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Differential Expression of Isoproterenol-Induced Salivary Polypeptides in Two Mouse Strains That Are Congenic for the H-2 Histocompatibility Gene Complex

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Abstract Two inbred mouse strains, A/Snell and A.Swiss, which were produced as congenic with regard to the H-2 histocompatibility gene complex, are homozygous for two different groups of isoproterenol-induced salivary polypeptides (IISP). These polypeptides, which have been considered as markers of the hypertrophic growth of the parotid acinar cells, are members of the complex family of salivary proline-rich proteins (PRP) on the basis of both their massive accumulation in the parotid acinar cells in response to chronic isoproterenol, secretory character, high solubility in trichloroacetic acid and metachromatic staining by Coomassie blue. IISP expressed in both mouse strains were identified by unidimensional SDS–polyacrylamide electrophoresis and Coomassie blue staining both in parotid gland homogenates and in whole salivas obtained from mice repeatedly stimulated at 24-h intervals with isoproterenol. Parotid glands from 40 mice (20 A/Snell and 20 A.Swiss) and salivas from 270 mice (200 A/Snell and 70 A.Swiss) were analyzed. One of the congenic strains (A/Snell) expressed five IISP (Mr 65, 61, 51.5, 38, and 37 kDa) and the other strain (A.Swiss) expressed six IISP (Mr 59, 57, 54.5, 46, 36, and 34 kDa). No inter-individual intra-strain variations were observed, thus defining strain-associated patterns of IISP (PRP). J. Cell. Biochem. 90: 945–956, 2003. © 2003 Wiley-Liss, Inc.

Key words: saliva; salivary gland; polypeptides; strain; mouse; isoproterenol; secretion; proline-rich proteins

Isoproterenol provokes marked trophic effects in the salivary glands of mouse and other rodents [Novi and Baserga, 1971; Barka and Burke, 1977]. This effect is far more evident in the parotid glands [Schneyer, 1962]. Concomitantly to the trophic effect in mouse parotids, isoproterenol induces in these glands a group of polypeptides [López Solís and Miranda Wilson, 1986; López Solís et al., 1987]. These polypeptides have been considered as markers of the trophic response in the parotid glands on the basis of the strict correlation between the intensity of their accumulation in the glands and the intensity of the experimentally induced gland enlargement [López Solís and Miranda Wilson, 1986; López Solís et al., 1987, 1990]. An additional feature of these polypeptides is their secretory character. Thus, once induced all of these can be observed in the fluid collected directly from cannulated hypertrophic parotid glands [López Solís et al., 1989]. These murine polypeptides (named as C, D, E, F, and G, Mr 65, 61, 51.5, 38, and 37 kDa) quite likely correspond to proline-glycine-glutamine-rich proteins (PRP) because they display both a metachromatic staining after Coomassie blue and a high

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solubility in trichloroacetic acid [Muenzer et al., 1979; Mehansho et al., 1983, 1987; Humphreys-Beher et al., 1987a; López Solís et al., 1993]. However, all those studies linking the expression of specific isoproterenol-induced parotid polypeptides, that are PRP, to the trophic response in those glands, have been carried out specifically in the murine model and by using a single mouse strain (A/Snell) [López Solís et al., 1987, 1989, 1990, 1993, 2003; González et al., 2000]. Salivary PRP have been described in man and in various rodent species, mainly rat and hamster [Mehansho et al., 1983, 1987; Ann et al., 1987; Azen et al., 1996]. These all seem to constitute a highly complex family of proteins [Ann et al., 1988; Azen, 1993; Kim et al., 1993]. A number of these have been sequenced in the past few years and a modular primary structure has been described [Clements et al., 1985; Ann et al., 1988; Kim et al., 1993; Castle and Castle, 1998]. Inducible or constitutive expression, multiple genes, alternative mRNA processing, and post-translational modifications have been mentioned as contributors to the PRP complexity [Maeda et al., 1985; Ann et al., 1987; Lyons et al., 1988; Layfield et al., 1992; López Solís et al., 1993; Bedi and Bedi, 1995; Miao et al., 1995; Ann et al., 1997; Zhou et al., 1997; Ann and Lin, 1998]. At least basic, acidic, and glycosylated subgroups of PRP have been recognized [Layfield et al., 1992; Bedi and Bedi, 1995]. Regarding their functions, some of the constitutively expressed human PRP seem to play an important role in saliva by binding Ca^{2+} and preventing its precipitation [Humphreys-Beher et al., 1987b]. They also may be involved in the formation of the acquired pellicle on the surface of teeth [Kousvelari et al., 1980; Bennick et al., 1983], in the differential bacterial colonization of the oral hard and soft surfaces [Amano et al., 1998; Carlen et al., 1998], and in neutralizing dietary tannins [Mehansho et al., 1983, 1987]. However, so far it is fully unclear how to associate that broad spectrum of possible general functions either with the molecular diversity of the PRP family in mammals or with the fine-tuning of their inducible expression in rodents [López Solís et al., 1993; Ann et al., 1997; Ann and Lin, 1998]. In the same direction, there is no clue regarding an eventual molecular mechanism linking the inducible expression of specific PRP and the induction of the trophic response in mice. The availability of animal models in which genetic variants of these proteins might be expressed would be highly relevant for the study of the role of those proteins in the organisms. Studies from other laboratories have shown differences between the isoproterenol-induced mRNA in parotid glands of rat and mouse [Ann et al., 1987]. Equally interesting is the fact that differences in the isoproterenol-induced mRNA have been observed in parotid glands obtained from different mouse strains [Ann et al., 1987]. However, no systematic analysis of eventual differences in the polypeptide components coded by this complex gene family within a single species has been reported. In the present study we have analyzed by unidimensional SDS–polyacrylamide gel electrophoresis the isoproterenol-induced polypeptides during the trophic response in the parotid glands of two old inbred (homozygous) mouse strains (A/Snell and A.Swiss). These strains were originally derivated as congenic for the H-2 histocompatibility gene complex loci, sharing the A/Snell chromosomal background and differing in a H-2 subregion ($H\text{-}2^a$ in A/Snell vs. $H\text{-}2^s$ in A.Swiss) [Klein, 1975; Altman and Dittmer Katz, 1979]. Parotid glands of each of the mouse strains expressed a group of unvariant new polypeptides in parallel to the trophic response to isoproterenol. However, marked differences between both groups of isoproterenol-induced parotid polypeptides were observed. Further analyses in the saliva collected from isoproterenol-stimulated mice of both inbred strains showed unequivocally the strain-associated profile of the isoproterenol-induced salivary polypeptides (IISP).

MATERIALS AND METHODS

Animals

Female or male mice of the A/Snell and A.Swiss strains, inbred in our laboratory since 1960 and weighing 24 ± 3 g were used when 3–4 months old. The animals were maintained on a 12 h light and 12 h dark schedule and fed ad libitum.

Induction of Salivary Polypeptides (IISP)

A 0.2 ml aliquot of a freshly prepared aqueous solution of isoproterenol was administered intraperitoneally (0.16 μmol/g body weight) once a day at 24-h intervals for at least 4–5 days when parotid or submandibular glands were the study material [López Solís et al., 1987] and for

1–3 days when saliva was going to be monitored [López Solís et al., 2003]. Control unstimulated mice consisted of animals that were injected with saline.

Salivary Tissue

Both control and experimental mice were organized in groups of at least four mice each. In those studies in which IISP were monitored in the salivary tissue, the animals were killed by cervical dislocation at the times indicated in the specific experiments and both parotid glands from each animal were dissected free of fat and lymph nodes and weighed in an analytical balance. Gland size was expressed as the average wet weight ± standard deviation. Parotid glands from individual animals were homogenized vigorously in 2.5 ml of 20 mM potassium phosphate/6.7 mM sodium chloride (pH 6.9, 20°C) using five strokes in a motor-driven Teflon-glass homogenizer, filtered through four layers of cheese-cloth laid at the bottom of a 5 ml disposable syringe, and adjusted to 4 ml with the same buffer. An aliquot was saved for protein quantitation and the rest of the homogenate was distributed in multiple aliquots and stored at −86°C until the day of the electrophoretic fractionation. In some experiments, both submandibular/sublingual and parotid glands were separately dissected from the same mice and processed in parallel.

Collection of Saliva

At about 24 h after the last of a series of isoproterenol administrations, a single dose of 10 μl of 4% pilocarpine was instilled directly in the mouse mouth (time zero). Once salivation became visible (at about 5 min), aspiration of the salivary fluid was initiated by means of a disposable tuberculine syringe fitted with a bent disposable tip [López Solís et al., 2001, 2003]. Saliva from every single mouse was accumulated in preweighed Eppendorf tubes that were maintained in ice. At the end of the collection procedure (at about 30 min), the Eppendorf tubes were weighed in order to estimate the amount of collected saliva by assuming a specific gravity of 1.00 g/ml. Routinely, over 250 μl of saliva per mouse were obtained by this procedure. A 10 μl aliquot of saliva was saved for protein quantitation and the rest of the sample was distributed into several aliquots and saved at −86°C until the electrophoretic fractionation.

Protein Content

Ten microliters aliquots of either saliva or whole salivary gland homogenates from every single mouse were spotted onto cellulose discs, fixed in 5% trichloroacetic acid (TCA) at 80°C, and washed successively in 5% cold TCA, 80% ethanol and 3:1 (v/v) ethanol/ether. The air-dried discs were incubated at 45°C in 0.25% Coomassie blue R-250 for 30 min, drained, and washed exhaustively in several changes of 7% acetic acid until the background was clear. The discs were dried, introduced into Khan tubes and eluted in 3 ml of 66% methanol–0.25% ammonia. Eluates were read at 610 nm in a double-beam Shimadzu UV-160 spectrophotometer. A standard curve was prepared by including discs containing 10–50 μg of bovine serum albumin. Blank discs contained no protein [Bramhall et al., 1969; Durham and López-Solís, 1979].

Protein Electrophoresis

Aliquots of whole mouse saliva containing 40–50 μg of protein or aliquots of whole salivary gland homogenates containing 50–100 μg of protein were mixed with sample buffer and electrophoresed in SDS–polyacrylamide slab gels (12%) as specified elsewhere [Laemmli, 1970; López Solís and Miranda Wilson, 1986]. Each sample to be electrophoresed corresponded to single animals. To calibrate individual electrophoretic separations, molecular weight standards were run in parallel. Following the electrophoretic separation, gels were fixed overnight in 15% isopropanol/10% acetic acid, rinsed twice in the same solution, and stained for at least 12 h in 0.25% Coomassie blue R-250 dissolved in 45% isopropanol/10% acetic acid. The gels were rinsed in 10% isopropanol/10% acetic acid until clear background. Finally, the gels were rinsed in three 5-min changes of distilled water and scanned in an AGFA-Snapscan 1236 device. Printing was carried out by using an Epson 910 printer.

Materials

Pilocarpine was obtained as a 4% solution from pharmaceutical suppliers (Licarpin TM, Saval Laboratories, Santiago, Chile). (+/−)-Isoproterenol- HCl (molecular weight 247.7), other reagent-grade chemicals and protein molecular weight standards for gel electrophoresis were acquired from Sigma (St. Louis, Mo).

Protein molecular weight standards were ovotransferrin (78 kDa), bovine seroalbumin (66 kDa), ovoalbumin (42.5 kDa), and carbonic anhydrase (30 kDa). Solvents used for gel processing were purchased from Merck (Santiago, Chile). Cellulose discs (Whatman grade 1; 2.5 cm diameter) were obtained from Whatman (Maidstone, England).

RESULTS

Trophic Effect of Isoproterenol on the Parotid Glands of A/Snell and A.Swiss Mice

A daily intraperitoneal dose of isoproterenol (0.16 μmol/g body weight) for 6-days provoked a marked enlargement of parotid glands in both mouse strains (Fig. 1). Thus, by the end of that treatment the parotid wet weight had increased at least over 2.5-fold with respect to unstimulated glands (Fig. 1A). An identical isoproterenol-induced trophic response was observed in the parotid glands of both mouse strains when the total content of glandular protein was assessed (Fig. 1B). During the whole period of treatment with isoproterenol no variation in the body weight of the animals was observed.

Effect of Isoproterenol on the Polypeptide Composition of Parotid Glands From A/Snell and A.Swiss Mice

The electrophoretic fractionation of polypeptides present in whole homogenates obtained from control unstimulated parotid glands of both mouse strains showed no differences. In both mouse strains, among the main polypeptide bands were the ones corresponding to two secretory proteins, namely, α-amylase or polypeptide A (55 kDa) and PSP or polypeptide B (21 kDa) (Fig. 2A,B). When whole homogenates were obtained from parotid glands of both strains of mice after a 4-day treatment with isoproterenol, the electrophoretic analysis showed the induction of marked changes in the polypeptide composition. In the A/Snell strain those changes involved basically the appearance of polypeptides C (Mr 64.500), D (Mr 60.000), E (Mr 51.500), F (Mr 37.000), and G (Mr 36.000) in addition to a significant diminution in the intensity of the normal polypeptide bands A (55 kDa) and B (21 kDa) (Fig. 2A). The polypeptide composition of the A/Snell submandibular salivary glands was altered only marginally after the treatment with isoproterenol. When observed, those minor changes were a tenuous and partial reflection of those observed in the parotid glands of the same animals, with the single exception of a weak polypeptide band of 40 kDa that was not observed in the parotid glands. In the A.Swiss strain, isoproterenol was also a powerful inducer of new polypeptides in the parotid glands, in addition to provoking a marked diminution in the glandular content of the normal secretory polypeptides A and B (Fig. 2B). At least six new polypeptide bands were observed in the whole homogenates of stimulated versus unstimulated A.Swiss parotid glands, namely P (Mr 59.000), Q (Mr 57.000), Q′ (Mr 54.500), R (Mr 46.000), S (Mr 36.000) and, T (Mr 34.000). Again, polypeptide B showed a significant diminution in intensity while polypeptide A became partially masked by some of the induced polypeptides. The intensities of

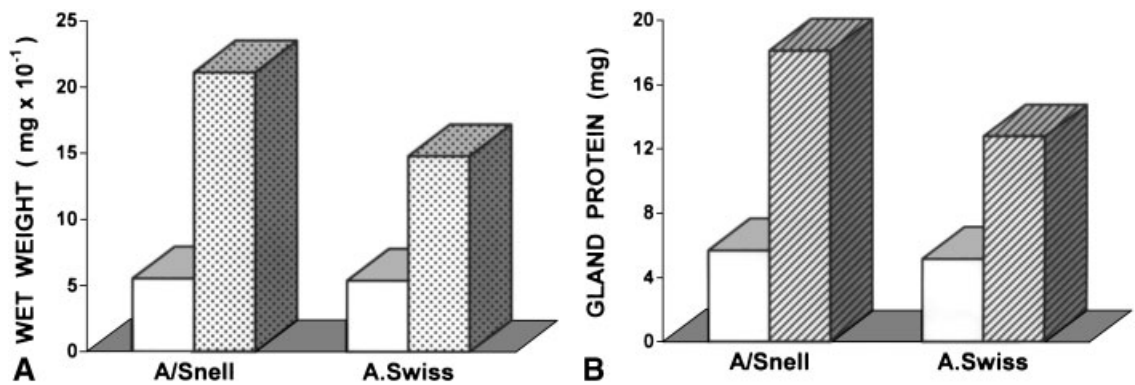

Fig. 1. Trophic effect of IPR on the parotid glands of A/Snell and A.Swiss mice. Groups of mice of each strain were injected with IPR (0.16 μmol/g body weight) at 24-h intervals for 6-days. At 24 h after the last administration of IPR, the animals were killed and both parotid glands from each animal were dissected, weighed, and processed for protein quantitation. Control mice consisted of four unstimulated animals. Wet weight (**A**) and protein content per pair of parotid glands (**B**) are shown as averages. Standard deviations were less than 10% of the averages. Unfilled columns: Controls; dashed columns: IPR.

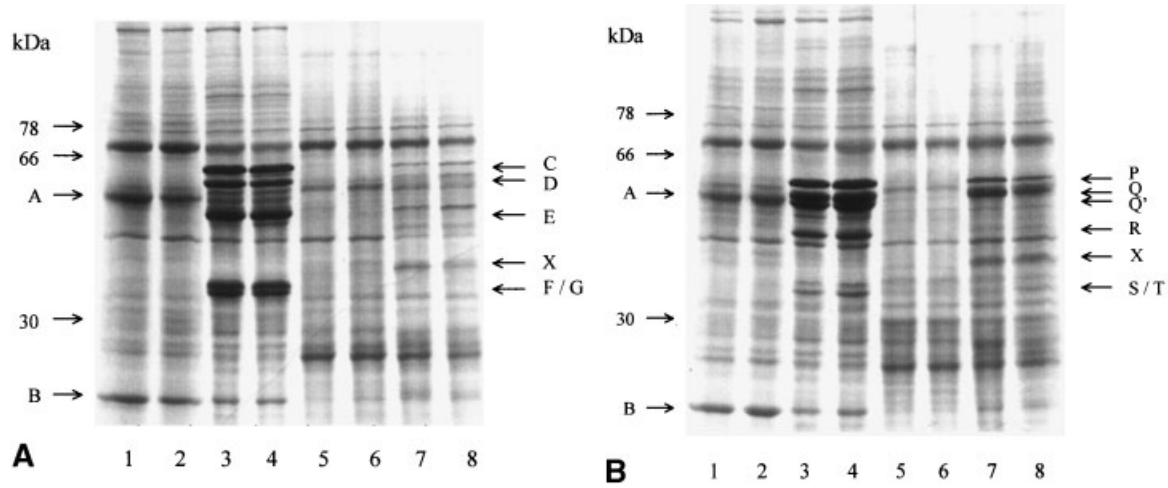

Fig. 2. Effect of chronic IPR on the polypeptide composition of salivary glands of the A/Snell and A.Swiss mouse strains. **A**: A/Snell strain aliquots of gland homogenates prepared from either parotid or submandibular/sublingual (sbm/sl) salivary glands of single mice were electrophoresed. Parotid glands (**lanes 1–4**) and sbm/sl glands (**lanes 5–8**) were dissected from control unstimulated mice (lanes 1, 2, 5, and 6) and from mice daily stimulated by IPR (0.16 μmol/g body weight/day) for 4-days (lanes 3, 4, 7, and 8). The gel is representative of an experiment comprising 20 control and 20 IPR-stimulated mice. Note in parotid glands the striking induction of polypeptides C, D, E, and the duplet F/G (Mr 64.5; 60; 51.5; 37, and 36 kDa) and the marked diminution of the normal secretory polypeptides A and B, while the rest of the parotid polypeptide components remain basically unaltered. By contrast, sbm/sl glands express in response to IPR only very minor qualitative changes in the polypeptide composition, most of which (with the single exception of polypeptide X, Mr 40 kDa) coincide with those induced in parotid glands. Standard molecular weights: ovotransferrine (78 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (30 kDa). **B**: A.Swiss strain. Lanes in the representative gel and the experimental conditions are the same as in A. Again, note the marked induction of polypeptides in parotid glands, the poor effect on the sbm/sl glands, the coincidence of some of the weakly induced polypeptide bands in these latter glands with the induced polypeptides in parotid glands and the induction of polypeptide X which seems to be specific for sbm/sl glands. Note the strikingly different profile of IPR-induced parotid polypeptides (P, Q, Q′, R, and the duplet S/T; Mr 59; 57; 54.5; 46; 36, and 34 kDa) with respect to the one induced in the A/Snell strain (strain-associated patterns).

the rest of the parotid polypeptides were unaltered after the treatment with isoproterenol. Submandibular salivary glands in this mouse strain also exhibited minor changes in the polypeptide composition and, when observed, they appeared mostly as an iteration at a much lower scale of the changes observed in parotid glands. Again, the exception was the weak isoproterenol-induced polypeptide band of 40 kDa that was not observed in the parotid homogenates (Fig. 2B). As a whole, the electrophoretic profile of the isoproterenol-induced parotid polypeptides in the A.Swiss mice was found to clearly differ from the one in the A/Snell mice. Thus, the isoproterenol-induced changes in the parotid polypeptide composition corresponded to a strain-dependent trait since after screening in this study 20 pairs of parotid glands from the same number of mice of each strain, these molecular differences were reproduced with no exception (pattern). Also, these results showed that the chronically isoproterenol-stimulated parotid tissue of both strains, at variance of the corresponding normal unstimulated tissue, differ to each other basically in the induced polypeptides (Fig. 3).

Secretory Character of the Isoproterenol-Induced Parotid Polypeptides in the A/Snell and A.Swiss Mouse Strains

SDS–polyacrylamide slab gel electrophoresis of isoproterenol-stimulated mouse parotid glands has proved to be advantageous for the identification of secretory polypeptides [López Solís and Miranda Wilson, 1986; López Solís et al., 1993]. To that end, the approach consisted in identifying polypeptide bands that diminish markedly in intensity or that are frankly lost from glands dissected within the first 1–2 h following the effect of a powerful secretagogue, as it is the case of either pilocarpine or isoproterenol. On the other hand, both morphological studies, α-amylase assays and electrophoretic studies have consistently indicated that isoproterenol is an effective inducer of the secretory response in normal mouse parotids and in parotid glands made hypertrophic by a chronic administration of the same β-adrenergic ago-

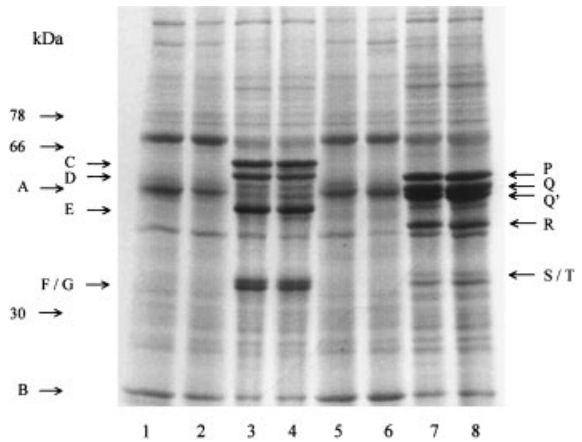

Fig. 3. Alignment of the polypeptide profiles of control and IPR-stimulated parotid glands of the A/Snell and A.Swiss mouse strains. Aliquots of whole homogenates prepared from parotid glands of single mice were electrophoresed. Glands were dissected either from unstimulated mice (**lanes 1**, **2**, **5**, and **6**) or from mice stimulated during 4 days with IPR (0.16 µmol/g body weight/day) (**lanes 3**, **4**, **7**, and **8**). Mice and parotid samples of both the A/Snell strain (lanes 1–4) and the A.Swiss strain (lanes 5–8) were handled in parallel. Secretory polypeptides A and B as well as most of the tisular parotid polypeptides from normal mice are common to both strains while the IPR-induced parotid polypeptides C–E (A/Snell) and P–R (A.Swiss) define the differences between both mouse strains. Resolution of this electrophoretic fractionation has not allowed to discriminate unequivocally between the duplets F/G (A/Snell) and S/T (A.Swiss).

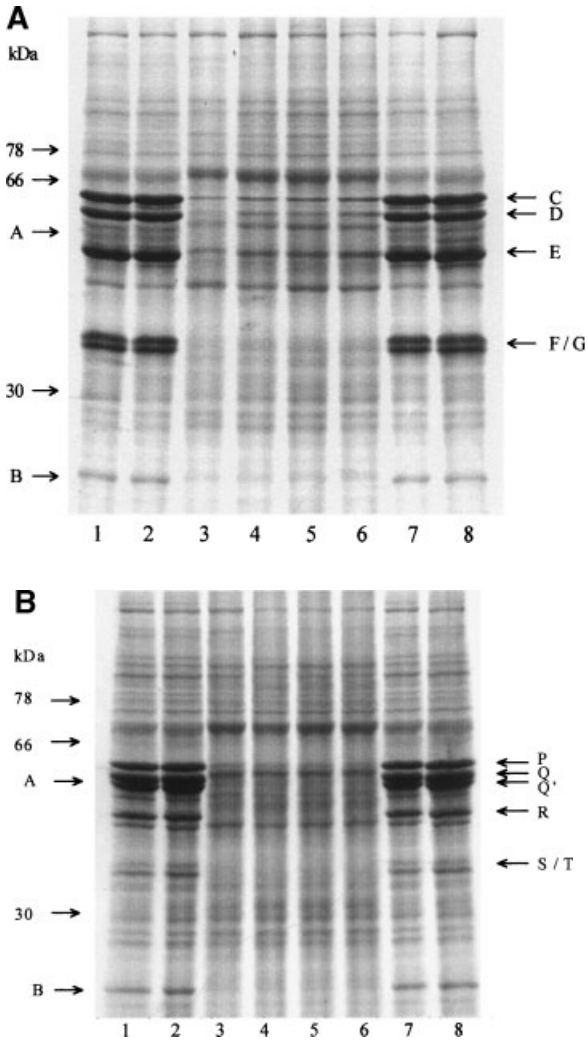

Fig. 4. Secretory behavior of the IPR-induced parotid polypeptides in the A/Snell and A.Swiss mice. Twelve mice of each strain were injected with IPR (0.16 µmol/g body weight) at 24-h intervals for 5-days. At 24 h after the fifth administration of IPR, four animals were killed and both parotid glands from each animal were dissected and processed for electrophoresis in order to confirm the induction of the strain-associated polypeptides (**lanes 1** and **2**). Simultaneously, the remaining eight mice were injected once again with the same dose of IPR (time 0). Four of these animals were sacrificed and parotid glands were dissected at 2 h (**lanes 3–6**) and the last four mice at 24 h (**lanes 7** and **8**). Representative gels of the experiment with mice of the A/Snell strain (**A**) and A.Swiss strain (**B**) are shown. Note in both strains the unequivocal presence of the IPR-induced parotid polypeptides in the glands after the fifth IPR stimulation (lanes 1 and 2) as well as their disappearance at 2 h (lanes 3–6) and reappearance at 24 h (lanes 7 and 8) after the sixth IPR stimulation.

nist [López Solís and Miranda Wilson, 1986; López Solís et al., 1989, 1993]. In the present study, groups of mice of the A/Snell and A.Swiss strains were subjected to successive stimulations by isoproterenol at 24-h intervals for 5-days in order to induce parotid glands to express the new polypeptides. At 24 h after the fifth administration of isoproterenol, 4 animals of both strains were sacrificed to confirm the presence of the corresponding induced polypeptides in the parotid glands (Fig. 4A,B) while a second larger subgroup of mice of each strain ($n = 8$) was injected once again (sixth injection) with isoproterenol (time 0). This latter subgroup of injected mice of each strain was in turn distributed into two halves to be sacrificed at 2 and 24 h after the sixth injection of isoproterenol, respectively. Immediately after the sacrifice, both parotid glands from each animal were removed and processed for the electrophoretic assay. In these studies, marked changes in the polypeptide composition of parotid glands, involving basically the isoproterenol-induced polypeptides, were observed. In effect, at 2 h after the sixth administration of isoproterenol, polypeptides C, D, E, F, and G in the A/Snell strain and polypeptides P, Q/Q′, R, S, and T in the A.Swiss strain practically disappeared from

the electrophoretograms. All those polypeptides fully reappeared in the parotid glands of the corresponding strains by 24 h after the sixth isoproterenol administration (Fig. 4A,B). Thus, the time-course of loss and recovery of the isoproterenol-induced polypeptides in the parotid glands of both mouse strains was identical to the one described previously for the normal secretory polypeptides A and B (see López Solís and Miranda Wilson, 1986), thus strongly suggesting that all of the induced polypeptides would have a secretory character.

Polypeptide Composition of Saliva From Chronically Isoproterenol-Stimulated A/Snell and A.Swiss Mice

In order to fully substantiate the secretory character of the isoproterenol-induced parotid polypeptides in both mouse strains, direct experiments were designed in order to establish the presence of those polypeptides in the corresponding salivas. To that end, groups of mice of the A/Snell and A.Swiss strains were subjected to a 3-day treatment with growth-inducing doses of isoproterenol given at 24-h intervals. At 23 h after the third isoproterenol stimulation, a single oral dose of pilocarpine (400 μg/mouse) was administered in order to provoke salivary secretion. Under those conditions, the secretory response of the hypertrophic salivary glands in both strains was intense and followed an identical time-course to that observed in mice that had not received previously an isoproterenol-treatment (normal salivary glands) [López Solís et al., 2001, 2003]. The electrophoretic separation in SDS–polyacrylamide gels and Coomassie blue staining of salivas, produced both by control mice (the ones stimulated only with pilocarpine) as well as by chronically isoproterenol-stimulated salivary glands followed by pilocarpine, showed the marked presence of the inducible polypeptides C–G in the A/Snell saliva (Fig. 5A) and the inducible polypeptides P–T in the A.Swiss saliva (Fig. 5B). The electrophoretic mobilities of those salivary polypeptides were the same as the ones displayed by the corresponding inducible polypeptides observed in homogenates of parotid glands obtained from the same animals used as donors of saliva (Fig. 5A,B). Accordingly, each one of the isoproterenol-induced parotid polypeptides in both mouse strains is an isoproterenol-induced salivary polypeptide or IISP.

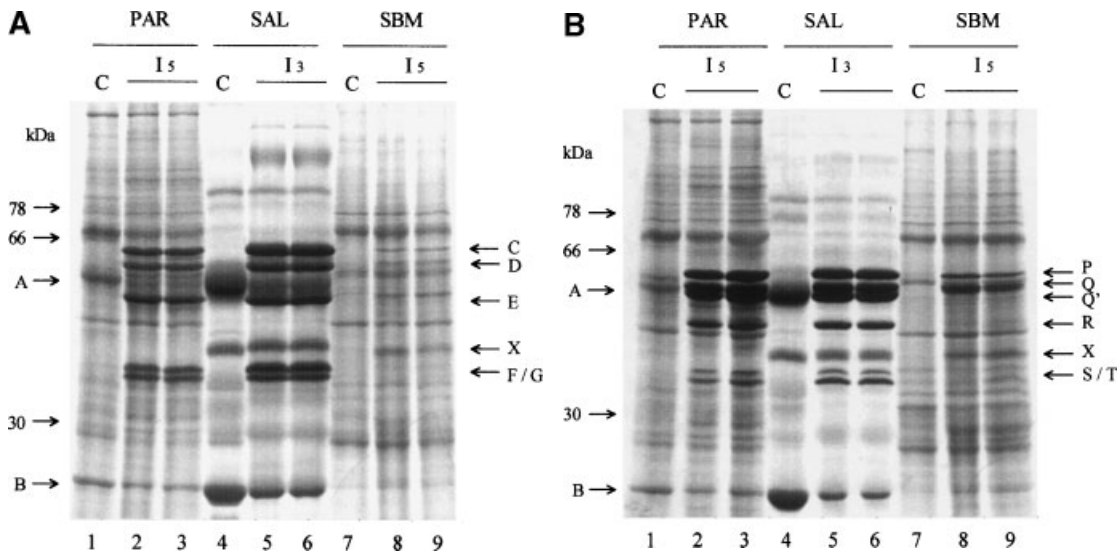

Fig. 5. Identification of the IPR-induced parotid polypeptides in the saliva of the A/Snell and A.Swiss mice. Mice of each strain were injected at 24-h intervals for 5-days with IPR (0.16 μmol/g body weight/day), killed 24 h after the fifth stimulation and both parotid and sbm/sl glands from each animal were dissected and processed for electrophoresis. Samples of saliva from all the animals were taken at 23 h after the third IPR-stimulation as described under ''Materials and Methods.'' Control parotid and sbm/sl salivary glands were taken from unstimulated mice. Control saliva was taken from mice that received a single oral administration of pilocarpine. In the representative gels, each lane corresponds to an independent sample. These were obtained from four different mice, namely, Mouse 1: **Lanes 1** and **7**; Mouse 2: **Lanes 2**, **5**, and **8**; Mouse 3: **Lanes 3**, **6**, and **9** and Mouse 4: **Lane 4**. Control saliva was taken from a different mouse at the beginning of the experiment. In the figures: **C**, control; IPR_{3d} and IPR_{5d}, three or five stimulations by IPR, respectively. Both normal and IPR-induced polypeptides, as well as molecular weight standards, are indicated. **A**, A/Snell strain; **B**, A.Swiss strain.

Differential Patterns of IISP in the Salivas of the A/Snell and A.Swiss Mouse Strains

Considering that each polypeptide assay was carried out routinely in salivas collected from individual mice, a screening addressed to identify eventual inter-individual variations in the profile of IISP within each of both strains was carried out. Insofar, such a screening has involved salivas from over 200 A/Snell and about 70 A.Swiss mice. The presence of the IISP C–G in the saliva of isoproterenol-stimulated A/Snell mice and the presence of the IISP P–T in the saliva of isoproterenol-stimulated A.Swiss mice were found to constitute unvariant features, that is, a strain-dependent or strain-associated pattern (Fig. 6A,B).

DISCUSSION

Two congenic mouse strains, constructed more than 40 years ago in a laboratory dedicated to the dissection of the H-2 major histocompatibility complex [Klein, 1975; Altman and Dittmer Katz, 1979] have been found to exhibit different patterns of IISP or, as would appear to be the equivalent in rodents, different patterns of salivary PRP [Muenzer et al., 1979]. Those strain patterns consisted of at least 5 polypeptides that can be resolved by unidimensional SDS–polyacrylamide gel electrophoresis. Given the inbreeding of both mouse strains for such a long period, it was expected that they displayed a very high index of homozigocity regarding the IISP. Such an expectation was confirmed on the basis that no polymorphic expression of those proteins within each of the analyzed strains could be observed. In other words, each of both mouse strains was shown to be genetically pure for a variant IISP phenotype.

Differences in the IISP expressed by the A/Snell and A.Swiss strains were firstly observed in the parotid tissue (Fig. 3). Although the identification of the IISP in that tissue was unequivocal, at least 4–8 days of successive daily stimulations with the β-adrenergic agonist were necessary to obtain a marked contrast

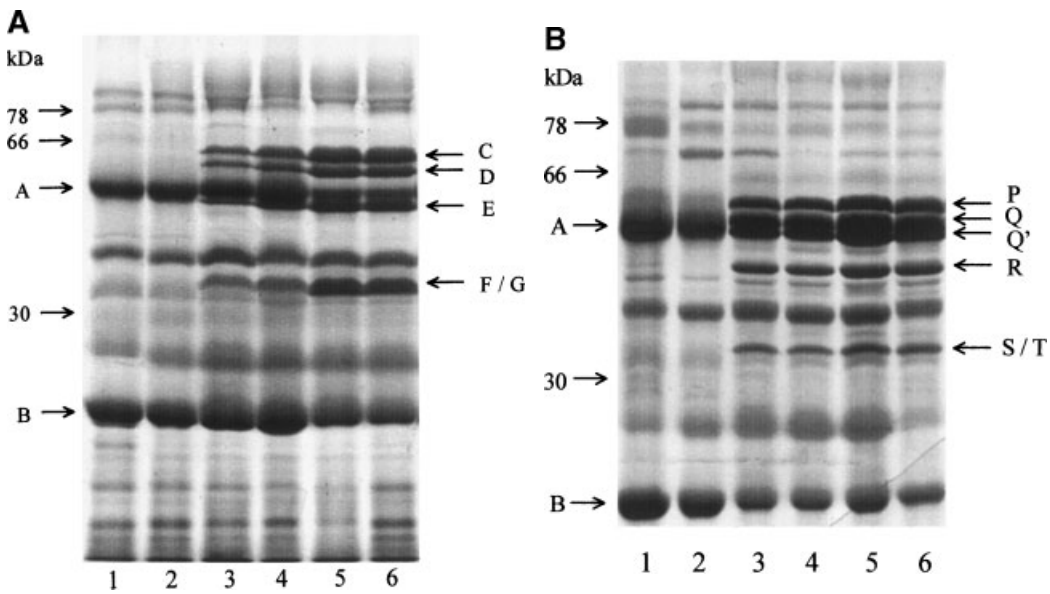

Fig. 6. Strain-associated patterns of IPR-induced salivary polypeptides (IISP) in the congenic inbred A/Snell and A.Swiss mouse strains. **A**: A/Snell strain. About 200 A/Snell mice were injected intraperitoneally at 24-h intervals with IPR (0.16 μmol/g body weight/day) for upto 3-days. At 24 h after the last IPR-stimulation, saliva from each animal was taken and an aliquot containing 50 μg protein was electrophoresed (**lanes 5** and **6**). Saliva from two control unstimulated A/Snell mice is included for comparison (**lanes 1** and **2**). In this strain, no variation in the group of IISP (C, D, E, F, and G) was observed (pattern). A single stimulation by IPR was shown to be sufficient to induce the whole group of strain-associated IISP (**lanes 3** and **4**). In the representative gel, both A/Snell IISP, normal secretory polypeptides A and B and molecular weight standards are indicated. **B**: A.Swiss strain. Lanes and experimental conditions are similar to those in A. In the representative gel, besides the two samples of saliva from control unstimulated A.Swiss mice (lanes 1 and 2), the polypeptide profile observed in four samples of saliva taken from equal number of 3-day IPR-stimulated mice are shown (lanes 3–6). In a total number of 77 A.Swiss mice, no variation in the group of IISP (P, Q, Q′, R, S, and T) was observed (pattern). A.Swiss IISP, normal secretory polypeptides A and B and molecular weight standards are indicated.

of the induced polypeptides with respect to the high number of non-inducible polypeptide components of the tissue (Figs. 2 to 4). Submandibular/sublingual salivary glands expressed only minor changes in the polypeptide composition after the isoproterenol stimulation and, when observed, they were roughly a faint and partial reproduction of the isoproterenol-induced changes in the parotid glands (Fig. 2A,B). The only exception in this respect was the isoproterenol-induced appearance of a weak band of 40 kDa in the submandibular/sublingual glands of both mouse strains without an equivalent in the parotid glands (Fig. 2A,B). At variance of the IISP polypeptides, the 40 kDa band is not metachromatically stained by Coomassie blue and it seems to be present in whole saliva obtained both from control unstimulated and from chronically isoproterenol-stimulated mice (Fig. 5A,B). Accordingly, that protein might well be constitutively expressed by some subtype of the abundant minor salivary glands and it would be inducible in submandibular/sublingual glands. Further studies to elucidate this point are necessary.

A highly advantageous reproduction of the differences between the IISP patterns of both strains was produced in comparing electrophoretograms of salivas, instead of parotid gland homogenates, obtained from isoproterenol-stimulated mice of the corresponding strains. The low background of noninducible polypeptides in saliva, derived from the very low number of abundant normally secretory polypeptides which additionally are strongly down-regulated after isoproterenol, was crucial in that respect (Fig. 5A,B). However, additional major advantages are derived from the use of saliva in the identification of the strain-associated IISP variants. For instance, stimulatory trials with daily isoproterenol for periods shorter than 2–3 days were sufficient for a clear induction and identification of the IISP (Fig. 5A,B). Besides, the identification of the IISP in saliva was carried out in animals that remained alive and healthy after their individual salivary molecular typing [López Solís et al., 2003]. Thus, those methodological advantages enhanced to a major extent our extensive populational study of the IISP phenotype, that involved almost 300 mice from both strains (Fig. 6A,B).

As in rat, hamster, and other rodent species, in both mouse strains the IISP phenotype is characterized by the induced expression of a group of several polypeptides [Muenzer et al., 1979; Ann et al., 1987; Mehansho et al., 1987]. In our study, five polypeptides were induced in the A/Snell mice (polypeptides C, D, E, F, and G) and six polypeptides in the A.Swiss mice (polypeptides P, Q, Q′, R, S, and T). With the equivocal exception of the apparently comigrating or closely migrating doublets F/G and S/T, the other polypeptide members of both sets of strain-associated mouse IISP are clearly distinguishable from each other. Thus, probably none of the identified IISP is shared by both strains (Fig. 3). Insofar, there is no evidence about the number of genes, molecular mechanisms and genetic relationships involved in the expression of these particular polypeptides. Whatever the number of genes coding for the IISP identified in both mouse strains, they would be present in each of them in a homozygous condition. Thus, at least two *IISP*-coding genes became segregated during the production of both the A/Snell and the allogeneic mouse strain used later to construct the congenic A.Swiss strain [Klein, 1975; Altman and Dittmer Katz, 1979]. Allelic or nonallelic relationships between those *IISP*-coding genes is a matter that still remains to be solved. However, given the congenic character of the A/Snell and A.Swiss strains, quite likely the gene or genes coding for C–G and P–T would be located in a single genetic region [Abbas et al., 1991]. Thus, the whole number of IISP expressed in each of the strains of the present study might well be the combined result of transcription of at least a single gene, alternative mRNA splicing and post-translational processing of the protein product or products as it has been described in regard to human PRPs [Maeda et al., 1985; Lyons et al., 1988; Azen et al., 1993, 1996; Kim et al., 1993].

In mouse, *PRP*-coding genes located in chromosomes 3 [Delhomme and Djian, 2000], 4 [Kim et al., 2002], 6 [Azen et al., 1989], 8 [Azen et al., 1984], and 15 [Yang and Mansour, 1999] have been identified. According to an earlier evidence derived from DNA analysis of somatic cell hybrids, genes coding for murine salivary PRP, such as the nonallelic *MP2* and *M14* genes, would be located in chromosome 8 [Azen et al., 1984; Ann et al., 1988]. However, more recent studies based on in situ hybridization and high resolution genetic mapping have assigned salivary *PRP* genes to chromosome 6 [Azen et al., 2000; Bachmanov et al., 2001]. In humans, loci

coding for various subfamilies of PRP-salivary proteins, have been mapped to proximal chromosome 12 (PRB1 through PRB4, PRH1, PRH2) and to distal chromosome 4 (PROL3 and PROL5) [Kim et al., 1993; Isemura and Saitoh, 1997]. Thus, different analytical strategies, different molecular probes used by various laboratories, different *PRP* genes actually located in different chromosomes and a combination of all these possibilities seem to underlie also the information presently available with regard to chromosomal location of *PRP*-coding genes in mouse. In this context, the availability of two pure mouse strains that are variants for the IISP character might become a highly valuable tool to carry out studies contributing to assess the number and location of the *IISP*-coding genes, and so of the productive *PRP*-coding genes, on the basis of experimental matings between individual mice previously typed for the IISP character. Such kind of information might become highly illustrative of part of the grounds sustaining the diversity of that group of proteins, at least within a single species.

Finally, the fact that two inbred mouse strains that were established as congenic for the *H-2* gene complex, differ in the quality of the IISP, might suggest a direct association between those molecular entities. However, at this preliminary stage, complexity seems to be the main and perhaps the only common component between both genetically defined systems. Thus, loci of the mouse *H-2* gene complex have been assigned to chromosome 17 [Watts et al., 1987] whereas, as mentioned above, certain genes coding for salivary PRP have been reported as being part of chromosome 6 [Azen et al., 1989, 2000; Bachmanov et al., 2001]. Interestingly, mouse salivary PRP loci in chromosome 6 are closely adjacent to the Natural Killer gene complex and differential susceptibility to lethal mousepox in two inbred mouse strains (DBA/2 and C57BL/6) has been associated to a gene or genes within that complex [Brownstein et al., 1991; Delano and Brownstein, 1995]. Again, the availability of pure mouse strains expressing genetic variants for PRP, such as the ones described in the present study, might be also used advantageously to address experiments to define linkage or independent assortment betwen genes coding for IISP and genes coding for H-2 and other functions of the immune system.

REFERENCES

Abbas AK, Lichtman AH, Pober JS. 1991. Cellular and molecular immunology: The major histocompatibility complex. Philadelphia: W.B. Saunders Co.

Altman PL, Dittmer Katz D. 1979. Inbred and genetically defined strains of laboratory animals. Part I: Mouse and rat. Bethesda, Maryland: Federation of American Societies for Experimental Biology.

Amano A, Shizukuishi S, Horie H, Kimura S, Morisaki I, Hamada S. 1998. Binding of *Porphyromonas gingivalis fimbriae* to proline-rich glycoproteins in parotid saliva via a domain shared by major salivary components. Infect Immun 66:2072–2077.

Ann DK, Lin HH. 1998. Transcriptional regulation of salivary proline-rich protein gene expression In: Kukuruzinska MA, Tabak LA, editors. Salivary gland biogenesis and function. Annals NY Acad Sci. 842:108–114.

Ann DK, Clements S, Johnstone EM, Carlson DM. 1987. Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. J Biol Chem 262:899–904.

Ann DK, Smith MK, Carlson DM. 1988. Molecular evolution of the mouse proline-rich protein multigene family. Insertion of a long interspersed repeated DNA element. J Biol Chem 5:10887–10893.

Ann DK, Lin HH, Kousvelari E. 1997. Regulation of salivary gland specific gene expression. Crit Rev Oral Biol Med 8(3):244–252.

Azen EA. 1993. Genetics of salivary protein polymorphisms. Crit Rev Oral Biol Med 4:479–485.

Azen EA, Carlson DM, Clements S, Lalley PA, Vanin E. 1984. Salivary proline-rich protein genes on chromosome 8 of mouse. Science 226:967–969.

Azen EA, Davisson MT, Cherry M, Taylor BA. 1989. *Prp* (proline-rich protein) genes linked to markers Es-12 (esterase-12), Ea-10 (erythrocyte alloantigen), and loci on distal mouse chromosome 6. Genomics 5:415–422. Erratum in Genomics 7:142 (1990).

Azen EA, Latreille P, Niece RL. 1993. *PRB1* gene variants coding for length and null polymorphisms among human salivary Ps, PmF, PmS, and Pe proline-rich proteins (PRPs). Am J Hum Genet 53:264–278.

Azen EA, Amberger E, Fisher S, Prakobphol A, Niece RL. 1996. PRB1, PRB2, and PRB4 coded polymorphisms among human salivary concanavalin-A binding, II-1 and Po proline-rich proteins. Am J Hum Genet 58:143–153.

Azen EA, Johnson EB, Sekhon GS. 2000. Assignment of the proline-rich protein gene Prp2 (alias D6Mit) to mouse chromosome 6G1 by in situ hybridization. Cytogenet Cell Genet 89:14–15.

Bachmanov AA, Li X, Li S, Neira M, Beauchamp GK, Azen EA. 2001. High-resolution genetic mapping of the sucrose octaacetate taste aversion (Soa) locus on mouse chromosome 6. Mamm Genome 12:695–699.

Barka T, Burke T. 1977. Secretory behaviour of hypertrophic and hyperplastic salivary gland. Histochem J 9:453–466.

Bedi GS, Bedi SK. 1995. Purification and characterization of rat parotid glycosylated, basic, and acidic proline-rich proteins. Prep Biochem 25:119–132.

Bennick A, Chau G, Goodlin R, Abrams S, Tustian D, Madapallimattam G. 1983. The role of human salivary

acidic proline-rich proteins in the formation of acquired dental pellicle in vivo and their fate after adsorption to the human enamel surface. Arch Oral Biol 28:19–27.

Bramhall S, Noack N, Wu M, Loewenberg JR. 1969. A simple colorimetric method for determination of protein. Anal Biochem 31:146–148.

Brownstein DG, Bhatt PN, Gras L, Jacoby RO. 1991. Chromosomal locations and gonadal dependence of genes that mediate resistance to ectromelia (mousepox) virus-induced mortality. J Virol 65:1946–1951.

Carlen A, Bratt P, Stenudd C, Olsson J, Stromberg N. 1998. Agglutinin and acidic proline-rich protein receptor patterns may modulate bacterial adherence and colonization on tooth surfaces. J Dent Res 77:81–90.

Castle D, Castle A. 1998. Intracellular transport and secretion of salivary proteins. Crit Rev Oral Biol Med 9(1):4–22.

Clements S, Mehansho H, Carlson DM. 1985. Novel multigene families encoding highly repetitive peptide sequences. J Biol Chem 260:13471–13477.

Delano ML, Brownstein DG. 1995. Innate resistance to lethal mousepox is genetically linked to the *NK* gene complex on chromosome 6 and correlates with early restriction of virus replication by cells with an NK phenotype. J Virol 69:5875–5877.

Delhomme B, Djian P. 2000. Expansion of mouse involucrin by intra-allelic repeat addition. Gene 252:195–207.

Durham JP, López-Solís RO. 1979. Effect of sodium hydroxyde upon protein binding to filter paper disks and its significance in the determination of protein in colorimetric and radioisotopic assays. Anal Biochem 100: 98–99.

González MJ, Peña y Lillo S, Alliende C, López Solís RO. 2000. Cell-enlargement related polypeptides are induced via β1-adrenoceptors in mouse parotids. Exp Mol Pathol 69:91–101.

Humphreys-Beher MG, Schneyer CA, Kidd VJ, Marchase RB. 1987a. Isoproterenol-mediated parotid gland hypertrophy is inhibited by effectors of 4β-galactosyltransferase. J Biol Chem 262:11706–11713.

Humphreys-Beher MG, Robinson GD, Al-Zahid S, Schneyer CA. 1987b. Neural and dietary modulation of proline-rich protein and 4β-galactosyltransferase biosynthesis in rat parotid glands. Arch Oral Biol 32:573–578.

Isemura S, Saitoh E. 1997. Nucleotide sequence of gene *PBI* encoding a protein homologous to salivary proline-rich protein P-B. J Biochem 121:1025–1030.

Kim HS, Lyons KM, Saitoh E, Azen EA, Smithies O, Maeda N. 1993. The structure and evolution of the human salivary proline-rich protein gene family. Mamm Genome 4:3–14.

Kim YS, Lewandoski M, Perantoni AO, Kurebayeshi S, Nakanishi G, Jetten M. 2002. Identification of Glis1, a novel Gli-related Kruppel-like zinc finger protein containing transactivator and repressor functions. J Biol Chem 23:30901–30913.

Klein J. 1975. Biology of the mouse histocompatibility-2 complex. Principles of immunogenetics applied to a single system. New York: Springer-Verlag.

Kousvelari EE, Baratz RS, Burke B, Oppenheim FG. 1980. Immunochemical identification and determination of proline-rich proteins in salivary secretion, enamel pellicle, and glandular tissue specimens. J Dent Res 59:1430–1438.

López Solís RO, Miranda Wilson DM. 1986. Changes in the polypeptide composition of mouse parotid glands after stimulation of secretion and DNA synthesis by isoproterenol. Biochim Biophys Acta 881:422–429.

López Solís RO, Alliende C, González J, Miranda D. 1987. Changes in the polypeptide composition related to the growth response in chronically-isoproterenol-stimulated mouse parotid glands. Biochim Biophys Acta 930:463–469.

López Solís RO, Miranda D, Alliende C, González J, Díaz N, Díaz H, Ruiz F. 1989. Secretory character of a group of isoproterenol-induced polypeptides in mouse parotid glands. J Cell Physiol 141:660–666.

López Solís RO, Miranda Wilson D, González Burgos J. 1990. Effect of isoproterenol analogs on the polypeptide composition of mouse parotid glands: Relationship to enhanced growth. Biochim Biophys Acta 1054:311–316.

López Solís RO, González MJ, Castillo L, Peña y Lillo S, Alliende C. 1993. Transcriptional and post-transcriptional regulation in the synthesis of isoproterenol-induced polypeptides in mouse parotid glands. Am J Physiol 265:G514–G520.

López Solís RO, Puente M, Wenk C, Morales I, Durán V. 2001. Characterization of mouse salivary polypeptide secretion after oral administration of pilocarpine. Rev Chil Hist Nat 74:193–203.

López Solís RO, Puente Díaz M, Morales Bozo I, Kemmerling Weis U, Díaz F. 2003. Early detection in saliva of polypeptides associated to isoproterenol-induced mouse parotid hypertrophy. Biochim Biophys Acta 1621: 41–47.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Layfield R, Bannister AJ, Pierce EJ, McDonald CJ. 1992. cDNA clones for mouse parotid proline-rich proteins. Eur J Biochem 204:591–597.

Lyons KM, Azen EA, Goodman PA, Smithies O. 1988. Many protein products from a few loci: Assignment of human salivary proline-rich proteins to specific loci. Genetics 120:255–265.

Maeda N, Kim H, Edwin EA, Azen EA, Smithies O. 1985. Differential RNA splicing and post-translational cleavages in the human salivary proline-rich protein gene system. J Biol Chem 260:11123–11130.

Mehansho H, Hagerman A, Clements S, Butler L, Rogler J, Carlson DM. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. Proc Nat Acad Sci USA 80:3948–3952.

Mehansho H, Ann DK, Butler LG, Rogler J, Carlson DM. 1987. Induction of proline-rich proteins in hamster salivary glands by isoproterenol treatment and an unusual growth inhibition by tannins. J Biol Chem 262:12344–12350.

Miao YJ, Subramaniam N, Carlson DM. 1995. cDNA cloning and characterization of rat salivary glycoproteins. Novel members of the proline-rich-protein multigene families. Eur J Biochem 228:343–350.

Muenzer J, Bildstein C, Gleason M, Carlson DM. 1979. Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats. J Biol Chem 254:5623–5628.

Novi AM, Baserga R. 1971. Association of hypertrophy and DNA synthesis in mouse salivary glands after chronic

administration of isoproterenol. Am J Pathol 62:295–308.

Schneyer CA. 1962. Salivary gland changes after isoproterenol-induced enlargement. Am J Physiol 203:232–236.

Watts S, Vogel JM, Harriman WD, Itoh T, Stauss HJ, Goodenow RS. 1987. DNA sequence analysis of the C3H H-2Kk and H-2Dk loci. Evolutionary relationships to *H-2* genes from four other mouse strains. J Immunol 139: 3878–3885.

Yang W, Mansour SL. 1999. Expression and genetic analysis of *prtb*, a gene that encodes a highly conserved proline-rich protein expressed in the brain. Dev Dyn 215: 108–116.

Zhou J, Wright PS, Wong E, Jessen K, Morand JN, Carlson DM. 1997. Cyclic AMP regulation of mouse proline-rich protein gene expression: Isoproterenol induction of AP-1 transcription factors in parotid glands. Arch Biochem Biophys 338:97–103.