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Studies on a Ca^{2+}- and Cyclic Nucleotide-Independent H1 Histone Kinase Purified from Rabbit Skeletal Muscle

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In an attempt to elucidate the regulatory mechanism of microsomal function by protein phosphorylation, one of the major protein kinases obtained during the preparation of the microsomal fraction of rabbit skeletal muscle was partially purified and characterized. This enzyme was a protein serine/threonine kinase and showed similar, but not completely same properties as those of Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C), judging from its elution profile from an anion-exchange column, molecular mass, responses to protein kinase activators or inhibitors and the substrate specificity. These results suggest a possible implication of this Ca^{2+}- and cyclic nucleotide-independent H1 histone kinase in protein phosphorylation of microsomal protein(s), although the exact role and the mechanism of regulation of this enzyme are not clear at this time.

Key words: H1 histone kinase; microsome; protein kinase C; protein phosphorylation; skeletal muscle (rabbit)

It has been well established that protein serine/threonine kinases play important roles in various cellular functions such as cell growth and differentiation or various metabolic and cell structural regulations (Edelman et al., 1987). For each protein kinase, a specific or multiple substrate proteins have been assigned according to its manner of substrate recognition as well as the topological arrangement of substrate and enzyme (Edelman et al., 1987). Judging from the universality of cellular regulation by protein phosphorylation and dephosphorylation reactions, this type of reactions must be detected in subcellar fractions such as microsome and mitochondria.

Previous reports indicate that a few protein kinase activities were detected in microsomal fraction (Edelman et al., 1987). On the other hand, Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) has been shown to be implicated in various cellular phenomena reflecting multiple subspecies of this class of enzyme (Nishizuka, 1995). In order to elucidate the protein kinase involved in the protein phosphorylation in microsomal fraction, a protein kinase havs been isolated during the preparation of this organelle from rabbit skeletal muscle in this report. After partial purification and characterization, this enzyme has been shown to possess similar properties as those of the catalytic fragment of protein kinase C (Takai et al., 1977; Hashimoto and Yamamura 1989). These results suggest that the protein kinase C or its related enzyme may have some target in

Abbreviations: BSA, bovine serum albumin; cdc2, cell division cycle 2; cAMP, cyclic AMP; CKI, casein kinase I; CKII, casein kinase II; CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide; CNBr, cyanogen bromide; MBP, myelin basic protein; M_r, relative molecular mass; protein kinase A, cyclic AMP-dependent protein kinase; protein kinase C, Ca^{2+}-phospholipid-dependent protein kinase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin layer chromatography; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TPCK, *L*-1-tosylamido-2-phenylethyl chloromethyl ketone

microsome although the physiological significance of the existence of this Ca^{2+}- and cyclic nucleotide-independent histone kinase is not well-established at this time.

Materials and Methods

Materials and chemicals

Rabbits, weighing 3 to 4 kg, were purchased from Kajitani Rabbit Farm (Shimane, Japan). Excised rabbit skeletal muscle was stored at –80˚C until use. DEAE-Sephacel, cyanogen bromide (CNBr)-activated Sepharose 4B, Sephacryl S-100, Sephacryl S-200, molecular mass markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration, Blue Dextran and CleanGel electrode strips (No. 18-1035-33) were obtained from Pharmacia Biotech (Uppsala, Sweden). H1 histone-Sepharose was prepared according to the manufacturer's instructions (Pharmacia Biotech). Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories (Richmond, CA). Silica gel 60 thin layer chromatography (TLC) plates (No. 5626) and cellulose TLC plates (No. 5716) were obtained from E. Merck (Darmstad, Germany). Cyclic AMP (cAMP)-dependent protein kinase (protein kinase A) was purified from bovine cardiac muscle as described previously (Rubin et al., 1974). Protein kinase C type I and type II was purified from rat brain as described previously (Sekiguchi et al., 1988). Casein kinase I (CKI) purified as described earlier (Liu et al., 1996) was a kind gift from Mrs. Y. Liu and Dr. H. Kohara in our laboratory. Casein kinase II (CKII) was purified as described previously (Hashimoto et al., 1995). Synthetic peptides, TTYADFIAS GRTGRRNAIHD [protein kinase A inhibitor peptide (Cheng et al., 1986)], RFARKGALR QKNV [protein kinase C inhibitor peptide (House and Kemp, 1987)], RRLSSL RASTSKA [S6 peptide analogue (Wettenhall et al., 1984)], and LRR ASLG [Kemptide] were prepared as described previously (Hashimoto et al., 1990a, 1995). The peptide which contains a phosphorylation site in the Na^+ channel α subunit (Trimmer et al., 1989), KKLGSKKPQK [Na^+ channel peptide], was synthesized by Tana Laboratories, L.C. (Houston, TX). The preparation of H1 and H2B histones from calf thymus was performed as described earlier (Hashimoto et al., 1976). *N*-(2-Aminoethyl)-5-chloroisoquinoline-8-sulfonamide (CKI-7), a selective casein kinase I inhibitor, and cell division cycle 2 (cdc2) kinase were purchased from Seikagaku Kogyo, Tokyo, Japan. [γ-^{32}P]ATP (> 185 TBq/mmol) was obtained from Amersham (Amersham, Bucks, United Kingdom). *L*-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, bovine brain calmodulin, bovine serum albumin (BSA; fatty acid-free), myelin basic protein (MBP), phosphoserine, phosphothreonine, phosphotyrosine and α-casein were obtained from Sigma (St. Louis, MO) . Phosphatidylserine (bovine brain) and diolein were purchased from Serdary Research Laboratories (London, ON, Canada). *N*-Bromosuccinimide and leupeptin were obtained from Wako Pure Chemical and the Peptide Institute, Osaka, Japan, respectively. P-81 Phosphocellulose paper and 3MM filter paper were purchased from Whatman (Kent, United Kingdom). Membrane filters (pore size, 0.45 μm) and PVDF membranes were obtained from Advantec Toyo (Tokyo) and Millipore (Bedford, MA), respectively.

Purification of H1 histone kinase

The H1 histone kinase was purified from rabbit skeletal muscle as described below. The majority of manipulations were carried out at 4˚C or on ice.

Step 1

Frozen rabbit skeletal muscle, a mixture of muscles isolated from the back, buttocks and limbs, (160 g) was cut into small blocks and ground in a meat grinder. The ground muscle was subsequently homogenized in 800 mL of buffer containing 5 mM imidazole-HCl (pH 7.4), 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride and 5 μg/mL leupeptin using a Polytron homogenizer (Model K) at speed 6 for 20 × 30 s bursts with 30 s intervals between

bursts. The homogenate was centrifuged at 7,000 × *g* for 10 min in an RF2A rotor (Himac CR21, Hitachi, Tokyo). The resulting pellets were re-homogenized in 800 mL of the same homogenizing buffer containing 0.5 mM Mg^{2+}-ATP at the same speed for 10 × 30 s bursts with 30 s intervals between bursts and re-centrifuged at 7,000 × *g* for 20 min. The supernatant was filtered though 4 layers of cheesecloth and centrifuged at 19,000 × *g* for 2 h in a JA-20 rotor (J2-21 M/E, Beckman, Palo Alto, CA). The resultant supernatant was re-filtered though another 4 layers of cheesecloth. This final supernatant was named Sup 3 and used for further purification. The precipitate obtained by this centrifugation corresponded to the microsome fraction reported by Inui and coworkers (1987). It is not clear at this time whether the proteins in Sup 3 were originally located on the surface of the microsome or released from this organelle.

Step 2

Sup 3 (670 mL) was adjusted to pH 7.5 with 0.5 M Tris-HCl (pH 7.5) and was loaded onto a DEAE-Sephacel column (2.5 × 4.5 cm) equilibrated with Buffer A [20 mM Tris-HCl (pH 7.5), 0.3 M sucrose and 10 mM 2-mercaptoethanol] containing 20 mM NaCl. The column was washed with the same buffer until the absorbance at 280 nm fell to zero. Proteins were eluted with 320 mL of Buffer A containing 100 mM NaCl at a flow rate of 45 mL/h, and 5 mL of each fraction was collected. When an aliquot of each fraction (20 μL) was assayed for H1 histone kinase activity, 2 active peaks were detected. The 2nd peak (120 mL) was dialyzed against 2 changes of Buffer A (5 L) containing 20 mM NaCl for 8 h. The 1st peak was not analyzed further.

Step 3

The dialyzed sample was applied to an H1 histone-Sepharose column (1.5 × 4.5 cm) equilibrated with Buffer A containing 20 mM NaCl. The column was washed with 150 mL of the same buffer and eluted stepwise with 100 mL each of Buffer A containing 100 mM, 250 mM and 500 mM NaCl, respectively. The flow rate was 25–35 mL/h and 5 mL fractions were collected. One major peak of enzyme activity was recovered in the fractions eluted with buffer containing 100 mM NaCl. The active fractions (41.3 mL) were pooled and dialyzed overnight against 5 L of Buffer B [5 mM potassium phosphate buffer (pH 7.5), 0.3 M sucrose and 10 mM 2-mercaptoethanol].

Step 4

The dialyzed one was applied to a hydroxyapatite column (0.9 × 1.3 cm) equilibrated with Buffer B. After washing the column with the same buffer, the enzyme was eluted stepwise with 20 mL each of Buffer B containing 100 mM and 300 mM potassium phosphate buffer (pH 7.5) in place of the 5 mM buffer. The flow rate was 17 mL/h and 1 mL fractions were collected. The active fractions (8.6 mL) were pooled and used for the present studies as a partially purified enzyme preparation.

Molecular mass analysis by gel filtration

An aliquot (0.5 mL, 100 μg protein) of the enzyme obtained from the hydroxyapatite column was applied to a Sephacryl S-200 column (0.8 × 45 cm) equilibrated with buffer A containing 400 mM NaCl. Proteins were eluted with the same buffer at a flow rate of 1 mL/h, and 0.5 mL fractions were collected into tubes in which ovalbumin had been added as a stabilizer at a final concentration of 0.1 mg/mL. After dialysis against Buffer A containing 20 mM NaCl, each fraction was assayed for the kinase activity. The elution positions of marker proteins were checked by SDS-PAGE. Molecular mass standards were as follows; Blue Dextran, void volume; human γ-globulin, 130,000 Da; BSA, 67,000 Da; ovalbumin, 43,000 Da; cytochrome *c*, 13,500 Da.

In-gel kinase assay

An aliquot of the purified enzyme (1.5 mU, 30 μL) from the hydroxyapatite column peak fraction was loaded on an SDS-PAGE slab gel. Either 150 μg/mL H1 histone or 150 μg/mL BSA (control protein) was included in the separation

gel just prior to the polymerization. After electrophoresis, the gel was treated as described by Kameshita and Fujisawa (1989) with slight modifications as indicated below. After renaturation, the gel was preincubated with 2 mL of incubation buffer [20 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$] at room temperature for 30 min. Phosphorylation of H1 histone within the gel was carried out by incubation of the gel at room temperature for 2 h with 2 mL of the incubation buffer in the presence of 50 μM non-radioactive ATP and 50 μCi [γ-^{32}P]ATP. After incubation, the gel was washed with 5% (w/v) trichloroacetic acid containing 1% (w/v) sodium pyrophosphate until the radioactivity of the washing solution became negligible. The washed gel was dried on Whatman 3MM filter paper and exposed to X ray film (New RX, Fuji Photo Film Co., Kanagawa, Japan) at –80˚C for 24 h or longer time. After the renaturing treatment, the lane of molecular mass markers was cut from the gel and stained with Coomassie Brilliant Blue.

In vitro kinase assay

H1 histone kinase activity was measured in a reaction mixture (50 μL) comprising 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 60 μg/mL H1 histone, 10 mM [γ-^{32}P]ATP ($5.0–7.0 \times 10^5$ cpm/nmol) and 20 μL of enzyme preparation. The differences in ionic strength due to sodium chloride or potassium phosphate at the different purification steps was not corrected for. In assays (except large scale incubation mixtures), 40 mM potassium phosphate derived from the enzyme source was constantly included in the reaction mixture. Phosphorylation reactions were initiated by the addition of [γ-^{32}P]ATP and incubation was usually performed for 30 min at 30˚C. The reaction was terminated by the addition of 25 μL of 5% (w/v) trichloroacetic acid. The mixture was centrifuged at $3,000 \times g$ for 10 min and 50 μL of the supernatant was spotted on to P-81 phosphocellulose paper (2 × 2 cm). These papers were then washed 4 times with 10 mL of 75 mM phosphoric acid per each paper for 2 min each wash. The incorporated radioactivity was determined with a Beckman-LS 5801 liquid scintillation counter by Cerenkov radiation. Control assays were performed in parallel where the substrate protein was omitted and these values were subtracted from those of the complete reaction system. One unit (1 U) of the protein kinase activity was defined as the amount of enzyme that catalyzed the transfer of 1 nmol phosphate into H1 histone under the standard assay conditions described above. This definition of enzyme activity unit was also applied to other protein kinases used in this study.

When the substrate specificity of H1 histone kinase was investigated, as well as the effects of Mg^{2+}, ionic strength and protein kinase modulators, assays were performed as described above, with individual modifications as indicated for each experiment. The concentrations of proteins and peptides used as substrates in this study were as follows: H2B histone, 60 μg/mL; MBP, 53 μg/mL; α-casein, 1 mg/mL; S6 peptide analogue, 0.1 mg/mL; Kemptide, 0.1 mg/mL; Na^+ channel peptide, 0.1 mg/mL. The reaction with α-casein was stopped by the addition of 2 mL of 10% (w/v) trichloroacetic acid. Acid-precipitates were collected on a membrane filter and washed with the same trichloroacetic acid solution. Radioactivity was measured as described above. For the determination of kinetic parameters with H1 histone, the kinase activities were determined as described above except that a) H1 histone concentrations were raised between 0.27 and 2.73 μM; b) 1.0 mU of the enzyme was employed; c) the incubation was carried out for 15 min.

For comparative experiments, the substrate specificity and the effects of protein kinase modulators were also examined with other representative protein kinases (protein kinase A, protein kinase C, cdc2 kinase, CKI and CKII). Protein kinase A, protein kinase C, CKI and CKII were assayed under the respective conditions described previously (Hashimoto et al., 1985, 1990b, 1995). cdc2 Kinase was assayed 30 min at 30˚C in a reaction mixture (50 μL) containing 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 10 μM [γ-^{32}P]ATP, 1 mg/mL H1 histone or other substrates as indicated above.

Tryptic peptide mapping

H1 histone (36 μg) was phosphorylated by the purified H1 histone kinase (1.0 mU) or other protein kinases; protein kinase A (3.0 mU), protein kinase C (0.96 mU) or cdc2 kinase (2 mU) under the respective reaction conditions described above except that a) the specific activity of [γ-^{32}P]ATP was increased to 2.4–3.0 × 10^6 cpm/nmol, b) the reaction mixture was scaled up by 3-fold (150 μL), c) the incubation time was 3–4 times longer. The reaction was stopped by the addition of Laemmli buffer, and the reaction mixtures were separated by SDS-PAGE (Laemmli, 1970). From an estimate of the radioactivity, 50–125 μmol of phosphate was incorporated per mol of H1 histone by each protein kinase. The radioactive H1 histone was extracted from the gel and digested overnight at 37˚C with TPCK-treated trypsin (final concentration 50 μg/mL) as described previously (Beemon and Hunter, 1978) with slight modifications. After freeze-drying, the tryptic peptides were dissolved in electrophoresis buffer (formic acid:acetic acid:H_2O = 5:15:80, v/v) and spotted on Silica gel 60 TLC plates. High voltage electrophoresis was performed at 1000 V for 50–60 min at 4˚C (Pharmacia Multiphor II flatbed electrophoresis apparatus) as described by Naka and coworkers (1983). For one-dimensional mapping, the dried plate was exposed to an X ray film for an appropriate time at –80˚C. For two-dimensional mapping, ascending chromatography was carried out at room temperature on the TLC plate with the buffer (n-butanol:acetic acid:pyridine:H_2O = 32.5:25:5: 20, v/v) as a solvent system. After chromatography, plates were exposed to X ray films as before.

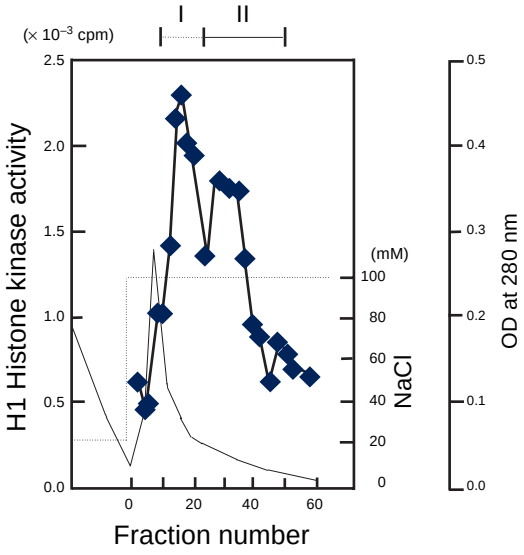

Fig. 1. Purification of H1 histone kinase by DEAE-Sephacel chromatography. Sup 3 fraction was chromatographed on a DEAE-Sephacel column and measurements of H1 histone kinase activity were performed as described under Materials and Methods. The 2 eluting peaks were named DEAE-I and DEAE-II, respectively. ◆, H1 histone kinase activity; —, absorbance at 280 nm; ---, NaCl concentration.

Phosphoamino acid analysis

Phosphorylation of H1 histone by the purified kinase and the separation by SDS-PAGE was performed as described under the previous section. The labeled H1 histone was extracted from the gel and precipitated with trichloroacetic acid as described by Beemon and Hunter (1978). The precipitate was washed and hydrolyzed in 6 N HCl for 2 h at 110˚C. The hydrolysate was lyophilized and resuspended in distilled water. An aliquot of this solution was spotted on a cellulose TLC plate together with 3 phosphoamino acids (50 nmol each). Electrophoresis was carried out at 1000 V for 1 h at 16˚C with the buffer (pyridine:acetic acid:H_2O = 1:10:189, v/v) on a Pharmacia Multiphor II flatbed apparatus as described by Mahoney and colleagues (1996). Positions of phosphoamino acids were visualized by ninhydrin spray and radioactive phosphoamino acids were detected by autoradiography.

Analysis of the phosphorylated domain of H1 histone by N*-bromosuccinimide cleavage*

The phosphorylation and subsequent purification of H1 histone were performed as indicated in the previous section. The extracted histone

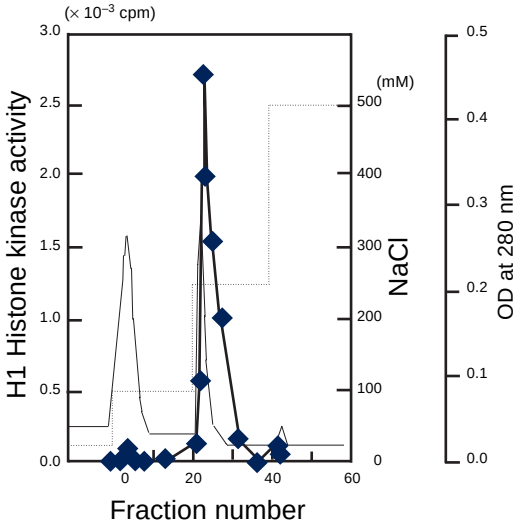

Fig. 2. Purification of H1 histone kinase by H1 histone-Sepharose chromatography. Purification of the DEAE-II fraction on a H1 histone-Sepharose column and measurements of H1 histone kinase activity were performed as described under Materials and Methods. ◆, H1 histone kinase activity; —, absorbance at 280 nm; ---, NaCl concentration.

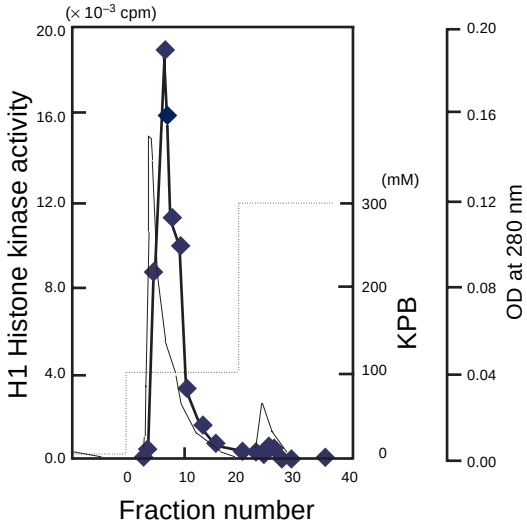

Fig. 3. Purification of H1 histone kinase by hydroxyapatite chromatography. Purification on a hydroxyapatite column of the active enzyme fraction obtained from the previous step (Fig. 2) and measurements of H1 histone kinase activity were performed as described under Materials and Methods. ◆, H1 histone kinase activity; —, absorbance at 280 nm; ---, concentration of potassium phosphate buffer (KPB).

was freeze-dried and resuspended in 0.8 mL of 1 N acetic acid with 2 mg of non-phosphorylated H1 histone as a carrier. *N*-Bromosuccinimide cleavage of the H1 histone was performed as described by Bustin and Cole (1969). The cleaved fragments were applied to a Sephacryl S-100 column (1.5 × 115 cm) equilibrated with 0.02 N HCl and eluted with the same solution at room temperature. The flow rate was 8.5 mL/h and 2 mL fractions were collected. The radioactivity of each fraction was monitored using a Beckman liquid scintillation counter in the Cerenkov mode and the absorbance at 230 nm of each fraction was measured with Hitachi 181 UV-VIS spectrophotometer. The amino-terminal and the carboxyl-terminal domains of H1 histone were confirmed by SDS-PAGE (15% polyacrylamide separating gel) and protein staining.

Other procedures and determinations

Unless otherwise indicated SDS-PAGE was performed in a mini-slab gel (8.5 cm × 6.5 cm × 0.1 cm) with 12.5% separating gel and 4.5% stacking gel by the method of Laemmli (1970). Electrophoresis was usually performed at 30 mA for 1 h. Protein was determined according to the method of Bradford (1976) using Bio-Rad reagent with BSA as a standard. Each experiment had been repeated at least 2 or 3 times and a representative result was presented.

Results

Purification of H1 histone kinase activity from rabbit skeletal muscle

Sup 3 fraction (670 mL, 502.5 mg protein) was used as the starting point for purification. It was adsorbed onto a DEAE-Sephacel column and proteins were eluted as broad 2 peaks with buffer containing 100 mM NaCl as shown in Fig. 1. They were named DEAE-I (fractions 8–25, 91.5 mL, 23.4 mg protein) and DEAE-II (fractions 26–48, 120.0 mL, 7.6 mg protein), respectively. The specific activity of the H1 histone

Table 1. Purification of H1 histone kinase from rabbit skeletal muscle

Step	Volume (mL)	Protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (-fold)
1. Sup 3 fraction	670	502.5	367	0.73	100.0	1.00
2. DEAE-Sephacel						
I	91.5	23.4	624	26.5	185	36
II	120.0	7.6	734	96.9	200	133
I + II	211.5	31.0	1358	43.9	385	60
3. H1 histone-Sepharose	41.3	1.95	440	225.5	120	309
4. Hydroxyapatite	8.6	0.75	297	396.6	81	543

Purification of H1 histone kinase and determination of enzyme activity and protein concentration were performed as described under Materials and Methods. Purification was repeated on several occasions and typical data are presented.

kinase in DEAE-II was 3.5 times higher than that of DEAE-I, and was therefore used for the next step in the purification. After decreasing the NaCl concentration to approximately 20 mM by dialysis, DEAE-II was applied to a H1 histone-Sepharose column. H1 histone kinase activity was eluted as a single peak with the buffer containing 250 mM NaCl as depicted in Fig. 2. The active peak fractions (fractions 23–30; 41.3 mL, 1.95 mg protein) were dialyzed and loaded to a hydroxyapatite column. The kinase activity this time was eluted with a buffer containing 100 mM potassium phosphate as indicated in Fig. 3. Further purification was attempted by several methods (gel filtration and cation exchange chromatography, affinity

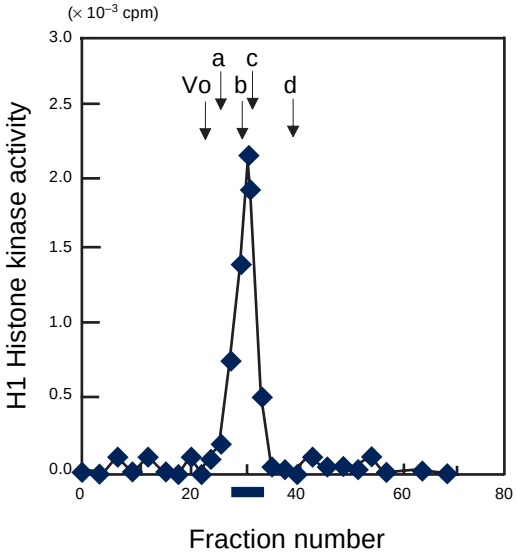

Fig. 4. Molecular mass analysis of H1 histone kinase by Sephacryl S-200 gel filtration chromatography. Gel filtration of the enzyme was performed and H1 histone kinase activity (◆) was assayed as described under Materials and Methods. Black bar indicates the peak fractions. The elution position of each molecular mass standard is pointed by an arrow. **Vo**, void volume determined using Blue Dextran; **a**, human γ-globulin; **b**, BSA; **c**, ovalbumin; **d**, cytochrome *c*.

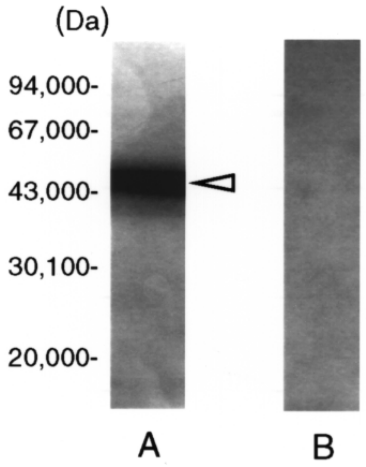

Fig. 5. Detection of H1 histone kinase activity by an in-gel assay method. The H1 histone kinase preparation was separated on SDS-PAGE containing either H1 histone (**A**) or BSA, as a control (**B**) and then the H1 histone kinase activity was detected as described under Materials and Methods. The position of the radioactive band with M_r 45,000 is indicated by arrow head. Numbers on the left side of the figure show the positions of molecular mass markers: phosphorylase *b* (94,000 Da), BSA (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,100 Da) and soybean trypsin inhibitor (20,000 Da).

chromatography on a heparin-Sepharose or a polylysine-agarose), but did not result in a significant increase in the specific activity of the enzyme (data not shown). Although the enzyme preparation was still contaminated with other proteins, the active fractions recovered from the hydroxyapatite column (fractions 4–12; 8.6 mL, 0.75 mg protein) was used for further analyses of this H1 histone kinase. A summary of the purification procedure is given in Table 1, indicating an approximately 540-fold purification from the Sup 3 fraction with an 81% overall recovery. Although the reason is not clear at present, the high recovery of enzyme activity may be due to the presence of an endogenous inhibitor in the starting Sup 3 fraction. The result shown in Fig. 4 depicts the elution profile of kinase activity from a Sephacryl S-200 gel filtration column. The molecular mass of the enzyme was estimated to be approximate 50,000 Da by comparison with the eluting positions of marker proteins. An SDS-PAGE analyses of the active fractions (fractions 29–35; black bar, Fig. 4) showed very faint protein band(s) around M_r 50,000 after staining with Coomassie Brilliant Blue (data not shown). However, radioactive H1 histone bands were detected on the autoradiogram of the gel after the active enzyme fractions were incubated with H1 histone and [γ-^{32}P]ATP and the reaction mixtures subsequently separated by SDS-PAGE (data not shown). In an attempt to obtain further information on the molecular mass of the H1 histone kinase, an in-gel assay method was employed, and Fig. 5 shows the existence of a radioactive protein band M_r ~45,000 in the gel containing H1 histone; this radioactive protein band does not appear to be due to autophos-

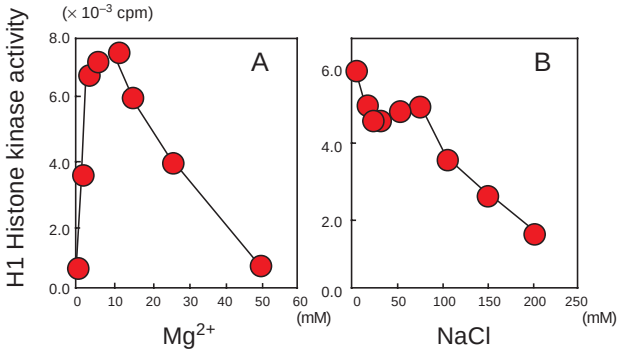

Fig. 6. Optimum concentration of Mg^{2+} and the effect of ionic strength on H1 histone kinase activity. Protein kinase activities were measured using 0.25 mU of H1 histone kinase as described under Materials and Methods except that the concentrations of Mg^{2+} (**A**) or NaCl (**B**) were changed as indicated.

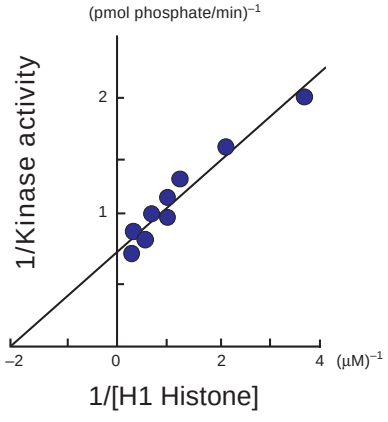

Fig. 7. Double-reciprocal plot of phosphorylation of H1 histone by H1 histone kinase. Protein kinase activities were measured using 1.0 mU of H1 histone kinase as described under Materials and Methods except that the incubation was performed under various concentrations (from 0.27 to 2.73 μM) of H1 histone for 15 min. The results are presented as a double-reciprocal plot.

Table 2. Effects of protein kinase activators and inhibitor on H1 histone kinase activity

Addition		Relative kinase activity (%)
None		100
cAMP	(0.4 mM)	115
$CaCl_2$ (0.2 mM) + Calmodulin	(10 μg/mL)	90
Heparin	(0.25 μg/mL)	107

cAMP, cyclic AMP.

H1 histone kinase activities were measured as described under Materials and Methods except that 0.3 mU of the enzyme was employed and protein kinase activators or inhibitor were added as indicated below. Results are presented as % activities taking that obtained in the absence of the modulator as 100.

phorylation. In contrast, no radioactive band was detected in the control gel. These results suggest that the purified H1 histone kinase is probably a monomeric protein with a molecular mass of 45,000–50,000 Da.

Enzymatic properties of the purified H1 histone kinase

The effect of Mg^{2+} on kinase activity was examined and the data in Fig. 6A indicate that the optimum concentration of Mg^{2+} was approximate 10 mM. Figure 6B depicts the effect of ionic strength on kinase activity. A small effect was observed at NaCl concentrations below 75 mM, but enzyme activity decreased rapidly above 100 mM NaCl.

A kinetic analysis of H1 histone phosphorylation was shown in Fig. 7. From the double-reciprocal plot, K_m and apparent Vmax values were calculated to be 0.51 μM and 1.36 pmol/min, respectively.

Table 3. Ca^{2+} and phospholipid requirement of H1 histone kinase and protein kinase C activities

Effector	Relative activity (%)	
	H1 histone kinase	Protein kinase C
+ Ca^{2+} + Phospholipid	100	100
+ Ca^{2+} – Phospholipid	73	9
+ EGTA + Phospholipid	108	71
+ EGTA – Phospholipid	97	11

Protein kinase C, Ca^{2+}-phospholipid-dependent protein kinase.

Protein kinase activities were measured as described under Materials and Methods except that i) 0.25 mU of the H1 histone kinase and 0.96 mU of protein kinase C were incubated for 30 and 10 min, respectively; ii) the final concentrations of Ca^{2+}, EGTA and phospholipid are 0.4 mM, 0.5 mM and 8 μg/mL phosphatidylserine plus 0.8 μg/mL diolein, respectively. Results are presented as % activities taking that obtained in the presence of Ca^{2+} and phospholipid as 100 for the respective protein kinases.

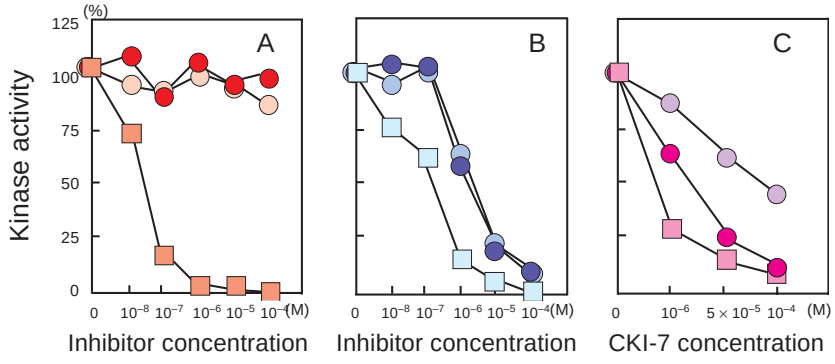

Fig. 8. Effects of various protein kinase inhibitors on H1 histone kinase activity. **A:** Effect of protein kinase A inhibitor peptide. H1 histone (60 mg/mL) was phosphorylated by H1 histone kinase (0.25 mU) or protein kinase A (1.2 mU) under the respective standard assay conditions described under Materials and Methods except that the inhibitor peptide was added at the indicated concentrations and 0.4 mM cAMP was included as indicated below. Results are presented as % activities taking that obtained in the absence of the inhibitor as 100. ● and ○, activities of H1 histone kinase in the presence and absence of cAMP, respectively; ■, activity of protein kinase A. **B:** Effect of protein kinase C inhibitor peptide. S6 peptide analogue (0.1 mg/mL) was phosphorylated by H1 histone kinase (0.35 mU) or protein kinase C (0.96 mU) under the respective standard assay conditions described under Materials and Methods except that the inhibitor peptide was added at the indicated concentrations and Ca^{2+} (0.4 mM) plus phospholipid (8 μg/mL phosphatidylserine plus 0.8 μg/mL diolein) or EGTA (0.5 mM) plus phospholipid suspension buffer were included as indicated below. Other conditions were same as indicated in (**A**). ● and ○, activities of H1 histone kinase in the presence of Ca^{2+} plus phospholipid and EGTA plus phospholipid suspension buffer, respectively; □, activity of protein kinase C. **C:** Effect of CKI-7 (inhibitor of CKI). H1 histone was phosphorylated by H1 histone kinase (0.25 mU) or protein kinase C (0.48 mU) and α-casein was phosphorylated by CKI (0.7 mU) under the respective standard assay condition described under Materials and Methods except that the inhibitor was added at the indicated concentrations. Other conditions were same as indicated in (**A**). ●, ○ and □: activities with H1 histone kinase, protein kinase C and CKI, respectively.

Table 4. Substrate specificity of H1 histone kinase and its comparison with other protein kinases

Substrate	Relative kinase activity (%)					
	H1 histone kinase	Protein kinase C	Protein kinase A	cdc2 Kinase	CKI	CKII
H1 histone	100	100	100	100	1	6
H2B histone	35	44	391	14	1	4
Myelin basin protein	153	110	19	ND	0	3
α-Casein	0	ND	ND	ND	100	100
S6 peptide analogue	310	271	530	6	1	1
Kemptide	30	13	680	0	8	3
Na^+ channel peptide	22	67	9	1	0	2

cdc2, cell division cycle 2; CKI, casein kinase I; CKII, casein kinase II; ND, not determined; protein kinase A, cyclic AMP-dependent protein kinase; protein kinase C, Ca^{2+}-phospholipid-dependent protein kinase.

Each protein kinase activity was measured as described under Materials and Methods except that the amount of enzyme and the incubation time were changed with respective enzymes as follows: H1 histone kinase, (0.3 mU, 30 min); protein kinase C, (0.96 mU, 10 min); protein kinase A, (2 mU, 5 min); cdc2 kinase, (0.3 mU, 30 min); CKI, (1.7 mU, 20 min); CKII, (0.8 mU, 20 min). Results are presented as % activities taking that obtained using H1 histone as 100 for H1 histone kinase, protein kinase C, protein kinase A and cdc2 kinase or using α-casein as 100 for CKI and CKII.

The effects of various modulators on protein kinase activities are shown in Tables 2 and 3, and Fig. 8. The H1 histone kinase activity was not significantly influenced by the addition of cAMP, $CaCl_2$ plus calmodulin or heparin (Table 2). The data in Table 3 indicate that the phosphorylation by the purified H1 histone kinase proceeded in Ca^{2+} and phospholipid-independent manner, in contrast to phosphorylation by protein kinase C. The kinase activity was not significantly influenced by the addition of protein kinase A inhibitor peptide irrespective of the presence or absence of cAMP (Fig. 8A). The reaction was strongly inhibited by the addition of protein kinase C inhibitor peptide although at concentrations one order of magnitude higher than those for protein kinase C itself (Fig. 8B). This inhibitory effect was equally observed irrespective of either the presence or absence of Ca^{2+} and phospholipid. The H1 histone kinase activity was moderately inhibited by CKI-7 (Fig. 8C), a relatively specific inhibitor of CKI. In comparison, CKI-7 only weakly inhibited protein kinase C (Chijiwa et al., 1989).

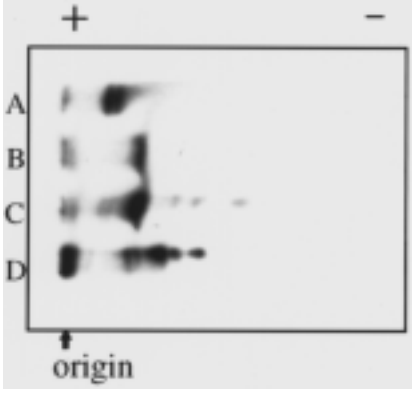

Fig. 9. One-dimensional peptide mapping of tryptic phosphopeptides derived from radioactive H1 histone. The sample phosphorylated each with cdc2 kinase (**A**); purified H1 histone kinase (**B**); protein kinase C (**C**); protein kinase A (**D**) was analyzed as described under Materials and Methods.

The substrate specificity of the purified H1 histone kinase was studied with various protein or peptide substrates and compared with that of other protein kinases (e.g., protein kinase A, protein kinase C, cdc2 kinase, CKI and CKII) (Table 4). MBP and S6 peptide analogue were phosphorylated more efficiently than H1 histone by our purified H1 histone kinase under the reaction conditions employed. In contrast, H2B histone, Kemptide (a peptide substrate for protein kinase A) and a Na^+ channel peptide were not such effective substrates for the H1 histone kinase, because the efficiencies in phosphoryla-

tion decreased to 22–35% compared with that of H1 histone. α-Casein (a standard substrate for casein kinase) was not phosphorylated by our kinase. From the results in Table 4, the substrate specificity of the purified H1 histone kinase was clearly different from that of protein kinase A, cdc2 kinase and CKI and CKII, but similar to that of protein kinase C.

To clarify the catalytic specificity of the H1 histone kinase, peptide mapping of the ^{32}P-labeled H1 histone was performed. At first, one-dimensional peptide mapping was examined using H1 histone preparations partially phosphorylated by the H1 histone kinase, protein kinase A, protein kinase C and cdc2 kinase, respectively, for comparative studies. The autoradiogram of the tryptic phosphopeptide maps is shown in Fig. 9. The pattern of the radioactive peptides obtained using the H1 histone kinase was different from those obtained by using protein kinase A and cdc2 kinase, but was similar to that obtained by using protein kinase C. In order to compare each functional specificity more precisely, the tryptic phosphopeptides obtained using H1 histone kinase and protein kinase C were examined using the two-dimensional peptide mapping. Both autoradiograms showed one major and several minor spots (Figs. 10A and B) and co-chromatography (Fig. 10C) indicates that the major spots are identical.

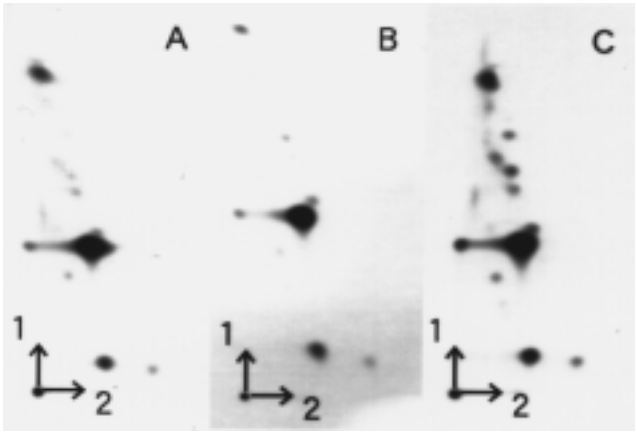

Fig. 10. Two-dimensional peptide mapping of tryptic phosphopeptides of radioactive H1 histone. Each H1 histone phosphorylated separately with either H1 histone kinase or protein kinase C was analyzed as described under Materials and Methods. Direction 1, high-voltage electrophoresis; Direction 2, ascending thin layer chromatography. **A:** H1 histone kinase; **B:** protein kinase C; **C:** co-chromatography of H1 histone kinase and protein kinase C.

Phosphoamino acid analysis of H1 histone indicated that radioactive phosphate was primarily incorporated into serine residue(s) (Fig. 11). When the radioactive H1 histone was chemically cleaved at Tyr-74 with *N*-bromosuccinimide, most of the radioactivity was recovered in a 15 kDa polypeptide derived from the carboxyl terminus (Fig. 12). Negligible radioactivity was detected on the 6 kDa polypeptide derived from the amino terminus. These results clearly indicate that the serine residue(s) in the carboxyl-terminal domain of H1 histone are phosphorylated by the purified H1 histone kinase. The high absorbance at 230 nm detected around the low molecular mass region is most likely due to unreacted reagent and acetic acid used as a solvent.

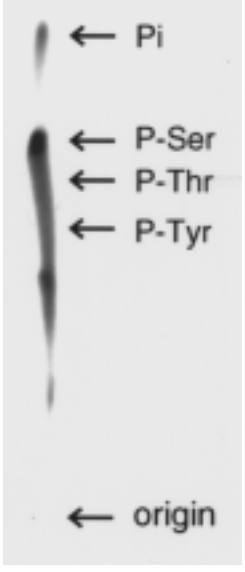

Fig. 11. Analysis of phosphorylated amino acid of H1 histone. H1 histone was phosphorylated by H1 histone kinase and phosphorylated amino acids of the modified histone were analyzed as described under Materials and Methods. The positions of phosphoamino acids are indicated on the right side of the figure. Pi, inorganic phosphate; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

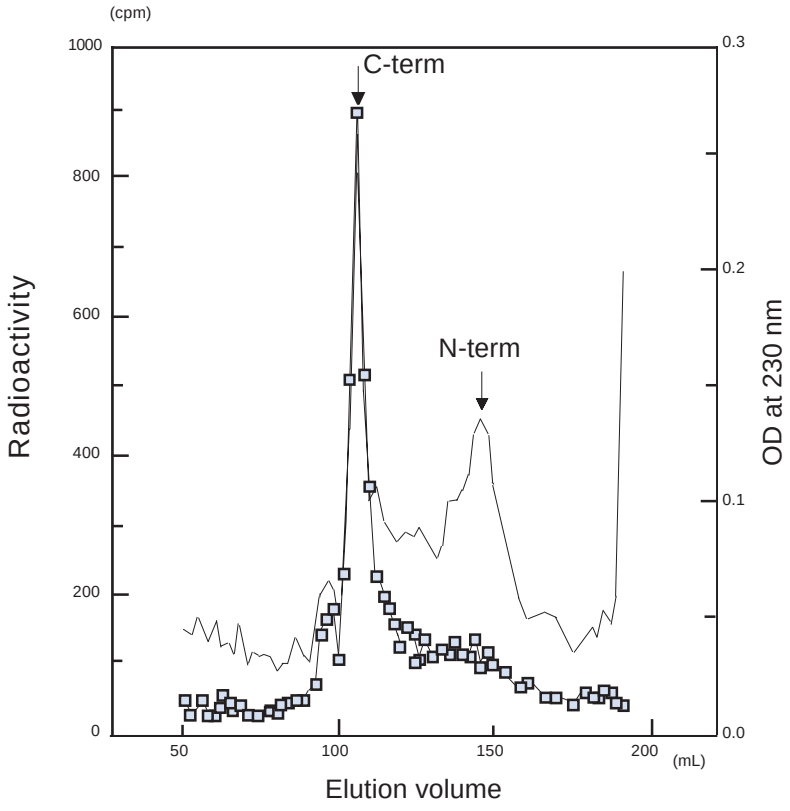

Fig. 12. Analysis of phosphorylated domain of H1 histone by *N*-bromosuccinimide treatment. Phosphorylated H1 histone was analyzed as described under Materials and Methods. The elution positions of carboxyl-terminal (C-term) and amino-terminal (N-term) domains are indicated by arrows. Γ, total radioactivity in each fraction; —, absorbance at 230 nm.

Discussion

In this paper, we reported the partial purification procedure and properties of one of the major H1 histone kinases recovered when the proteins obtained during the preparation of the microsomal fraction of rabbit skeletal muscle were separated on the DEAE-Sephacel column (Fig. 1). After the anion-exchange column, the enzyme was further purified by the column chromatographies on H1 histone-Sepharose and hydroxyapatite (Figs. 2 and 3). Finally, this kinase was purified by about 540-fold with an approximate yield of 80% (Table 1), although the final preparation did not seem to be homogeneous. The molecular mass of the purified H1 histone kinase was estimated to be 45,000–50,000 Da by gel-filtration and in-gel assay methods. Consistent with this results, the molecular mass of 47,700 Da was also calculated with its Stokes radius and sedimentation coefficient (Siegel and Monty, 1966) (data not shown).

In order to obtain further information on the enzymatic properties of this H1 histone kinase, the effects of various types of protein kinase modulators (Tables 2 and 3, and Fig. 8), the substrate specificity (Table 4) and the catalytic specificity (Figs. 9–11) were examined and compared with those of the representative protein serine/threonine kinases. The results obtained in these experiments revealed that the features of the purified H1 histone kinase were clearly different from those of protein kinase A, cdc2 kinase, CKI, CKII and calmodulin-dependent protein kinase, but similar to those of protein kinase C except for the requirement for Ca^{2+} and phospholipid on this kinase activity. The K_m value for H1 histone by the histone kinase was slightly lower than that by protein kinase C (Hashimoto et al., 1996). The analyses on the phosphopeptides and the phosphoamino acid demonstrated that the H1 histone kinase phosphorylated serine residue(s) of the carboxyl-terminal region generated by *N*-bromosuccinimide treatment as well as protein kinase C

(Jakes et al., 1988). These results suggest a possibility that the purified enzyme may be a proteolyzed product of protein kinase C (Takai et al., 1977; Hashimoto and Yamamura, 1989). However, some properties of this kinase were distinguishable from those of the proteolyzed protein kinase C. For example, the H1 histone kinase was recovered from the DEAE-Sephacel column by the buffer at pH 7.5 containing 100 mM NaCl, whereas the catalytic fragment of protein kinase C is usually eluted from the same anion-exchange column by the buffer containing higher salt concentrations (Takai et al., 1977).

The catalytic fragment of protein kinase C was usually produced by the limited proteolysis of this enzyme by some protease such as calpain (Kishimoto et al., 1983) or trypsin (Huang et al., 1989) in in vitro reaction systems as well as cellular systems stimulated with tumor-promoting phorbol ester such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Nishizuka, 1989). Although the physiological significance on the generation of the catalytic fragment of protein kinase C has not been well confirmed, it has been assumed to be temporarily-produced intermediate during the process of down-regulation (Nishizuka, 1989) or an active kinase with a special role for recognizing specific protein(s) for cellular regulation (Pontremoli et al., 1987). In the case of protein kinase C, the translocation from cytosol to membrane or other organelle has been demonstrated after stimulation with various effectors (Disatnik et al., 1994). It seems to be plausible to assume that the translocated kinase may be subjected to limited proteolysis by endogenous protease.

It has been well known that protein kinase C plays important roles in signal transduction pathways through phosphorylation of various intracellular and membrane-bound proteins as well as protein kinase A (Nishizuka, 1989). Although the relation between the H1 histone kinase and protein kinase C is not clear at this time, we expect that the histone kinase may participate in the regulation of some microsomal functions. Further purification and structural analysis as well as the studies on the mechanism of activation and the survey for its physiological target(s) seem to be important for more detailed understanding of this H1 histone kinase.

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