Cellular Phosphorylation of an Acidic Proline-Rich Protein, PRP1, a Secreted Salivary Phosphoprotein†

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ABSTRACT: Phosphorylation of many secreted salivary proteins is necessary for their biological functions. Identification of the kinase, which is responsible for in vivo phosphorylation, is complicated, because several of the protein phosphorylation sites conform both to the recognition sequence of casein kinase 2 (CK2) and Golgi kinase (G-CK), which both are found in the secretory pathway. This study was undertaken to determine the kinase recognition sequence in a secreted proline-rich salivary protein, PRP1, and thereby identify the responsible kinase. This was done by transfecting a human submandibular cell line, HSG, and a kidney cell line, HEK293, with expression vectors encoding wild-type or mutated PRP1. It was shown that phosphorylation occurred only at the same sites, Ser8 and 22, as in PRP1 purified from saliva. Phosphorylation at either site did not depend on the other site being phosphorylated. The sequence surrounding Ser8 has characteristics of both CK2 and G-CK recognition sequences, but destruction of the CK2 recognition site had no effect on phosphorylation, whereas no phosphorylation occurred if the G-CK recognition sequence was altered. The sequence surrounding Ser22 did not conform to any known kinase recognition sites. If Ser22 was mutated to Thr, no phosphorylation was seen, and a cluster of negatively charged residues at positions 27−29 was identified as part of the enzyme recognition site. Ser22 may be phosphorylated by a G-CK that recognizes an atypical substrate sequence or by a novel kinase. No difference in phosphorylation was seen between undifferentiated and differentiated HSG cells.

Saliva is usually supersaturated with respect to the basic calcium phosphate salts that form dental enamel, a property which provides protection for the teeth. An undesirable side effect of this is that unwanted precipitation of solid calcium phosphate may occur in the salivary glands, the fluid of the mouth, or on dental enamel (*1*). However, this is avoided by the presence of proteins such as statherin (*2*), a phosphoprotein that prevents calcium phosphate precipitation and growth of hydroxyapatite crystals (*1*). The latter activity is shared by acidic proline-rich proteins (APRPs)[1] (*1*, *3*). Statherin and APRPs loose these activities upon dephosphorylation (*1*). Moreover, the ability of APRPs to bind calcium and thereby aid in maintaining the concentration of ionic calcium in saliva also depends on phosphorylation of the proteins (*4*, *5*). In addition, dephosphorylation of APRPs decreases their binding to hydroxyapatite (*6*), thus preventing them from taking part in formation of dental pellicle, the thin layer of adsorbed proteins found on teeth which may provide protection against demineralization (*7*). While these biological functions of APRPs and statherin depend on phosphorylation of the proteins, the process whereby it occurs is not well understood.

Two enzymes have been implicated in phosphorylation of secreted proteins: Golgi kinase (G-CK) and casein kinase 2 (CK2) (*8*, *9*). While CK2 has been extensively characterized (*10*), G-CK has not been purified to homogeneity or cloned.

To elucidate phosphorylation of secreted salivary proteins, we have studied phosphorylation of APRPs, the most abundant phosphoproteins in saliva. A crude protein kinase from simian parotid glands was shown to phosphorylate APRPs at serines 8 and 22, the only residues that are phosphorylated in vivo (*11*), but other residues were phosphorylated as well, probably because of exposure of the substrate to enzymes in the preparation that it would not encounter during synthesis in the glands (*12*). Subsequently, a partially purified enzyme was prepared from human sublingual glands that phosphorylated a peptide substrate with the same sequence as found at Ser8 in APRPs. This enzyme preparation shared characteristics with both CK2 and G-CK, suggesting that the sublingual kinase had unique properties or that a mixture of kinases was present in the preparation (*13*). The latter possibility is supported by the fact that Ser8, one of the phosphorylated residues, is located in the sequence 8-SQED-11 which is in agreement with the SXE/S(P) substrate recognition sequence for G-CK (*8*) as

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[1] Abbreviations: APRP, acidic proline-rich protein; CK2, casein kinase 2; DAB, diaminobenzene; DMEM, Dulbecco's minimum essential medium; ECL, enhanced chemiluminesence reagent; FCS, fetal calf serum; G-CK, Golgi kinase; Gla, γ-carboxyglutamic acid; PBS, phosphate-buffered saline; PRP1, proline-rich protein 1; RIPA, 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS; S(P), phosphoserine; TBS-T, 20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20; Y(P), phosphotyrosine.

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well as the S/TXXD/E/S(P)/Y(P) recognition sequence for CK2 (*14*). The other phosphorylated residue, Ser22, is located in the sequence 22-SEQF-25, which does not agree with the substrate recognition sequence of either kinase or of any other kinase, supporting the possibility that the sublingual kinase has unique properties.

To identify the substrate recognition sequence for the kinase that phosphorylates secreted salivary proteins in vivo, we have studied cellular phosphorylation of PRP1, an APRP, in tissue culture. For these experiments, we have transfected a human submandibular cell line (HSG) (*15*) with a vector encoding PRP1 or a mutated PRP1. By mutating selected residues in the sequences surrounding the phosphorylation sites in PRP1, it can be determined if for example CK2 or G-CK is responsible for phosphorylation. HSG cells have an undifferentiated appearance when grown on plastic, but when plated on matrigel, they form structures similar to salivary gland acini (*16*). It is therefore of interest to evaluate if the morphological differentiation is accompanied by an increase in protein kinase activity. To evaluate if the kinase has a more widespread occurrence, additional experiments were done with a human kidney cell line (HEK 293), since the kidney is also known to secrete phosphoproteins (*17*). The pattern of phosphorylation was identical in HEK293 cells, indicating that the kinase is also present in cells derived from other tissues that secrete phosphoproteins.

MATERIALS AND METHODS

Materials. All reagents for recombinant DNA work were of molecular biology grade and were purchased from New England Biolabs (Mississauga, ON, Canada) and LifeTechnologies Inc. (Burlington, ON, Canada). Restriction enzymes and buffers were purchased from New England Biolabs (Mississauga, ON, Canada). Plasmid DNA purification kits, Qiafilter, and Qiagen Maxi-prep kits were provided by Qiagen (Mississauga, ON, Canada), and GeneClean DNA purification kits were obtained from Bio101, Inc. (La Jolla, CA).

The oligonucleotides used in PCR-mediated mutagenesis and in sequencing reactions were synthesized on a Pharmacia LKB Gene Assembler automated oligosynthesizer at the Hospital for Sick Children Biotechnology Service Centre (Toronto, ON, Canada) and purified using an OPC cartridge. The plasmid vector pCRII-PRP1, containing the coding sequence for PRP1, an APRP, cloned in the commercially available vector pCRII (Invitrogen, Carlsbad, CA) was a gift from Dr. D. Dickinson, University of Texas, Houston, TX, and pRhR1100, which contained the coding sequence for human prorenin (*18*), was provided by Dr. T. Reudelhuber, Institute for Clinical Research, Montreal, PQ, Canada.

Polyacrylamide gel electrophoresis and Western transfer experiments were performed using the Mini-protean Cell and Transfer Units (Bio-Rad, Mississauga, ON, Canada).

Electrophoresis-grade reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Biochemicals (Mississauga, ON, Canada). Reagents for Western blot experiments including diaminobenzene (DAB) or enhanced chemiluminescence reagent (ECL) were purchased from Amersham Biochemicals (Oakville, ON, Canada) and Bio-Rad Biochemicals (Mississauga, ON, Canada). Radioisotopes were obtained from ICN Biochemicals (Montreal, PQ, Canada).

A

Primer name	Primer sequence (5'....3')
S8A-r	GGACTTAGATGAAGATGTCGCGCAAGAAGACGTTCCC
S8E-r	GGACTTAGATGAAGATGTCGAACAAGAAGACGTTCCC
S8T-r	GGACTTAGATGAAGATGTCACCCAAGAAGACGTTCCC
S22A-r	GGAGGAGACGCGGAGCAGTTCATAGATGAGG
S22E-r	GGAGGAGACGAAGAGCAGTTCATAGATGAGG
E10D-r	GGACTTAGATGAAGATGTCAGCCAAGACGACGTTCCC
D11A-r	GGACTTAGATGAAGATGTCAGCCAAGAAGCCGTTCCC
S22T-r	GGAGGAGACACTGAGCAGTTCATAGATGAGGAGCG
D27A,E28A,E29A-r	GAGCAGTTCATAGCTGCGGCGCGTCAGGGACCACC
Forward flanking	CGAGCTCGGATCCACTAGTAACGGCCGCCAG
Reverse flanking	GGGGGATGTGCTGCAAGGCGATTAAGTTGGG

B

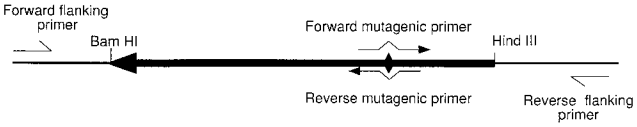


FIGURE 1: (A) Sequences of reverse mutagenic and flanking primers used in PCR-mediated site-directed mutagenesis. Primers containing the sequences of the coding strand are shown. Forward mutagenic primers (not shown) contained the sequence complementary to the reverse primers. The triplet codon encoding the mutated amino acid is underlined. (B) Schematic illustration of the location of the primers on the template. The heavily drawn arrow illustrates the location of PRP1 coding region. The site of mutation is indicated (♦).

All cell culture media preparations and culture dishes were purchased from LifeTechnologies (Burlington, ON, Canada) and Sarstead Inc. (St. Leonard, PQ, Canada). Protease inhibitors (Complete inhibitor tablets) were obtained from Boehringer Mannheim (Laval, PQ, Canada). HEK293 cells were provided by Dr. A. Castle, and the human submandibular gland cell line (HSG) was obtained from Dr. M. Hoffman, NIDR, Bethesda, MD. Matrigel and Dispase were purchased from Collaborative Research (Bedford, MA).

PRP1 was purified from human parotid saliva (*3*) and dephosphorylated as described (*19*). An anti-Ty polyclonal Goat IgG which only recognizes the nonphosphorylated proline-rich Ty repeat region of the protein was prepared previously (*20*). The Ty region is distinct from that containing Ser8 and Ser22. Therefore, the antibodies do not recognize the region subject to phosphorylation where all the mutations were carried out. Rabbit anti-cystatin antiserum was a gift from Dr. L. Tabak, University of Rochester, Rochester, NY.

Expression Vector and Mutagenesis of PRP1 Coding Sequence. A transfection vector was constructed by digesting the pRhR1100 vector with *Hin*dIII and *Bam*H1 to remove the prorenin coding region and create a cloning site for the PRP1 coding region. The plasmid pCRII-PRP1 was digested with the same enzymes to obtain a fragment with cohesive ends that contained the region encoding PRP1. This fragment was then ligated into the linearized pRhR1100 vector. The resulting plasmid is referred to as pPRP1.

Site-directed mutagenesis of the PRP1 coding sequence was performed using a PCR overlap strategy (*21*), using Taq rather than Vent polymerase. The template was PCRII-PRP1. The sequences of the flanking and mutagenic primers are given in Figure 1, which also shows schematically the location of the hybridization sites of the primers on the template. If two sites were mutated, a PCR product contain-

ing one of the desired mutations was used as template to introduce the second mutation. The resultant mutant PRP1 cDNA was digested with *Hin*dIII and *Bam*H1 and ligated into the linearized pRhR1100 expression vector. DNA sequencing by the method of Sanger (*22*) was performed to verify that the desired mutations were introduced.

Cell Culture. Human submandibular gland cells (HSG) were grown in either uncoated plastic dishes or dishes coated with matrigel in 1:1 DMEM/F-12 medium supplemented with 5% FCS and antibiotics. Human embryonic kidney cells, HEK293, were cultured in uncoated plastic dishes in DMEM supplemented with 5% FCS and antibiotics (100 μg/mL penicillin and streptomycin and 0.5 μg/mL gentamycin). Both cell lines were maintained in a humidified incubator at 5% CO_2 atmosphere at 37 °C.

For culture of HSG cells on matrigel, 6-well tissue culture plates were coated with matrigel (1 mL/well), which had been diluted according to the manufacturers instructions. HSG cells (0.4×10^6 cells) that had been transfected 1 day before with pRhR1100 in which the prorenin coding region had been replaced with the wild-type PRP1 or mutated PRP1 coding region were plated on the matrigel-coated wells and incubated for 48 h to allow differentiation. Cells were harvested after 30 min incubation in Dispase.

Transfection. Transient transfections of HSG and HEK293 cell lines were carried out using a calcium phosphate method (*23*). Subconfluent cells grown in 6 cm plastic culture dishes were transfected with 10 μg of plasmid DNA containing the coding sequence for either wild-type or mutated PRP1, or with vector alone. Cells were incubated for 4 h before replacing the medium. Exogenous expression of PRP1 was analyzed 24−72 h posttransfection. For analysis of secreted proteins, cells were incubated in serum-free medium for the last 8−16 h.

Metabolic Radiolabeling with [^{32}P]Orthophosphate. Phosphorylation of PRP1 was studied in HSG or HEK293 cells 48 h posttransfection. Prior to labeling, confluent monolayers were rinsed in phosphate-free DMEM and incubated for 1 h. Cells were then incubated for 3−6 h with 0.4 mCi/mL of carrier-free [^{32}P]orthophosphate in phosphate-deficient DMEM. For analysis of secreted protein, the cells were further incubated in complete medium for 3 h.

Immunoprecipitation. Samples of media which had been desalted by dialysis against water were immunoprecipitated with 1 μg of anti-Ty IgG for 16 h at 5 °C. After adding 30 μL of a 1:1 suspension of protein-G agarose, samples were further incubated for 2 h and washed eight times in ice-cold RIPA buffer. Immunoprecipitated protein was eluted in 2× SDS−PAGE sample loading buffer by heating at 100 °C for 5 min.

Electrophoresis and Blotting. Samples were resolved by SDS−PAGE on 12% gels using the method of Laemmli (*24*). Alternately, electrophoresis was performed on 10% gels in the same buffer system, but leaving out SDS (native−PAGE). Resolved proteins were transferred electrophoretically onto a nitrocellulose membrane.

Immunodetection and Protein Quantitation. To visualize and quantitate electroblotted PRP1, the nitrocellulose membranes with the electroblotted proteins were incubated for 1 h in blocking buffer consisting of 5% fatfree milk solution in TBS-T, followed by overnight incubation in blocking buffer containing 1 μg/mL of goat anti-Ty IgG. After washing six times for 5 min in TBS-T, the blots were incubated for 1 h with 1:500 diluted rabbit anti-goat IgG conjugated to peroxidase, followed by six more washes in TBS-T. The immunoreactive proteins were visualized with DAB or ECL. For quantitation, a set of standards of native and dephosphorylated PRP1 was similarly subjected to electrophoresis and Western blotting. The bands were scanned and quantitated using Iplab Gel software.

Autoradiography. To quantify the radioactivity incorporated in immunoprecipitated proteins, nitrocellulose membranes containing electroblotted proteins were exposed to a phosphoimager screen (Molecular Dynamics) for 2−3 days. Quantitation was performed using a Storm phosphoimager using ImageQuant Software and the extent of phosphorylation expressed in pixels.

Quantitation of Protein Phosphorylation. To quantitate protein phosphorylation, immunoprecipitated protein was subjected to SDS−PAGE and transferred to nitrocellulose membranes. The electroblotted proteins were first quantitated for phosphorylation by autoradiography and then for protein by Western blotting. The extent of phosphorylation was expressed as pixels per unit mass of protein. For comparison, the extent of phosphorylation of wild-type PRP1 was arbitrarily set at 100% and phosphorylation of the various mutated PRP1 expressed in the same experiment as percent of that of the wild-type PRP1. Each experiment was done three times. The difference in phosphorylation between wild-type and each of the mutant PRP1 was calculated and differences in phosphorylation evaluated by means of a *t*-test.

RESULTS

Expression of PRP1 in HSG Cells. HSG cells grown on plastic showed a typical cobblestone pattern, but when the cells were grown on matrigel, a different appearance as described (*25*) was seen. After 24 h of incubation, the cells were arranged into a mesh-like structure around spaces which were devoid of cells. At 48 h, this network of cells had broken into clumps of cells which remained up to 72 h. HSG cells grown on plastic or matrigel for 72 h were lysed and analyzed for the presence of cystatin, a marker of submandibular gland differentiation. SDS−PAGE and Western transfer using antibodies to cystatin demonstrated the presence of cystatin in cells grown on matrigel, but not in cells that had been cultivated on plastic in agreement with previous observations (*25*) (results not shown).

To evaluate expression of PRP1, HSG cells were transfected with pPRP1 or pRhR1100. Following incubation for 64 h, media were collected from the cultures and analyzed for expression of PRP1 by SDS−PAGE and Western blot using antibodies to PRP. As can be seen from Figure 2, there was no expression of PRP1 in cells that been transfected with pRhR1100, but those transfected with pPRP1 and grown either on plastic or matrigel secreted PRP1.

Localization of Phosphorylated Residues in PRP1. To demonstrate that cellular phosphorylation resulted in the same residues being phosphorylated as seen in the protein secreted from the glands, a series of plasmids containing mutated coding regions for PRP1 was constructed (Table 1). HSG cells grown on uncoated dishes or dishes coated with matrigel were transfected with the following vectors: (1) pPRP1 encoding wild-type PRP1; (2) pPRP1,8E, where the coding

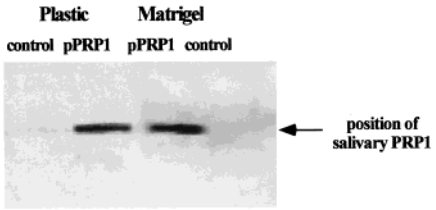


FIGURE 2: Expression of PRP1 in HSG cells. Anti-Ty Western blot analysis of proteins secreted from HSG cells transfected with the pPRP1 or control (pRhR1100) plasmids. Five hours after transfection, the cells were plated on plastic or matrigel-coated dishes and cultured for 48 h. At this time, new serum-free medium was added and the cells further incubated for 16 h before collecting cell media for analysis of secreted proteins.

region for PRP1 had been mutated so Ser8 would be replaced with Glu; (3) pPRP1,22E, where Ser22 would be replaced by Glu; and (4) pPRP1,8A,22A, where both Ser8 and 22 would be replaced by Ala. The transfected cells were incubated in the presence of [^{32}P]orthophosphate, and the secreted protein was recovered by immunoprecipitation. Analysis of these proteins by SDS−PAGE and Western blot (Figure 3, panels A and B) demonstrated the presence of a single phosphorylated immunoreactive protein with the same mobility as that of PRP1 isolated from saliva. Figure 3, panels A and B, also shows the extent of phosphorylation of PRP1. From these data, the level of phosphorylation could be calculated as described in the Materials and Methods (Figure 3C). An evaluation of the data in Figure 3C of the HSG cells grown on plastic or matrigel showed that the support medium had no effect on the extent of phosphorylation of either wild-type or mutated PRP1. There was a significant difference ($p < 0.001$) in the level of phosphorylation of wild-type PRP1 and PRP1,22E, which only contains the Ser8 phosphorylation site, of 37%, and the difference between the phosphorylation of wild-type PRP1 and PRP1,8E, containing only the Ser22 phosphorylation site, of 72%, was also significant ($p < 0.001$). Moreover, there was a significant difference ($p < 0.001$) between the phosphorylation of PRP1,22E and PRP1,8E. If both Ser8 and 22 were replaced with Ala (pPRP1,8A,22A), no phosphorylation was seen. These results show that both Ser8 and 22, but no other residues, were phosphorylated by the cells. Thus, the pattern of phosphorylation was the same as seen in PRP1 secreted from salivary glands in vivo, demonstrating the usefulness of the cells in studying phosphorylation of PRP1.

The lower level of phosphorylation of the mutant pPRP1,-8E than that of pPRP1,22E also suggests that at least Ser22 is not completely phosphorylated, in contrast to PRP1 isolated from saliva in which both residues are fully phosphorylated (*11*).

To further evaluate the effect of culture conditions on phosphorylation of secreted PRP1, HSG cells grown on uncoated plastic dishes and dishes coated with matrigel were transfected with the vector pPRP1. The cells were cultured in the absence of [^{32}P]orthophosphate, and the secreted proteins were subjected to native PAGE whereby unphosphorylated PRP1, as well as PRP1 that has been phosphorylated at one or two residues, can be separated. Figure 4A shows a Western blot of the secreted proteins developed with anti-Ty IgG. The identity of the bands could be determined by comparing their mobilities to those of samples of native PRP1 and dephosphorylated PRP1 (not shown). The relative amounts of protein in the bands were determined by scanning the blot, and the results are tabulated in Figure 4B.

The validity of quantitating phosphorylated and dephosphorylated PRP1 with anti-Ty IgG was demonstrated in a separate experiment in which triplicate samples of amounts of native and dephosphorylated PRP1 ranging from 5 to 50 μg were subjected to Western blotting and developed with anti-Ty IgG. A students *t*-test showed no significant difference between the band intensities of corresponding amounts of native and dephosphorylated PRP1, demonstrating the validity of using the antibodies for quantitation of native as well as dephosphorylated PRP1 (result not shown).

It can be seen that PRP1 secreted from the cells consists of a mixture of protein phosphorylated at both Ser8 and 22, protein in which only one residue is phosphorylated and unphosphorylated protein. There was no significant difference in phosphorylation between cells grown on plastic and matrigel.

Phosphorylation of PRP1 Mutants with Altered Ser8 Target Sequence. To evaluate the substrate sequence requirements of the kinase that phosphorylates Ser8, a series of mutants were constructed in all of which Ser22 had been mutated to Glu so that only Ser8 would be phosphorylated. The mutated vectors are listed in Table 1 together with partial amino acid sequences of the mutated proteins they encode. The vectors include (1) pPRP1,22E, which would lead to replacement of Ser22 with Glu; (2) pPRP1,8T,22E, where Ser 8 would be replaced with Thr in addition to the replacement of Ser22 with Glu; (3) pPRP1,10D,22E, where Glu10 would be replaced with Asp in addition to the mutation of Ser22 to Glu; and (4) pPRP1,11A,22E, where Asp11 would be substituted by Ala in addition to the mutation of Ser22.

Figure 3, panels A and B, shows the results obtained with these mutants. Quantitation of the data (Figure 3C) indicated that the level of phosphorylation of PRP1,22E was decreased by 37% reflecting phosphorylation of only Ser8. The level of phosphorylation of PRP1 secreted from cells transfected with the vectors pPRP1,22E and pPRP1,11A,22E were not significantly different, indicating that substitution of Asp11 with Ala had no effect on phosphorylation of Ser8. For CK2 to phosphorylate Ser8, a negatively charged residue is required at position 11, so it is unlikely that CK2 phosphorylates this residue. In contrast, there was a drastic decrease in phosphorylation when Ser8 was replaced with Thr, or Glu10 was substituted with Asp, as shown by the low level of phosphorylation of mutant PRP1 secreted from cells transfected with either pPRP1,8T,22E or pPRP1,10D,22E. Since G-CK phosphorylates Thr very poorly if at all and requires Glu in position 10 in order to phosphorylate Ser8 (*8*), it can be concluded that PRP1 is phosphorylated by a kinase with the same substrate recognition sequence as G-CK. Growing cells on plastic or matrigel had no effect on the relative levels of phosphorylation.

Phosphorylation of PRP1 Mutants with Altered Ser22 Target Sequence. To evaluate the substrate sequence requirements of the kinase that phosphorylates Ser22, mutants were constructed in which Ser8 had been altered to Ala so that only Ser22 would be phosphorylated. The mutated vectors are listed in Table 1 together with partial amino acid

Table 1 [a]

vector	partial sequence of wild-type and mutant PRP1																					
	8 Ser[b]	9 Gln	10 Glu	11 Asp	12 Val	13 Pro	14 Leu	15 Val	16 Ile	17 Ser	18 Asp	19 Gly	20 Gly	21 Asp	22 Ser[b]	23 Glu	24 Gln	25 Phe	26 Ile	27 Asp	28 Glu	29 Glu
PPRP1 (wild-type)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PPRP1,8A	Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pPRP1,22A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ala	-	-	-	-	-	-	-
pPRP1,8A,22A	Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	Ala	-	-	-	-	-	-	-
pPRP1,8E	Glu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pPRP1,22E	-	-	-	-	-	-	-	-	-	-	-	-	-		Glu	-	-	-	-	-	-	-
pPRP1,8T,22E	Thr	-	-	-	-	-	-	-	-	-	-	-	-	-	Glu	-	-	-	-	-	-	-
pPRP1,10D,22E	-	-	Asp	-	-	-	-	-	-	-	-	-	-	-	Glu	-	-	-	-	-	-	-
pPRP1,11A,22E	-	-	-	Ala	-	-	-	-	-	-	-	-	-	-	Glu	-	-	-	-	-	-	-
pPRP1,8A,22T	Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	Thr	-	-	-	-	-	-	-
pPRP1,8A,27A,28A,29A	Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Ala	Ala	Ala

[a] Partial amino acid sequence of wild type and mutant PRP1 encoded by the vectors listed in the left column. [b]The amino acids that are phosphorylated in the native secreted protein. Amino acid changes from the wild-type PRP1 are shown. (-) No changes.

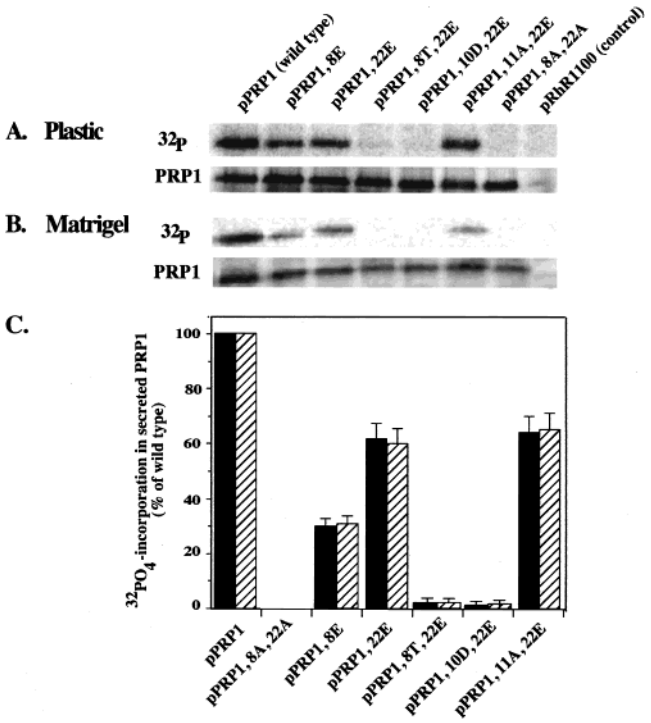
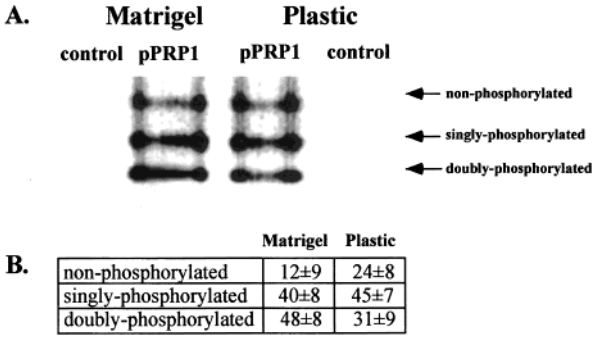


FIGURE 3: Location of phosphorylation sites in PRP1 expressed in HSG cells and effect of mutations of the serine 8 substrate recognition sequence. Phosphorylation of wild-type and mutant PRP1 expressed in HSG cells grown either on plastic or matrigel and transfected with the vectors indicated at each lane in panels A and B and at the bars in panel C. For explanation of the vectors see Table 1. Cells were incubated with [^{32}P]orthophosphate for 3 h before collecting secreted protein for 3 h. Immunoprecipitated PRP was resolved by SDS−PAGE on 12% gels and transferred onto nitrocellulose paper. (A) Autoradiogram (^{32}P) and anti-Ty Western blot (PRP1) of the same nitrocellulose membrane containing proteins secreted from HSG cells grown on plastic. (B) Autoradiogram (^{32}P) and anti-Ty Western blot (PRP1) of the same nitrocellulose membrane containing proteins secreted from HSG cells grown on matrigel. (C) Bar diagram showing the level of phosphorylation of PRP1 mutants relative to the wild-type PRP1. Solid bars represent PRP1 secreted from cells grown on plastic; crosshatched bars represent PRP1 secreted from cells grown on matrigel. The mean of three experiments is shown. The error bar shows SD.



B.

	Matrigel	Plastic
non-phosphorylated	12±9	24±8
singly-phosphorylated	40±8	45±7
doubly-phosphorylated	48±8	31±9

FIGURE 4: Relative levels of PRP1 phosphorylated at 1 or 2 sites in HSG cells. Extent of phosphorylation of PRP1 secreted by HSG cells which had been transiently transfected with pPRP1 or control plasmids and cultured on plastic or matrigel-coated dishes. (A) Anti-Ty Western blot analysis of proteins secreted from transfected HSG cells for 16 h, 2 days posttransfection. Concentrated samples were resolved by native−PAGE on 10% gels and transferred onto nitrocellulose paper. Gel electrophoretic positions of the PRP1 species were determined from the migration of markers of chemically dephosphorylated PRP1 and native PRP1 purified from saliva. (B) Quantitation of the amount of nonphosphorylated and phosphorylated PRP1, expressed as a percentage of total secreted PRP1. The mean ± SD of three experiments is shown.

sequences of the proteins they encode. The vectors include (1) pPRP1,8A, which leads to replacement of Ser8 with Ala; (2) pPRP1,8A,22T, where Ser 22 is replaced with Thr in addition to the replacement of Ser8 with Ala; and (3) pPRP1,8A,27A,28A,29A, where Asp27, Glu28, and Glu 29 all would be replaced with Ala in addition to the mutation of Ser8 to Ala.

Figure 5, panels A and B, shows the results obtained with cells grown on plastic or matrigel, respectively. From these data, the level of phosphorylation could be calculated (Figure 5C). It can be seen that growing cells on matrigel had no effect on the extent of phosphorylation, and the level of phosphorylation seen with pPRP1,8A was decreased by 72% compared to pPRP1 reflecting phosphorylation only of Ser22. Compared to cells transfected with pPRP1,8A, there was almost no phosphorylation of PRP1 secreted from cells that had been transfected with pPRP1,8A,22T. Interestingly, mutant PRP1 secreted from cells that had been transfected with pPRP1,8A,27A,28A,29A showed only 3% phosphorylation, indicating that a major component of the substrate recognition sequence is located in the Asp27−Glu29 sequence.

Expression of PRP1 in HEK293 Cells. The ability of HEK293 cells transfected with wild-type pPRP1 to express and secrete PRP1 is illustrated in Figure 6. A Western blot analysis of medium from cultures of transfected cells

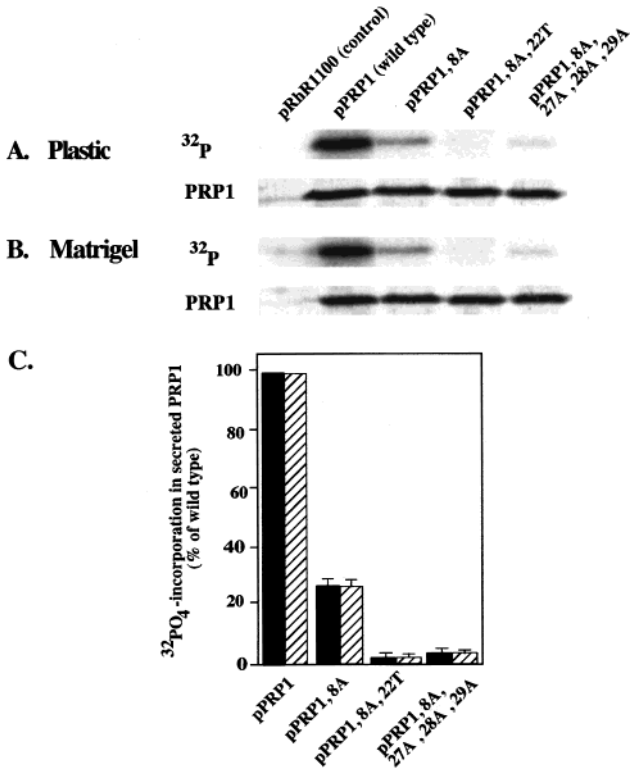


FIGURE 5: Effect of mutations of the serine 22 substrate recognition sequence. Phosphorylation of wild-type and mutant PRP1 expressed in HSG cells grown either on plastic or matrigel and transfected with the vectors indicated at each lane in panels A and B and at the bars in panel C. For explanation of the vectors see Table 1. Cells were incubated with [^{32}P]orthophosphate for 3 h before collecting medium containing secreted protein for 3 h. Immunoprecipitated PRP was resolved by SDS−PAGE on 12% gels and transferred onto nitrocellulose paper. (A) Autoradiogram (^{32}P) and anti-Ty Western blot (PRP1) of the same nitrocellulose membrane containing proteins secreted from HSG cells grown on plastic. (B) Autoradiogram (^{32}P) and anti-Ty Western blot (PRP1) of the same nitrocellulose membrane containing proteins secreted from HSG cells grown on matrigel. (C) Bar diagram showing the level of phosphorylation of PRP1 mutants relative to the wild-type PRP1. Solid bars represent PRP1 secreted from cells grown on plastic; crosshatched bars represent PRP1 secreted from cells grown on matrigel. The mean of three experiments is shown. The error bar shows SD.

demonstrated that they secreted an immunoreactive protein of the same size as PRP1 isolated from saliva (Figure 6A). Similarly, cells transiently transfected with pPRP1 in the presence of [^{32}P]orthophosphate were shown to secrete ^{32}P-labeled immunoreactive protein with the same mobility as PRP1 (Figure 6B).

To confirm that PRP1 secreted from HEK293 cells had the same pattern of phosphorylation as PRP1 secreted from salivary glands, HEK293 cells were transfected with pPRP1 and labeled with [^{32}P]orthophosphate. The observed pattern of phosphorylation was the same as that seen with HSG cells (Figure 7, panels A and B). PRP1 secreted from cells that had been transfected with pPRP1,8A,22A in which the Ser at both phosphorylation sites in PRP1 would be mutated to Ala was not phosphorylated. Transfection with pPRP1,8A and pPRP1,22A, which abolish the phosphorylation sites at Ser8 and 22, respectively, led to a level of partial phosphorylation of secreted PRP1 similar to that observed in HSG cells (Figure 7C). Compared to wild-type pPRP1, the levels of phosphorylation obtained with pPRP1,8A and pPRP1,-

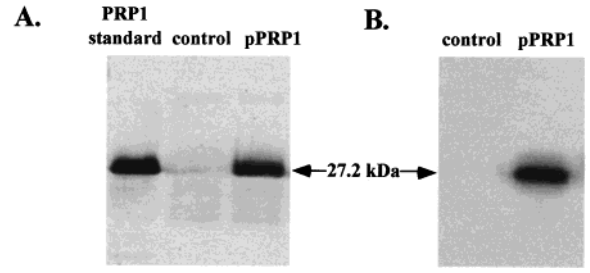


FIGURE 6: Expression and phosphorylation of PRP1 in HEK293 cells. (A) Anti-Ty Western blot analysis of secreted protein from HEK293 cells transiently transfected with a plasmid construct containing PRP1 cDNA (pPRP1) or with a control (pRhR1100) plasmid lacking the PRP1 coding region. One day posttransfection, cells were incubated in serum-free medium for 16 h, at which time the medium was collected. Media samples were resolved by SDS−PAGE on 12% gels and transferred to a nitrocellulose membrane which was developed with antibodies to PRP. The arrow shows the position of a PRP1 marker. (B) Autoradiography of ^{32}P-labeled PRP1 secreted from transfected HEK293 cells. Cells transfected with pPRP1 or pRhR1100 as a control were incubated with [^{32}P]-orthophosphate for 3 h and for a further 3 h in the absence of [^{32}P]-orthophosphate before collecting the cell medium. PRP1 was immunoprecipitated and subjected to SDS−PAGE on 12% gels and autoradiography.

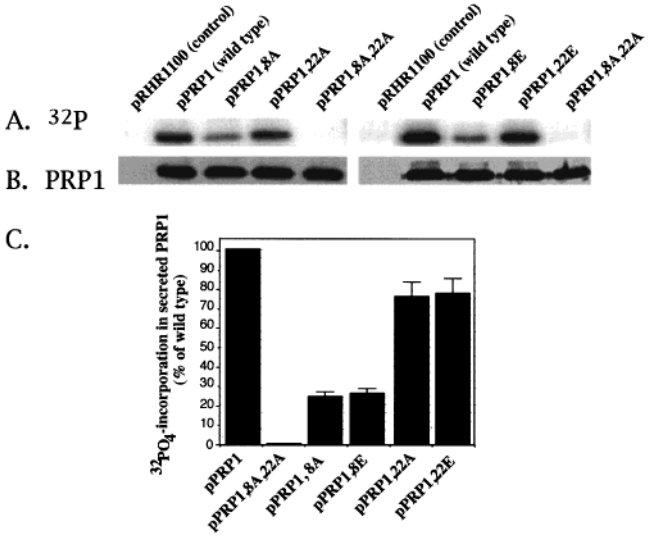
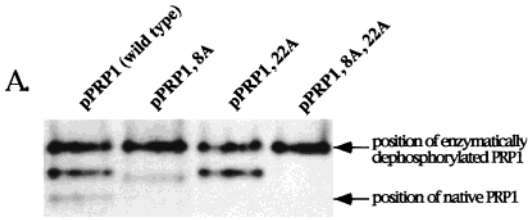


FIGURE 7: Location of phosphorylation sites in PRP1 expressed in HEK293 cells. ^{32}P-Incorporation into wild-type and mutant secreted PRP1. HEK293 cells were transfected with the vectors indicated at each lane in panels A and B and the bars in panel C. The results of two experiments are shown. The five left lanes in Figures A and B constitute one experiment and the five right lanes in Figures A and B constitute a second experiment. For description of the vectors see Table 1. Secreted PRP1 was collected from the medium of cells which had been incubated with $^{32}PO_4$. For experimental details see the text. Immunoprecipitated PRP1 was resolved by SDS−PAGE on 12% gels and transferred onto nitrocellulose membrane. (A) Autoradiographic analysis of the nitrocellulose membrane. (B) Anti-Ty Western blot (on the same nitrocellulose membrane as used in panel A). (C) Bar diagram showing the extent of phosphorylation of mutant PRP1 relative to the wild-type. The mean of three experiments is shown. The error bar shows SD.

22A were significantly lowered by 64% ($p < 0.001$) and 23% ($p < 0.05$), respectively. Additional transfections with the vectors pPRP1,8E and pPRP1,22E gave results identical to those obtained with pPRP1,8A and pPRP1,22A, respectively, showing that the presence of a negative charge at one phosphorylation site did not affect phosphorylation of the other site.



B.

	pPRP1 (wild type)	pPRP1,8A	pPRP1,22A
non-phosphorylated	59±6	87±8	53±6
singly-phosphorylated	30±8	13±6	27±7
doubly-phosphorylated	11±7		

FIGURE 8: The relative levels of PRP1 phosphorylated at 1 or 2 sites in HEK293 cells. HEK293 cells were transfected with the vectors indicated at each lane in panel A. (A) Anti-Ty Western blot of secreted PRP1 resolved by native−PAGE on 12% gels. Arrows indicate the electrophoretic mobilities of standards of PRP1 purified from saliva and dephosphorylated PRP1. (B) Quantitation of phosphorylated and nonphosphorylated PRP1, expressed as a percentage of total secreted PRP1. The mean ± SD of three experiments is shown.

To further evaluate the pattern of phosphorylation of PRP1, media were collected from HEK293 cells transfected with wild-type pPRP1, pPRP1,8A, pPRP1,22A, and pPRP1,8A,-22A. The media samples were subjected to native−PAGE followed by Western blot using anti Ty IgG (Figure 8). Comparing Figures 4A and 8A, it can be seen that the patterns of phosphorylation of PRP1 secreted from HEK293 and HSG cells were similar. Both cell types secreted PRP1 that had been phosphorylated at two or one residue as well as unphosphorylated PRP1. However, the extent of phosphorylation was significantly greater in HSG cells as illustrated by 31 or 48% of PRP1 secreted from HSG cells being phosphorylated at two residues, whereas only 11% of the protein secreted from HEK293 cells was phosphorylated at both Ser8 and 22 (Figures 4B and 8B) ($p < 0.025$ and $p > 0.001$, respectively). Moreover, it can be seen (Figure 8) that transfection with pPRP1,22A led to more extensive phosphorylation than that obtained with pPRP1,8A. The level of phosphorylation of Ser8 was significantly higher than that of Ser22 ($p < 0.025$), in agreement with the results shown in Figure 7.

To evaluate the substrate recognition sequence responsible for phosphorylation of Ser8, HEK293 cells were transfected with the vector pPRP1,11A,22E, which would lead to destruction of the substrate recognition sequence for CK2 at the remaining Ser8 phosphorylation site. This had no effect on phosphorylation, since it was not significantly different from that of cells transfected with pPRP1,22E, which would lead to phosphorylation of only Ser8 (Figure 9). In contrast, HEK293 cells transfected with either pPRP1,8T,22E or pPRP1,10D,22E led to secretion of insignificantly phosphorylated PRP1 (Figures 9). In both of these proteins, the recognition sequence for G-CK has been destroyed. Together, these experiments indicate that an enzyme related to G-CK and not CK2 is responsible for phosphorylation of PRP1 at Ser8 in transfected HEK293 cells.

To evaluate the substrate sequence requirements for Ser22, HEK293 cells were transfected with pPRP1,8A, which led to a 73% decrease in phosphorylation of PRP1 when compared to cells transfected with wild-type pPRP1 (Figure 10). In contrast, cells transfected with pPRP1,8A,22T secreted insignificantly phosphorylated protein (Figure 10)

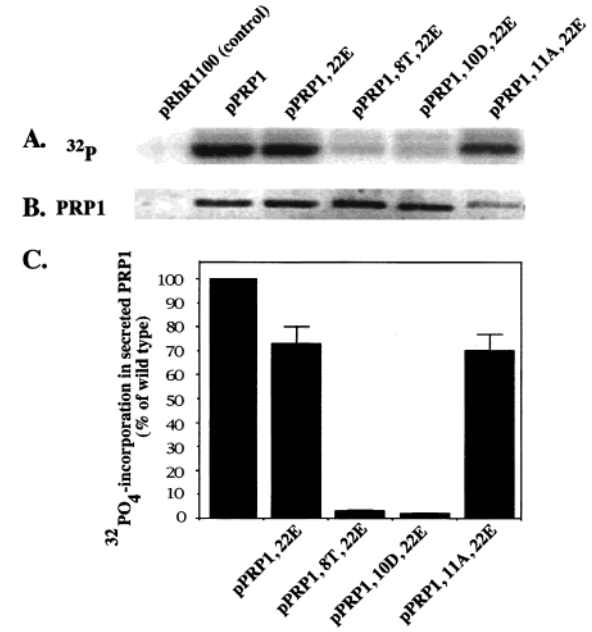


FIGURE 9: Effect of mutating the serine8 substrate recognition site in PRP1 expressed in HEK293 cells. ^{32}P-Labeling of secreted wild-type and mutated PRP1 with altered target sequence around serine 8. HEK293 cells were transfected with the vectors indicated at each lane in panels A and B and the bars in panel C. For description of the vectors see Table 1. Secreted PRP1 was collected from transfected cells which had been incubated with $^{32}PO_4$ as described in the text. Immunoprecipitated protein was resolved by SDS−PAGE on 12% gels and transferred onto nitrocellulose membrane. (A) Autoradiography of the nitrocellulose membrane. (B) Anti-Ty Western blot of the same membrane as shown in panel A. (C) Bar diagram showing the levels of ^{32}P-incorporation into mutant PRP1 relative to the wild-type PRP1. The mean ± SD of three experiments is shown.

and a similar low level of phosphorylation was seen in transfection experiments with pPRP8A,27A,28A,29A. Thus, the substrate recognition requirements for phosphorylation of Ser22 are similar in HSG and HEK293 cells and they include the 27AspGluGlu29 sequence.

DISCUSSION

At present, there is a lack of well-established salivary gland cell lines that are suitable for studying synthesis and posttranslational modifications of secreted proteins. The immortalized HSG cell line is derived from submandibular intercalated duct cells (*15*). During salivary gland development, intercalated duct cells are believed to give rise to acinar cells, the site of synthesis of most secreted salivary proteins including PRP1. When grown on matrigel, HSG cells form discrete spherical structures similar to the acini seen in salivary glands and show evidence of functional differentiation by their ability to secrete cystatin, a protein that is secreted from salivary glands in vivo (*25*). Moreover, under these culture conditions, increased activity of the amylase promoter has been reported (*26*). In view of the morphological and possibly functional differentiation of HSG cells grown on matrigel, it was of interest to evaluate if there was a change in APRP expression and G-CK activity in cells grown on matrigel. While we found expression of cystatin in cells cultured on matrigel, there was no evidence of synthesis of APRPs, although they are synthesized in submandibular glands (*27*). As judged by phosphorylation

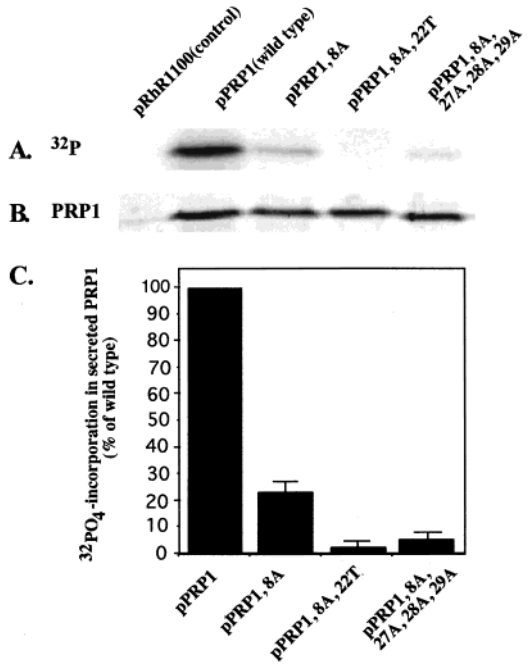


FIGURE 10: Effect of mutating the serine 22 substrate recognition site in PRP1 expressed in HEK293 cells. ^{32}P-Labeling of secreted wild-type and mutated PRP1 with altered target sequence around serine 22. HEK293 cells were transfected with the vectors indicated at each lane in panel A and B and the bars in panel C. For description of the vectors see Table 1. Secreted PRP1 was collected from transfected cells which had been incubated with $^{32}PO_4$ as described in the text. Immunoprecipitated protein was resolved by SDS−PAGE on 12% gels and transferred onto nitrocellulose membrane. (A) Autoradiography of the nitrocellulose membrane. (B) Anti-Ty Western blot of the same membrane as shown in panel A. (C) Bar diagram showing the levels of ^{32}P-incorporation into mutant PRP1 relative to the wild-type PRP1. The mean ± SD of three experiments is shown.

of expressed PRP1, the G-CK activity was not affected by the growth conditions of the cells.

To evaluate if Golgi kinase is responsible for phosphorylation of protein secreted from other tissues, we used HEK293 cells. Several lines of evidence show the relevance of these kidney derived cells. G-CK activity has been demonstrated in rat kidneys (*8*) and in rat kidney cell lines other than HEK293 (*17*). The secreted matrix Gla protein which is phosphorylated at three serine residues located in the G-CK substrate recognition sequence SXE/S(P) is expressed in the kidney (*17*). Furthermore the phosphoprotein, osteopontin, which is secreted from tubular kidney cells, has been shown to be a substrate for G-CK (*28*), and epithelial kidney cells have the potential to differentiate into tubular cells (*29*).

The ability of HSG as well as HEK293 cells to phosphorylate heterologously expressed PRP1 makes them useful in studies on phosphorylation of this protein as well as other proteins, and suggests that the responsible kinase may have a wider tissue distribution and may be responsible for phosphorylation of other secreted phosphoproteins.

In the present study, we have employed polyclonal antibodies that only react with the nonphosphorylated C-terminal proline-rich Ty region of PRP1, a region distinct from that containing Ser8 and Ser22. Therefore, the antibodies do not recognize the region subject to phosphorylation, where all the mutations were done, and consequently, there was no difference in the reactivity of native and dephosphorylated PRP1 with anti-Ty IgG.

While PRP1 secreted from salivary glands is fully phosphorylated at Ser8 and 22 (3), only partial phosphorylation of these residues is seen in the cultures of transfected HSG and HEK293 cells. While 11% of PRP1 secreted from HEK293 cells was phosphorylated at both residues, 31 and 48% of HSG cells grown on plastic or matrigel, respectively, were phosphorylated at both Ser8 and 22. The higher level of phosphorylation seen in HSG cells compared to HEK293 cells make them particularly useful in these studies.

Interestingly, less G-CK activity has been found in the kidney than in mammary glands (*8*), suggesting that cells which are specialized for exocrine secretion may have higher levels of expression of G-CK. The inability to obtain complete phosphorylation of Ser8 and 22 in this study may be a function of the cell culture. For example, it is possible that in glandular acinar cells, APRPs such as PRP1 are further phosphorylated in the trans-Golgi network. Support for this possibility is the observation that in mammary glands phosphorylation of α- and γ-casein apparently occurs in the cis-Golgi complex while β-casein phosphorylation takes place in the trans-Golgi network (*30*, *31*).

The sum of percentages of protein phosphorylation of the single mutants pPRP1,8A and pPRP1,22A (103%) is not significantly different from the phosphorylation of wild-type PRP1 (100%) in HEK293 cells (Figure 7), and the same result was obtained when the sum of phosphorylation of the mutants pPRP1,8E and pPRP1,22E (104%) was compared with wild-type pPRP1 phosphorylation (100%) in HEK293 cells (Figure 7). Thus, there is no evidence of cooperativity in phosphorylation or hierarchical phosphorylation, that is phosphorylation of one site is dependent on another site being phosphorylated, as reported for bone and dentine extracellular proteins (*9*). The lack of effect of substituting either Ser8 or 22 with Glu rather than Ala further demonstrates that the presence of a negative charge at one phosphorylation site does not affect phosphorylation at the other site.

In HSG cells (Figure 3), the sum of phosphorylation of pPRP1,8E and pPRP1,22E was 92 ± 6.6%. Evaluation of difference in phosphorylation between the wild-type and the sum of phosphorylation of pPRP1,8E and pPRP1,22E showed a significant difference ($p < 0.05$), so the possibility that cooperativity may have a small effect on phosphorylation in HSG cells cannot be excluded.

From the levels of phosphorylation attained in mutants pPRP1,8A and pPRP1,8E, which only have the Ser22 phosphorylation site, and in pPRP1,22A and pPRP1,22E, which only have the Ser8 phosphorylation site (Figures 3 and 7), it can be seen that phosphorylation of Ser22 is less effective than phosphorylation at Ser8.

Since Thr is a good substrate for CK2, but a very poor substrate for G-CK, the absence of phosphorylation in the mutant PRP1,8A,22T suggests that the kinase phosphorylating Ser22 may be related to G-CK. Alternately, Ser22 may be phosphorylated by a kinase that equally recognizes serine and threonine. Since several protein phosphatases show preference for phosphothreonine rather than phosphoserine *(32)*, the lack of phosphorylation of PRP1,8A,22T may be due to extensive dephosphorylation of initially phosphorylated PRP1,8A,22T compared to PRP1,8A. PRP1 lacks Glu in position *n* (phosphorylated residue) + 2 (residue 24) as

required for recognition by G-CK (8), but it also lacks a negatively charged residue in position $n + 3$ as required for recognition by CK2 (*14*). In fact, the target sequence surrounding Ser22 is not in agreement with that of any known kinase. In unpublished experiments, we used a peptide containing a sequence surrounding Ser22 (18-Gly-Gly-Asp-Ser-Glu-Gln-Phe-Ile-26) as substrate for a partially purified salivary gland kinase (*13*). No phosphorylation was seen indicating that a critical part of the substrate recognition sequence is located elsewhere in the protein. The drastically decreased phosphorylation seen in the mutant PRP1,8A,27A,-28A,29A (Figures 5 and 10) indicates that the acidic residues in positions 27−29 contain a critical part of the substrate recognition sequence. Thus, Ser22 may be phosphorylated by a novel kinase, although it cannot be excluded that Ser22 is contained in an atypical substrate sequence recognized by G-CK. To solve this problem, it will be important to further delineate the Ser22 substrate recognition site so a specific substrate can be made and used to identify the responsible kinase.

While this study has demonstrated cellular phosphorylation of Ser8 by a G-CK, the enzyme responsible for in vivo phosphorylation remains to be identified. Saliva contains several other phosphoproteins including statherin (*2*), basic PRPs (*33*), histatin 1 (*34*), and cystatin S (*35*) and many of the phosphorylation sites are surrounded by sequences that could be recognized both by G-CK and CK2. Identification of the salivary gland kinase that phosphorylates PRP1 including its purification or cloning is therefore of wider interest. Since no other G-CK has been purified to homogeneity or cloned, this would be of general interest for understanding phosphorylation of many phosphoproteins secreted from other tissues. The possibility that Ser22 is phosphorylated by a novel protein kinase is also intriguing and warrants further investigation.

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