorporated/h per mg of chromatin DNA. The maximal stimulation of phosphorylation of spermine-binding protein in the presence of spermine was observed at a spermine concentration between 0.6 mM and 1.0 mM. The apparent K_a for spermine in this reaction was 0.11 mM. Under the various conditions employed by us the rates of reaction were linear for 20–30 min either in the absence or in the presence of 1.0 mM-spermine with 50 μg of spermine-binding protein/ml in the reaction mixture (Fig. 3*a*).

At a fixed reaction time of 15 min, varying the concentration of spermine-binding protein from 10 to 100 μg/ml in the reaction mixture did not show any evidence of deviation from linearity (Fig. 3*b*). An estimation of K_m value for spermine-binding protein with this enzyme system was not possible, as sufficiently high concentrations of this protein in the reaction were not feasible.

The phosphorylation of spermine-binding protein was optimal at pH around 7.0–7.4. It declined sharply at pH 7.65, but rose rapidly to another maximum at pH 7.87, followed by a marked decline at higher pH values (Fig. 3*c*). The pH–activity profile for the phosphorylation of spermine-binding protein is similar to that observed for

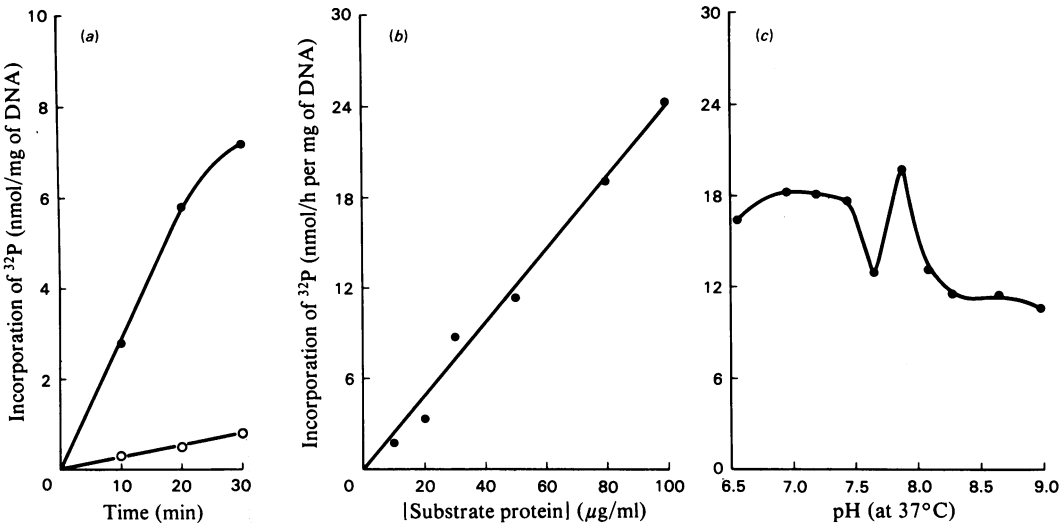

Fig. 3. *Phosphorylation of spermine-binding protein by rat ventral-prostate chromatin-associated protein kinase activity: (a) time course of phosphorylation, (b) effect of varying the concentration of spermine-binding protein, and (c) effect of pH on the phosphorylation of spermine-binding protein*

Tris/HCl buffers of pH values 6.55, 6.96, 7.17, 7.45, 7.65, 7.87, 8.07, 8.28, 8.65 and 8.97 (measured at 37°C) were added to a final concentration of 30mM. All other experimental conditions were the same as described in the Materials and methods section. ○, No spermine; ●, 1mM-spermine.

Table 2. *Phosphorylation of spermine-binding protein catalysed by protein kinase activity associated with chromatin and non-histone protein fraction*

Rat liver or ventral-prostate chromatin or non-histone protein fraction derived from the latter was used as the source of protein kinase activity. All other experimental details were the same as described in the Materials and methods section.

Protein kinase source	Incorporation of ^{32}P into spermine-binding protein (nmol/h per mg of enzyme protein*)	
	No spermine	+1mM-Spermine
Rat liver chromatin	0.42	5.4
Rat ventral-prostate chromatin	0.48	6.2
Rat ventral-prostate non-histone protein fraction	0.29	16.1

* Enzyme protein refers to protein in chromatin or non-histone protein fraction.

the phosphorylation of dephosphophosvitin mediated by prostatic chromatin (Ahmed & Wilson, 1975).

A comparison of chromatin preparations derived from prostate and liver showed that cyclic AMP-independent protein kinase activity from both these sources was equally effective in mediating spermine-stimulated phosphorylation of spermine-binding protein, so that the percentage stimulation evoked by spermine was similar for either of the preparations (Table 2). Calculations of recovery of protein kinase activity capable of phosphorylating spermine-binding protein revealed that the non-histone protein fraction (Ahmed *et al.*, 1978) contained all the chromatin-associated protein kinase active toward spermine-binding protein.

Phosphorylation of spermine-binding protein by purified cyclic AMP-independent protein kinases

As described in the Materials and methods section, two cyclic AMP-independent protein kinases purified from prostate or liver nuclei (termed N1 and N2) and two cyclic AMP-independent protein kinases purified from liver cytosol (termed C1 and C2) were tested for their ability to phosphorylate purified spermine-binding protein. As shown in Table 3, the phosphorylation was mediated to various degrees by all four of the cyclic AMP-independent protein kinases. How-

Table 3. *Phosphorylation of spermine-binding protein by various cyclic AMP-independent protein kinases*
The cytosolic and nuclear cyclic AMP-independent protein kinases were the same as described in the Materials and methods section. All protein kinase assays were carried out in the presence of 1 mM-spermine, which stimulated the reactions by 2–6-fold. The amounts of spermine-binding protein and casein in the reaction mixture were 50 μg/ml and 5 mg/ml respectively. All other experimental details were the same as described in the Materials and methods section.

Protein kinase	Protein kinase activity (nmol of ^{32}P incorporated/h per mg of enzyme protein)	
	Towards spermine-binding protein	Towards casein
Rat liver cytosolic cyclic AMP-independent protein kinase (C1)	10940	23750
Rat liver cytosolic cyclic AMP-independent protein kinase (C2)	52600	67500
Rat liver nuclear cyclic AMP-independent protein kinase (N1)	28800	37000
Rat liver nuclear cyclic AMP-independent protein kinase (N2)	76000	74000
Rat ventral-prostate cyclic AMP-independent protein kinase (N2)	90000	95000

Table 4. *Comparison of the effects of various polyamines and related compounds on chromatin-associated protein kinase activity towards spermine-binding protein as substrate*
Rat ventral-prostate chromatin was used as the source of protein kinase activity. All other experimental details were the same as given in the Materials and methods section.

Addition	Protein kinase activity (nmol of ^{32}P incorporated/h per mg of chromatin protein)	Percentage change
None	1.0	—
1.0 mM-Spermine	6.8	+580
2.0 mM-Spermidine	3.7	+270
2.0 mM-N^8-Acetylspermidine	1.2	+20
4.0 mM-1,6-Hexanediamine	1.9	+90
4.0 mM-Cadaverine	1.6	+60
4.0 mM-Putrescine	1.8	+80
0.5 mM-Methylglyoxal bis(guanylhydrazone)	0.1	−90

ever, as described above, the apparent K_m for spermine-binding protein for the nuclear-associated enzymes (i.e. with chromatin as the source of enzyme activity), due presumably to the presence of N1 and N2 protein kinases (Goueli *et al.*, 1980), appeared to be somewhat high. Therefore, in order to examine further the question of the physiological relevance of the activity of various protein kinases *in vitro*, experiments were undertaken to determine the kinetic properties of the purified cytosolic cyclic AMP-independent protein kinase C2. The results demonstrated that the apparent K_m for spermine-binding protein towards this enzyme was 0.75 μM in the presence or in the absence of 1 mM-spermine. The $V_{max.}$ was reached at a concentration of spermine-binding protein near 7.1 μM (i.e. 250 μg of protein/ml), and was enhanced in the presence of spermine. These results indicate that protein kinase C2 may be at least one of the protein kinases involved in the physiological phosphorylation of spermine-binding protein.

Effect of polyamines and other analogues on the phosphorylation of spermine-binding protein

A comparison of the relative effect of spermine and other polyamines as well as a number of aliphatic amines on the phosphorylation of spermine-binding protein is given in Table 4. Spermine was the most effective polyamine in stimulating the phosphorylation of spermine-binding protein. In the various experiments described above, the stimulation of phosphorylation of spermine-binding protein in the presence of spermine varied from 4- to 20-fold. Spermidine, cadaverine, putrescine and 1,6-hexanediamine were significantly less effective than spermine. Acetylation of spermidine at the N-8 position resulted in further marked decrease in its ability to stimulate phosphorylation of spermine-binding protein. Methylglyoxal bis-(guanylhydrazone) {1,1′-[(methylethanediylidene)-dinitrilo]diguanidine} was inhibitory in this protein kinase reaction.

Effect of castration on the phosphorylation of spermine-binding protein mediated by prostatic chromatin-associated protein kinase reaction

Prostatic chromatin prepared from rats castrated 48h previously demonstrated a marked decline in its ability to phosphorylate spermine-binding protein (Table 5). This is in accord with our previous demonstration of the rapid loss of prostatic chromatin-associated protein kinases active towards phosvitin and non-histone proteins after androgen deprivation (Ahmed & Ishida, 1981; Ahmed & Wilson, 1975, 1978; Goueli *et al.*, 1980).

Effect of spermine-binding protein on the phosphorylation of other proteins

We have previously shown that phosphorylation of chromatin-associated proteins catalysed by endogenous cyclic AMP-independent protein kinase(s) is stimulated by polyamines (Ahmed *et al.*, 1978, 1979, 1981, 1983*a*,*b*). The results in Figs. 4(*a*) and 4(*b*) show that spermine-binding protein was without any influence on the phosphorylation of chromatin-associated proteins. Thus, whereas spermine-binding protein was a very good substrate for cyclic AMP-independent protein kinase reactions, it was without any effect on the phosphorylation of other phosphoproteins, i.e. it did not appear to enhance the stimulatory effects of polyamines in this reaction, and, further, it was not phosphorylated by effector-mediated protein kinases, as mentioned above.

Phospho amino acid analysis of ^{32}P-labelled spermine-binding protein

Spermine-binding protein was labelled with ^{32}P in the presence of spermine (with the aid of chromatin-associated protein kinase activity), and subjected to acid hydrolysis for analysis of phospho amino acids. The only ^{32}P-labelled amino acid detected was phosphoserine. There was no indication of ^{32}P incorporation into tyrosine or threonine.

Table 5. *Effect of castration on prostate chromatin-associated protein kinase activity towards spermine-binding protein as substrate*

Chromatin preparations were from nuclei of ventral-prostatic tissue from normal or castrated rats as shown. All other experimental details were as given in the Materials and methods section. Protein kinase activities are given as means ± S.D. The $t_{0.01}$ value in the absence of spermine was calculated to be 3.35 (Table $t_{0.01} = 2.9$), whereas in the presence of spermine $t_{0.01}$ was calculated to be 4.42 (Table $t_{0.01} = 3.06$) for protein kinase activity of chromatin from normal and castrated rats.

Source of prostate chromatin	Protein kinase activity (nmol of ^{32}P incorporated/h per mg of chromatin protein)	
	No spermine	+1 mM-Spermine
Normal rats	0.67 ± 0.22	7.6 ± 1.7
48h-castrated rats	0.25 ± 0.04	3.5 ± 1.0

Discussion

The results described above clearly indicate that spermine-binding protein exists as a phosphoprotein *in vivo*, and that its phosphorylation is sensitive to the androgenic status of the animal. *In vitro*, the phosphorylation of this protein is mediated primarily by cyclic AMP-independent (i.e. with no apparent effector requirement) protein kinases (both the nuclear-associated and cytosolic), but not by the cyclic AMP-dependent protein kinase or the Ca^{2+} calmodulin-dependent protein kinase or the Ca^{2+} phospholipid-dependent protein kinase. On the basis of the available data concerning the affinity of various protein kinases for spermine-binding protein as substrate it would appear that the protein kinase C2 (from the cytosol) is physiologically relevant in phosphorylating this protein in the intact cell. The apparent K_m for spermine-binding protein towards this enzyme was 0.75 μM, which is at least an order of magnitude lower than the estimated concentration of this protein in the cell (i.e. about 10 μM, assuming a uniform intracellular distribution). At present it is not clear whether or not the other cyclic AMP-independent protein kinases active towards spermine-binding protein *in vitro* play any physiological role in its phosphorylation *in vivo*. Further work is needed on the kinetics of these enzymes for the phosphorylation of this substrate. However, it may be noted that, if the spermine-binding protein were compartmentalized in the cell (as is often the case with biological molecules), the concentration at a particular site would be substantially higher, making it feasible for other protein kinases also to phosphorylate the protein. It is also not clear at the present whether the sites of phosphorylation are the same or different for each of the four cyclic AMP-independent protein kinases. Some of the general characteristics of the chromatin-associated protein kinase-mediated phosphorylation of spermine-binding protein were similar to those observed for phosvitin as substrate, e.g. pH–activity profile and kinetics of ATP requirement (Ahmed & Wilson, 1975; Goueli & Ahmed, 1983). However, some marked differences were also noted, such as activation of the reaction at lower concentrations of $MgCl_2$ and a lack of requirement for salt in the reaction with spermine-binding protein as the substrate. These observations reflect the variations in the kinase reaction that relate to

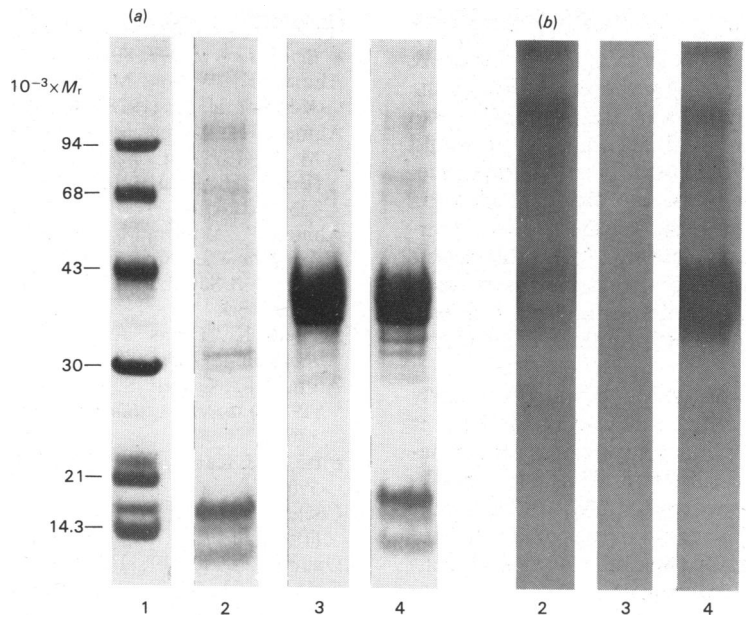

Fig. 4. *Effect of spermine-binding protein on the phosphorylation of chromatin-associated proteins*
A 8 μg portion of chromatin protein, or 30 μg of spermine-binding protein, or the two together (as indicated below), were incubated for 15 min at 37°C in a reaction medium containing 30 mM-Tris/HCl buffer, pH 7.45 (at 37°C), 5 mM-$MgCl_2$, 1 mM-dithiothreitol, 1 mM-spermine and 0.01 mM-[γ-^{32}P]ATP (about 1.0 μCi/nmol). At the end of the reaction the samples were prepared for gel electrophoresis and autoradiography as indicated in the Materials and methods section. Similar experiments were performed with other protein kinases such as cyclic AMP-dependent protein kinase, Ca^{2+} + calmodulin-dependent-protein kinase and Ca^{2+} + phospholipid-dependent protein kinase to phosphorylate spermine-binding protein. Since no radioactivity was detected in spermine-binding protein in these reactions, the autoradiographic data are not included. (*a*) Coomassie-Blue-stained gels: lane 1, standard M_r marker proteins; lane 2, 8 μg of chromatin protein; lane 3, 30 μg of spermine-binding protein; lane 4, 8 μg of chromatin protein and 30 μg of spermine-binding protein. M_r values are indicated at the left. (*b*) Autoradiogram of duplicate gels shown in (*a*).

the nature of the protein substrate. The general data on the kinetics of phosphorylation of spermine-binding protein by chromatin were in accord with the possibility that the chromatin-associated protein kinase activity was due to the presence of more than one enzyme active towards acidic-protein substrates (Ahmed & Wilson, 1975; Goueli *et al.*, 1980; Goueli & Ahmed, 1983), as confirmed from experiments with the purified nuclear cyclic AMP-independent protein kinases (N1 and N2), which are most probably responsible for the chromatin-associated activity.

Although spermine-binding protein is present primarily in the prostatic tissue as compared with other tissues (Hiipakka *et al.*, 1984), its phosphorylation *in vitro* was mediated equally effectively by the prostatic or liver cyclic AMP-independent protein kinase activity. In each case the phosphorylation of spermine-binding protein was markedly stimulated in the presence of spermine. The effect of spermine was not specific, since spermidine could substitute for it. Free multivalent cationic charge appeared to be necessary for the stimulatory effect on phosphorylation of spermine-binding protein, since acetylation of spermidine (resulting in a decrease in cationic charges) abolished the stimulation attributed to spermidine. Indeed, phosphorylation of spermine-binding protein was stimulated by the inert cation cobalt(III) hexaammine as effectively as by spermine (K. Ahmed, S. A. Goueli & H. G. Williams-Ashman, unpublished work), as observed for other protein kinase reactions (Ahmed *et al.*, 1983*b*). Thus, taken together, the results indicate that stimulation of the phosphorylation of spermine-binding protein elicited by polyamines was primarily due to their charge properties and probably a consequence of their interaction with spermine-binding protein so as to produce a favourable conformation of this substrate for the phosphorylation reaction, as proposed previously for the mechanism of polyamine stimulation of certain protein kinase reactions (Ahmed *et al.*, 1978, 1983*a*,*b*).

The activity of the protein kinases that phosphorylate spermine-binding protein (especially protein kinase C2 and N2) decreases rapidly (in

the prostate) on androgen deprivation in rats (Goueli *et al.*, 1980; Tse *et al.*, 1984; Goueli & Ahmed, 1984). Since, under these conditions, the spermine-binding activity of the protein is also lost, along with its rapid degradation, it would appear that it is the dephospho form of spermine-binding protein that is rapidly degraded on androgen withdrawal in the animals. These observations indicate that the cyclic AMP-independent protein kinase(s) involved in the phosphorylation of spermine-binding protein may play a role in the stability of this protein *in vivo* (via phosphorylation).

Finally, it is noteworthy that, despite their fairly wide substrate activity, the effector-dependent protein kinases were inactive towards spermine-binding protein as a substrate. Thus the remarkable specificity of the cyclic AMP-independent protein kinases in this regard may provide a useful tool in investigations of the cyclic AMP-independent protein kinase reactions.

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