Serine-578 Is a Major Phosphorylation Locus in Human Plasma Plasminogen†

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ABSTRACT: It has been reported that human plasminogen (HPg) exists in plasma in a phosphorylated form. We now document that both major glycoforms of plasma HPg contain a phosphoserine residue in their latent protease chains, as revealed by quantitative protein phosphate determinations and ^{31}P-NMR analysis. The sequence location of the phosphoserine residue was established by time-of-flight matrix-assisted laser desorption ionization with delayed extraction mass spectrometric analysis of peptides resulting from complete tryptic and cyanogen bromide digests of the latent protease chain of HPg. Confirmation of the presence of organic phosphate in the identified peptide was obtained by determination of the resulting mass shift after treatment of the peptide with alkaline phosphatase. The data show that Ser^{578} is a major phosphorylation site in HPg.

HPg[1] is the zymogen form of the serine protease, HPm, the major enzyme responsible for eliciting the fibrinolytic response. The mature human-derived zymogen contains 791 amino acids in a single polypeptide chain (Forsgren et al., 1987), along with two sites of glycosylation. One glycan is present at Thr^{346}, a site that contains the disaccharide GalNAc-Gal, with microheterogeneity in sialic acid additions to this core unit (Hayes & Castellino, 1979a,c). Additionally, a consensus sequence, Asn-Arg-Thr, suitable for assembly of N-linked carbohydrate, is present at Asn^{289} (Hayes & Castellino, 1979b). Two major glycoforms of plasminogen have been identified and isolated (Brockway & Castellino, 1972). These differ on the basis of glycosylation at Asn^{289}, one form not containing a saccharide chain at this location, and the other form containing biantennary glycan, with variable sialylation (Hayes et al., 1975; Hayes & Castellino, 1979a,b; Powell & Castellino, 1983). The extent and nature of the glycosylation at Asn^{289} significantly influences the activation properties of HPg (Takada & Takada, 1983; Edelberg et al., 1990; Mast et al., 1991; Davidson & Castellino, 1993; Pirie-Shepherd et al., 1995, 1996), as well as its stability in plasma (Siefring & Castellino, 1974).

Another post-translational modification of HPg has been reported to involve sites of phosphorylation. On the basis of reactivity of HPg with specific phospho (P) amino acid monoclonal antibodies, it was concluded that both P-Thr and P-Tyr were present in this protein (Barlati et al., 1995). Similarly, phosphorylation was also found to exist on two other enzymes involved in fibrinolysis, *viz.*, uPA and tPA (Mastronicola et al., 1990; Barlati et al., 1991, 1995). This modification appears to result in functional consequences, at least in the case of uPA, in its HPg activation-related properties (Takayashi et al., 1992b) and the sensitivity of this enzyme to its naturally occurring inhibitors (Mastronicola et al., 1992).

In order to understand more fully the relationships between phosphorylation of HPg and HPm and their functions, the sites of protein phosphorylation must first be rigorously determined. Therefore, we have undertaken an investigation to identify the protein sequence locations of phosphorylated amino acids in HPg and have successfully accomplished this goal. This communication provides a summary of these findings.

MATERIALS AND METHODS

Proteins and Protein Fragments. The two major glycoforms of HPg were isolated by affinity chromatography, as previously described (Brockway & Castellino, 1972). Their concentrations were calculated from the absorbancies at 280 nm, using an ϵ (0.1%, 280 nm, 1 cm) of 1.70, which was determined from the amino acid composition of this protein (Edelhoch, 1967).

For generation of the heavy and light chains of HPg, the zymogen (0.25 mg/mL) was first activated with low-molecular-weight urokinase (Abbott Laboratories, North Chicago, IL) at a protein to enzyme ratio of 200:1 (mol:mol). The buffer used was 10 mM Na-Hepes/100 mM NaOAc/1 μg/mL aprotinin (Sigma Chemical Co., St. Louis, MO), pH 7.4. The reaction was maintained at room temperature for 50−60 min with gentle stirring. The time course of activation was monitored by SDS−PAGE and these conditions were found to fully activate HPg into HPm. Following this, guanidine-HCl was added to the reaction mixture to a final concentration of 4 M. The solution was then purged with N_2 for 30 min at room temperature. Next, dithiothreitol (Aldrich Chemical Co., Milwaukee, WI) was added to a final concentration 20 mM. The subsequent reaction was retained under N_2 for another 3−4 h, after which time it was terminated by dialysis against four exchanges of 4 L of 7% HOAc. The reduced HPm sample was then concentrated and loaded onto a Sephadex G-200 column (2 cm × 100 cm), previously equilibrated in 7% acetic acid. The flow rate was 7 mL/h. Column fractions were monitored

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[1] Abbreviations: HPg, human plasma plasminogen; HPm, human plasmin; uPA, urokinase-type plasminogen activator; tPA, tissue-type plasminogen activator; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TOF-MALDI-DE-MS, time-of-flight matrix-assisted laser desorption ionization with delayed extraction mass spectrometry; RP-HPLC, reverse phase-high performance liquid chromatography; NCS, *N*-chlorosuccinimide; MAb, monoclonal antibody.

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by absorbancies at 280 nm and SDS−PAGE. Two major fractions were obtained, corresponding to the heavy and light (protease) chains of HPm.

Tryptic Digestion of the HPm Protease Chain. The isolated protease chain of HPm was lyophilized and resuspended in 6 M guanidine-HCl/0.002 M EDTA/0.5 M Tris-HCl, pH 8.1, after which dithiothreitol was added to a final concentration of 0.020 M. The resulting solution was maintained at room temperature, under N_2, for 30 min. After this time, iodoacetic acid (recrystallized from hexane) was added to a final concentration of 41 mM. The pH was adjusted to 8.0 with ammonia, and the reaction allowed to proceed for 4 h under N_2 in the dark. The solution was then dialyzed against 1 mM HCl. After dialysis against 1 mM HCl and lyophilization, the reduced and S-carboxymethylated protease chain was digested with 1-chloro-3-(tosylamino)-7-amino-2-heptanone hydrochloride-treated trypsin (Sigma) at 37 °C at a 1:10 (mol:mol) enzyme to protein ratio in a solution of 50 mM NH_4HCO_3. After a 4 h incubation period, a second identical aliquot of trypsin was added and the digestion was continued for an additional 12 h.

Peptide Mapping by HPLC. The tryptic digestion mixture was injected onto a RP-HPLC C_4 column (214 TP54, 4.6 × 250 mm, Vydac, Hesperia, CA). The peptides were separated using a linear gradient from 94% solvent A (0.1% TFA acid in H_2O)/6% solvent B (0.1% TFA acid in CH_3CN) as the start solution, to 40% solvent A/60% solvent B as the limit solution. The gradient was applied for 54 min at a flow rate of 0.5 mL/min. The eluted peaks were detected by their absorbancies at 220 nm. The molecular mass of each fraction (0.25 mL) was evaluated by TOF-MALDI-DE-MS. Further purification by RP-HPLC of one of these peptides (no. 15) on a C_4 column was performed using a linear gradient from 75% solvent A/25% solvent B (start solution) to 65% solvent A/35% solvent B (limit solution) over 30 min at a flow rate of 0.5 mL/min. The material in the lone fraction was then pooled for dephosphorylation and molecular mass analysis.

Amino Acid Sequence Analysis. The methods employed in this laboratory for solid phase automated amino-terminal amino acid sequence determinations have been described in detail in a previous report (Chibber et al., 1990).

CNBr Digestion of the HPm Protease Chain. The isolated protease chain of HPm was dissolved in 70% HCOOH at a concentration of *ca.* 3 mg/mL. A 50-fold (w:w) excess over protein of CNBr was added to the solution and the reaction allowed to proceed for 12−14 h. Aliquots were removed for TOF-MALDI-DE-MS analysis to monitor the progress of the reaction, in order to confirm its completion over the time period indicated. The reaction was terminated by addition of H_2O.

After completion of the reaction, the solution was lyophilized and the components of the mixture separated on Sephadex G-25 (1 cm × 60 cm, in 5% HOAc). The products present in the eluate were identified by TOF-MALDI-DE-MS.

NCS Cleavage (Shechter et al., 1977). The peptide was dissolved at a concentration of approximately 0.7 mM in 50% HOAc. A volume of 3 mL of this protein solution was then mixed with 0.1 mL of NCS (0.3 M in DMF). The reaction was allowed to proceed at room temperature for 30 min and terminated by the addition of *N*-acetyl-L-methionine. The resulting sample was directly subjected to TOF-MALDI-DE-MS analysis.

Enzymatic Dephosphorylation of Peptides (Mellgren et al., 1977). The selected peptides were dephosphorylated with calf intestinal mucosa alkaline phosphatase (Sigma). An aliquot of peptide (5−10 pmol) was dried and redissolved in 20 μL of 25 mM NH_4HCO_3, pH 7.9, and 5 μL of alkaline phosphatase (125 units). The reaction was allowed to incubate at 37 °C for 1 h.

Phosphate Analyses. The determination of the amounts of organic phosphate in proteins and peptides was performed as previously described (Ames, 1966) with minor operational adjustments. Specifically, a solution of $NaH_2PO_4 \cdot H_2O$ (98.6 mM) was employed as the stock standard solution. HPg samples were exhaustively dialyzed against 0.1% TFA. The standard solution and protein samples were dried overnight in clean Pyrex test tubes at 60 °C. A volume of 0.1 mL of ashing reagent [10% $Mg(NO_3)_2 \cdot 6H_2O$ in 95% ethanol] was added to each tube, which was then heated over a flame until the brown fumes disappeared and a white residue remained. After the tubes were cooled to room temperature, 0.3 mL of 0.5 N HCl was added, the tubes were capped with marbles and then heated in a boiling water bath for 15 min to hydrolyze to phosphate any pyrophosphate formed in the ashing step. After cooling, 0.7 mL of the phosphate reagent (1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate·$4H_2O$ in 1 N H_2SO_4, freshly mixed) was added into each tube and incubated for 20 min in a 45 °C water bath. Absorbances were determined at 820 nm, and the colors were stable for several hours. A standard curve was constructed each time the assay was performed and the amount of phosphate in each protein sample was calculated from the resulting absorbance and the standard curve. No significant inorganic phosphate background was observed. To ascertain that the glassware used was free of phosphate, especially from detergents, new Pyrex tubes were used each time and were not reused for this assay.

TOF-MALDI-DE-MS. Mass spectral data were obtained on a PerSeptive Biosystems (Framingham, MA) Voyager-DE BioSpectrometric Workstation equipped with a nitrogen laser (337 nm, 4 ns pulse). The accelerating voltage of the ion source was 20 kV. Data were acquired with a 20 MHz digitizer. The matrix used for the peptides was α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid was used for the P-6-$(Man)_5$ (Bretthauer et al., 1973) and $(Man)_5$ standards. The matrix material was dissolved in aqueous CH_3CN (33%, v/v) to give a saturated solution at 20 °C. A volume of 0.5 μL of matrix was mixed with 0.5 μL of sample prior to loading on the plate.

^{31}P-NMR (Brauer & Sykes, 1984). The ^{1}H-coupled-^{31}P-NMR spectra of HPg and its component protease chain were acquired on a Varian 500 MHz NMR in 8% acetic acid/10% ^2H_2O, using a 10 mm probe. An internal capillary standard of 85% H_3PO_4 was employed. A pulse of 17 s was implemented with an acquisition time of 1.64 s and a delay time of 1.0 s. A total of 25 370 transients were accumulated. The sample concentration was 5−10 mg/mL.

Western Analysis with Anti-Phosphoamino Acid Antibodies. Western blotting was accomplished using anti-phosphoserine (anti-P-Ser), anti-phosphothreonine (anti-P-Thr), and anti-phosphotyrosine (anti-P-Tyr) MAbs (Sigma) at 1:2000 dilutions in 1% milk. The buffer employed was 10 mM Tris-HCl/150 mM NaCl, pH 7.4. For the competitive

assay, the same protein sample was transferred onto three membranes and incubated with anti-P-Ser MAb alone and with anti-P-Ser in the presence of 20 mM P-Thr and 20 mM P-Tyr, respectively.

RESULTS

Quantitative organic phosphate determinations have been carried out on both HPg glycoforms (HPg I and HPg II). On the basis of duplicate analyses of five different protein concentrations of three independent assays, we found that HPg I contains 0.98 ± 0.03 mol of phosphate/mol of protein and HPg II contains 0.99 ± 0.03 mol of phosphate/mol of protein. Similar values were found for the HPm light chain, and the heavy chain contained <0.2 mol of phosphate/mol of peptide. Thus, it is clear that HPg contains 1 mol equiv of a phosphate group that appears to reside on its latent light chain and that the two glycoforms are identical in that regard. Western immunoblotting of the HPg with anti-P-Ser antibodies yielded a positive stain in HPg, which was not present when the antibody staining was performed in the presence of the free amino acid P-Ser. The amino acids, P-Thr and P-Tyr, did not displace the anti-P-Ser antibody in this experiment. This finding is suggestive of the specificity of the reaction with P-Ser. When this latter experiment was performed on the component HPm chains, the protease chain, but not the nonprotease chain, provided the same results as observed with HPg. Thus, we concluded that P-Ser was present on the latent protease chain of HPg.

These findings were confirmed by analysis of the isolated protease chain by TOF-MALDI-DE-MS. In this case, a single species was observed of molecular mass 25 286 Da, which corresponds exactly to the theoretical molecular mass of the phosphorylated light chain of HPg, *viz.*, 25 286 Da. After dephosphorylation with alkaline phosphatase, the molecular mass was reduced to 25 188, which is less than 0.06% deviation from the theoretical molecular mass (25 206) of the apo-light chain of HPm. In some preparations of HPm light chain, a lower molecular mass species was observed. Its dephosphorylated form possessed a molecular mass of 15 025. The amino-terminal amino acid sequence of this material was found to be VVGG, which was the same as that of the HPm light chain. Assuming, then, that its molecular basis was due to plasminolytic cleavage at the carboxy-terminus of the HPm light chain, this peptide would uniquely correspond to residues $V^{562}-K^{698}$, which possesses a theoretical molecular mass of 15 031.4. Thus, a HPm sensitive site in its protease chain has been uncovered at $K^{698}-E^{699}$. Since this material was phosphorylated and contained an intact amino-terminus, we have combined it in some experiments with the intact HPm light chain.

Regarding the nature of the phosphorylated amino acid(s) on the HPg, previous qualitative work with anti-phosphoamino acid antibodies of this type had indicated that only P-Thr and P-Tyr were present on HPg (Barlati et al., 1995). When these latter antibodies were examined in our system, reactivity was also observed with HPg, and with the heavy chain of HPm, despite the fact that direct phosphate determinations did not reveal significant levels of phosphate on the HPm heavy chain. Also, a control experiment with phosphorylase a, which is well-known to contain only a P-Ser residue, also reacted positively with all three phosphoamino acid antibodies. Thus, while it is possible that the HPm heavy chain contains low levels of P-Thr, P-Tyr, and/or P-Ser, and in fact such an observation has been made for P-Ser by more direct methods (Pirie-Shepherd et al., 1997), we were left at this point with reservations regarding the specificity of these antibodies for the phosphorylated amino acids in these proteins.

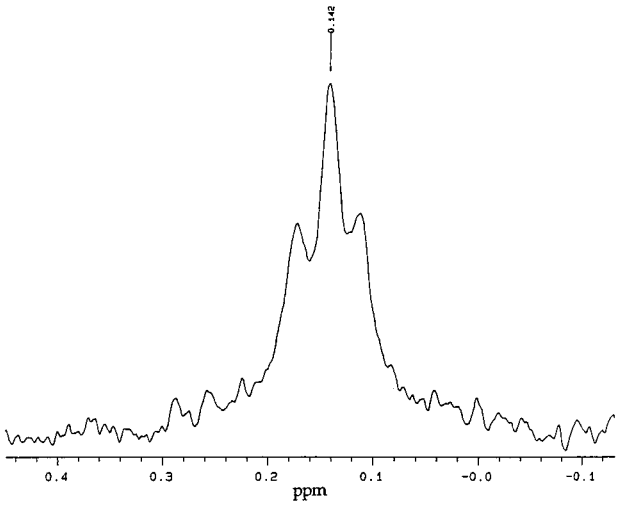

FIGURE 1: ^{1}H-coupled-^{31}P-NMR spectrum of the protease chain of HPg I. The spectrum was acquired on a 500 MHz NMR, in 8% HOAc/10% $^{2}H_2O$/82% H_2O, using a 10 mm probe. An internal capillary standard of 85% H_3PO_4 was used for concentration calibration and to set the reference point a 0 ppm and removed prior to acquiring the spectrum. A pulse of 17 s was employed with an acquisition time of 1.64 s and a delay time of 1.0 s. A total of 25 370 transients were accumulated. The sample concentration was 5.1 mg/mL. The chemical shifts are referenced to a H_3PO_4 standard.

Since the identity of the phosphorylated amino acid(s) in the HPg latent light chain was not fully clarified by use of the anti-phosphoamino acid antibodies, we decided to rigorously identify the amino acid by ^{31}P-NMR. First, each HPg glycoform was converted into the respective two-chain HPm. Following reduction and alkylation, the light (protease) chains were isolated by gel filtration. The ^{1}H-coupled-^{31}P spectrum obtained on the HPg I-derived latent protease chain is shown in Figure 1. Similar spectra were obtained for intact HPg I and HPg II and the latent protease chain of HPg II. The triplet of the fully protonated phosphate that was observed is highly characteristic of the presence of P-Ser (Brauer & Sykes, 1984), since the phosphate is split by the two protons present on the β-carbon. On the basis of these same considerations, the phosphate of P-Thr would exist as a more upfield-shifted doublet (split by a single proton) at approximately 0.9−1.0 ppm at acid pH, and the ^{1}H-coupled-^{31}P resonance of P-Tyr would give rise to a further upfield-shifted singlet, at approximately 4.0−4.1 ppm, at acid pH. A similar spectrum for P-Arg would be present much farther upfield (*ca.* 6.0−6.1 ppm) than that of P-Tyr (Brauer & Sykes, 1984). Thus, no evidence of P-Thr or P-Tyr was found in these analyses. Additionally, using this method, the molar stoichiometry of the P-Ser to the HPm protease chain was approximately 1.2:1, clearly demonstrating that a P-Ser in the light chain of HPm is the major amino acid that is phosphorylated in both HPg I and HPg II. Separate ^{31}P-NMR experiments on the isolated heavy chains of HPm did not result in observable phosphate signals. Thus, levels of phosphorylation in the heavy chain region, if at all present, are much less than in the light chain.

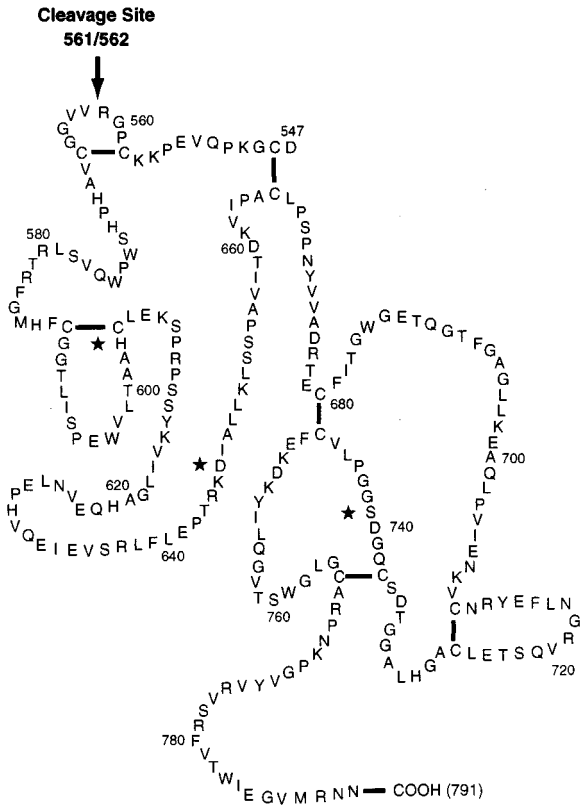

FIGURE 2: Amino acid sequence of the human plasmin protease chain. The sequence shown begins at Asp^{547} and the diagram illustrates the $R^{561}-Val^{562}$ activation cleavage site. The protease (light) chain sequence begins at Val^{562}. The serine protease catalytic triad is located at His^{603}, Asp^{646}, and Ser^{741}. The phosphorylated serine is Ser^{578}.

In order to reveal the sequence position of the P-Ser in the HPm light chain, the reduced and alkylated polypeptides were subjected to tryptic digestion and the peptides separated by RP-HPLC. The amino acid sequence of this peptide chain is provided for reference in Figure 2. The HPLC peptide profile obtained for HPg I is shown in Figure 3A. Each fraction (0.25 mL) of this tryptic digest was subjected to molecular weight analysis by TOF-MALDI-DE-MS. The data of Table 1 show that only one of the peptides (no. 15), of calculated molecular mass 2 174.5, and uniquely corresponding to residues $Val^{562}-Arg^{580}$, possessed a molecular mass of approximately 80 Da higher (its Na^+ adduct at 2 279.6 Da and its $2Na^+$ adduct at 2 310.4) than expected (Table 1 and Figure 4A). After further purification of this peptide (Figure 3B) and treatment with alkaline phosphatase, its molecular mass was reduced to 2 173.4, plus its Na^+ (2 195.5) and $2Na^+$ (2 217.2) adducts (Figure 4B clearly illustrates the Na^+ adduct). Similar data were obtained for HPg II when its light chain was subjected to this same analysis. Positive control experiments were designed to demonstrate the effectiveness of this mass spectrometric strategy. Here, samples of P-6-$(Man)_5$ and its alkaline phosphatase-cleaved products were similarly analyzed. It was found that P-6-$(Man)_5$ possessed a molecular mass distribution of 933.42 and 955.2, corresponding to the Na^+ and $2Na^+$ adducts of the phosphate group (calculated $M + 2H^+ = 908.7$). Determination of the molecular mass of the alkaline phosphatase-treated product yielded a single species at 853.3, corresponding extremely well to the theoretical mass ($M + Na^+ = 851.4$) of $(Man)_5$.

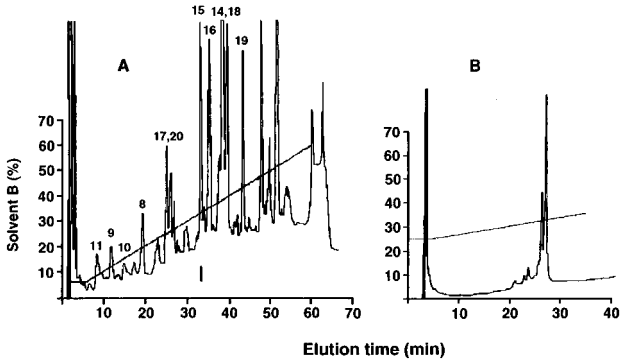

FIGURE 3: HPLC separation of the tryptic peptides of the reduced and S-carboxymethylated protease chain of HPg. (A) The tryptic digest was applied to a C4 column. The peptides were separated using a linear gradient from 94%/6% to 40%/60% solvent A [0.1% TFA in H_2O/solvent B (0.1% TFA in CH_3CN)] over a 54 min time period at a flow rate of 0.5 mL/min. The peaks were detected by the absorbancy at 220 nm. Each fraction (0.25 mL) was evaluated by TOF-MALDI-DE-MS. The numbers above the peaks refer to those in Table 1. The elution position of the phosphate-bearing peptide is indicated by the vertical bar. (B) Further purification of peptide P15 on the C4 column. The linear gradient employed in this case was from 75% A/ 25% B to 65% A/ 35% B over a time of 30 min, at a flow rate of 0.1 mL/min. The full scale absorbance was 0.1.

Confirmation that phosphorylation occurred at the region of the molecule encompassing Ser^{572} and Ser^{578} was obtained by use of another cleavage strategy. The HPm I (or HPm II) protease chain was digested with CNBr. This treatment should result in three peptides, corresponding to amino acid sequences of $V^{562}-M^{585}$, $H^{586}-M^{788}$, and $R^{789}-N^{791}$. After gel filtration, TOF-MALDI-DE-MS analysis provided major fractions with a molecular masses of 22 095 and 2 740.2. These correspond closely to nonphosphorylated $H^{586}-M^{788}$ (calculated, 22 083.4), and phosphorylated $V^{562}-M^{585}$ (calculated, 2 741.1), with homoserine lactone in place of the COOH-terminal Met residues. The other expected CNBr-derived peptide, $R^{789}NN$ (calculated molecular mass, 403.4 Da) was not observed by this type of mass spectrometric analysis. Minor fractions (<10% total) with molecular masses of 24 873, 15 088, 12 401, and 7 314 were also observed. Employing molecular mass and NH_2-terminal sequence analyses, these fractions were found to most likely correspond to the following peptides; phospho-$V^{562}-M^{788}$ (calculated, 24 853.5, Na^+ adduct, 24 876.5), phospho-$V^{562}-K^{698}$ (calculated, 15 111.5), $H^{586}-K^{698}$ (calculated, 12 341.3, Na^+ adduct, 12 364.3), and $V^{720}-M^{788}$ (calculated, 7 286.3, Na^+ adduct, 7 309.3), with homoserine lactone substituted for terminal Met residues. This latter peptide probably resulted from a plasminolytic cleavage at $R^{719}-V^{720}$. After dephosphorylation of the original CNBr-derived 2 740 Da peptide with alkaline phosphatase, a single species, at molecular mass 2 660.4 resulted, which corresponds almost exactly to that of the dephospho-peptide, $V^{562}-M^{585}$ (calculated molecular mass, 2 661.2). Thus, from an independent approach, we find that one of the two Ser residues, *viz.*, Ser^{572} or Ser^{578}, is phosphorylated in HPg. Similar data were obtained for analyses conducted with the HPg II protease chain.

To determine which of these two Ser residues was the modified amino acid, the above CNBr-derived phosphopeptide was treated with NCS/HOAc. The reaction mixture was then analyzed by TOF-MALDI-DE-MS, and the data obtained are presented in Figure 5. Assuming the proposed

Table 1: Tryptic Peptides Isolated from HPg and Identified by TOF-MALDI-MS

peptide	*m/z*[a] theory	*m/z*[a] observed	sequence[b]
1	146.2		K^{645}
2	246.2		$N^{790}N$
3	261.3		$D^{751}K$
4	275.3		$T^{581}R$
5	360.4		$V^{777}SR$
6	548.6		$V^{709}\overline{C}NR$
7	671.8		$D^{646}IALLK^{651}$
8	875.0	876.7	$L^{638}FLEPTR^{644}$
9	898.0	898.5	$Y^{713}EFLNGR^{719}$
10	921.0	921.6	$\underline{S}^{608}PRP\underline{SS}YK^{615}$
11	1030.2	1033.5	$\overline{L}^{652}\underline{SS}P\overline{A}VITDK^{661}$
		1055.6[c]	
12	1140.3		$E^{699}AQLPVIENK^{708}$
13	1237.5		$F^{780}VTWIEGVMR^{789}$
14	1772.0	1776.1	$V^{662}IPACLP\underline{S}PNYVVADR^{671}$
15	2174.5	2279.6[c]	$V^{562}VGGC\overline{V}AHPH\underline{S}WPWQV\underline{S}LR^{580}$
	2310.4[d]		
16	2374.5	2377.7	$T^{678}ECFITGWGETQGTFGAGLLK^{698}$
17	2496.8	2501.2	$V^{616}ILGAHQEVNLEPHVQEIEV\underline{S}R^{637}$
18	2693.1	2696.2	$Y^{753}ILQGVT\underline{S}WGLGCARPNKP\overline{G}VYVR^{776}$
19	2864.2	2866.8	$F^{583}GMHFC\overline{G}GTLI\underline{S}PEWVLTAAHCLEK^{607}$
20	3241.4	3245.4	$V^{720}Q\underline{S}TELCAGHL\overline{A}GGTD\underline{S}CQGD\underline{S}GGPLVCFEK^{750}$

[a] The theoretical molecular masses were increased by 59 for each Cys residue to account for the CH_2COOH group added as a result of the alkylation modification. [b] The amino acid sequence begins at Glu^1 of human plasminogen. [c] Na^+ adduct. [d] $2Na^+$ adduct.

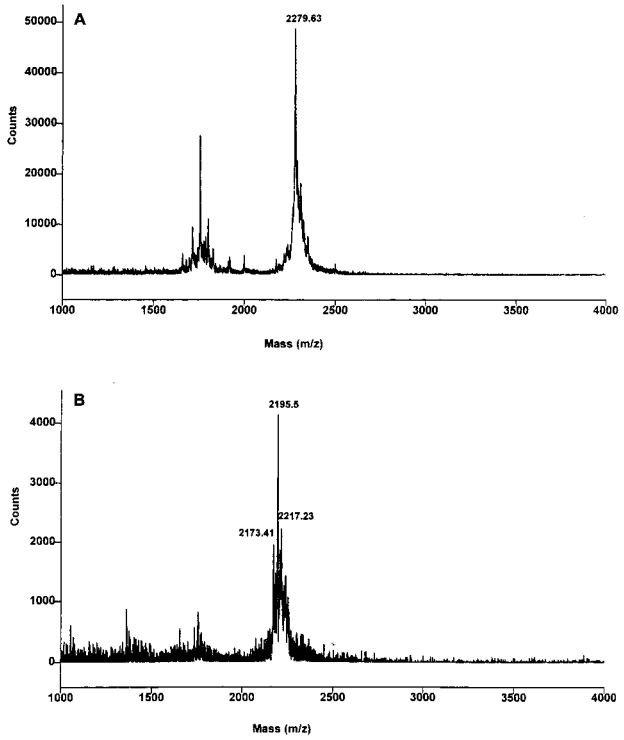

FIGURE 4: TOF-MALDI-DE-MS of reduced and carboxymethylated tryptic peptide P15. (A) The intact peptide. The observed molecular mass was 2279.9, consistent with the Na^+ adduct of the tryptic peptide P15, *viz.*, V^{562}VGGC(Cm)VAHPHSWPWQVSLR^{580}, containing one phosphate group (theoretical mass, 2277.5 Da). (B) The sample in panel A, after treatment with alkaline phosphatase. The peak at mass 2195.9 Da is the Na^+ adduct of dephosphopeptide P15 (theoretical mass, 2197.5).

chemistry for the Trp cleavage for purposes of molecular mass calculations (Shechter et al., 1977), possible apopeptides of this digestion would be $V^{562}-W(O)^{573}$ (calculated molecular mass, 1 323.5), $V^{562}-W(O)^{575}$ (calculated molecular mass, 1 545.8), and $Q^{576}-$M(homoserine lactone)^{585} (calculated molecular mass, 1 147.3). The major products observed possess molecular masses of 1 226.8, 1 542.6, and 1 565.4. These peptides uniquely correspond to $Q^{576}-$M(homoserine lactone)^{585}, which is phosphorylated at Ser^{578}, nonphosphorylated $V^{562}-W(O)^{575}$, and the Na^+ adduct of nonphosphorylated $V^{562}-W(O)^{575}$. After treatment with alkaline phosphatase, only the 1 226.8 peptide showed a decrease in its molecular mass to a value of 1 146.7, a value nearly identical to the dephosphorylated $Q^{576}-$M(homoserine lactone)^{585}. The only other mass species noted possessed a molecular mass of 1 560.0, which is the Na^+ adduct of nonphosphorylated $V^{562}-W(O)^{575}$, and was unchanged from the mass of this species prior to alkaline phosphatase addition. Interestingly, the peptide that would have resulted from NCS-based cleavage at W^{573} was not observed, a result demonstrating that only one of these two closely spaced W residues was reactive with NCS during this time period. Since only a single residue of phosphate was found in this $V^{562}-W^{575}$ peptide, this entire set of cleavage and mass analyses are entirely and uniquely consistent with Ser^{578} as the major phosphorylation locus of HPg.

DISCUSSION

Previous studies have shown that fibrinolytic components, plasma HPg, and tumor cell-derived uPA and tPA, existed as phosphorylated proteins (Mastronicola et al., 1990; Barlati et al., 1991, 1995; Takayashi et al., 1992a). While these investigations have provided confirmatory evidence of the existence of phosphorylated forms of these proteins, the identity and location within the proteins of the amino acids that are modified in this manner are unknown or unclear. Conflicting evidence has appeared regarding this point in the case of the most intensely studied protein of this group, uPA. Specifically, it was originally reported that approximately 20−50% of the intracellular and secreted pro-uPA was modified by phosphorylation in both human epidermoid carcinoma A431 and human fibrosarcoma HT1080 cells. Additionally, only Ser residues in the latent nonprotease (A) and protease (B) chains of these proenzymes were

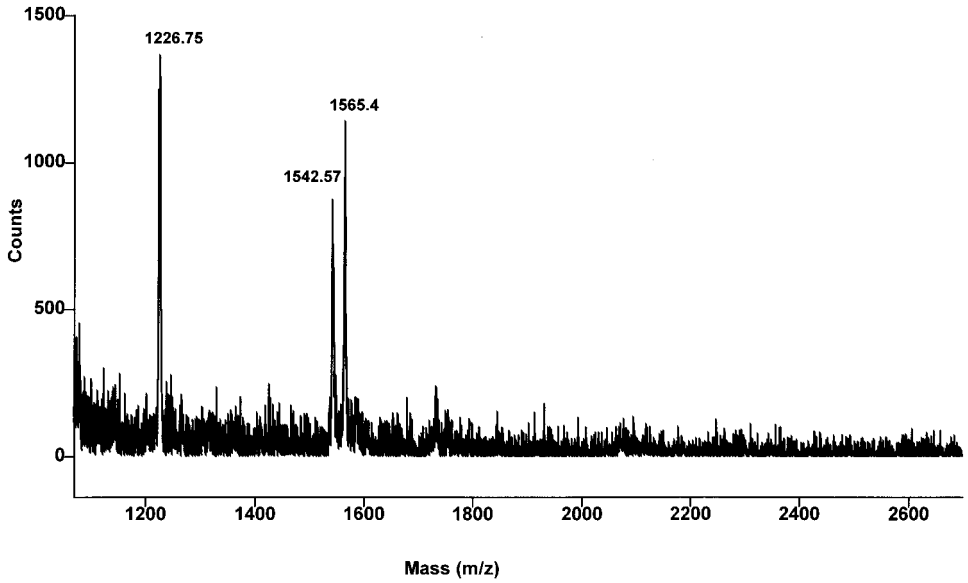

FIGURE 5: TOF-MALDI-DE-MS of the CNBr-derived phosphopeptide of the HPm protease chain after treatment with NCS. The mass peak of 1542.7 Da corresponds to the peptide fragment, V^{562}VGGCVAHPHS^{572}WPW(O)^{575} (theoretical mass, 1545.8 Da), and its Na^+ adduct is observed at molecular mass, 1565.4. These peptides are not phosphorylated. The peak at mass 1226.8 corresponds to the fragment Q^{576}VS^{578}LRTRFGM(homoserine lactone)^{585}, with one phosphate group (theoretical mass, 1227.3 Da).

found to be modified in this manner (Mastronicola et al., 1990). On the other hand, another investigation demonstrated that uPA secreted from HT1080 cells apparently contained a considerable number of P-Tyr residues, particularly in the protease (B) chain of this protein (Barlati et al., 1991). Further confounding the issue was the later discovery that while both P-Ser and P-Tyr residues were observed in uPA bound to surface receptors of pharangeal carcinoma Detroit 562 metastatic cells (Peterson et al., 1971), the P-Tyr residues were localized exclusively in the nonprotease (A) chain of the protein, and all six Tyr residues present therein were modified in this manner (Takayashi et al., 1992a). Finally, in a recent study, it was found that P-Ser, P-Thr, and P-Tyr were present in uPA, P-Thr, and P-Tyr in plasma-derived HPg and P-Ser and P-Tyr in human melanoma tPA (Barlati et al., 1995). These conflicting results were primarily based upon reaction of the relevant proteins with anti-phosphoamino acid antibodies. It is not known whether the differences were due to differing quality and/or nonspecificity of the antibodies employed or due to the types of kinases presented by viral oncogenes that may exist in different tumor cells. In any case, these uncertainties led us to examine the suitability of more direct approaches to reveal the exact locus of the phosphorylated sites on plasma HPg. Only with this type of information can we rationally proceed to functional investigations on *in vivo* and *in vitro* structure−function relationships on the role of phosphorylation in these systems.

Quantitative analysis of the organic phosphate released from HPg demonstrated clearly that the equivalent of a single phosphate residue was present in both glycoforms of plasma HPg and was primarily contained in the latent protease (light) chain of this protein. The ^{1}H-coupled-^{31}P-NMR spectrum of the protease chain displayed the triplet uniquely characteristic of P-Ser residues (Figure 1). Similar data were obtained when HPg I, HPg II, and the latent protease chain of these proteins were analyzed in this manner. Thus, we conclude from these data that HPg contained one residue of P-Ser in its latent protease chain as the major phosphorylation event. We cannot completely rule out small levels of phosphorylated amino acids in the HPm heavy chain, but, if present, these would not be major loci in mature plasma HPg. Digestion of this HPm light chain with trypsin, and analysis of the molecular masses of the peptides obtained by RP-HPLC (Figure 3), revealed that only one peptide (no. 15), composed of V^{562}−VGG(Cm-Cys)VAHPHS^{572}WPWQV-S^{578}LR, possessed a size that was larger by the mass of one phosphate group than expected from theoretical mass calculations of the peptides (Table 1). Treatment of peptide no. 15 with alkaline phosphatase reduced the molecular mass to the expected value of the apopeptide (Figure 4), thus demonstrating that the peptide was indeed modified with phosphate.

Since the amino acid sequence of the HPm protease chain contains only two residues of Met, a larger scale purification of the putative phospho-peptide was possible after cleavage with CNBr. In this case, the three peptides that result have greatly different molecular masses, thus simplifying their separation. Using this method with routine separation procedures led to isolation of the phospho-peptide, which encompassed residues V^{562}−M(homoserine lactone)^{585}. Two residues of Ser are present in this peptide, *viz.*, Ser^{572} and Ser^{578}. Unfortunately, the amino acids situated between these two Ser residues did not lend themselves to ready cleavage strategies. Thus, we relied on a more rarely employed chemical cleavage procedure with NCS, which should cleave the peptide at the carboxy-terminal side of Trp. Mass analysis of the peptide mixture subsequent to NCS cleavage (Figure 5) showed clearly that two peptides were formed, providing three mass species, one containing Ser^{572}, *viz.*, V^{562}−VGGCVAHPHS^{572}WPW(O)^{575}, its Na^+ adduct, and another containing Ser^{578}, *viz.*, Q^{576}VS^{578}LRTRFGM-(homoserine lactone)^{585}. Only this latter peptide contained a mass shifted by the size of one residue of phosphate. Thus, it is clear that the major phosphorylated amino acid in plasma HPg is Ser^{578}. The residue Thr^{581} cannot be phosphorylated in this latter peptide, since we found that the stoichiometry of phosphate/HPg was approximately 1.0, and that, from ^{31}P-NMR analysis, the relevant phosphorylated residue must be a Ser.

This direct finding that Ser^{578} is the phosphorylated site in HPg is not in agreement with previous work that excluded the presence of P-Ser in plasma HPg and which demonstrated that only P-Thr and P-Tyr were present as the phosphorylated residues in HPg (Barlati et al., 1995). While small levels of these residues in this protein cannot be excluded, especially since our studies on HPg with anti-P-Thr and anti-P-Tyr antibodies yielded faint reactivities with these antibodies, we currently attribute these latter findings to minor cross-reactivities of these commercial antibody preparations to other kinds of residues within HPg. In any case, if present at all, P-Thr and P-Tyr are very minor components of the molecular population of HPg, are not observed in the ^{1}H-coupled-^{31}P-NMR spectrum, and such minor phosphorylation is not observed in the mass analyses of the tryptic peptides containing Thr and Tyr that have been isolated.

During the mass analysis of the tryptic peptides of the HPg light chain, we did not find significant amounts of nonphosphorylated peptide no. 15. Thus, it is likely that Ser^{578} bears the only phosphate group present on the light chain of HPm. However, small amounts of this apopeptide, or of other phosphorylated peptides, present in the minor fractions of the HPLC chromatogram of Figure 3, cannot be entirely ruled out. Again, if present, such peptides would be minor components and would not influence the interpretation in a major manner. Only one Ser-containing protease chain-derived peptide, $V^{777}SR$, was not analyzed by this procedure, since it is too small to be identified by TOF-MALDI-DE-MS analysis. However, since the HPm light chain contains only one residue of phosphate, and since Ser^{578} appears to be fully phosphorylated, it is highly unlikely that Ser^{778} could contain significant amounts of phosphate.

The role for this P-Ser in HPg and/or HPm function awaits elucidation. It is possible that a relationship exists between phosphorylation and HPg secretion. With respect to uPA in this regard, at least part of the uPA produced in both A431 and HT1080 cancer cells was found to be phosphorylated intracellularly and secreted (Mastronicola et al., 1990), and receptor-bound uPA isolated from Detroit 562 cells was shown to be phosphorylated at Tyr residues. This reaction was catalyzed by the viral *src* gene product pp60 and phosphorylation on Ser residues was catalyzed by protein kinase C (Takayashi et al., 1992a). Since these cell types secrete a mixture of different types of phosphorylated species, as well as nonphosphorylated uPA molecules, phosphorylation does not appear to be a necessary step for secretion of this protein. However, the role and temporal nature of phosphatase action in relationship to the possible production of the variety and types of phosphorylated uPA and their contributions to production of nonphosphorylated uPA have not been studied.

In the case of uPA, phosphorylation was found to significantly influence its enzymatic properties. While P-uPA from tumor cells possessed only a 2−3-fold higher k_{cat}/K_m than nonphosphorylated human urinary uPA toward small synthetic substrates, its k_{cat}/K_m was nearly 15-fold lower for HPg activation. Additionally, the ability of P-uPA to interact with its naturally occurring inhibitors, plasminogen activator inhibitor (PAI)-1 and PAI-2, was diminished by 3−4-fold (Takayashi et al., 1992a). This latter property would serve to increase the activity of uPA *in vivo*, and perhaps contribute to the proteolytic activity and subsequent enhanced invasiveness of metastatic cells.

Phosphorylation is a fundamentally important event in regulating the *in vivo* and *in vitro* biological activity of proteins. Understanding the functional roles of phosphorylation in these proteins is dependent upon knowledge of the molecular details of the protein phosphorylation sites, which we have provided herein in the case of plasma HPg.

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