Induction of Proline-rich Glycoprotein Synthesis in Mouse Salivary Glands by Isoproterenol and by Tannins*

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Glycoproteins which contain about 45 mol % proline were dramatically induced in mouse parotid and submandibular glands by isoproterenol treatment, but these unusual proteins were not detected in control animals. These acid-soluble substances were obtained by extracting tissues with 10% trichloroacetic acid, as reported previously for isolating proline-rich proteins from rat submandibular glands (Mehansho, H., and Carlson, D. M. (1983) J. Biol. Chem. 258, 6616-6620). Three major proline-rich glycoproteins were induced in parotid glands with apparent molecular weights of 66,000 (GP-66p), 45,000 (GP-45p), and 27,000 (GP-27p), whereas only one such protein was expressed by the submandibular glands (66,000 (GP-66sm)). Both GP-66p and GP-66sm contained about 19% carbohydrate with the following molar ratios, respectively: GalNAc, 1.0, 1.0; Gal, 1.6, 2.3; GlcNAc, 0.6, 1.1; sialic acid, 0.8, 1.9. The peptide chains of GP-66p and GP-66sm appear to be identical by amino acid compositions, glycophorin analysis, and preliminary amino acid sequencing data. Northern blot analysis of RNAs from parotid glands of normal and isoproterenol-treated rats, probed with a 32P-labeled proline-rich protein cDNA, confirmed that control animals were devoid of mRNAs encoding these proteins and that isoproterenol treatment dramatically induced expression of these genes. Feeding sorghum high in tannins caused changes in the parotid glands similar to those observed upon isoproterenol treatment, as noted earlier with rats (Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., and Carlson, D. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3948-3952). These glycoproteins have high affinities for tannins as demonstrated by competitive binding curves.

Several unusual related proteins high in proline, glycine, and glutamine occur in the parotid and submandibular glands and in the secretions of these glands of various animals (1-3) see Ref. 3 for a review). About 70% of the protein content of human parotid gland secretions is comprised of these proline-rich proteins (3). Only small amounts (<10% of the total soluble protein) are generally found in rat salivary glands (1, 2), but a dramatic increase is observed upon treatment with isoproterenol with the synthesis of a complement of over 10 related proteins (1, 2, 4). The proline-rich protein genes of the mouse were located on chromosome 8 by Southern blot analysis of HindIII restriction digests of DNAs from a series of Chinese hamster/mouse hybrid cell lines, probed with 32P-labeled proline-rich protein cDNAs (5). These proline-rich protein genes appeared to be dormant, however, since these proteins were not detected in salivary glands of control mice (6). As observed previously in rats, isoproterenol caused a dramatic induction of proline-rich proteins in the mouse parotid and submandibular glands (6). These studies report the isolation and characterization of the predominant glycoproteins induced by isoproterenol treatment and confirm earlier studies on rats (7) that feeding sorghum high in tannins mimics the effects of isoproterenol by the morphological and biochemical changes of the parotid glands of mice, but not of the submandibular glands.

EXPERIMENTAL PROCEDURES

Materials—All reagents were of highest purity available and were purchased from commercial sources unless otherwise indicated. The following substances were purchased from the respective companies: nick-translation system, L-[3,4-3H]proline (40-60 Ci/mmol), and L-[35S]methionine (600-1500 Ci/mmol), Amersham Radio Chemicals; DL-isoproterenol HCl, Pronase (type VI), Sigma; Sephadex G-50, Pharmacia Fine Chemicals; Bio-Gel A-1.5 m, Bio-Gel P-2, molecular weight standards, Bio-Rad; ENHANCE, New England Nuclear; reticulocyte lysate system, Bethesda Research Laboratories; nitrocellulose, Schleicher and Schuell. Sorghum varieties with high tannin (Savanna) and low tannin (RS-610) were grown at the Purdue University Agronomy Farm. Tannin Analysis was done by using the modified vanillin assay (8). The tannin contents expressed in catechin equivalents are equal 0.8 and 7.7% for RS-610 and Savanna, respectively.

Isoproterenol Treatment—Male BALB/cAnNHsd BR mice (6-8 weeks) fed Purina Lab Chow ad libitum were utilized as tissue donors. Isoproterenol was administered as described by Muenzer et al. (1). Each mouse received daily injections, intraperitoneally, of 2 mg of dl-isoproterenol-HCl in 0.2 ml of 0.14 M NaCl for 10 days, unless otherwise indicated.

Feeding Trials—Mice (4-5 weeks) were maintained on Purina Lab Chow for 4 days before starting the feeding experiments. Sorghum grains were ground and incorporated into diets as described previously (7). Feed and water were provided ad libitum. At the end of the feeding experiments, weight gain was measured.

Isolation of Proline-rich Glycoproteins—Mice were anesthetized with sodium pentobarbital and killed by exsanguination. The parotid and submandibular glands were removed, stripped of connective tissues, and weighed. The acid-soluble proline-rich glycoproteins were isolated as described by Mehansho and Carlson (2). Tissues were homogenized in 10% trichloroacetic acid. The trichloroacetic acid-soluble fraction, which contained most of the proline-rich proteins, was fractionated on a Bio-Gel A-1.5m column.

Amino Acid Analysis—Protein samples (0.1-0.3 mg) were hydro-
lyzed as described earlier (9). Analysis was performed on a Durrum autoanalyzer.

**Protein Determination**—Proline-rich proteins are generally very low in aromatic amino acids (3, 9), and they were quantitated by absorbance at 230 nm. The calculated extinction coefficient for GP-66sm (E280) is 20.0.

**Carbohydrate Analysis**—Neutral and amino sugars were assayed by gas chromatography of the alditol acetates as described previously (10) with slight modifications (2). Sialic acid was estimated by the thiobarbituric acid procedure after acid hydrolysis (11).

**Preparation of Glycopeptides**—GP-66p and GP-66sm were treated with Pronase (12), and glycopeptides were separated on a Sephadex G-50 column as described earlier (2). The glycopeptide peaks were identified by the phenolsulfuric acid method (13). Fractions which contained carbohydrate were pooled, lyophilized, and desalted on Bio-Gel P-2. Further separations of the glycopeptides were carried out by reverse phase HPLC as described by Mahoney and Hermodson (14). Analyses were done on a preparative SynChropak RP-8 column. Elution was accomplished by using 0.1% trifluoroacetic acid in H2O (v/v) as a primary mobile phase and 0.1% trifluoroacetic acid in methanol (v/v) as a secondary mobile phase.

**Isolation and Cell-free Translation of mRNAs**—Total RNA was isolated according to the method of Chirgwin et al. (15). Cell-free translations were performed as described previously (4) with both [35S]methionine and [3H]proline using the reticulocyte lysate system (16). The proline-rich proteins were precipitated by using 10% trichloroacetic acid containing 1% phosphotungstic acid.

**Northern Hybridization**—RNAs from parotid and submandibular glands of normal and isoproterenol-treated mice were separated on methyl mercury hydroxide agarose gels (17). Following electrophoresis, RNAs were transferred to nitrocellulose (18). Hybridization was performed as described previously (19) with a 32P-labeled nick-translated rat proline-rich protein cDNA clone, pRP33 (20).

**SDS-Polyacrylamide gel Electrophoresis**—Gel electrophoresis was carried out as described by Laemmli (21). Proteins were precipitated with acetone, dissolved in sample buffer, and heated in a boiling water bath for 5 min. Electrophoresis was on 12% polyacrylamide gels unless specified. Fixation and staining were accomplished as described elsewhere (2, 22) with a slight modification. Overnight destaining with the formaldehyde-containing solution described by Steck et al. (22) removed stains resulting from the glycoprotein bands. The gels were, therefore, immersed first in the destaining solution 30 to 60 min and then left overnight in water. Glycoproteins were detected by the periodic acid-Schiff’s staining procedure (23). Radioactivity on gels was identified by fluorography using ENHANCE (24).

**RESULTS**

**Effect of Isoproterenol**—Treatment with isoproterenol for 10 days increased the weights of the parotid and submandibular glands by 3.5- and 2.2-fold, respectively. Sublingual glands were essentially unchanged. To determine the effects of isoproterenol treatment on proline-rich protein synthesis, the trichloroacetic acid-soluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis. As illustrated in Fig. 1A, the acid-soluble fraction of the parotid glands of isoproterenol-treated mice contained at least 3 major proteins (GP-66p, GP-45p, and GP-27p). Submandibular gland extracts contained only one protein, GP-66sm. These acid-soluble components from both parotid and submandibular glands are glycoproteins, and they stain very strongly with periodic acid-Schiff’s reagent (Fig. 1B). As demonstrated both by Coomasie Blue and by periodic acid-Schiff’s stainings, these glycoproteins were not detected in either the parotid or submandibular glands of normal mice.

The acid-soluble fraction from parotid glands of isoproterenol-treated mice was chromatographed on Bio-Gel A-1.5m. Seven ml (about 15 mg/ml, based on 230-nm absorption) of the acid-soluble extract of parotid glands of isoproterenol-treated mice was chromatographed on a column (2 x 100 cm) of Bio-Gel A-1.5m. The column was equilibrated and eluted with 25 mM Tris-HCl, pH 7.4, containing 0.14 M NaCl. Five-ml fractions were collected. Analysis of fractions 57 to 80 by SDS-polyacrylamide gel electrophoresis (50 μg protein/lane) is shown in the inset. Fractions 57–63 (GP-66p) and 72–75 (GP-27p) were combined for further studies.

![Fig. 1. SDS-polyacrylamide gel electrophoresis of trichloroacetic acid extracts.](image)

![Fig. 2. Chromatography of the trichloroacetic acid-soluble components from mouse parotid glands on Bio-Gel A-1.5m.](image)
mainly GP-27p, with minor contamination, and these were combined. GP-45p has not been adequately resolved as yet. Similar fractionation on Bio-Gel A-1.5m was also performed on the acid-soluble components from the submandibular glands (Fig. 3). Fractions of peak I were combined into four portions as indicated and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3, inset). Portions 1, 2, 3, and 4 had identical electrophoretic mobilities and stained for both protein and carbohydrate (not shown). Peak I contained only GP-66sm. Amino acid compositions of each of the four fractions were the same (analyses not shown).

**Compositional Analysis**—The amino acid compositions of GP-66p, GP-66sm, and GP-27p are presented in Table I. As observed previously with proline-rich proteins from rat salivary glands (2, 9), these proteins are unusually high in proline, glutamine and glutamic acid, and glycine. They lack aromatic and sulfur-containing amino acids. GP-66p and GP-66sm have the same amino acid compositions. Comparing the probably carbohydrate linkages, GP-27p has more aspartic acid and less threonine than GP-66p. Carbohydrate compositions, given in Table II, show that GP-66p, GP-66sm, and GP-27p contain 19.9, 19.2, and 9.1% carbohydrate, respectively. Since both GP-66p and GP-66sm contain N-acteylgalactosamine and lack mannose, the carbohydrates are likely O-linked oligosaccharides. GP-27p lacks sialic acid but has fucose. From the sugar composition, it is likely that the carbohydrate moieties of GP-27p are mixtures of both O- and N-linked oligosaccharides.

**Glycopeptide Isolation and Analysis**—GP-66p and GP-66sm were digested with Pronase, and the glycopeptides were separated by Sephadex G-50 chromatography. From each glycoprotein, glycopeptides with apparent molecular weights of about 2000–3000 were obtained (data not shown). Further separations of these glycopeptides by reverse phase HPLC are shown in Fig. 4. Both GP-66p and GP-66sm contain four major components. Amino acid analyses of these glycopeptides are presented in Table III. Respective peaks from GP-66p and from GP-66sm have similar amino acid compositions. Aside from the relative increases in threonine, which is presumably involved in the sugar to protein linkages, the amino acid compositions of these glycopeptides are similar to those of the glycoproteins (Table I). Carbohydrate compositions of the glycopeptide fractions obtained from HPLC are shown in Table IV. Molar ratios of the various sugars in each of the fractions are similar. Equimolar amounts of GalNAc and Thr were found in each glycopeptide fraction (Table IV).
isolated by HPLC as described in Fig. 4. See “Experimental Procedures” for details.

Proteins labeled with \([3H]\)proline from translations with normal or parotid glands of isoproterenol-treated rats and mice. Induction of proline-rich proteins is the band at about 1300 nucleotides. Additional mRNAs are present in smaller amounts, and present upon treatment with isoproterenol. Several new proteins are sized upon treatment with isoproterenol. Gene expression in the parotid glands similar to those seen upon isoproterenol treatment. There is glandular hypertrophy and a dramatic induction of proline-rich protein synthesis (Table V). The acid-soluble components of parotid glands of mice fed RS-610 (low-tannin sorghum) and Savanna (high-tannin sorghum) were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 7). The patterns and apparent molecular weights of the glycoproteins induced are the same as those of IPR-treated animals. Induction of proline-rich proteins was not detected in the submandibular glands (data not shown) by feeding high-tannin sorghum.

**Binding of Proline-rich Proteins to Tannins**—Proteins high in proline and hydroxyproline, such as gelatin and proline-rich proteins, have high affinities for tannins. The initial evidence has been presented that diets of sorghum high in tannins induce proline-rich proteins in rats (7). The initial weight loss experienced by animals on the high-tannin diet was caused by the tannins binding to proteins in the saliva, preventing their absorption. This led to decreased food intake and weight loss.

**Discussion**

Proline-rich proteins are the major components of salivary secretions of humans (3). The apparent unique occurrence of proline-rich proteins in saliva suggests functional roles for these unusual proteins in the oral cavity. The potential functions in calcium binding, hydroxyapatite binding, inhibition of hydroxyapatite formation, and formation of the acquired dental pellicle are all discussed by Bennick (3). Recently, evidence has been presented that diets of sorghum high in tannins induce proline-rich proteins in rats (7). The initial weight loss experienced by animals on the high-tannin diet was caused by the tannins binding to proteins in the saliva, preventing their absorption. This led to decreased food intake and weight loss.
Regulation of Gene Expression in Salivary Glands

**FIG. 6.** Northern blot analysis of mouse parotid and submandibular gland RNAs. RNAs (10 µg) from parotid (PAR) and submandibular (SUB) glands of normal (NOR) and isoproterenol-treated (IPR) mice were electrophoresed on 1.5% agarose gels containing 5 mM methyl mercury and blotted onto nitrocellulose filters. The blot was probed with 32P-labeled pRP33 (20) in hybridization solution at 40 °C. The blot was washed and autoradiographed. Molecular weight markers were from a HaeIII digest of φX174.

**TABLE V**

| Diet*     | Glands  | Proline-rich proteins** |
|------------|---------|-------------------------|
|            | Parotid | Submandibular | Parotid | Submandibular |
| Rat chow   | 4.0     | 6.0          | 0       | 20.0          |
| RS-610     | 3.6     | 6.0          | 6.0     | 20.0          |
| Savanna    | 9.9     | 6.5          | 103.9   | 26.0          |

* RS-610 and Savanna are low (0.2%) and high (7.7%) tannin sorghums, respectively.
** Values are averages of 5 mice obtained by pooling glands.

sorghum diet was reversed at 3 days, at the time when the induction of proline-rich proteins was maximal. Recent results clearly show that tannins, or tannic acids, are responsible for the increased expression of the proline-rich protein gene family.4

Upon treatment of rats with the β-agonist isoproterenol, proline-rich proteins increase dramatically and may comprise more than half of the total soluble protein of the parotid glands (1, 2). The isoproterenol-treated rat and now the isoproterenol-treated mouse are considered to be excellent models to study the overall responsiveness of gene expression to catecholamines. Cell-free translations of proline-rich proteins from RNAs prepared from rat (4) and monkey (26) salivary glands have been performed. Since many of the proline-rich proteins are soluble in 10% trichloroacetic acid (2,9), either 10% trichloroacetic acid plus 1% phosphotungstic acid or acetone should be used to precipitate the translation products (4).

Plasmids containing cDNAs encoding proline-rich proteins have been isolated from a cDNA library prepared from RNAs from parotid glands of isoproterenol-treated rats, and the nucleotide sequence of one of these cDNAs, pRP33 (primer extended), has been determined (20). It encodes an acidic proline-rich protein, which is likely Ipr-1A2 (1, 9). The first
13 N-terminal amino acids are highly hydrophobic and may be part of a signal peptide. The amino acid sequence derived from pRP33, beginning at proline 80, contains six tandemly repeat regions of 18 to 19 amino acids. The high homology in both the nucleotide and amino acid sequences in the repeat region suggests that this protein, and possibly other proline-rich proteins, may have evolved by duplication of internal portions of a progenitor gene.

Variable amounts of proline-rich proteins are found in rat parotid glands (1, 2), and generally the amounts tend to increase with age. Presently we believe that the diets of control animals may induce the synthesis of the proline-rich proteins. The proline-rich protein genes of the mouse, however, seem to be dormant or are not normally expressed. Neither proline-rich proteins nor proline-rich protein mRNAs were detected in mouse salivary glands, but both were dramatically induced by isoproterenol treatment and by feeding high-tannin sorghum. Three major proline-rich glycoproteins were induced in the parotid glands of IPR-treated mice, GP-66p, GP-45p, and GP-27p. One major glycoprotein was induced in submandibular glands, GP-66sm. The amino acid compositions of GP-66p and GP-66sm and their respective glycopeptides prepared by Pronase digestion are similar and suggest high homologies between these two proteins. Preliminary studies on sequence analysis of the glycopeptide fractions also indicate close similarities for the peptide chains of these two glycopeptides. As in the protein encoded by pRP33 and GP-158 (2), repeating peptide units comprise a large segment of the proteins of GP-66p and GP-66sm. Each of these glycoproteins is about 20% carbohydrate. GP-66sm has about one additional residue each of sialic acid and galactose per glycopeptide moiety. Unlike GP-158, which was isolated and characterized from submandibular glands of isoproterenol-treated rats (2), GP-66sm and GP-66p contain GalNAC and undoubtedly contain o-glycosidic linkages. This is supported by the high amounts of threonine and essentially equimolar amounts of GalNAC and threonine in the glycopeptide fractions. The apparent sizes of the glycopeptides were 2000 to 3000. Structural analysis of the oligosaccharides, linkage sites, and total sequence analysis of the proteins are currently being investigated.

Three major proline-rich protein mRNAs from parotid glands and one from submandibular glands of IPR-treated mice were identified by Northern blot analysis and these likely code for the major proteins induced. A longer exposure of the Northern blots showed about 6 or 7 mRNAs from the parotid glands. Even with the prolonged exposure, no proline-rich protein mRNAs were detected in glands from control animals. Plasmid pRP33 has been used to screen a human gene library for proline-rich protein gene sequences (27) and to probe Southern blots of DNAs from mouse and hamster cells and from mouse/hamster somatic cell hybrids (5). Repetitive sequences characteristic of proline-rich proteins were derived from the human genomic clones (27). The proline-rich protein gene family of the mouse has definitely been assigned to chromosome 8 (5). Southern blots of the hamster DNA clearly show cross-hybridization with pRP33 and, therefore, likely contain a proline-rich gene(s). However, hamsters do not respond to diets of high-tannin sorghum, and animals maintained on high-tannin sorghum appear healthy but fail to gain weight.4

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