CLONING AND FUNCTIONAL STUDY OF PORCINE PAROTID HORMONE, A NOVEL PROLINE RICH PROTEIN

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Running Title: Cloning and function of porcine parotid hormone

A parotid gland hormone that stimulates intradental fluid movement is believed to play a significant role in maintaining the vitality of dentin. This hormone has been purified from porcine parotid glands and partially sequenced in our previous study. We now report the cloning and functional study of porcine cDNAs that code for this hormone and its complete amino acid sequence. Three cDNA clones were isolated from a porcine parotid cDNA library. The last 30 amino acids encoded by two of the cDNAs agreed with amino acid sequence of the isolated parotid hormone. In situ hybridization and immunohistochemical staining demonstrated that the acinar cells of the parotid glands were the primary location for both parotid hormone related mRNAs and the translation products. A 216-bp fragment of the cDNA that contains the coding sequence for the porcine hormone was subcloned into an expression vector, and the protein expression was detected by immunoblot analysis and quantified by ELISA. In addition, the 30 amino acid parotid hormone was synthesized. Both the expressed and synthetic proteins were biologically active, in that they enhanced intradental fluid movement as measured by intradental dye penetration.

A new insight into the fundamental basis of resistance to cariogenesis is provided by research in dentinal ultrastructure and internal dynamics. Odontoblastic cells at the pulp-dentin junction have cytoplasmic extensions extending within canaliculi that stretch from the pulp/pre-dentin area to the dentin/enamel junction. These canaliculi are filled with dentinal fluid (1, 2). Accordingly, dentin can be regarded as a barrier to bi-directional diffusive and convective fluid movement between the oral environment and the underlying pulp (3, 4). The inward diffusive passage of bacterial cytotoxic products is opposed by a continuous outward flow of dentinal fluid that normally is maintained by an elevated hydrostatic pressure within the tooth. This movement may represent a physiological means by which the integrity of avascular dental tissues is maintained despite the potential for ingressive diffusion of exogenous substances (5-7). Therefore, natural resistance of teeth to decay may involve maintaining adequate endogenous centrifugal dentinal fluid movement (DFM)1. DFM can be visualized with an intradental dye penetration (IDDP) assay in which a fluorescent dye, acriflavine hydrochloride, is injected intraperitoneally into rats, and the magnitude of dye penetration into the dentinal tubules is evaluated in sectioned molar teeth.

The significance of DFM in relation to dental health is suggested by caries studies done in rats that show an inverse relationship between the cariogenicity of a high sucrose diet and certain dietary supplements that stimulate DFM (8). The observation that the supplements are ineffective in stimulating DFM in parotidectomized animals (8, 9) implies that they exercise their cariostatic effect through mechanism(s) involving the parotid glands. A factor in parotid glands that stimulates DFM when infused into rats has been purified from porcine parotid tissue, and its partial amino acid sequence was determined (10). This factor, designated as parotid hormone (PH), is a moderately basic, small glycoprotein. Its amino acid composition and partial sequence indicate a molecule particularly rich in proline and glycine (10).
Parotid glands secrete a number of salivary proline rich proteins (PRPs) that represent a unique family of molecules. More than 20 PRPs have been described in humans. They are classified as either acidic or basic on the basis of their charge, and some are modified by glycosylation or phosphorylation (11). Typically, these proteins contain 25-45% proline, 18-22% glutamine, and 18-22% glycine, with acidic PRPs containing 9-11% aspartate and basic PRPs containing 7-10% lysine plus arginine (12). Sequences of human, rat and mouse PRP genes exhibit a high degree of similarity (12, 13). This high conservation of sequence and structure of PRP genes and PRPs argues for specific biological functions for these unusual proteins. For example, the proline residues in the PRPs appear to bind tannins that are potentially harmful compounds present in food (14). Other functions, such as calcium binding, hydroxypatite binding, and formation of the dental acquired pellicle, have been attributed to acidic PRPs (15, 16); while antiviral activity has been demonstrated with basic PRPs (17, 18). In addition, there are reports of a decrease in PRPs, including basic PRPs, under several conditions (19-21), each of which is associated with increased caries susceptibility (22-24).

A comparison with reported PRPs suggests that porcine PH is unique in its amino acid sequence and that its amino acid composition hints at possible linkage with basic PRPs. To better understand the relationship between PH and PRPs, and the functional aspects of PH, further characterization of porcine PH is needed. We now report the isolation and sequence of several full-length cDNAs related to porcine PH, the tissue distribution of the PH mRNA and proteins, and the expression of the PH encoding cDNAs in transfected COS-7 cells. PH was also commercially synthesized. The function of the expressed proteins and the synthetic protein was assessed with the IDDP assay, and the degree of stimulation was compared with that induced by feeding, a potent physiological stimulus for endogenous PH secretion (25).

Materials and Methods

Tissue Collection - Three-month-old male pigs were sacrificed with an overdose of Nembutal.
Construction and Screening of cDNA Library -
The porcine parotid cDNA library was constructed using SuperScript Plasmid System (Life Technologies) per manufacturer's instructions. Because feeding and isoproterenol treatment increase porcine PH release (26, unpublished data), parotid samples were collected from two young male pigs, one stimulated by feeding, and the other treated with daily injections of isoproterenol (0.6 mg/kg) for 4 days. Total RNA was isolated with RNAzol B solution (Tel-Test, Inc.), and the poly A + messenger RNA was purified from total RNA with the Oligotex spin column (Qiagen). About 3 µg of mRNA was used to construct the cDNA library. The library was probed with a 20-base oligonucleotide, PHIA (5'-GCICCICGGIGCIMGICC-3'), corresponding to the N-terminal sequence of the isolated porcine PH. Deoxynosine was incorporated into positions where the degeneracy was higher than 2.

DNA Sequencing of Positive cDNA clones -
The cDNA inserts of positive clones were sequenced from both directions, and the partial sequences were analyzed for the location of the site complementary to PHIA. Three full-length cDNAs: D2A, TP23, and SL44-1 were taken through the process of progressive unidirectional deletions with the Erase-a-Base System (Promega) to obtain a series of overlapping clones (each overlap was at least 50-100 nucleotides long). The representative clones containing appropriate truncations were sequenced, and the sequences were compiled to obtain the complete sequence of the original clone.

In situ Hybridization -
The various porcine tissue samples, harvested and fixed in 4% PFA in PBS, were dehydrated, embedded in paraffin, and cut into 5 µm sections. Adjacent sections were hybridized with either the antisense probe, or the sense probe, the latter serving as a negative control. A plasmid containing a partial sequence of TP23, approximately 300 bp in size, was used to generate the sense and antisense single-strand RNA probes. The slides were hybridized at 60°C with the riboprobe (2 x 10⁶ cpm/ml), and the posthybridization wash was carried out at 65°C. The images of in situ hybridization specimens were captured with a video camera linked to a computer. The image revealed by blue Hoechst (a nuclear dye) fluorescence was digitally superimposed with a darkfield image. The silver grains were pseudocolored to yellow to enhance the contrast, using the Adobe Photoshop program.

Immunohistochemistry -
Immunohistochemical studies for the presence of PH were performed by indirect immunoperoxidase staining on 5 µm PFA-fixed/paraffin-embedded tissue sections. Adjacent sections were reacted with the polyclonal antibody made against the isolated porcine PH (PH-Ab) (28), and with normal rabbit serum serving as the control. Antigen retrieval was performed by incubating the sections at room temperature for 15 min in 2 N HCl. A DAKO LSAB+ Kit was used for immuno-reactions. Peroxidase blocking reagent was first applied to the sections for 15 min to quench endogenous peroxidase activity. Tissue sections were then taken through the following incubation steps: blocking in 5 % nonfat milk 10 min, PH-Ab (1:50) 1 hr at 35°C, biotinylated anti-rabbit IgG 30 min at 35°C, and peroxidase-conjugated streptavidin 30 min. For color development, AEC (3-amino, 9-ethyl-carbazole) chromogen plus H₂O₂ substrate (BioGenex Supersensitive Detection Kit) was applied to the tissue sections. Hematoxylin was used for histological counterstaining.

Southern Blot Analysis of Genomic DNAs -
Genomic DNAs were extracted from porcine and rat parotid tissues, and human genomic DNA was purchased from Roche. Porcine genomic DNA, 10 µg, was digested in separate reactions with restriction endonuclease BamHI, EcoRI, Hind III, Pst I, Sac I, and Xba I. Human and rat genomic DNAs were digested with EcoRI I. The restriction products were separated through a 0.7% agarose gel, and transferred to a Duralose-UV membrane. The membrane was hybridized at 68°C in the QuickHyb Hybridization Solution (Stratagene) with a 300 bp, [α-³²P] dCTP random-primed cDNA fragment of TP23. The post-hybridization wash was carried out first in 2x SSC and 0.1% SDS twice for 15 min each at room temperature. For high-stringency wash, the membrane was washed once for 60 min at 60°C, with 0.1 x SSC and 0.1% SDS. Autoradiography was performed using a Kodak Biomax MR film.

PCR Cloning of the cDNA for Porcine PH -
A deletion clone of TP23 was used as the PCR
template, which was obtained earlier during the process of generating progressive unidirectional deletions with the Erase-a-Base system. This truncated clone (DT 11-18) contains the coding capacity of the last 72 amino acids from clone TP 23, and includes the 30 amino acids of PH and two of the repeats upstream. The 5’ forward primer PHC-5 was designed corresponding to the cDNA sequence of DT 11-18 encoding the first five amino acids of the first 21 amino acid repeat (APPGA), and a 5’ addition of a Nde I site (5’-CATATGGCCCCACCTGGTGC-3’). The 3’ primer was the M13 reverse sequencing primer complementary to the sequence present in the cloning vector pSPORT 1. The PCR products were purified and ligated with the pCR-Blunt vector (Invitrogen), and introduced into DH 5α cells. The presence of the PH cDNA insert in plasmid pCR-D 15 was confirmed by DNA sequencing.

Coupled in vitro Transcription/Translation of the PH Encoding cDNAs - The cDNA insert of TP 23 was subcloned into the vector pET-28a(+) (Novagen), which features an N-terminal HisTag sequence downstream from a T7 promoter. The fusion was confirmed to be in-frame with the HisTag by DNA sequencing. Plasmid pET-28a(+) - TP 23 was then subjected to the TNT Quick Coupled Transcription/Translation System (Promega), and the biotinylated lysine-tRNA complex was included in the translation procedure according to the manufacturer’s instructions. The translation products were analyzed using 12% SDS-PAGE and Western blotting. The biotinylated proteins were visualized using the Transcend Colorimetric Detection System (Promega).

The cDNA insert of pCR-D 15 was released by digestion with Nde I and Not I and subcloned into the corresponding sites in vector pET-28a(+). The fusion construct pET-28a(+) - D 15 was in-frame as confirmed by DNA sequencing, which was then subjected to the TNT system. The translated products were separated in a 12% SDS polyacrylamide gel, and transferred to a NitroBind nitrocellulose membrane. The detection of the expressed proteins was performed by Western blot analysis using Immun-Star substrate with enhancer as presented previously.

Quantifying Porcine PH Expression in the Cell Lysate with ELISA - A direct competitive ELISA was developed with a polyclonal antibody, PH-Ab, that had been previously characterized (28) and used routinely in the PH RIA (26, 29). Porcine PH that was used for amino acid sequence determination served as immobilized tracer and standard. Immulon 4 HBX flat bottom microtiter plates (Dynex Technologies) were coated with 1.3 ng PH/50µl. The primary rabbit PH-Ab was used at a dilution of 1:30,000. Goat anti-rabbit IgG peroxidase conjugate (BioRad), 1:2,000 dilution, was the secondary antibody. Color development
was accomplished with the enzyme substrate o-phenylenediamine (product P-9187, Sigma), and the optical density of each well was read at 450 nm.

Nonspecific values were subtracted from all readings. Displacement curves were obtained by plotting the logit-transformed ratio B/B₀ (sample binding value/maximum binding) versus the log of the PH standard doses. A least-squares regression of the log-logit data was performed and the resulting equation of the standard curve was used to calculate the titers of the cell lysate samples.

Functional Determination of the Expressed and Synthetic PH with IDDP Assay in Rats - The intradentinal dye penetration assay was used to determine the presence of porcine PH in the cell extracts and its function in rats. Male Sprague-Dawley rats, 21 days old, were obtained from Harlan (San Diego, CA), and fed for 1 week on pelleted Purina rat chow. The rats were kept in wire bottom cages to prevent coprophagy and ingestion of wood chips, particularly during fasting. The rats were divided into four groups with at least 6 animals in each group: Group 1: fasting only (negative control), Group 2: fed only (positive control), Group 3: fasting plus intravenous infusion with the cell lysate obtained from pCEP4 transfected COS-7 cells, and Group 4: fasting plus intravenous infusion of the cell lysate obtained from pCEP4-His-D₁₅ transfected COS-7 cells. All of the experiments were conducted on the rat’s 28th day after one week of adaptation to their environment. Pentobarbital sodium (4 mg/100g body weight) was administered intraperitoneally to anesthetize the animals.

After fasting, the rats in Group 1 were anesthetized and injected intraperitoneally with the fluorescent dye, acriflavine hydrochloride, dissolved in saline (5 mg/ml/100 g body weight). Thirty minutes after dye injection the rats were decapitated, their upper and lower jaws were quickly removed and frozen on dry ice. The frozen jaws from each animal were wrapped, coded, and kept at –80°C until sectioned. The rats in Group 2 were fed pellets of Purina rat chow for exactly 15 min before the above procedure. The rats in Group 3 and 4 were infused with 10 µl of cell lysate in 0.1 ml saline administered as a bolus infusion into the left subcalvian-external jugular vein junction after dye injection. Their jaws were collected 30 min later.

In a separate study, the activity of synthetic PH was also assessed. The 30 amino acid peptide of PH was commercially synthesized by Biopeptide, Co. LLC. (San Diego, CA). The synthetic PH was purified by HPLC using column Phenomenex Proteo C18, with 0.1% TFA in H₂O as solvent A, and 0.1% TFA in Acetonitrile as solvent B. The gradient was 15-45%B in 30 min, with a flow rate of 1ml/min, and the absorbance was monitored at 215 nm. It was found that the retention time for the synthetic PH peaked at 10.82 min, and the molecular weight by electro-spray mass spectrometry analysis was 2,858.

The synthetic PH was tested at the dose of 0.5 ng because this dose falls in the upper range of the dose response curve tested in the original PH isolation study (10). Two groups of male Sprague-Dawley rats were housed similarly as above, and after fasting overnight, the rats in Group 5 were anesthetized and injected with acriflavine, and infused with 0.1 ml of saline as described previously, while the rats in Group 6 were infused with 0.5 ng of synthetic PH dissolved in 0.1 ml saline. Thirty minutes after the infusion, the rats were killed, and their jaws were collected.

Dye penetration within dentinal tubules was measured by fluorescence microscopy in thin slices of the molar teeth following the procedure described by Leonora et al. (30). The positive response was defined as the presence of fluorescent dye in the dentinal tubules within a strictly defined area beneath the bottom of the occlusal groove. Each occlusal groove area was photographed using a digital camera, and evaluation of dye penetration into dentinal tubules was calculated from these pictures. Scoring was done without knowledge of treatment.

**Data Analysis** - The intradentinal dye penetration score was calculated as an IDDP ratio expressing the number of occlusal grooves under which fluorescence was observed divided by the total number of occlusal grooves examined for a given animal. Results were presented as the mean ± standard error of the IDDP ratios. Statistical comparisons among several treatment groups were made by analysis of variance (ANOVA), and multiple comparisons were performed...
with Bonferroni test when the ANOVA showed an overall significance. Values of p less than 0.05 were considered significant.

RESULTS

The complete amino acid sequence for parotid hormone isolated and purified from porcine parotid glands was found to be 30 amino acids in length as follows: NH$_2$-Ala-Pro-Pro-Gly-Ala-Arg-Pro-Pro-Gly-Pro-Pro-Pro-Pro-Pro-Gly-Pro-Pro-Pro-Pro-Pro-Gly-Pro-Pro-Pro-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Ser-Pro-Pro-Arg-Pro-Pro-Pro-Gly-Pro-Pro-Pro-Pro-Pro-Gln-COOH.

To identify the cDNA sequence encoding PH, a porcine parotid cDNA library was constructed and screened with 32P-labeled oligonucleotide probe PHiA, which corresponded to the N-terminal sequence of the isolated porcine PH. Twenty positive clones were randomly selected with the insert size ranging from 400 bps to 2300 bps. Three of the full-length cDNA clones, TP$_{23}$, SL$_{44}$, and D$_2$A, were chosen for complete sequence analysis.

The nucleotide sequences of D$_2$A and TP$_{23}$ contained open reading frames encoding polypeptides of 511 (48.5 kDa) and 566 amino acids (53.2 kDa), respectively. The complete nucleotide and the deduced amino acid sequences for these two cDNAs are presented in Fig. 1A and 1B. The sequence of the D$_2$A cDNA was identical to that of the TP$_{23}$ cDNA with the exception that D$_2$A cDNA had a 243-bp “deletion” in the 3’ region of the sequence.

The cDNA insert of SL$_{44}$ contained the complete coding sequence for a putative 62.3 kDa proline-rich protein (676 amino acids). The nucleotide sequence of SL$_{44}$ had an 89.6% identity with TP$_{23}$ (Fig. 1A). The derived amino acid sequences of the two cDNAs were 88.3% identical (Fig. 1B). While the TP$_{23}$ and D$_2$A messages may have been derived from the same gene, SL$_{44}$ appeared to originate from a different transcript. The differences between SL$_{44}$ and TP$_{23}$ were distinct, and probably not introduced by insertion, deletion, or alternative splicing. Both sequences contained the same 21-amino acid repeat with the prototype sequence of PPPGPPPPGPPPGPAPPGAR. However, a close inspection reveals that the codon usage was somewhat different. The last 30 amino acids encoded by the cDNA insert of both TP$_{23}$ and SL$_{44}$ were identical to the amino acid sequence of the isolated porcine PH.

In order to determine the tissue specific expression of the PH genes, the RNA and protein expression patterns of the TP$_{23}$ cDNA were examined. In situ hybridization of parotid gland slices (Fig. 2B) with the riboprobe showed a high density of silver grains over acinar cell cytoplasm, with occasional grains over nuclei. Silver grains were also observed in some of the serous cells in the sublingual glands, while the submandibular glands were negative. No significant silver grains were seen in other porcine tissues such as liver, pancreas, small intestine, trachea, lungs, kidney, hypothalamus, pituitary, and skeletal muscles (not shown).

Immunohistochemical staining of parotid gland slices with PH-Ab was most intense over acinar tissue (Fig. 2C). There was minimal staining in the ductal and other non-acinar areas. Some of the serous cells from the sublingual glands were also stained, correlating with the results from the in situ hybridization.

The genomic organization of the PH genes was explored using a 300 bp cDNA fragment from TP$_{23}$. Two major hybridization bands were observed in porcine genomic DNA restricted with BanHI, Hind III, Sac I, and Xba I, and five bands were detected after restriction with EcoR I or Pst I (Fig. 3). When the membrane was reprobed with the 1.7 kb cDNA insert from TP$_{23}$, a similar hybridization pattern was observed, but the intensity of the hybridization bands became more equal (not shown). There was no hybridization of the cDNA probe with human and rat genomic DNAs, even when the posthybridization washing stringency was lowered to wash in 2x SSC and 0.1% SDS twice for 15 min each at room temperature.

Full length cDNA TP$_{23}$ was subcloned into the expression vector pET-28a(+), and the resulting plasmid DNA pET-28a(+)-TP$_{23}$ was used in the TNT Quick Coupled Transcription/Translation System to obtain cell free protein synthesis. Separation of the translation mixture revealed one major product with a molecular weight of 100 kDa, which is much higher than 58.7 kDa deduced from the DNA sequence (Fig. 4A). A Western blot using PH-Ab revealed a similar result (not shown).
When a 216 bp cDNA, encoding the last 72 amino acids from TP23, which includes the 30 amino acids of PH and two of the 21-amino acid repeats upstream, was subcloned into pET-28a(+), the resulting plasmid DNA pET-28a(+)-D15 was used in the cell-free protein synthesis system. The Western blot analysis using PH-Ab showed one major product with an estimated molecular weight of 13 kDa (Fig. 4C, lane 1), which is larger than the size predicted from the sequence (9 kDa).

Fig. 4B shows the gel separation of expressed proteins recognized by PH-Ab in COS-7 cells, detected by colorimetric reaction. In pCEP4-TP23 transfected cells, a major band was seen with an estimated molecular weight of 110 kDa, which is much larger than the predicted 53.2 kDa. In the culture medium, a major band was seen at the position of 107 kDa. Several bands of smaller size were also observed. A band at 125 kDa was present in both pCEP4 transfected and pCEP4-TP23 transfected cell lysates, suggesting a cross-reaction with PH-Ab.

Fig. 4C also shows the Western blot of the proteins over expressed in COS-7 cells recognized by PH-Ab 72 hours after transfection and detected by chemiluminescence reaction. In cells transfected with pCEP4-His-D15, three major protein bands were present ranging from 5 to 13 kDa. Bands of similar size were also present in the culture medium.

The expressed PH and its related products in the cell lysate were quantified with ELISA. ELISA displacement curves for PH standard and for serial dilutions of pCEP4-His-D15 transfected cell lysate are presented in Fig. 5. Cells transfected with pCEP4-His-D15 DNA were harvested 72 hours after DNA addition, and 1 ml of cell lysate was obtained from one culture dish (100 mm) where cells were about 90% confluent. Under these culture conditions, the concentration of the expressed PH in the pCEP4-His-D15 transfected cell lysate was 1ng/µl. The proteins recognized by PH-Ab in the pCEP4 transfected cell lysate were not detectable.

The dentinal fluid stimulating activity of the expressed and synthetic PH was assessed with the IDDP assay in rats. On average, twelve to sixteen occlusal grooves were photographed and scored for each animal. The IDDP ratio was calculated and presented in Table 1. Consistent with previous studies (25), intradental dye penetration in the molar teeth of rats fed for 15 min was significantly greater than in fasting animals. The response in rats infused with the cell lysate obtained from pCEP4-His-D15 DNA transfected COS-7 cells was also significantly higher than that in the control animals. The difference between dye penetration scores in fasting animals and animals injected with the cell lysate from COS-7 cells transfected with vector alone was not statistically significant.

DISCUSSION

Screening of porcine parotid cDNA library with the oligonucleotide probe corresponding to the partial sequence of the isolated porcine PH resulted in the identification of numerous full-length cDNAs. Three full-length cDNAs described in this study encode polypeptides consisting of a series of domains that are characteristic of proline-rich proteins: signal sequence, transition region, highly repeated proline-rich domain, and C-terminal domain (12). The deduced amino acid sequences of D2A, TP23, and SL44-1 cDNAs presented in Fig. 7 show these four domains with some variations being observed in the repeat regions. These porcine proteins are particularly rich in proline (52-61%), while glutamine and glutamate (2-5%) are much less prominent as compared to PRPs in humans, rats, and mice. They also contain relatively high amounts of arginine and lysine (5.6-7.4%), suggesting that they are basic PRPs. Similar to human PRPs, these porcine PRPs contain 21-amino acid repeats, but with the prototype sequence of PPPPPPPPPPPPPPPAPPGAR. Variance in one or two amino acids is presented in some of the repeats. There are 12 repeats in D2A, 14 in TP23, and 26 in SL44-1. While most of these repeats are tandemly arranged, there are sequences within the repeat region that do not fit the 21 amino acid repeats. Among those, stretches of
sequences rich in lysine are present within, and at the end of the repeat region in TP23 and D2A. The lysine rich sequence, as underlined in Fig. 7, is also observed in SL44-1. With all these variations in the repeat region, the structure of porcine PRPs appears to be more complex than that of humans.

Genbank database search failed to identify significant sequence homology between the porcine cDNAs, including TP23 and SL44-1, and any known nucleotide sequence with the exception of the 5′-flanking sequence and the putative signal peptide region. The amino acid sequence of the putative signal peptide derived from these porcine cDNAs is very similar to the consensus sequence of the signal peptide for human PRPs (91% identity), but the identity in the repeat region is less than 50% (Fig. 8A). In contrast, similarities between rat, mouse, and human sequences are higher than 70%. There appears to be little conservation or identity in the carboxyl-terminal regions of the PRPs within and across species (Fig. 8A). In the C-terminal region of D2A, there is a cluster of various non-aromatic amino acids, while in TP23 and SL44-1, remnants of the repeat regions are present.

In human basic PRP genes, sites for the restriction enzyme BstN I occur in the repeat regions, approximately every 63 bases (31). The typical BstN I repeats are also present in the repeat region of D2A, TP23 and SL44-1, suggesting the possibility that these cDNAs belong to salivary basic PRP multigene families. BstN I cuts the DNA sequence C-C-A/T-G-G, which is within one of the coding sequences for the dipeptide Pro-Gly, C-C-N-G-G-N. Fig. 8B shows the DNA sequence and the deduced amino acid sequence of TP23 compared to the consensus nucleotide and amino acid sequences from several basic PRPs (31, 32). In one 21-amino acid repeat of porcine PRPs, the dipeptide Pro-Gly encoding sequence occurs four times, and the BstN I cutting site three times.

Human proline rich proteins are divided into two subfamilies according to Maeda (33). One group contains Hae III repeats coding for acidic PRPs and the other group contains BstN I repeats coding for basic and glycosylated PRPs. There are six loci controlling the synthesis of human salivary PRPs, including two loci forming the Hae III-type subfamily and four loci forming the BstN I-type subfamily. In Maeda’s study, when the Southern blots containing EcoR I digested human DNA was hybridized with a probe made from each of the two types of repeats, both probes showed hybridization to six identical EcoR I fragments under non-stringent conditions. Since the cDNA insert of TP23 contained BstN I-type repeats, hybridization pattern was compared with that of human basic PRP genes. Digestion with EcoR I or Pst I generated more hybridizing fragments in porcine genomic DNA than with other restriction enzymes, suggesting that there are cutting sites within the genes for these two enzymes. Since all other enzyme digests resulted in two major hybridizing bands, the basic porcine PRP genes recognized by the TP23 cDNA probe appear to be localized in two loci, instead of four as seen in humans.

The fact that no hybridization was observed with the porcine cDNA probe to human and rat genomic DNAs suggests that the porcine basic PRP genes are considerably different from these species. These data call into question the conclusion that the nucleotide sequences of PRPs are well conserved across species as suggested by cross-hybridization among the rat cDNAs (12), by hybridization of the rat pRP33 to human and mouse cDNAs (12), and by hybridization of the human PRP1 to rabbit cDNAs (34).

Porcine basic PRPs were constitutively expressed, as is the case in humans. Distribution studies demonstrated that under unstimulated condition there was an abundant synthesis of PH-related proteins in porcine parotid glands, minimal expression in sublingual glands, and none in submandibular glands. This pattern is similar to the distribution of basic PRPs in humans and rabbits. In both species, basic PRPs are present in parotid glands and in a few sublingual demilune cells but not in the submandibular glands (34, 35).

Similar to PRPs reported in several other studies (36, 37), the protein(s) encoded by TP23 DNA migrated slower in SDS-PAGE than expected from their molecular weight. It is likely that the band at 110 kDa in plasmid pCEP4-TP23 transfected cell extracts was the expressed full length protein, and the band seen in the culture medium at 107 kDa was probably the secreted form. The smaller protein bands may be the result of post-translational modifications and/or post-secretory cleavage.

The cloning of cDNAs that encode porcine PH has led to the discovery of several
features unique to porcine PRPs. These porcine PRPs have particularly high proline content with much less glutamine and glutamate as compared to human, rat or mouse PRPs. Their sequences are significantly different from PRPs found in other species. However, it is possible that there are families of PRPs in porcine parotid glands that can be recognized by the probes from human or rat PRPs, and the “basic PRPs” found in this study have no counterpart genes in human and rat.

The isolated porcine PH was the most active peptide purified from parotid extracts (10) and it may represent the mature protein processed from a larger precursor. Since the last 30 amino acid sequence encoded by TP23 and SL441 cDNAs is in agreement with the sequence obtained from the isolated PH, it is very likely that porcine PH is derived from the 3’ end of these cDNA clones in a manner similar to how many human basic PRPs are derived (38).

Our functional study data provided further evidence that porcine PH is most likely derived from the 3’ regions of these cDNAs. As indicated by an increase in IDDP, the infusion of a PH containing protein extract, produced from pCEP4-His-D15 transfected COS-7 cells stimulated DFM in rats. The magnitude of the response was comparable to that induced by feeding, a physiological stimulus for PH secretion. The increase in IDDP to the infused proteins suggested that the effect was systemically mediated. This strengthens the putative concept of the endocrine nature of PH. Whether the infused expression factor(s) was working directly or indirectly on the dental target tissue is presently unknown. The possibility exists that the stimulating effect may have occurred through an unidentified mechanism.

The PH encoding cDNA carried on pCEP4-His-D15 resulted in the expression of three major species of proteins as determined by immunoblot analysis. This suggests that each protein, either alone or in combination with each other, might have biological activity. Among the three major proteins over expressed, at least two of them have molecular weights smaller than the full-length protein synthesized in the cell-free system, suggesting that a proteolytic cleavage occurred after expression. Further studies are required to determine whether an endo-proteolytic cleavage, a nonspecific proteolytic degradation, or other post-translational modifications were responsible for generating these different products. The fact that the synthetic peptide was active in stimulating IDDP suggests that at least the last 30 amino acid derived from pCEP4-His-D15 was responsible for this IDDP stimulating activity.

To facilitate purification and identification of the expressed PH, the HisTag-encoding DNA sequence was fused to the 5’ end of the PH encoding cDNA. Remarkably, the HisTag was not found attached to the expression product that prevented its purification. Presumably, post-translational or endo-proteolytic cleavage of the expression product had removed the HisTag; therefore, the whole cell lysate was used for protein analysis and testing for the IDDP-stimulating activity. The cell lysate obtained from the mock-transfected cells was used as a negative control, which demonstrated no significant effect on IDDP. However, the possibility of some factor in the cell lysate that could have altered the stimulatory effect of the expressed PH cannot be ruled out.

During the past decades, it has become increasingly apparent that the physiological role of the parotid glands is more complex than their recognized salivary function (9, 10, 26, 39-43). Our data suggest that parotid acinar cells possess, in addition to salivary function, an endocrine function by secreting PH that is involved in inhibiting cariogenesis. To our knowledge, this may be the first example of a single cell type, at least among the major salivary glands, that possesses intracellular mechanism(s) that regulates two important complex functions (endocrine and exocrine). Morphologically, these cells appear well suited to regulate both functions (44). Furthermore, studies have shown that parotid glands are able to secrete transgene-encoded proteins into serum as well as saliva (45, 46). If our assumption is correct that parotid acinar cells possess a dual function, it implies that these cells have at least two transport mechanisms directing the proper distribution of molecules associated with each function, e.g., salivary PRPs are directed toward the apical end of the cells and PH to the basal portion to be secreted directly into the blood. How the molecular sorting is accomplished needs to be explored. Interestingly, several studies suggest that the proline-rich domain of proteins associated with the parotid glands may have a role in sorting and regulating the secretion of parotid
proteins (47). Ultimately, this would lead to the segregation of hormonal and salivary molecules into the appropriate pathways.

In summary, we have identified the cDNAs from porcine parotid glands that encode the amino acid sequence of a protein previously isolated from porcine parotid glands that possesses DFM-stimulating activity. The protein(s) expressed by these cDNAs in the transfected COS-7 cells and the synthetic protein mimic the activity of the endogenously secreted PH in response to the physiological stimulus of feeding. Our present data provide further confirmation that the parotid glands, in addition to their salivary function, possess an endocrine function that participates in systemic mechanism(s) directed towards preventing cariogenesis and even possibly integrating mechanism(s) associated with nutrient homeostasis since secretion of PH is initiated with the onset of nutrient intake (48).

REFERENCES


**FOOTNOTES**

* We thank Dr. Larry Sandberg for his help in amino acid sequencing, Dr. Paul McMillan for his help in immunohistochemistry studies and Dr. George Bagi for his help in Western blotting studies. This work was partially sponsored by the Department of the Army, Cooperative Agreement Number (DAMD17-97-2-7016), and the content of this paper does not necessarily reflect the position of the government or NMTB, and no official endorsement should be inferred.

The nucleotide sequence reported in this paper has been submitted to the GenBank under GenBank Accession Number AY035847, AY035848 and AY035849.

1The abbreviations used are:
ANOVA, analysis of variance; DFM, dentinal fluid movement; IDDP, intradentinal dye penetration; PFA, paraformaldehyde; PH, parotid hormone; PH-Ab, polyclonal antibody against porcine PH; PRP, proline-rich protein; PTH, phenyl-thio-hydantoin; TFA, trifluoroacetic acid.

**FIGURE LEGENDS**

Fig. 1. Alignment of nucleotide and deduced amino acid sequences of D2A, TP23 and SL44-1 cDNAs. The alignments were generated using the Clustal W program. A, The nucleotide sequences are numbered beginning with the first nucleotide of each cDNA, and the nucleotide position numbers are given on the left of the sequences. The putative coding sequences are in capital cases, and the stop codons are in bold letters. The 243-bp nucleotide sequence that was “missing” in D2A is marked by *. The 300 bp fragment of TP23 that was used for in situ hybridization and Southern blot analysis is underlined. Black background and gray shading indicate identical and similar nucleotides, respectively. B, The deduced amino acid sequences are numbered beginning with the initiator methionine, and the numbers on the left of the sequences indicate the amino acid position. The amino acids conserved are shown on a black background, and similar residues are indicated by gray shading. The last 30 amino acids of TP23 and SL44.
that are identical to the isolated porcine parotid hormone are in bold letters.

**Fig. 2.** Localization of PH gene transcripts by in situ hybridization and the PH proteins by immunohistostaining in porcine parotid, sublingual and submandibular glands. *A*, Hematoxylin & eosin staining provides histological reference. *B*, In situ hybridization of parotid hormone mRNA. *C*, Immunocytochemical localization of parotid hormone with PH-Ab. The magnification levels are indicated on the right. PA, parotid glands; SL, sublingual glands; SM, submandibular glands.

**Fig. 3.** Southern blot analysis of pig, human and rat genomic DNAs. Genomic DNAs were digested with the restriction enzyme indicated above each lane. The membrane was probed with the 300-bp cDNA fragment from clone TP23. The sizes of marker DNAs (in kb) are shown on the left.

**Fig. 4.** Western blot analysis of proteins encoded by the cDNA insert of TP23 or D15 produced in vitro using a cell free system and in vivo using transfected COS-7 cells. *A*, The cDNA insert of plasmid pET-28a(+)TP23 was transcribed and translated in vitro in the presence of biotinylated lysine. Lane 1 represents the translation product detected by the Transcend Colorimetric Detection system. The sizes of protein molecular weight markers are given at the left in kDa. *B*, Western blot analysis of proteins expressed in COS-7 cells detected by PH-Ab. Lane 1 represents the cell extracts obtained 72 hr after the transfection with pCEP4 vector. Lane 2 to 4 represents the cell extracts collected 24, 48 and 72 hr after the transfection with plasmid pCEP4-TP23, respectively. Lane 5 represents the culture medium collected 72 hr after the transfection with pCEP4 vector, and Lane 6 to 8 represents the culture medium collected 24, 48, and 72 hr after the transfection with pCEP4-TP23. The sizes of protein molecular weight markers are indicated at the right in kDa. *C*, cell-free translation of the cDNA insert of plasmid pET-28a(+)D15 using the TNT system (lane 1), and COS-7 cells 72 hours after the transfection with plasmid pCEP4 (lanes 2, cell lysate, and 3, culture medium) or pCEP4-His-D15 (lanes 4, cell lysate, and 5, culture medium). The proteins were detected with PH-Ab. Molecular weight scale is indicated at the left.

**Fig. 5.** Quantification of PH in the cell lysate using ELISA. ELISA displacement curves for porcine PH (filled circle) and pCEP4-His-D15 transfected COS-7 cell lysate (open circle) are presented. Serial dilutions were tested in duplicates. The log-logit transformed data and the computed linear regression were plotted for each hormone preparation.

**Fig. 6.** Intradentinal dye penetration in sagittal tooth sections from rats. The intradentinal dye penetration assay was carried out on six groups of rats as detailed in the experimental procedures. The positive response was defined as the presence of fluorescent dye in the dentinal tubules within a strictly defined area beneath the bottom of the occlusal groove (O.G.), delineated by two imaginary lines as shown in the photomicrograph. *A*, A negative occlusal groove section from a rat which was fasted overnight without any treatment. *B*, A positive molar section from a rat which was fed for 15 min before teeth were collected. Fluorescence was observed in the dentin area beneath the bottom of the occlusal groove due to stimulation of DFM (transporting acriflavine hydrochloride). *C*, A negative molar section from a rat which was injected with 10 µl of pCEP4 transfected COS-7 cell lysate; and *D*, a positive molar section from a rat which was injected with 10 µl of pCEP4-His-D15 transfected COS-7 cell lysate. Fluorescence was observed in the dentin area beneath the bottom of the occlusal groove. *E*, A negative tooth section from a rat infused with 0.1 ml of saline, and *F*, a positive section from a rat treated with 0.5 ng of synthetic PH, in which the dentinal tubules showed a brilliant green fluorescence of acriflavine hydrochloride.

**Fig. 7.** Four domains of amino acid sequences derived from the cDNA inserts of D2A (*A*), TP23 (*B*), and SL44-1(*C*). The derived amino acid sequence of each cDNA is arranged to align identical regions. Variations in the repeat regions are indicated in bold letters, and the lysine rich segments shared by all three cDNAs are underlined.
Fig. 8. Comparison of porcine PRPs with PRPs from other species. A, Comparison of signal peptide, repeat, and C-terminal sequences of human, rat, mouse proline-rich proteins to those derived from the cDNA inserts of TP₂₃, SL₄₄-1, and D₂A (denoted as porcine PRPs). The sequences for human acidic and basic proline rich proteins are consensus sequences, and those for rat or mouse are from individual cDNAs. Sequences are aligned to give maximum identities, and the conserved amino acids across species are highlighted. The similarities between porcine PRPs and human basic PRPs are also underlined. S, signal peptide; R, proline rich repeat; C, carboxyl-terminal region. B, Comparison of consensus DNA and amino acid sequences of TP₂₃ and D₂A cDNAs (from BstN I repeats) to consensus DNA and amino acid sequences of the repeat region from basic PRPs described by others (31, 32). The nucleic acid codes are: R=G A, Y=T C, K=G T, M=A C, S=G C, W=A T, B=G T C, and V=G C A. The potential BstN I restriction sites (CCA/TGG) are underlined.
Table 1. Effects of feeding and infusion of synthetic, expressed and isolated porcine parotid hormone on intradentinal dye penetration in rat molar teeth.

The intradentinal dye penetration (IDDP) assay was performed on 28 days old male Sprague-Dawley rats. The rats were divided into six groups and the number of animals in each group is presented in parentheses. The bioassay was carried out as detailed in the experimental procedures. The data for Group 7 and 8 were complied from a previous study (10). The IDDP ratio was calculated for each animal, and presented as mean ± standard error for each group. Statistical comparisons among several treatment groups were made by ANOVA, and multiple comparisons were performed with Bonferroni test. The \( p \) values for comparisons between groups are presented.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dietary State</th>
<th>PH Source</th>
<th>Dose (ng)</th>
<th>IDDP Ratio</th>
<th>(N)</th>
<th>Statistical Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>fasting</td>
<td>-</td>
<td>-</td>
<td>0.187 ± 0.056</td>
<td>(6)</td>
<td>1 vs. 2 ( p=0.015 )</td>
</tr>
<tr>
<td>2</td>
<td>fed</td>
<td>endogenous secretion</td>
<td>-</td>
<td>0.567 ± 0.088</td>
<td>(9)</td>
<td>2 vs. 3 ( p=0.004 )</td>
</tr>
<tr>
<td>3</td>
<td>fasting</td>
<td>mock expressed cell lysate</td>
<td>-</td>
<td>0.135 ± 0.040</td>
<td>(6)</td>
<td>3 vs. 4 ( p=0.001 )</td>
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<tr>
<td>4</td>
<td>fasting</td>
<td>cDNA expressed cell lysate</td>
<td>10</td>
<td>0.660 ± 0.097</td>
<td>(6)</td>
<td>4 vs. 1 ( p=0.004 )</td>
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<tr>
<td>5</td>
<td>fasting</td>
<td>(saline)</td>
<td>-</td>
<td>0.219 ± 0.095</td>
<td>(6)</td>
<td>5 vs. 6 ( p=0.007 )</td>
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<td>6</td>
<td>fasting</td>
<td>synthetic peptide (saline)</td>
<td>0.50</td>
<td>0.524 ± 0.063</td>
<td>(6)</td>
<td>7 vs. 8 ( p=0.001 )</td>
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<tr>
<td>7*</td>
<td>fasting</td>
<td>porcine parotid glands (saline)</td>
<td>-</td>
<td>0.163 ± 0.026</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>8*</td>
<td>fasting</td>
<td>porcine parotid glands</td>
<td>0.54</td>
<td>0.378 ± 0.030</td>
<td>(17)</td>
<td></td>
</tr>
</tbody>
</table>

* see reference 10, Table 5 and Fig. 4
Figure 5

- pCEP4-His-D15-transformed COS-7 cell lysate—serial dilutions

Logit \( \frac{B}{B_0} \)

- pg porcine PH (log scale)
A. D₂A

Signal Sequence:  MLPILLSVALALLSSA₁⁶

Transition Region:  RSPFFDLEDANSNSAEKFLRPPPPGGPPPPPEE₅¹
SQGEHQRPRPPGDGPEQGPAPPGR₇₈

Repeat Region:  PPPGPPPPGPAPPGR₉₉
PPPAPPAPPAPPAPPGR₁₂₀
PPPAPPAPPAPPAPPGR₁₄₁

PPPAPPAGGLQQGPAPSHV₁₆₂
GPKKKPPPPAGHPPRPPPP₁₈₂
ANESQPPGRPPPPSPP₂₀₀
ANDSQEGSPPS²₁₁

ADGPQQPAPSPGDKKPAPPAGPPPPPPPPG²₄₅
PPPAPPAPPGR²₅₇

Repeat Region:  PPPAPPAPPAPPAPPGR²₇₈
PPPAPPAPPAPPAPPAPPHGR₂₉₉
PPPAPPAPPAPPAPPAPPGR₃₂₀
PPPAPPAPPAPPAPPAPPGR₃₄₁
PPPAPPAPPAPPAPPAPPGR₃₆₂
PPPAPPAPPAPPAPPAPPGR₃₈₃
PPPAPPAPPAPPAPPAPPGR₄₀₄
PPPAPPAPPAPPAPPAPPGR₄₂₅
PLPPAPPAPPAPPAPPGR₄₄₆

PLPPAPP₄₅₄

ADEPQQPAPSPGDKPAPPAGPPPPPPPPG₄₈₈

C-terminal:  IQGKMSAKTPVLRAVTLECDG₅₁₁
B. TP$_{23}$

Signal Sequence: MLPILLSVALLALSSA$^{16}$

Transition Region: RSPFFDLEDANSNSAEKFLRPPPGGPPRPPPPPEE$^{51}$
SQGEHQQKRPRPPGDGPLQGPAPPGAR$^{78}$

Repeat Region: PPPGPPPPGPPPPGPPAPPGAR$^{99}$
PPGPPPPGPPPPGPPAPPGAR$^{120}$
PPGPPPPGPPPPGPPAPPGAR$^{141}$

PPGPPPPPAGGLQQGPAPSHV$^{162}$
GPKKKPPPGAGHPRPQPPPP$^{182}$
ANESQPGPRPPGPPSSP$^{200}$
ANDSQEGSSPS$^{211}$

ADGPPQGPAPSGDKKKPPPAGPPPPPPPPPG$^{245}$
PPGPPAPPGAR$^{257}$

Repeat Region: PPPGPPPPGPPPPGPPAPPGAR$^{278}$
PPGPPPPGPPPPGPPAPPGAR$^{299}$
PPGPPPPGPPPPGPPAPPGAR$^{320}$
PPGPPPPGPPPPGPPAPPGAR$^{341}$
PPGPPPPGPPPPGPPAPPGAR$^{362}$
PPGPPPPGPPPPGPPAPPGAR$^{383}$
PPGPPPPGPPPPGPPAPPGAR$^{404}$
PPGPPPPGPPPPGPPAPPGAR$^{425}$
PLPGPPPPGPPPPGPPAPPGAR$^{446}$

PPGPPPPP$^{454}$
ADEPQGPAPSGDKKKPPPAPGPPPPPPPPPG$^{488}$
PPGPPAPPGAR$^{500}$
FRPGPPPPGPPPPGPPAPPGAR$^{521}$
PPLGGPPPPGPPPPGPPAPPGAR$^{542}$

C-terminal: PPPGPPPPPAGPSRARPPPPPPPPPG$^{566}$
C. SL44-1

Signal Sequence: MLPILLSVALLALSSA

Transition Region: RSPFFDLEDANSNSAEKFLRPPGGPPRPPPPEE SQGEGHQKRPRPPGDPEQGPAPPGAR

Repeat Region: PPPGPPPPGPAPPDPAR PPPGPPPPGPAPPDPAR PPPGPPPPGPAPPDPAR PPPGPPPPGPAPPDPAR PPPGPP216

C-terminal: PPPGPPPPGPAPPDPAR
## Figure 8

### A.

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<td><strong>MLILLSVALLALSSA</strong></td>
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<tr>
<td>S\textsubscript{porcine} PRPs:</td>
<td><strong>MLILLSVALLALSSA</strong></td>
</tr>
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<td><strong>PPPGQPPGPPPGPAPFGAR</strong></td>
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<td><strong>PQPGKPQGPPQGPPQQRP</strong></td>
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<td><strong>PQPGKPQGPPQGPPQQKP</strong></td>
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<td><strong>GNQPPQAPAGQPPGPQGRAPSPPG</strong></td>
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<td><strong>GNPQGPPQGRPGPQ</strong></td>
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<td>C\textsubscript{rat pRP33:}</td>
<td><strong>PAQDATHEQPSYLWFSS</strong></td>
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### B.

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<td><strong>CCTCSTCCGGAAGCCGAGACCGACCCGCAACGCCCAGAGGCCAACGCCCAGAGCCACAGTCCCA</strong></td>
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Cloning and functional study of porcine parotid hormone, a novel proline rich protein
Qian Zhang, Aladar A. Szalay, Jean-Marc Tieche, Eru Kyeyune-Nyombi, John F. Sands,
Kerby C. Oberg and John Leonora

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