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Mass Spectrometric Analysis of Phosphoserine Residues Conserved in the Catalytic Domain of Membrane-Bound Guanylyl Cyclase from the Sea Urchin Spermatozoa

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ABSTRACT—We have developed a large-scale purification method of the phosphorylated form (131 kDa) of membrane-bound guanylyl cyclase (mGC) from *Hemicentrotus pulcherrimus* spermatozoa. The purified mGC contained 26.0 ± 1.3 moles of phosphate/mol enzyme (mean ± S.D., n = 6). Phosphorylated peptides were isolated from the trypsin digest of the carboxymethylated *H. pulcherrimus* sperm mGC by affinity chromatography on a Chelating Sepharose Fast Flow column, and the peptides were then subjected to mass spectrometric analysis and determination of phosphoserines, after the conversion of phosphoserines to S-ethylcysteines by amino acid analysis. Based on the observed mass number and the content of phosphoserine, serine residues at positions 561, 565, 652, 722, 740, 755, 894, 897, 914, 918, 927, 930, 951, and 985, in addition to two residues among those at positions 666, 670, and 671, were shown to be phosphorylated. They are all located in the intracellular region (kinase-like and catalytic domains). Notably, serine residues at positions 894, 918, 927, and 930, that are conserved in the sequence of mammalian mGCs and medaka fish-eye-specific mGCs, are phosphorylated in the sea urchin sperm mGC.

INTRODUCTION

Guanylyl cyclase [GTP pyrophosphate-lyase (cycling), EC 4.6.1.2] is found in various cellular compartments as soluble and/or particulate forms, and catalyzes the formation of cGMP and inorganic pyrophosphate from GTP (Mittal and Murad, 1982). For over twenty years, it has long been known that cGMP concentrations can be increased by a wide variety of agents (Goldberg and Haddox, 1977). Critical functions of cGMP have been reported in photo-transduction (Stryer, 1991; Winquist *et al*., 1984; Waldman *et al*., 1984) and in the actions of several peptide factors (Hamet *et al*., 1984). Following homogenization of sea urchin spermatozoa, all or most enzyme activity is recovered in particulate fractions (Radany *et al*., 1983). This suggests that sea urchin sperm guanylyl cyclase exists primarily in the membrane-bound form. The membrane-bound guanylyl cyclase (mGC) is composed of an extracellular region, a single membrane-spanning region, and an intracellular region. The intracellular region consists of two clearly defined domains, the first of which is a protein kinase-like regulatory domain, and the second, a cyclase catalytic domain. Both domains are highly conserved among invertebrates and vertebrate (Garbers, 1992; Garbers and Lowe, 1994). The binding of sperm-activating peptides to the sperm surface receptors has been reported to cause a marked and rapid increase and subsequent rapid decrease in cGMP concentration in sperm cells (Kopf *et al*., 1979; Suzuki, 1990). The transient increase in cGMP concentration has been attributed to transient activation and subsequent inactivation of the guanylyl cyclase, which is closely linked to the state of the enzyme (Garbers, 1989). A specific sperm-activating peptide such as SAP-IIA isolated from the egg jelly of the sea urchin *Arbacia punctulata* causes an initial transient activation of homologous sperm mGC and subsequent inactivation, which is closely linked to the state of phosphorylation of the enzyme (Ramarao and Garbers, 1985; Suzuki *et al*., 1984). The *A. punctulata* sperm mGC contains up to 17 mol phosphates/mol enzyme, all on serine residues, but after treatment of the spermatozoa with SAP-IIA, the number of phosphoserines decreases to less than 2 mol phosphates/mol enzyme (Vacquier and Moy, 1986). The specific activity of the phos-

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phorylated form of *Strongylocentrotus purpuratus* sperm mGC is 5-fold higher than that of the dephosphorylated form (Ramarao and Garbers, 1988). We purified both phosphorylated and dephosphorylated forms of mGC from *Hemicentrotus pulcherrimus* spermatozoa and showed that the phosphorylated form had a higher level of activity than the dephosphorylated form (Harumi *et al.*, 1992). Atrial natriuretic peptide receptor/guanylyl cyclase has been shown to desensitize in response to the peptide in a manner similar to sperm-activating peptides (Potter and Garbers, 1992). The intracellular region of mGCs is highly conserved between sea urchins and mammalians (Garbers, 1992; Garbers and Lowe, 1994). However, little is known about sites of phosphorylation and dephosphorylation, despite their apparent importance in the regulation of enzyme activity. These sites have been difficult to analyze by means of common protein chemistry methodology because of an insufficient amount of the necessary enzyme. As far as we know, sea urchin spermatozoa are the most adequate material for such analysis since we could obtain 2.6 mg (about 20 nmol) of mGC from 10 g wet weight of spermatozoa (Shimizu *et al.*, 1996).

In order to obtain a deeper understanding of the activation/inactivation mechanisms by phosphorylation/dephosphorylation of mGC, we used *H. pulcherrimus* sperm mGC with known primary structures to carry out mass spectrometric analysis of phosphoserine-containing peptides isolated from the phosphorylated form of mGC. Here, we report that at least four conserved serine residues located in the catalytic domain are phosphorylated.

MATERIALS AND METHODS

Materials

The sea urchins (*H. pulcherrimus*) were collected at the coast near Noto Marine Laboratory, Kanazawa University, Japan. Spermatozoa were obtained by intracoelomic injection of 0.5 M KCl and were collected as "dry". Trypsin was purchased from Sigma Chemical Co. and acetonitrile (ACN) of HPLC grade was obtained from Wako Pure Chemical Industries Ltd. A site-directed antibody against the synthetic peptide with the sequence KPPPQKLTQEAIEIAANRVIPDDV corresponding to the residues 1102 through 1125 of *H. pulcherrimus* sperm mGC was made by Shimizu *et al.* (1996). Other chemicals of analytical grade were obtained from Wako Pure Chemical Industries Ltd, Nacalai Tesque Inc. or Sigma Chemical Co.

Purification of the phosphorylated form of mGC from *H. pulcherrimus* spermatozoa

In a previous study, we purified the phosphorylated form (active form) of *H. pulcherrimus* sperm mGC (Harumi *et al.*, 1992). However, the method used in the study was not adequate for large scale purification because thus purified mGC lost enzyme-bound phosphates and activity gradually during the storage. This might be due to action of a protein phosphatase(s) which is associated tightly with the mGC. Therefore, dry sperm, the starting material for mGC purification, were heat-treated in order to inactivate the endogenous protein phosphatase which dephosphorylates sperm mGC. A test tube containing about 15 g dry sperm was placed in a boiling water bath at 100°C for 10 min and then the test tube was cooled down by placing it in an ice bath for 10 min. The boiled dry sperm were suspended in 30 ml of distilled water (DW) and kept in a freezer (–40°C) until use. Two ml of the suspension was mixed with 2 ml of 20% SDS and vortexed vigorously under occasional heating. The suspension was centrifuged at 10,000 × g for 10 min. The resulting supernatant was mixed with an equal volume of the sample buffer without SDS, and was applied on a preparative SDS-PAGE system model 491 Prep-Cell (BioRad) using a 6% polyacrylamide gel. Five ml of the fractions were collected and every third fraction was subjected to Western blot analysis using anti-*H. pulcherrimus* sperm mGC antiserum (Towbin *et al.*, 1979). Remaining fractions containing mGC were pooled and used for further experiments.

Carboxymethylation of mGC

To avoid the formation of inter- and/or intra-disulfide bonds that may interfere with purification of mGC, the enzyme (6 mg protein in 30 ml of 8 M urea) isolated by Prep-Cell was reduced with 2-mercaptoethanol (2.5 mg/ml) and carboxymethylated with iodoacetic acid (6.25 mg/ml) according to the method of Crestfield *et al.* (1963). After the reaction, the solution was dialyzed extensively with 0.2 M acetic acid and DW. It was then concentrated with Amicon Diaflow Cell RK 52 using a YM-30 membrane and subjected to HPLC on a TSK-Gel G6000PW column (7.5 × 300 mm, TOSOH K. K.) equilibrated with 50 mM ammonium bicarbonate (pH 8.5).

Proteolytic digestion of mGC

The mGC (1.3 mg protein) purified by HPLC was digested for 20 hr at 37°C with trypsin (enzyme/substrate: 1/20, w/w) in 50 mM ammonium bicarbonate (pH 8.5) containing 2 mM $CaCl_2$. The digest was applied to a Superdex Peptide HR10/30 column (Pharmacia), which was developed at the flow rate of 0.5 ml/min with 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl at 30°C. Fractions containing peptides were pooled and dried, and then dissolved in buffer A (1 M NaCl, 50 mM MES, pH 5.5).

Metal ion affinity chromatography on a Fe (III) immobilized Chelating Sepharose Fast Flow column

The affinity chromatography was carried out according to the method of Muszynska *et al.* (1992). The degassed Chelating Sepharose Fast Flow gel (Pharmacia) suspended in DW was packed into the column (v_t = 1 ml), which was equilibrated with a few column volumes of 20 mM iron (III) chloride solution. Excess Fe (III), (unbound or loosely bound), was removed from the column by washing it with 10-15 column volumes of DW, buffer C (1 M NaCl, 100 mM Tris-HCl, pH 7.5) and buffer A, consecutively. Then, the sample was applied to the column, which was washed with 10 ml of buffer A, and then developed with 10 ml of buffer B (1 M NaCl, 50 mM MES, pH 6.5) at room temperature and at a flow rate of 15 ml/hr. Phosphorylated peptides were eluted with a continuous pH gradient formed by the gradual mixing of 17 ml of buffer C with 17 ml of buffer B, and finally with buffer D (1 M NaCl, 100 mM Tris, pH 8.0). Fractions of 2 ml were collected, and those containing phosphorus were pooled and dried. The residue was dissolved in 5% ACN in 0.1% trifluoroacetic acid (TFA) for the next HPLC.

Separation of peptides by HPLC

Peptides were separated by HPLC using a Hitachi model L-6400 chromatography system on a Superiorex ODS column (Shiseido), which was developed with a linear gradient of ACN (5 - 60%) in 0.1% TFA at a flow rate of 1 ml/min at 40°C. The column effluent was monitored by A_{215} with a Hitachi L-4200 UV-VIS detector. Fractions containing phosphorylated peptides were rechromatographed under the same conditions as descibed above.

Conversion of phosphoserine to S-ethylcysteine

The phosphorylated peptide was dissolved in 50 μl of a reaction solvent consisting of ethanethiol (60 μl), DW (200 μl), dimethylsulphoxide (200 μl), ethanol (100 μl) and 5 N NaOH (65 μl), and incubated for 1 hr at 50°C under nitrogen gas (Holmes, 1987). Incubations were terminated by the addition of 10 μl acetic acid and cooling. The mix-

ture was dried after having been diluted to 1 ml by adding DW.

Analysis of amino acid composition

The peptide was hydrolyzed with constantly boiling HCl at 110°C for 20 hr. The hydrolysate was dried and dissolved in 100 μl of coupling solution (ethanol: 0.1 M boric acid buffer, pH 9.0 : PITC, 79 : 20 : 1, v/v/v). The mixture was incubated at room temperature for 15 min and then dried. The residue was dissolved in 100 μl of sample buffer (3% ACN in 50 mM sodium phosphate buffer, pH 6.5, containing 100 mM sodium perchlorate) and submitted to HPLC on a reverse-phase column (TSKgel ODS 80 TM, 4.6 × 150 mm) equilibrated with the sample buffer. It was developed for 21 min at a flow rate of 1 ml/min at 40°C using a linear gradient of ACN (3 - 50%) in a 50 mM sodium phosphate buffer (pH 6.5) containing 100 mM sodium perchlorate. The column effluent was monitored by the absorbance at 254 nm.

Mass spectrometry

The purified peptide was subjected to the analysis by a matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry on a Voyager PR (PerSeptive Biosystems) with sinapinic acid as a matrix.

Other methods

The phosphate content of *H. pulcherrimus* sperm mGC and its proteolytic fragments were determined by the method of Buss and Stull (1983) as previously described (Harumi *et al.*, 1992). SDS-PAGE was carried out as described by Laemmli (1970). The gel was silver-stained by Morrissey's method (1981). Amino acid sequence analysis was carried out using an Applied Biosystems model 476A Sequencer. Using bovine serum albumin as a standard, protein concentration was determined according to a method developed by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

In order to conduct a large-scale purification of mGC from *H. pulcherrimus* spermatozoa, we were especially careful to avoid the contamination of protein phosphatase, which seems to be tightly associated with the guanylyl cyclase. To this end, we used as starting material the boiled spermatozoa with almost all enzymes inactivated for the purification of mGC. The mGC isolated by preparative SDS-PAGE was carboxymethylated with iodoacetic acid under reduced conditions and then subjected to gel filtration chromatography on a TSK-Gel G6000PW column to remove excess SDS in the enzyme preparation (Fig. 1A). Thus purified mGC migrated to the position corresponding to the phosphorylated form (131 kDa) of *H. pulcherrimus* sperm mGC on SDS-PAGE (Fig. 1A, insert). The mGC then reacted with site-directed anti-*H. pulcherrimus* sperm mGC antibodies (not shown). By this method, we could obtain 3.8 mg (about 30 nmol) of the mGC from about 15 g wet weight of spermatozoa. The mGC contained 26.0 ± 1.3 mole phosphate/mol enzyme (mean ± S.D., n = 6), comparable to those reported in a previous paper (Harumi *et al.*, 1992). The mGC was then subjected to tryptic digestion and the digest was applied to a Superdex Peptide HR10/30 column (Fig. 1B). The fractions combined as three parts (I, II, III) were pooled, dried, and dissolved in buffer A, respectively. The pooled fractions were then applied to a Chelating Sepharose Fast Flow column. As shown in Fig. 2, each frac-

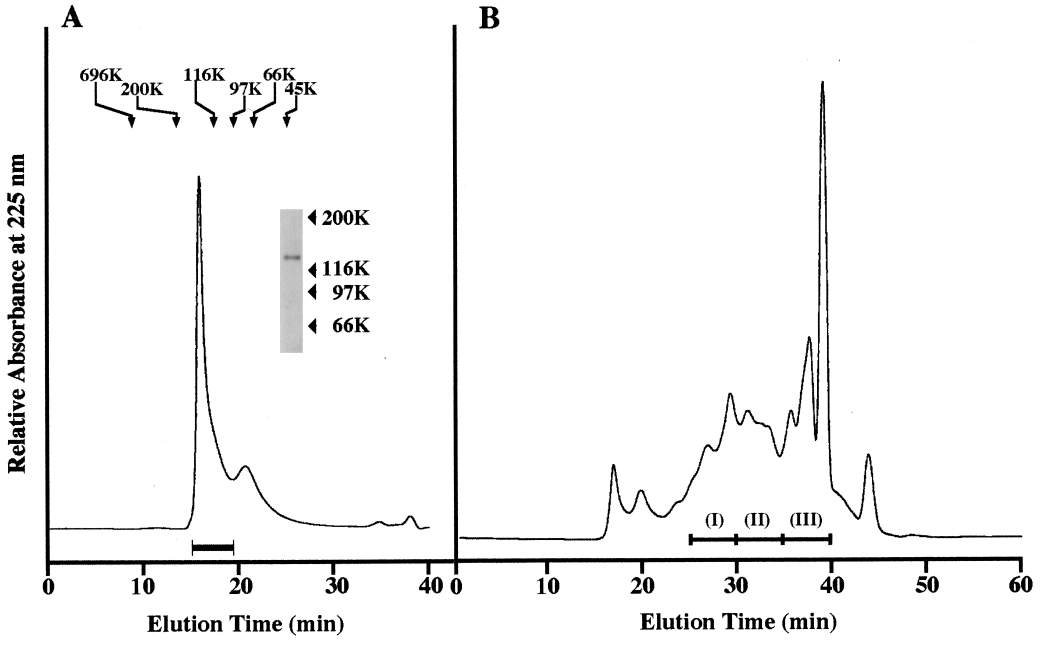


Fig. 1. Gel filtration profiles. (**A**) *H. pulcherrimus* sperm mGC (200 μg) obtained from the Prep-Cell was applied to a TSK-Gel G6000PW column equilibrated with 50 mM ammonium bicarbonate (pH 8.5) and eluted with the equilibration solution at a flow rate of 0.5 ml/min at 30°C. Fractions of 0.5 ml were collected and the peak fraction (20 μl) was analyzed by SDS-PAGE with a 7.5% gel (insert). (**B**) The tryptic digest of the cyclase was applied to a Superdex Peptide HR10/30 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 200 mM NaCl and eluted with equilibration solution at a flow rate of 0.5 ml/min at 30°C. Fractions of 0.5 ml were collected and fractions 26 - 30 (I), 31 - 35 (II), and 36 - 40 (III) were pooled, separately. The column effluent was monitored by the absorbance at 225 nm.

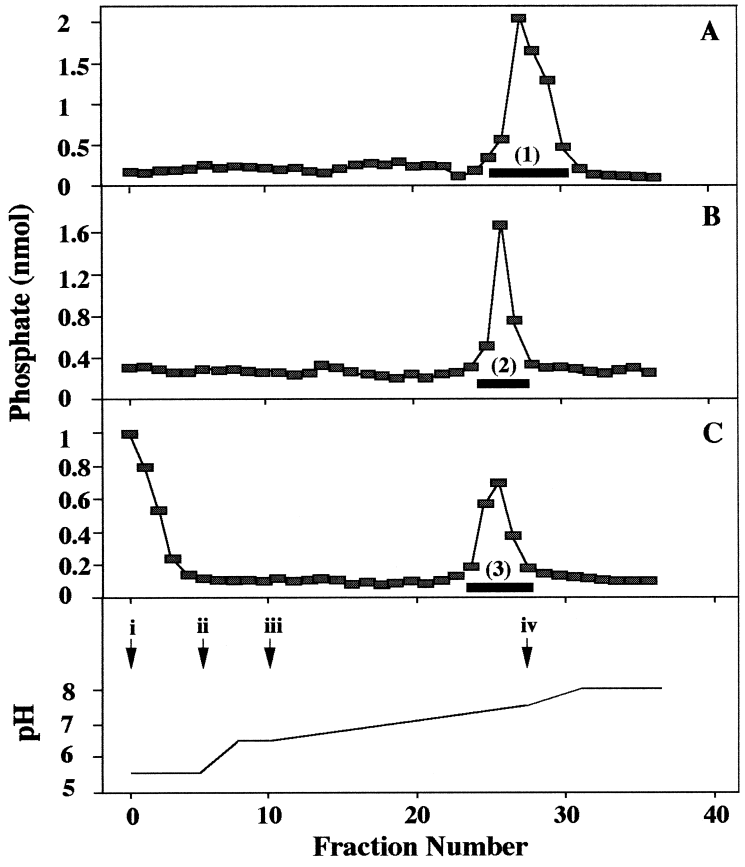


Fig. 2. Affinity chromatography of the phosphate-containing fractions on a Chelating Sepharose Fast Flow column. The pooled fractions (I), (II), and (III) obtained from gel chromatography on the Superdex column were subjected to affinity chromatography. **A**, fraction (I); **B**, fraction (II); **C**, fraction (III). Arrows indicate buffer change points: i = buffer A; ii = buffer B; iii = pH gradient of buffers B and C; iv = buffer D. Fifty microliter aliquot of each fraction was analyzed for the phosphate content. In each chromatography, phosphate-containing fractions were pooled, and are designated by the number in parenthesis with a bold-bar.

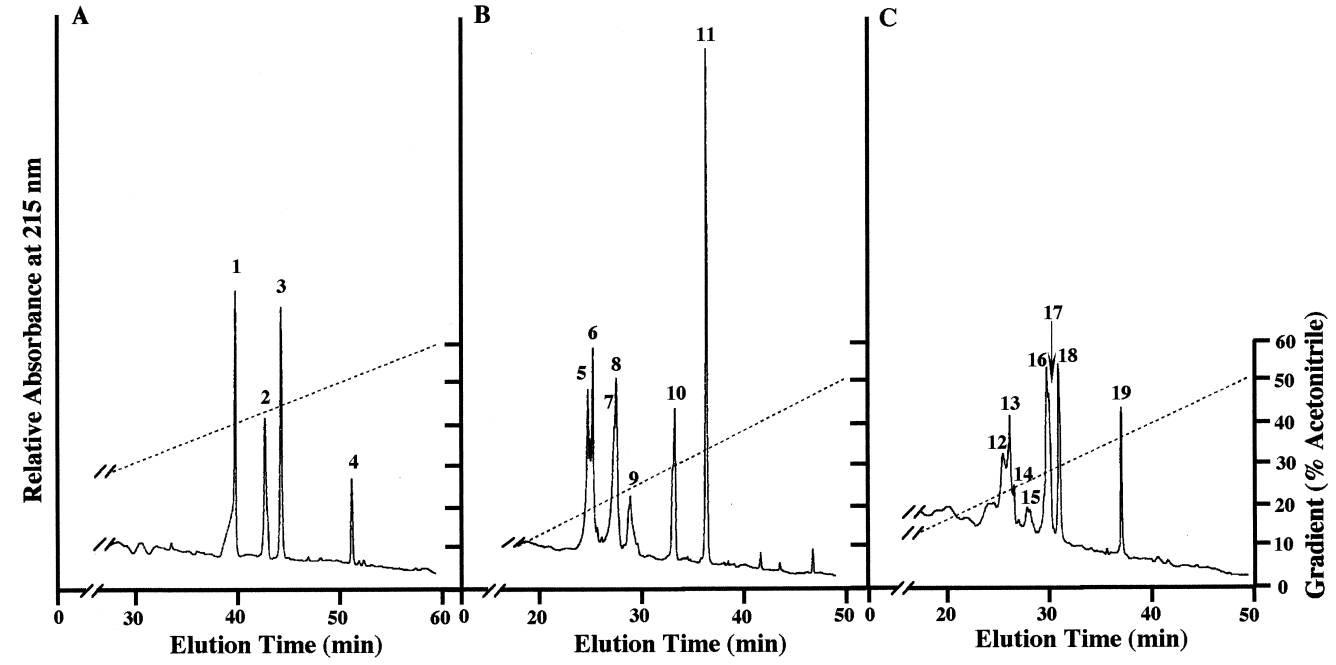


Fig. 3. HPLC profiles of phosphate-containing fractions. Phosphate-containing fractions (1), (2), and (3) obtained from affinity chromatography were subjected to HPLC on a Superiorex ODS column, using a linear gradient of ACN from 5 to 60% in 0.1% TFA at 40°C. (**A**) phosphate-containing fraction (1); (**B**) phosphate-containing fraction (2); (**C**) phosphate-containing fraction (3). The column effluent was monitored by absorbance at 215 nm.

tion gave essentially a single phosphate-containing peak. The peaks (2) and (3) from the pooled fractions (II) and (III), respectively, were eluted in the pH ranges of 7.3 - 7.5. In contrast, the peak (1) from the pooled fraction (I) was eluted as a rather broad peak in the pH ranges of 7.4 - 8.0. The fractions designated as (1), (2), and (3), which correspond to peaks (1), (2), and (3), respectively, were saved and dried, respectively. The residue was dissolved in 5% ACN in 0.1% TFA, and then subjected to HPLC on a reverse-phase column. Four peptides (Fig. 3A), seven peptides (Fig. 3B), and eight peptides (Fig. 3C) were obtained from the fractions (1), (2), and (3), respectively. Each peak peptide fraction was rechromatographed on the same column under the same conditions as above. The content of phosphoserine residues in each peptide fraction was determined by amino acid analysis after they converted to S-ethylcysteine residues (Table 1). Mass spectrometric analysis of these peptides yielded to a single protonated molecular ion $(M + H)^+$ and a typical mass spectrum is shown in Fig. 4A. However, peptide peak 3 produced two protonated molecular ions of 1402.60 and 2759.65 (Fig. 4B). Two peptides in peptide peak 3 were separated by HPLC after conversion of phosphoserine residues into S-ethylcysteines. Amino acid analysis showed that both peptides contained 2 S-ethylcysteine residues (Fig. 5 and Table 1). Based on the observed mass number and the content of phosphoserine, we assigned the position of phosphoserine and located each phosphoserine-containing peptide by referring to the deduced amino acid sequence of *H. pulcherrimus* mGC (Table 2). For example, the peptide in peak 11 obtained by the first HPLC contains 2 S-ethylcysteine residues, and the observed mass number is 1734.67. The calculated mass numbers of the peptide fragment (IVGFTALSAASTPIQV) found in the amino acid sequence (residues 920 to 935) of *H. pulcherrimus* mGC are 1573.70 without 2 phosphates, and 1733.70 with 2 phosphates. The latter mass number is consistent with the peptide fragment containing 2 phosphoserines. Therefore, we assigned the serines at positions 927 and 930 as phosphorylated. However, the peptide in peak 14 obtained by the first HPLC contains 2 S-ethylcysteine residues and the observed mass number of the peptide is 1186.92. The calculated mass number of the peptide fragment (KLDSMFLSS) found in the amino acid sequence of the mGC is 1185.90, which corresponds to that of the peptide fragment with 2 phosphoserine residues and 1 serine residue. The amino acid analysis also reveals that the peptide contains 2 S-ethylcysteine residues and 1 serine. Thus, in this experiment, assignment of phosphoserine was not successful.

In the present study we identified the positions of 14 phosphoserine residues, about half of the expected number. The difference of the number between identified and expected phosphoserine residues may be attributable to insufficient isolation of phosphoserine-containing tryptic peptides and/or the presence of phosphothreonine residues. To clarify these problems, we shall continue the isolation and analysis of other phosphate-containing tryptic peptides.

It should be mentioned that serine at positions 561, 565,

Table 1. Amino acid compositions of S-ethylcysteine-containing peptides purified by affinity chromatography and subsequent HPLC

Amino acids	Peaks obtained by 1st HPLC									Peaks obtained by 2nd HPLC					
	peak 2	peak 3-1	peak 3-2	peak 5	peak 8	peak 11	peak 14	peak 18	peak 19	peak 5	peak 7	peak 8	peak 12	peak 15	peak 16
Asp (D)	139 (2)	56 (2)	–	78 (2)	31 (1)	–	66 (1)	37 (1)	32 (1)	–	53 (1)	123 (2)	–	200 (2)	–
Glu (E)	324 (5)	27 (1)	71 (2)	–	34 (1)	52 (1)	–	–	37 (1)	–	–	–	–	–	257 (4)
Ser (S)	–	–	–	–	–	–	65 (1)	–	184 (5)	–	–	56 (1)	–	–	–
Gly (G)	–	31 (1)	30 (1)	–	67 (2)	120 (1)	–	–	39 (1)	76 (1)	–	–	–	–	–
His (H)	60 (1)	–	37 (1)	–	–	–	–	–	74 (2)	56 (1)	–	–	126 (2)	–	40 (1)
Thr (T)	61 (1)	–	36 (1)	–	–	115 (1)	–	–	–	–	–	–	–	–	40 (1)
Ala (A)	–	–	74 (2)	–	–	222 (3)	–	–	–	89 (1)	–	46 (1)	100 (1)	42 (1)	45 (1)
Pro (P)	55 (1)	–	99 (3)	–	37 (1)	87 (1)	–	–	–	–	–	–	–	–	76 (1)
Arg (R)	65 (1)	–	34 (1)	–	–	–	–	–	–	–	–	–	–	–	126 (2)
Tyr (Y)	84 (1)	–	–	45 (1)	30 (1)	–	–	–	–	63 (1)	–	–	–	89 (1)	–
Val (V)	–	23 (1)	37 (1)	40 (1)	–	122 (2)	–	39 (1)	–	–	64 (1)	–	–	–	–
Met (M)	–	47 (2)	39 (1)	–	–	–	41 (1)	–	–	–	95 (2)	26 (1)	–	–	–
S-ethyl C	38 (1)	39 (2)	52 (1)	42 (1)	21 (1)	100 (2)	96 (2)	49 (2)	–	44 (1)	38 (1)	85 (2)	37 (1)	39 (1)	31 (1)
Ile (I)	–	–	87 (3)	–	34 (1)	193 (2)	–	69 (2)	40 (1)	–	–	97 (1)	–	135 (2)	–
Leu (L)	92 (2)	–	150 (5)	–	–	110 (1)	114 (2)	–	37 (1)	58 (1)	–	197 (3)	53 (1)	–	132 (2)
Phe (F)	43 (1)	25 (1)	–	–	–	61 (1)	53 (1)	71 (2)	–	–	–	45 (1)	–	–	–
Lys (K)	–	27 (1)	31 (1)	–	–	–	55 (1)	–	69 (2)	148 (1)	51 (1)	–	–	–	106 (1)

Amino acid compositions are expressed as pmol and numbers in parentheses refer to the most probable number of residues in a peptide. Tryptophan and cysteine were not determined. S-ethyl C denotes S-ehtylcysteine converted from phosphoserine.

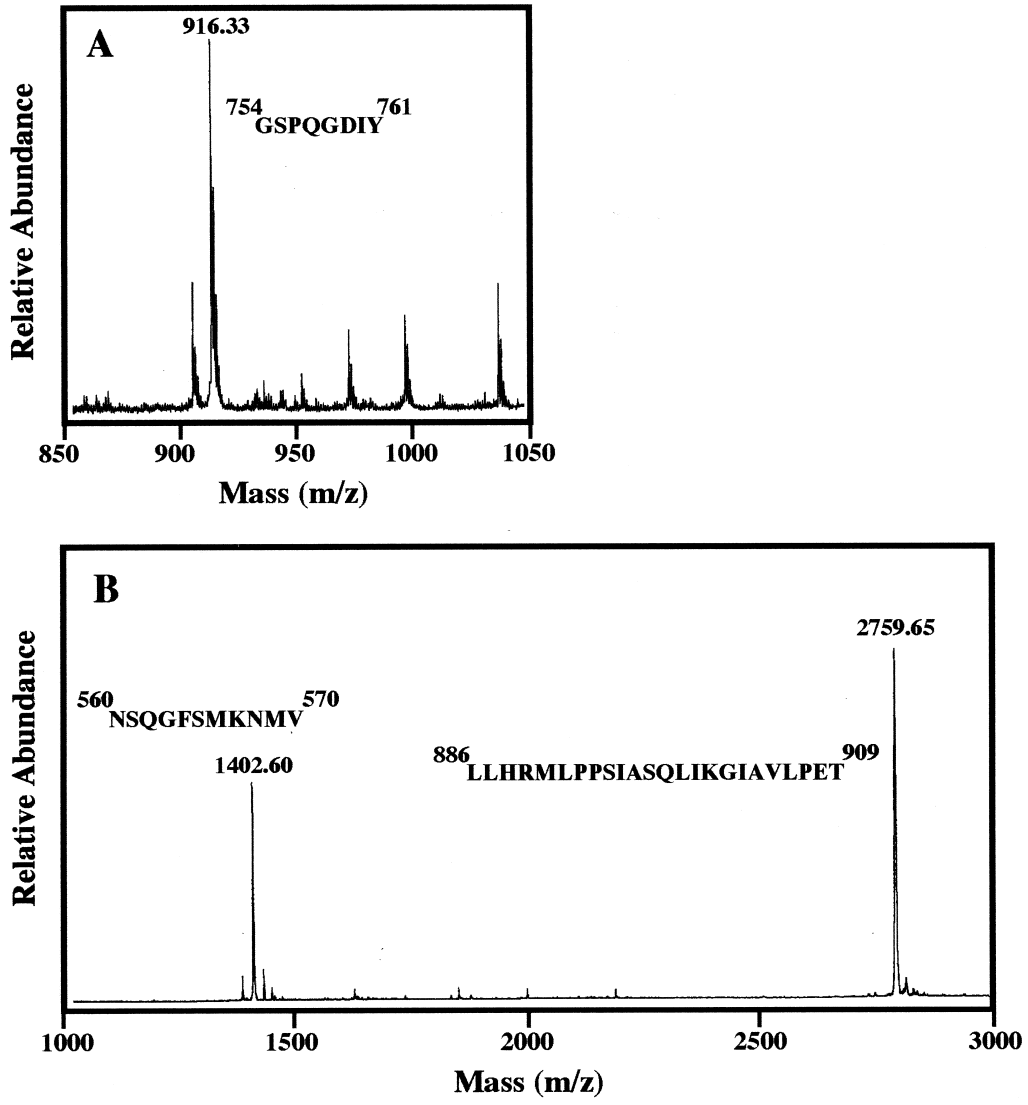


Fig. 4. The MALDI time-of-flight mass spectra of HPLC peaks. Peptides were subjected to mass spectrometry (**A**, peak 8; **B**, peak 3). The number on the peak denotes the mass number of the protonated molecular ion $[M + H]^+$. The amino acid sequences (insert) were assigned by the mass number and phosphate content.

652, 666, 740, 755 772, 894, 897, and 951 are phosphorylated. In each of these cases, serine is located in or near "turn" region of the predicted secondary structure (Fig. 6A). Furthermore, serine residues at the positions 894, 918, 927, and 930 in the catalytic domain are phosphorylated, and each of these is conserved in vertebrate mGCs except GC-C (Fig. 6A and B). The loss of phosphates associated with mGCs due to the binding of a specific sperm-activating peptide or an atrial natriuretic peptide has been reported to correlate with a decrease in enzyme activity (Harumi *et al.*, 1992; Potter and Garbers, 1992; Potter, 1998; Ramarao and Garbers, 1985; Vacquier and Moy, 1986). Therefore, we presume that it is not a far-flung speculation that the loss of charge in or near the "turn" region affects the stability of the secondary and tertiary structures of the enzyme and such a loss of charge causes the decrease in enzyme activity. At the present, however, we do not know which phosphoserines are dephosphorylated. However, one should make note of a recent study by Potter and Hunter (1998), who reported that reductions in the phosphorylation state of GC were caused by mutation of any one of the following phosphorylated serine or threonine residues into alanine residues: 4 serine residues at positions 497, 502, 506, and 510, and 2 threonine residues at 500 and 513 (within the ATP-binding site in the kinase-like domain of mature mammalian GC-A expressed in HEK 293 cells). Threonine residue at position 500 in mammalian GC-A corresponds to phosphoserine at position 565 in *H. pulcherrimus* sperm mGC. By continuing identification of the rest of phosphoserine and the sperm-activating peptide-inducible dephosphorylating sites using *H. pulcherrimus* sperm mGC, we may be able to provide useful data to support the hypothesis that guanylyl cyclase-linked receptors are regulated by phosphorylation/dephosphorylation.

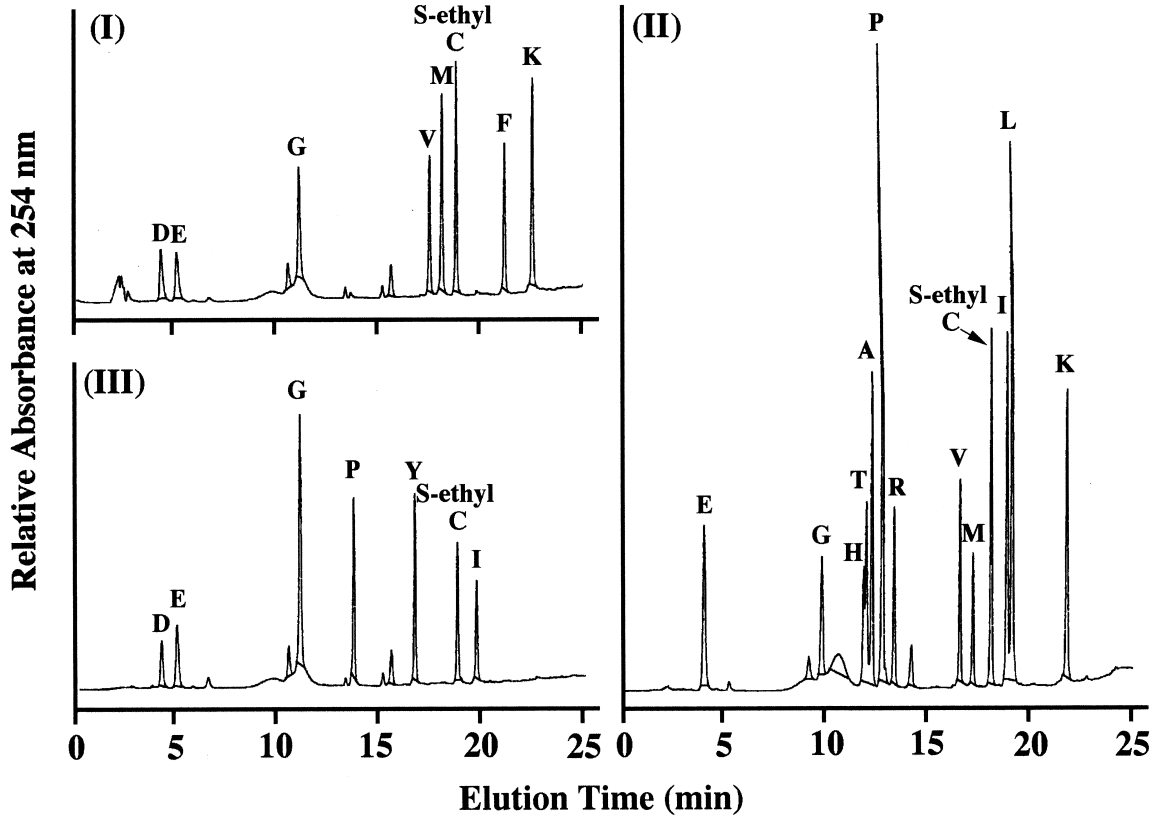


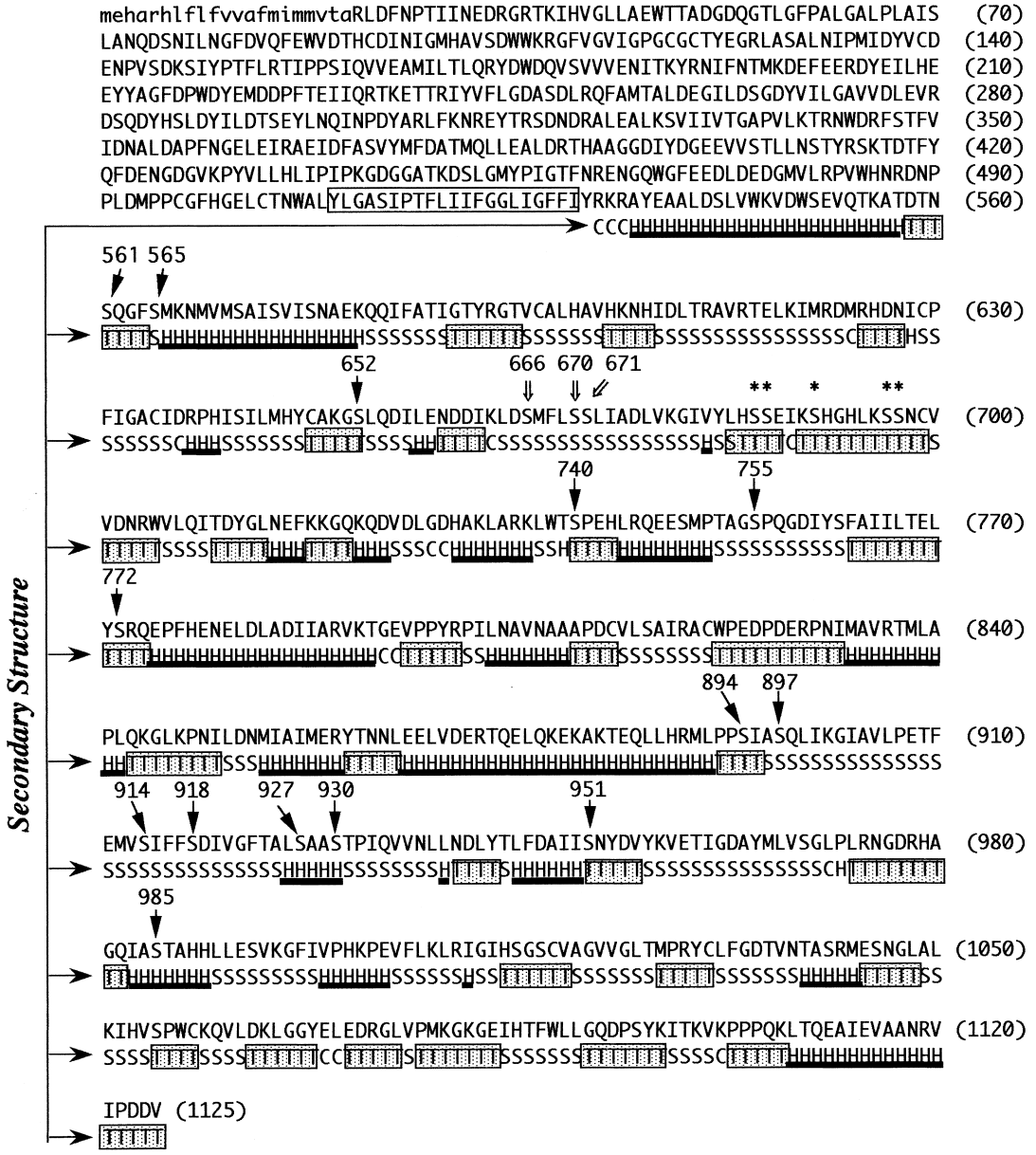
Fig. 5. The amino acid analysis of phosphoserine-containing peptides. As shown in Fig. 4B, the HPLC peak 3 contained two peptides with a molecular mass of 1402.60 or 2759.65. Peptides containing phosphoserine, which were converted to S-ethylcysteine, were separated from each other by HPLC on a Superiorex ODS column with a linear gradient of ACN from 5 to 60% in 0.1% TFA. The peptides with a molecular mass of 1402.60 (peak 3-1), 2759.65 (peak 3-2), and 916.33 (peak 8) were analyzed for the amino acid composition. (**I**), chromatogram for peak 3-1; (**II**), chromatogram for peak 3-2; (**III**), chromatogram for peak 8.

Table 2. Amino acid sequence of phosphoserine-containing peptides identified by mass spectrometry and amino acid analysis

Observed mass number $(M + H)^+$	Molecular mass calculated from predicted sequence		Predicted sequence	S-Ethylcysteine determined by amino acid analysis (residues/peptide)	Numbers of peak in HPLC
	without phosphate	with phosphate			
1402.60	1241.60	1400.60	(560) NSQGFSMKNMV (570)	1.5	3 (3-1)
788.992	708.00	788.0	(565) SMKNMV (570)	0.7	7*
958.098	877.10	957.10	(646) HYCAKGSL (653)	0.8	5*
1186.92	1025.90	1185.90	(663) KLDSMFLSS (671)	1.7	14
1471.69	1310.70	1470.70	(664) LDSMFLSSLIAD (675)	1.7	8*
1510.72	1509.70		(685) SSEIKSHGHLKSSN (698)[#]	0.0	19
1961.17	1880.20	1960.20	(734) ARKLWTSPEHLRQEE (748)	0.5	16*
916.33	835.30	915.30	(754) GSPQGDIY (761)	0.5	8
2088.20	2007.00	2087.20	(768) TELYSRQEPFHEHELD (783)	0.7	2
2759.65	2598.70	2758.70	(886) LLHRMLPPSIASQLIKGIAVLPET (909)	1.5	3 (3-2)
1087.52	926.50	1086.50	(914) SIFFSDIV (921)	1.5	18
1734.67	1573.70	1733.70	(920) IVGFTALSAASTPIQV (935)	1.8	11
990.199	909.20	989.20	(947) DAIISNYD (954)	0.7	15*
677.15	596.10	676.10	(951) SNYDV (955)	1.0	5
745.849	664.80	744.80	(985) STAHHL (990)	0.7	12*

[#] This peptide did not contain phosphoserine although it was isolated by affinity chromatography of the trypsin digest of the phosphorylated *H. pulcherrimus* sperm mGC on a Chelating Sepharose Fast Flow column. The numbers of peaks with asterisks (*) denote peptides obtained from the second HPLC.

A



B

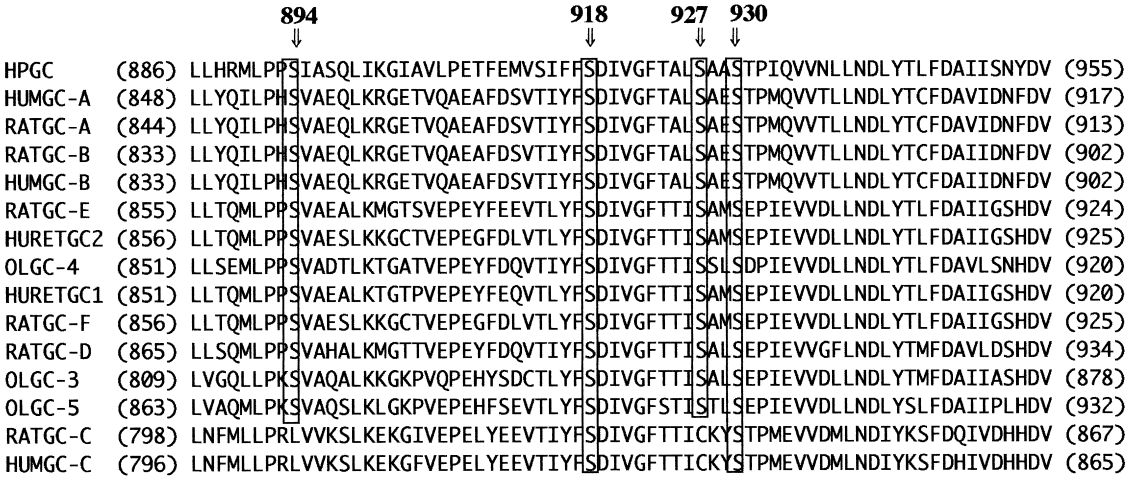


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Fig. 6. (**A**) Amino acid sequence of *H. pulcherrimus* sperm mGC. The residue number is listed in parentheses on the right hand side. Phosphorylated serines are indicated by solid arrows with residue numbers. Two of three serine residues indicated by open arrows with numbers are phosphorylated. The serine residues with asterisks (*) at positions 685, 686, 690, 696, and 697 are not phosphorylated. Residues 600 - 720 and 860 - 1090 correspond to the kinase-like and catalytic domains, respectively. The secondary structure was predicted by Chou and Fasman (1978) using DNasis software: α-helix is indicated by H with bold underlines; turns are shown by a "T" with shaded-boxes; S and C denote sheet and coil, respectively. The signal sequence is indicated by small letters and the transmembrane sequence is boxed. (**B**) Sequence alignment of the region containing conserved serine residues. A part of the catalytic domain of *H. pulcherrimus* sperm mGC is compared with the sequences of human GC-A (HUMGC-A, S04459; Lowe *et al*., 1989), GC-B (HUMGC-B, S05514; Chang *et al*., 1989), GC-C (HUMGC-C, A40940; de Sauvage *et al*., 1991), retinal GCs (HURETGC1, M92432; Shyjan *et al*., 1992; HURETGC2, L37378; Lowe *et al*., 1995), rat GC-A (S03348; Chinkers *et al*., 1989), rat GC-B (A33300; Schulz *et al*., 1989), rat GC-C (A36292; Schulz *et al*., 1990), rat GC-D (L37203; Fülle *et al*., 1995), rat GC-E (A55915; Yang *et al*., 1995), rat GC-F (B55915; Yang *et al*., 1995), and medaka fish eye GCs (OLGC-3, AB000899; OLGC-4, AB000900; OLGC-5, AB000901; Seimiya *et al*., 1997). Conserved serine residues are boxed and also indicated by open arrows with numbers.

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