Biochem. J. (1985) **227**, 271–276
Printed in Great Britain

Phosphorylation of high mobility group 1 protein by phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig testis

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(*Received 8 October 1984/Accepted 30 November 1984*)

Phospholipid-sensitive Ca^{2+}-dependent protein kinase was partially purified from total particulate fraction of pig testis. The enzyme phosphorylated high mobility group 1 protein (HMG 1), one of the major chromatin-associated non-histone proteins. Other HMG proteins (HMG 2, 14 and 17) were not phosphorylated by the enzyme. Exhaustive phosphorylation of HMG 1 revealed that 1 mol of phosphate was incorporated/mol of HMG 1. The apparent K_m value for HMG 1 was 3.66 μM. 1,3-Diolein stimulated the phosphorylation at 10 μM-Ca^{2+} in the presence of phosphatidylserine. The phosphorylation of HMG 1 was inhibited by adriamycin, an inhibitor of spermatogenesis.

Phospholipid-sensitive Ca^{2+}-dependent protein kinase (C-kinase) was originally described by Takai *et al.* (1979). The enzyme is selectively activated by the presence of both Ca^{2+} and phospholipid. Diacylglycerol increases the affinity of the enzyme for Ca^{2+} as well as for phospholipid, thereby rendering the enzyme active at physiological Ca^{2+} concentrations (Kishimoto *et al.*, 1980). The enzyme is reported to be present in a wide variety of tissues of different phyla in the animal kingdom (Kuo *et al.*, 1980; Kikkawa *et al.*, 1982). Davis & Clark (1983) have recently reported the presence of the enzyme in corpus luteum and have suggested that the enzyme is involved in the regulation of the functions in the gonadal tissues. Several proteins have been identified as substrates for the enzyme. In particular, identification of eukaryotic initiation factor 2 (Schatzman *et al.*, 1983*a*) and ribosomal protein S6 (Le Peuch *et al.*, 1983) led to the study of functional role of the enzyme in the regulation of protein synthesis.

Recently, we reported that pig testis contains C-kinase and its substrates in total particulate fraction, particularly in nuclei of germ cells (Kimura *et al.*, 1984). At least four proteins (of 83, 33, 26 and 21 kDa) were detected in the nuclei as substrates for the enzyme. Of these substrates, the 26 kDa protein had a similar mobility in SDS/polyacrylamide-gel electrophoresis to high mobility group 1 protein (HMG1), which is one of the major chromatin-associated nonhistone proteins. We therefore examined the ability of C-kinase to phosphorylate HMG 1. Here, we report that C-kinase from pig testis phosphorylates HMG-1 prepared from pig testis and that the phosphorylation is inhibited by adriamycin, an inhibitor of spermatogenesis.

Abbreviations used: HMG 1, high mobility group 1 protein; SDS, sodium dodecyl sulphate.

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Experimental

Materials

Phosphatidylserine (bovine brain), 1,3-diolein and lysine-rich histone (histone H1, type III-S) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Affigel 10 was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Phenylmethanesulphonyl fluoride and marker proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, α-chymotrypsinogen, myoglobin and cytochrome *c*) for M_r determination on SDS/polyacrylamide gels were from Boehringer Mannheim Biochemicals (Tokyo, Japan). Adriamycin (doxorubicin hydrochloride) and histone H3 were generously given by Dr. A. Miyagawa, Kyowa Hakko Kogyo Co. and Dr. O. Inanami, Department of Veterinary Radiation Biology, Hokkaido University, respectively. Carrier-free ^{32}P was obtained from Japan Atomic Energy Research Institute (Tokaimura, Japan).

Methods

C-kinase from total particulate fraction of pig testis was solubilized by 0.1% Triton X-100 and 1.2mM-EGTA, and the solubilized enzyme was chromatographed on DEAE-cellulose and Sephacryl S-200 as described by Kimura *et al.* (1984). The enzyme was further purified by phosphatidylserine–Affigel 10 affinity chromatography according to the method of Schatzman *et al.* (1983*b*), with a slight modification. A column (15cm × 8cm) of phosphatidylserine–Affigel 10 was washed with 2M-NaCl, instead of 1M-NaCl. Washing with 2M-NaCl resulted in the co-elution of the enzyme and a 26kDa protein (presumably HMG 1). After washing with 2M-NaCl followed by washing with 3mM-EGTA, the enzyme, almost completely free of the 26kDa protein, was eluted by a combination of 2mM-EDTA, 10mM-EGTA and 2M-NaCl. The active fractions were dialysed and concentrated by DEAE-cellulose chromatography (Wise *et al.*, 1982). By following the procedures described above, C-kinase from testis homogenate was purified by about 500-fold. Specific activity of the purified enzyme was 0.3–0.4μmol/min per mg of protein, when lysine-rich histone was used as substrate. The enzyme was dependent on both Ca^{2+} and phosphatidylserine, and was stimulated about 20-fold by the two cofactors. Purity was 60–70% as judged by SDS/polyacrylamide-gel electrophoresis and its M_r was estimated to be 56000 by electrophoresis. Although we have not yet characterized the particulate enzyme, its properties seem to be similar to those of the cytosolic enzyme (Wise *et al.*, 1982; Kikkawa *et al.*, 1982; Schatzman *et al.*, 1983*b*).

HMG proteins were prepared from pig testes according to the method of Goodwin *et al.* (1975). HMG 1 was separated from the other HMG proteins by chromatography on CM-Sephadex C-25 (Sanders, 1977).

C-kinase activity was assayed as previously described (Katoh *et al.*, 1981*a*; Kimura *et al.*, 1984). Briefly, standard reaction mixture in a final vol. of 0.2ml contained: 25mM-Tris/HCl (pH7.5), 10mM-$MgCl_2$, 0.25mM-EGTA, 40μg of substrate (lysine-rich histone or HMG proteins), 50μM-[γ-^{32}P]ATP containing (1–5) × 10^5c.p.m. and the appropriate amount of enzyme protein. When present, $CaCl_2$ was 0.5mM and phosphatidylserine was 5μg. The reaction was started by the addition of ATP and carried out for 5–10min at 30°C, and the phosphorylated protein was precipitated with 5% (w/v) trichloroacetic acid containing 0.25% tungstate, with bovine serum albumin (0.65%) as a carrier protein. Phosphorylation of HMG proteins, SDS/polyacrylamide-gel electrophoresis, and subsequent autoradiography were carried out as described previously (Katoh *et al.*, 1981*b*; Kimura *et al.*, 1984).

Phosphatidylserine–Affigel 10 was prepared according to the method of Schatzman *et al.* (1983*b*). Amino acids were measured with a Hitachi-803 amino acid analyser. Samples were hydrolysed for 24h at 110°C. The endogenous phosphate content in HMG 1 was determined by the method of Fiske & SubbaRow (1925), after ashing the protein by the method of Ames (1966). RNA and DNA in HMG 1 were first separated according to the method of Munro & Fleck (1966). RNA and DNA contents were measured by the methods of Lin & Schjeide (1969) and Dische (1930), respectively. Protein was determined by the method of Bradford (1976) with ovalbumin as the standard protein. [γ-^{32}P]ATP was prepared as described by Post & Sen (1967). Ca–EGTA buffer was prepared as described by Bartfai (1979) using a dissociation constant (K_d) value of $4.4 \times 10^6 M^{-1}$.

Results

Fig. 1(*a*) shows SDS/polyacrylamide-gel electrophoresis of HMG proteins isolated from pig testis. Four visible protein bands corresponding to HMG 1, 2, 14 and 17 were observed. The likely identity of HMG proteins was established by their electrophoretic migration between histones H1 and H3 in SDS gels (Bhorjee, 1981). Also, their M_r distribution in SDS gels was in good agreement with that determined by Bustin *et al.* (1978).

Experiments with the HMG proteins as substrates revealed that C-kinase phosphorylated several proteins, including those of 33, 26, 21 and 17kDa (Fig. 1*b*). There was no detectable endogenous phosphorylation of the enzyme in the presence of Ca^{2+} and phosphatidylserine without the added HMG proteins (lane 5). Although only four HMG proteins were detected by Coomassie Brilliant Blue staining, proteins other than HMG proteins also appeared as substrates for the enzyme. Of these substrates, proteins of 33, 26 and 21kDa appeared to be the same as the substrates detected in testis nuclei (Kimura *et al.*, 1984). The 33 and 17kDa proteins had similar mobilities to those of histones H1 and H3, respectively. The 21kDa protein did not correspond to any bands of HMG proteins or histones. The 26kDa protein had the same mobility as that of HMG 1, suggesting that HMG 1 was substrate for the enzyme. In contrast, phosphorylation bands corresponding to HMG 2, 14 and 17 were not observed.

Phosphorylation of HMG 1 by C-kinase was confirmed by experiments with the isolated HMG 1 as substrate (Fig. 2). HMG 1 was phosphorylated by the enzyme in the presence of both Ca^{2+} and phosphatidylserine, but HMG 2 was not. Identifi-

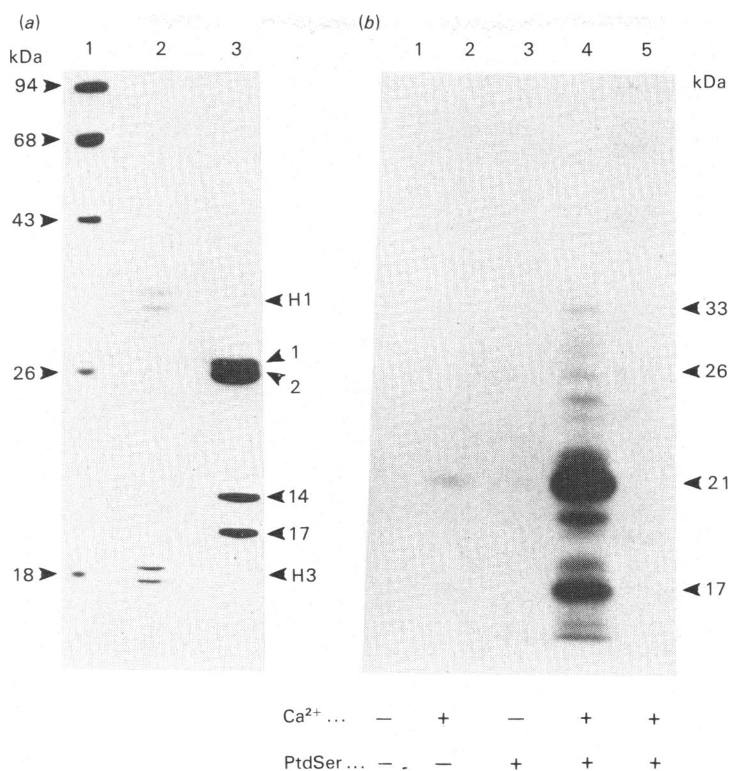

Fig. 1. *Phosphorylation of HMG proteins by C-kinase*
HMG proteins (unfractionated, 180 μg) were incubated with C-kinase (0.1 μg) in a total volume of 0.2 ml in the absence or presence of $CaCl_2$ (0.5 mM) or phosphatidylserine (PtdSer, 5 μg). The amount of HMG proteins applied was 30 μg/lane. The gel used was 15%. (*a*) Proteins stained with Coomassie Brilliant Blue: 1, marker proteins; 2, histones H1 and H3; 3, HMG proteins. (*b*) Autoradiogram. In lane 5, HMG proteins were not included so as to show there was no detectable endogenous phosphorylation of C-kinase in the presence of Ca^{2+} and phosphatidylserine.

cation of HMG 1 and 2 was established by amino acid analysis of the proteins. The compositions of HMG 1 and 2 from pig testis were similar to those from rooster testis (Chiva & Mezquita, 1983) and from calf thymus (Goodwin *et al.*, 1975). The phosphorylation of HMG 1 was not affected by the addition of HMG 2 (results not shown), which was closely associated with HMG 1 in nucleosomes (Shooter *et al.*, 1974; Javaherian *et al.*, 1978). The addition of 1,3-diolein stimulated the phosphorylation of HMG 1 at 10 μM-Ca^{2+} (Table 1). At higher Ca^{2+} concentration (250 μM), 1,3-diolein had little effect of stimulating the phosphorylation of HMG 1.

The endogenous phosphate content of HMG 1 was determined to be 0.313 mol/mol of HMG 1. The contents of RNA and DNA in HMG 1 were negligible (0.00052 mol of nucleotide/mol of HMG 1 for RNA and 0.0069 mol/mol for DNA, assuming that the mean nucleotide M_r of RNA and DNA were 339 and 327, respectively), indicating that the phosphate content in HMG 1 was derived from

Table 1. *Effect of 1,3-diolein on the phosphorylation of HMG 1 by C-kinase at different Ca^{2+} concentrations*
HMG 1 (40 μg) was incubated with C-kinase (0.3 μg) in a total vol. of 0.2 ml in the absence or presence of $CaCl_2$, phosphatidylserine (PtdSer, 5 μg) or 1,3-diolein (1 μg). Ca–EGTA buffer was used to make 10 μM-Ca^{2+} (Bartfai, 1979). Free Ca^{2+} concentration at 0.5 mM-$CaCl_2$ and 0.25 mM-EGTA was calculated to be 250 μM. EGTA (0.25 mM) was included in all assays except for the assays at 10 μM-Ca^{2+}, as described in the Experimental section.

Addition	Protein kinase activity (pmol/min)
PtdSer	1.1
Diolein	4.5
PtdSer + diolein	4.4
Ca^{2+} (10 μM) + PtdSer	6.8
Ca^{2+} (10 μM) + diolein	5.2
Ca^{2+} (10 μM) + PtdSer + diolein	12.1
Ca^{2+} (250 μM) + PtdSer	14.5
Ca^{2+} (250 μM) + diolein	5.2
Ca^{2+} (250 μM) + PtdSer + diolein	13.5

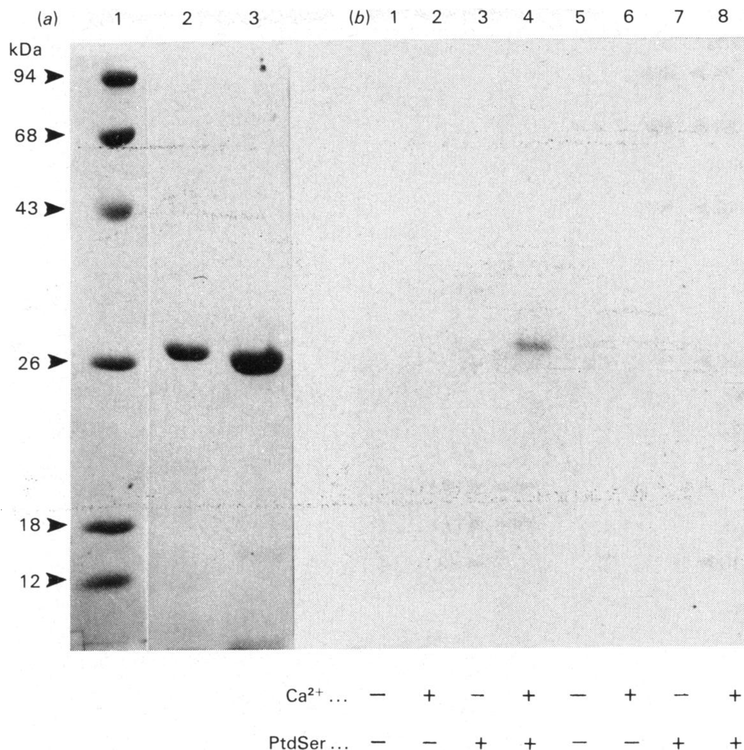

Fig. 2. *Phosphorylation of HMG 1 by C-kinase*

Experimental conditions were the same as in Fig. 1, except that concentration of HMG 1 in the reaction mixture was 60 μg/0.2 ml and the amount of HMG 1 applied was 10 μg/lane. (*a*) Proteins stained with Coomassie Brilliant Blue: 1, marker proteins; 2, HMG 1; 3, HMG 2. (*b*) Autoradiogram: 1–4, HMG 1; 5–8, HMG 2.

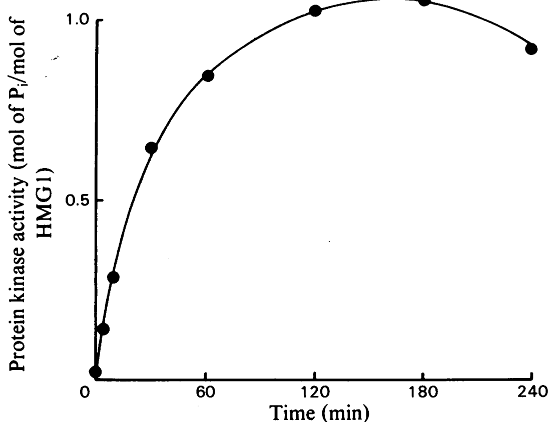

Fig. 3. *Stoichiometry of phosphorylation of HMG 1 by C-kinase*

HMG 1 (18.7 μg or 0.719 nmol) was incubated with C-kinase (0.6 μg) in a total volume of 0.2 ml in the presence of phosphatidylserine (5 μg) and $CaCl_2$ (0.5 mM). The concentration of ATP used was 100 μM, instead of 50 μM, in order to maximize the phosphorylation of the protein (Katoh *et al.*, 1983). The data presented were corrected for the blank values seen in the absence of Ca^{2+}. The M_r of HMG 1 used for calculating stoichiometry was 26000.

HMG 1 binding phosphate, but not from nucleotide contaminants in HMG 1. C-kinase was able to incorporate about 1 mol of phosphate/mol of HMG 1 (Fig. 3). The apparent K_m value for the phosphorylation of HMG 1 was determined to be 3.66 μM. Although we have not yet completely purified the enzyme, $V_{max.}$ was calculated to be 0.085 μmol/min per mg of protein. This value was 10–15% of that obtained using lysine-rich histone as substrate.

Phosphorylation of HMG 1 by the enzyme was inhibited by adriamycin. Adriamycin inhibited in a dose-dependent way Ca^{2+}-stimulated activity of the enzyme, but did not inhibit the basal activity. The IC_{50} value (concentration causing 50% inhibition) was 40 μM, and the value was similar to those obtained with histone H1 and cardiac endogenous substrates (Katoh *et al.*, 1981*a*).

Discussion

In the present studies, we have shown that HMG 1 prepared from pig testis incorporated a stoichiometric amount of phosphate when incubated with C-kinase from total particulate frac-

tion of pig testis. The apparent K_m value (3.66 μM) is comparable with K_m values reported for most known effective substrates of C-kinase, i.e., troponin I (3.4 μM; Katoh *et al.*, 1983), ribosomal protein S6 (0.8 μM; Le Peuch *et al.*, 1983) and retinoid-binding proteins (5.1–6.2 μM; Cope *et al.*, 1984). Although we have not yet completely purified the testis enzyme, its $V_{max.}$ (0.085 μmol/min per mg) is also comparable with the reported values of the respective substrates. The 26 kDa protein, presumably HMG 1, appears to be tightly bound to C-kinase during the purification procedure of the enzyme (see the Experimental section). By phosphatidylserine–Affigel 10 affinity chromatography, the 26 kDa protein could be removed by washing with 2 M-NaCl, resulting in significant loss of the enzyme. By washing with 1 M-NaCl (the procedure originally reported by Schatzman *et al.*, 1983*b*), the 26 kDa protein could not be removed completely, although the washing did not result in the loss of the enzyme. Thus, HMG 1 has high affinity for C-kinase and serves as a good endogenous substrate for the enzyme. Other than HMG 1, C-kinase also phosphorylated several proteins in the HMG protein fraction (Fig. 1). In particular, the 21 kDa protein was the most predominant substrate for the enzyme in the fraction, at least under the conditions used in this experiment. It remains to be clarified whether the 21 kDa protein is an unidentified non-histone protein or a breakdown product from HMG protein or histone. The relatively weak phosphorylation of HMG 1 in the fraction, as compared with the other phosphoproteins, may be explained by the presence of histone contaminants, because histones interact with HMG 1 (Shooter *et al.*, 1974).

HMG proteins consist of four main proteins, HMG 1, 2, 14 and 17. Among these, HMG 14 and 17 have been reported to be phosphorylated by several protein kinases, including cyclic nucleotide-dependent protein kinases (Walton *et al.*, 1982) and casein kinase II (Inoue *et al.*, 1980). However, the enzyme responsible for the phosphorylation of HMG 1 has not yet been reported, in spite of the fact that HMG 1 is a phosphoprotein (Goodwin *et al.*, 1975). HMG 1 is thought to be correlated with RNA synthesis. HMG 1 interacts with DNA through ionic bonding between the basic amino acids of the proteins and the phosphate groups of the DNA (Shooter *et al.*, 1974). Several investigators have reported the functional roles of HMG 1 in nucleosomes. Javaherian *et al.* (1978) have shown that HMG 1 and 2 have higher affinity for single stranded DNA rather than for double stranded DNA, and are thereby involved in the unwinding activity of DNA. Shastri *et al.* (1982) and Cockerill & Goodwin (1983) have reported that HMG 1 and 2 inhibit the digestion of single stranded DNA by S-1 nuclease and have suggested that these proteins may function *in vivo* by protecting regions of altered DNA conformation. HMG 1 and 2, as discussed above, are similar in their structures and functions. It is worth noting that C-kinase selectively phosphorylates HMG 1, but not HMG 2. The phosphorylation by C-kinase may be useful in distinguishing the functional roles of HMG 1 and 2.

In testis, HMG 1 is thought to be involved in regulation of spermatogenesis. HMG 1 and 2 are present in germ cells, particularly in the spermatids, but absent from mature spermatozoa obtained from epididymis (Chiva & Mezquita, 1983). It is noteworthy that the change in HMG 1 content during spermatogenesis coincides with the distribution of C-kinase and its endogenous substrates in the testis. As we reported recently (Kimura *et al.*, 1984), C-kinase and its substrates, including the 26 kDa protein, were present in germ cells, but little in matured spermatozoa. Because of the importance of Ca^{2+} for the regulation of spermatogenesis (Feinberg *et al.*, 1983), it is conceivable that C-kinase is involved in the regulation of spermatogenesis through phosphorylation of HMG 1. The phosphorylation of HMG 1 was inhibited by adriamycin. Lu & Meistrich (1979) have reported that adriamycin is extremely cytotoxic to the testis. It remains to be clarified whether the inhibition by adriamycin is related to the cytotoxicity in spermatogenesis.

We thank Dr. C. Mukuria in our Department for his critical reading of the manuscript. We are also grateful to Dr. D. Endoh, Department of Veterinary Radiation Biology, Hokkaido University, for granting us permission to use the radioisotope facilities at his laboratory.

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