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Formation of salivary-mucosal pellicle: the role of transglutaminase

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The present investigation was carried out to identify salivary components of mucosal pellicles *in vivo* and explore further the mechanism of interaction between salivary molecules and buccal epithelial cells. By using specific antisera and immunoprotein blotting, high-(MG1) and low-(MG2) molecular-mass salivary mucins, amylase, salivary cystatins and proline-rich proteins were detected within mucosal pellicle *in vivo*. In addition, the data indicated that the mucins and proline-rich proteins could be cleaved into lower-molecular-mass products, whereas the proline-rich proteins could also be cross-linked into higher-molecular-mass complexes. The role of buccal epithelial cell transglutaminase in these interactions was further studied by utilizing purified iodinated amylase, neutral cystatin SN and acidic proline-rich proteins 1 and 3 (APRP1 and 3). After incubation with buccal epithelial cells *in vitro* ^{125}I-labelled APRPs appeared to undergo a greater degree of cross-linking than ^{125}I-labelled cystatin SN, as determined by SDS/PAGE/autoradiography. Amylase did not appear to be cross-linked at all. Recovery of ^{125}I-labelled APRPs and ^{125}I-labelled cystatin SN with epithelial cell envelopes after repeated extraction suggested that both molecules were cross-linked to envelope proteins, but that ^{125}I-labelled APRPs were cross-linked to a greater degree than ^{125}I-labelled cystatin SN. Cross-linking in buccal epithelial cell preparations was inhibited by an excess of methylamine hydrochloride, a transglutaminase substrate. In a further assessment of amylase, cystatin and APRPs as transglutaminase substrates, only APRP3 and a partially purified preparation of APRPs acted as an amine acceptor for the cross-linking of [^{14}C]methylamine by purified transglutaminase, as determined by SDS/PAGE/fluorography. This reaction was completely inhibited by excess EDTA. The combined data from this study suggest that during mucosal pellicle formation multiple components of saliva adsorb to buccal epithelial cell surfaces, and that, within this group, selected components are enzymically cross-linked by an epithelial transglutaminase and/or proteolytically cleaved into smaller fragments.

INTRODUCTION

Increasing evidence suggests that pellicles, derived from saliva, play a significant role in the maintenance and microbial colonization of oral surfaces (Levine *et al.*, 1985, 1987*a*,*b*; Mandel, 1987; Sreebny & Broich, 1987; Tabak & Bowen, 1989). Present knowledge of pellicle formation and function has been primarily derived from the study of acquired enamel pellicle and its role in tooth remineralization and microbial colonization (Moreno & Zahradnick, 1979; Rolla, 1983; Al-Hashimi & Levine, 1989; Gibbons, 1989). Pellicles, however, have also been detected on oral bacteria as well as dental materials and are now thought to form on all oral surfaces (Douglas & Russell, 1984; Levine *et al.*, 1985; Edgerton *et al.*, 1989). In recent work, we have begun to utilize the concepts derived from enamel pellicle studies as a basis for characterizing the formation of pellicles on oral mucosal surfaces, i.e. the mucosal pellicle (Bradway *et al.*, 1989).

Enamel pellicles consist of an organic film, approximately 1 μm in thickness, which are thought to form through selective high-affinity saliva–surface adsorption followed by additional intermolecular saliva protein interactions (Mayhall, 1970; Hay, 1973; Sonju & Rolla, 1973; Bennick *et al.*, 1979; Juriaanse *et al.*, 1981; Tabak *et al.*, 1982; Levine *et al.*, 1985; Al-Hashimi & Levine, 1989). The constituents of salivary pellicles possess structural domains which function as receptors for bacterial organisms and/or may collectively endow the attendant pellicle with characteristics such as moisture retention, lubrication and barrier protection (Edwards, 1978; Tabak *et al.*, 1982; Hatton *et al.*, 1985; Levine *et al.* 1987*a*,*b*). Significant evidence now suggests that selective modification of pellicle constituents by host and bacterial enzymes may alter their functional characteristics and thus the function of the pellicle (McBride & Gisslow, 1977; Ellen *et al.*, 1980; Hatton *et al.*, 1985; Gibbons *et al.*, 1988; Gibbons, 1989).

In previous studies of mucosal pellicle formation, we have reported that components of human parotid saliva (HPS) adsorb to buccal epithelial cells, but after adsorption some components are cleaved into smaller fragments, presumably by the abundant proteinases in saliva (Bradway *et al.*, 1989). Interestingly, additional evidence suggests that some salivary components are also covalently cross-linked by an epithelial isoenzyme of transglutaminase (endo γ-glutamyl ϵ-lysine aminoacyltransferases; EC 2.3.2.13). Transglutaminases catalyse the formation of intermolecular cross-links between the γ-carboxy group of glutamine and the ϵ-amine of lysine residues through amide bonds (Williams-Ashman & Canellakis, 1980). Transglutaminases may potentially react with any endoglutamine residue within a substrate protein, but the reactivity of any one glutamine residue may be partially regulated by flanking amino acid residues and, as of yet undefined, conformational constraints (Folk, 1977;

Abbreviations used: APRPs, acidic proline-rich proteins; DTT, dithiothreitol; HPS, human parotid saliva; HSMSL, human submandibular–sublingual saliva; MG1 and MG2, the high- and low-molecular-mass salivary mucins respectively; TBS, Trisma-bufferd saline (0.02 M-Tris/HCl, pH 7.5, containing 0.154 M-NaCl); FMLP, *N*-formylmethionyl-leucylphenylalanine.

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Bowness *et al.*, 1987; Simon & Green, 1988). Potentially, transglutaminases as well as proteinases associated with buccal epithelial cells may selectively process mucosal pellicle constituents and ultimately affect the mucosal pellicle function. The purpose of this study is to identify salivary components in mucosal pellicle and assess their reactivity with a previously reported transglutaminase. Our findings could provide some insight into the selectivity involved in the adsorption or cross-linking of salivary components during mucosal pellicle formation.

MATERIALS AND METHODS

Materials

The following reagents were purchased from the indicated sources: En^{3}Hance and [^{14}C]methylamine (48 mCi/mmol) were obtained from NEN Research Products/DuPont, Boston, MA, U.S.A. Nitrocellulose-blotting membranes were all obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A. DEAE-cellulose was obtained from Whatman Ltd., Maidstone, Kent, U.K. Sephadex G-25, Sepharose CL-2B and electrophoresis calibration kits (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soyabean trypsin inhibitor, 20 kDa; α-lactalbumin, 14.4 kDa) were purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Na[^{125}I] (13–17 mCi/μg) was purchased from Amersham, Arlington Heights, IL, U.S.A. All other reagents were obtained from either J. T. Baker, Philipsburg, NJ, U.S.A. or Fisher Scientific Co., Rochester, NY, U.S.A. Purified guinea-pig liver transglutaminase was generously provided by Dr. Soo Il Chung (Laboratory of Cellular Development and Oncology, National Institute of Dental Research, Bethesda, MA, U.S.A.). Goat anti-[acidic proline-rich protein 3 (APRP3)] was generously provided by Dr. A. Bennick (Department of Biochemistry, University of Toronto, Toronto, Ont., Canada). Monoclonal antibody to neutrophil *N*-formylmethionyl-leucylphenylalanine (FMLP) receptors was generously provided by Dr. E. DeNardin (Department of Oral Biology, State University of New York, Buffalo).

Preparation of purified salivary molecules

Starting materials for the purification of salivary molecules were derived from gustatory stimulated HPS and human submandibular–sublingual saliva (HSMSL) which were collected as previously described (Shomers *et al.*, 1982). A non-glycosylated isoenzyme of salivary amylase (approx. 56 kDa) was purified from HPS as described by Scannapieco *et al.* (1989); cystatin SN (14.1 kDa) was isolated from HSMSL as described by Al Hashimi *et al.* (1988); and the acidic proline-rich proteins (APRP1 and APRP3) were isolated from HPS by a method modified from the procedure of Hay *et al.* (1988). Briefly, HPS (1 litre) was fractionated on a column (1.5 cm × 120 cm) of DEAE-cellulose utilizing a linear gradient consisting of 500 ml of 100 mM-Tris/HCl, pH 7.6, and 500 ml of the same buffer with 100 mM-NaCl. APRPs were eluted between 40 and 80 mM-NaCl and were further purified by repeated recycling over a H5/5 Mono Q anion-exchange column (Pharmacia LKB Biotechnology Inc.) developed (for 45 min at 1 ml/min) with a 0.235–0.250 M-NaCl gradient in 10 mM-Tris/HCl, pH 8, utilizing an f.p.l.c. system (Pharmacia). The purity of each salivary protein was confirmed by amino acid analysis (Al-Hashimi *et al.*, 1988), f.p.l.c./Mono Q, SDS/PAGE on 10% gels (Laemmli, 1970) and anionic PAGE on 7.5% gels (Davis, 1965).

Iodination of purified salivary components

APRPs were iodinated by methods previously described (Bradway *et al.*, 1989). Briefly, 100 μg of APRPs was derivatized at 4 °C in 100 μl of 0.1 M-borate buffer, pH 8.6, with 100 μg of Bolton–Hunter reagent (*N*-succinimidyl 3,4-dihydroxyphenylpropionate; Pierce, Rockford, IL, U.S.A.). The derivatized APRPs as well as 100 μg each of amylase and cystatin SN were individually iodinated at room temperature by the chloramine-T method (Greenwood *et al.*, 1963). The specific radioactivity of APRP1 and APRP3 was approx. 2 μCi/μg of protein. Amylase and cystatin SN each had specific radioactivity of approx. 1 μCi/μg of protein. All iodinated samples were stored at 4 °C before use.

Preparation of antisera

Antiserum to APRP1 was prepared in New Zealand White rabbits. The rabbits were initially primed with subcutaneous injections of 1 mg of antigen/ml in phosphate-buffered saline (10 mM-Na_2HPO_4/NaH_2PO_4, pH 7.2, containing 0.154 M-NaCl) emulsified in a 1:1 dilution of Freund's complete adjuvant (Gibco BRL, Gaithersburg, MD, U.S.A.). Subsequent injections of antigen, emulsified 1:1 in Freund's incomplete adjuvant, were given at 21 and 30 days. Two weeks after the final immunization, blood was obtained by cardiac puncture and the serum was evaluated for reactivity to the purified antigens as well as to intact ductal salivas by immunoprotein blotting. Rabbit antiserum to salivary cystatin SN was prepared in rabbits as described by Shomers *et al.* (1982), and monoclonal antibodies were raised to purified salivary mucin glycoproteins (MG1 and MG2) as previously described by Cohen *et al.* (1990).

Detection of individual salivary components bound to buccal epithelial cells

Mucosal pellicle in our studies is defined as the constituents of saliva which bind to epithelial cells in buffers with the same ionic strength as saliva. Thus buccal epithelial cells are collected in a buffer with the pH and ionic strength of stimulated HPS [0.021 M-Na_2HPO_4/NaH_2PO_4, pH 7.3, containing 36 mM-NaCl and 0.96 mM-$CaCl_2$ (Bennick & Cannon, 1978)]. The collection of buccal epithelial cell and subsequent extractions were performed as previously described by Bradway *et al.* (1989). For extraction, approx. 4×10^6 cells were heated at 100 °C for 2 min in 200 μl of 0.1 M-Tris/HCl, pH 7.5, containing 1% SDS and 0.095 M-dithiothreitol (DTT). Total protein in each of the extracts was determined by the method of Hill & Straka (1988) utilizing a bicinchoninic acid protein assay kit with BSA as standard protein (Pierce). Portions of extract were separated by SDS/PAGE on 10% gels with a 3% stacking gel under reducing conditions as described by Laemmli (1970). The extracts were then transferred to nitrocellulose in 0.25 M-Trisma Base [tris(hydroxymethyl)-aminomethane]/0.25 M-glycine/20% (v/v) methanol utilizing a TE70 semi-dry transfer unit (Hoefer Scientific, San Francisco, CA, U.S.A.) at 100 mA for 45 min. Transfers to be allowed to react with anti-amylase and anti-cystatin SN were blocked with 0.05% Tween 20 in Trisma-buffered saline (0.02 M-Tris/HCl, pH 7.5, containing 0.154 M-NaCl; TBS) while transfers to be allowed to react with anti-APRP were blocked with 5% BSA (Sigma, St. Louis, MO, U.S.A.) in TBS. Transfers were then incubated for 2 h at 25 °C with the appropriate dilution of antiserum (see Figure legends) in TBS containing 0.1% BSA and washed in three changes (10 ml) of TBS. Subsequently, the blots were incubated for 1 h at 25 °C in TBS containing 0.1% BSA and 1:3000 dilutions of horseradish peroxidase conjugates of either goat anti-rabbit or goat anti-mouse IgG (Bio-Rad Laboratories). The blots were washed as before in TBS and developed with 4-chloro-1-naphthol as recommended by the manufacturer (Bio-Rad Laboratories). Freshly collected HPS and HSMSL (approx. 20 μg of protein/sample) or column fractions of HPS enriched in APRP1 and APRP3 (1 μg of each) were used as positive controls. Foot callus epithelium, prepared as previously

described (Bradway *et al.*, 1989) was used to produce an extract of epithelial cells which had not previously been exposed to saliva. Non-immune rabbit sera, hybridoma supernatants, monoclonal antibodies to non-saliva antigens and secondary antibody HRP-conjugate alone were used as negative controls.

Interaction of purified salivary components with epithelial cells and epithelial cell envelopes

Four duplicate samples of buccal epithelial cells (approx. 5×10^5 cells) were divided into 1.5 ml polypropylene microfuge tubes and centrifuged at 13000 *g* for 4 min at room temperature. The experimental group was resuspended in 40 μl of 0.1 M-Tris/HCl, pH 7.5, containing 1.25 mM-EDTA and 1.25 mM-DTT as well as 12.5 mM-$CaCl_2$. The control group was suspended in the same buffer containing 62.5 mM-methylamine hydrochloride (Sigma) to inhibit transglutaminase activity. Both groups of cells were then preincubated for 15 min at 37 °C. After preincubation, 2×10^6 c.p.m. of ^{125}I-labelled amylase, ^{125}I-labelled cystatin SN, ^{125}I-labelled APRP1 or ^{125}I-labelled APRP3 was added in 10μl of 0.1 m-Tris/HCl, pH 7.5, to give final concentrations of 50 mM-methylamine hydrochloride, 1 mM-EDTA, 1 mM-DTT and 10 mM-$CaCl_2$ in either control or experimental reaction mixtures. After incubation, the cells were recovered at 13000 *g* for 4 min, and the reaction supernatants were saved. The cells were then extracted at 100 °C for 2 min in 30 μl of 0.1 M-Tris/HCl, pH 7.5, containing 1% SDS with 0.05 M-DTT and the extracts were recovered by centrifugation at 13000 *g* for 4 min at room temperature. The cell extracts and the original reaction supernatants containing equal amounts of radioactivity were analysed by SDS/PAGE on 10% gels. The gels were dried and autoradiography was performed at −70 °C utilizing Kodak X-O-Mat AR radiographic film and a high-speed calcium tungstate intensifying screen (Eastman Kodak Company, Rochester, NY, U.S.A.).

Cross-linking of purified salivary components to epithelial cell envelopes was assessed after recovery of the epithelial envelopes from the same epithelial cells incubated with ^{125}I-labelled salivary molecules in the above experiments. The envelopes were prepared by repeated extraction of the epithelial cell pellets with 4% SDS/10% 2-mercaptoethanol at 65 °C. After each extraction, the cells were recovered by centrifugation at 13000 *g* for 4 min at room temperature. Extraction of soluble iodinated salivary components was monitored by γ counting and determined to be complete when radioactivity could no longer be detected in extraction buffer supernatants. Extraction of all soluble radioactivity generally required 72 h.

Interaction of purified salivary components with purified transglutaminase

To determine the reactivity of salivary components with transglutaminase, cross-linking of [^{14}C]methylamine to purified salivary components by purified liver transglutaminase was assessed by SDS/PAGE/fluorography. In these experiments samples containing 1 nmol each of amylase, cystatin SN and APRP3 or 15 μg of partially purified APRP preparation were incubated in a final volume of 30 μl of 0.1 M-Tris/HCl, pH 7.5, with 50 nCi of [^{14}C]methylamine (5 μl of 48 mCi/mmol, dried from ethanol), 1 mM-EDTA, 1 mM-DTT, 10 mM-$CaCl_2$ and 2.5 μg of transglutaminase. As a negative control, DTT and $CaCl_2$ in an identical group of samples was substituted with 50 mM-EDTA which acts as a transglutaminase inhibitor at this concentration. All samples were incubated at 37 °C for 4 h after which the reaction was quenched with 30 μl of 0.1 M-methylamine. The total reaction mixture was freeze-dried, resuspended and subjected to SDS/PAGE on 10% gels. The gels were treated with En^{3}Hance and examined by fluorography.

RESULTS

Detection of salivary components in extracts of buccal epithelial cells

Immunoblot analysis with specific antisera to salivary components showed that multiple components of saliva could be detected in extracts of buccal epithelial cells. Monoclonal antisera to salivary mucins MG1 and MG2 (both at 1:1000) reacted with extract components (Fig. 1, lanes b and d respectively) which correspond to MG1 and MG2 in HSMSL (Fig. 1, lanes a and c respectively). Anti-MG1 also reacted with components at approx. 100 kDa and approx. 15 kDa, and anti-MG2 reacted with an additional component of approx. 17 kDa. These findings suggest that the mucins had undergone some cleavage into smaller fragments, presumably by enzymes in the buccal epithelial cell preparations. Polyclonal antiserum to salivary amylase (1:1500) reacted with two components of approx. 56–60 kDa in HPS which correspond to non-glycosylated and glycosylated isoenzymes of amylase (Fig. 2, lane a). Only the lower-molecular-mass isoenzyme was detected in buccal epithelial cell extracts (Fig. 2, lane b). Anti-cystatin (1:3000) reacted with a single band (approx. 14 kDa) in both HSMSL (Fig. 2, lane c) and in buccal epithelial cell extracts (Fig. 2, lane d). Anti-APRP (1:500) reacted with APRP1 or APRP3 in HPS fractions highly enriched in these components (Fig. 3, lanes a and b respectively). This antiserum reacted with two bands in HSMSL and multiple bands in HPS (Fig. 3, lanes c and d respectively) which have relative mobilities comparable with those of proline-rich proteins. Interestingly, anti-APRP reacted with multiple components in epithelial cell extracts (Fig. 3, lane e) which had relative mobilities similar to native proline-rich proteins as well as mobilities both higher and

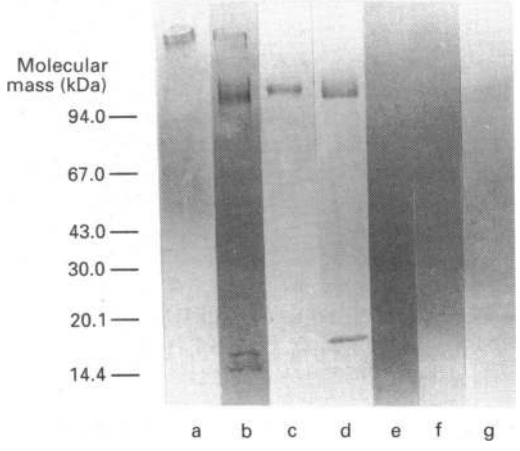

Fig 1. Immunoblot detection of salivary mucins in oral epithelial cell extracts

The reaction of anti-MC1 and anti-MG2 (both at 1:1000) with extracts of buccal epithelial cells was compared with that of freshly collected HSMSL. Anti-MG1 and anti-MG2 reacted with components of epithelial extracts (lanes b and d respectively) which corresponded to MG1 and MG2 in HSMSL (lanes a and c respectively). Both antisera reacted with lower-molecular-mass components not detected in fresh saliva. Myeloma supernatants (lane e) and monoclonal antibody to neutrophil FMLP receptors (lane f) did not react with buccal epithelial cell extracts. Anti-MG1 and anti-MG2 monoclonal antibody did not react with extracts of foot callus (representative reaction with anti-MG1, lane g). Each lane contains 24 μg of protein.

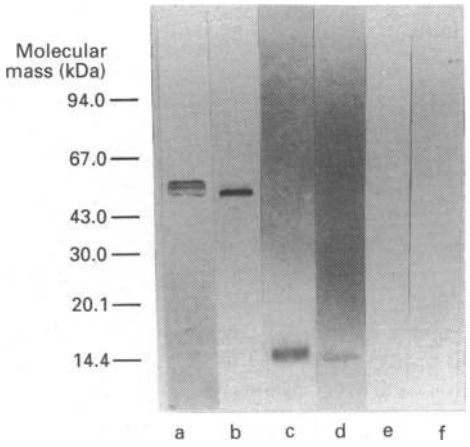

Fig 2. Immunochemical detection of salivary amylase and cystatin in oral epithelial cell extracts

The reaction of rabbit anti-amylase (1:1500) and rabbit anti-cystatin SN (1:3000) with buccal epithelial cell extracts was compared with their reaction to HSMSL. Anti-amylase reacted with two bands in saliva corresponding to the glycosylated (upper) and non-glycosylated (lower) isoenzymes (lane a). The same antiserum reacted with only one band in buccal epithelial cell extracts (lane b). Anti-cystatin SN antiserum reacted with a single band of the same relative mobility in saliva and buccal epithelial cell extracts (lanes c and d respectively). Non-immune rabbit antiserum did not react with either buccal (lane e) or callus (lane f) epithelial cell extracts. Each lane contains 24 μg of protein.

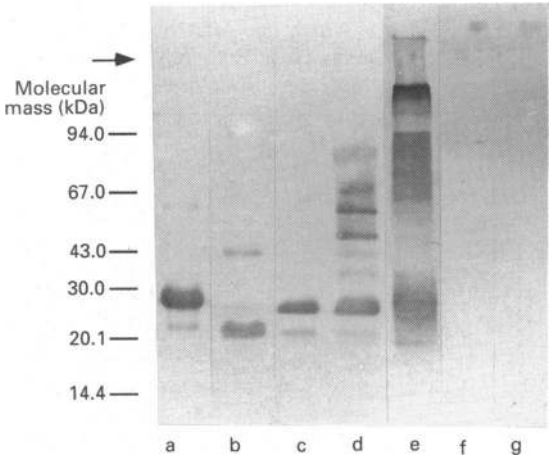

Fig 3. Immunochemical detection of APRPs in buccal epithelial cell extracts

The reaction of anti-APRP (1:500) with buccal epithelial cell extracts (lane e) was compared with APRP1- and APRP3-enriched fractions (lane a and b respectively) as well as HSMSL and HPS (lane c and d respectively). Anti-APRP reacted with multiple components in buccal epithelial cell extracts (lane e) including high-molecular-mass components which were dispersed throughout the stacking gel (arrow). Non-immune rabbit serum did not react with extracts of buccal epithelial cells (lane f) and anti-APRP did not react with callus extracts (lane g). Each lane contains 24 μg of protein.

lower than those of the native proteins. One of these components was found dispersed throughout the 3% stacking gel, indicating a molecular mass many times greater than any proline-rich protein found in saliva (Bennick, 1987). Similar gel profiles were

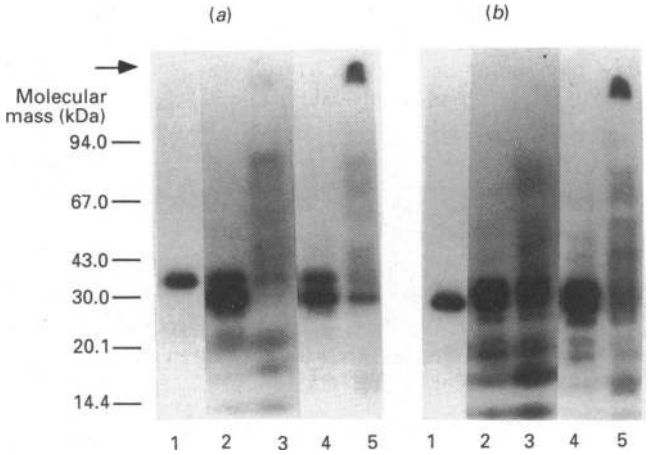

Fig 4. Incubation of APRP1 (*a*) and APRP3 (*b*) with buccal epithelial cells

Purified ^{125}I-labelled APRP1 and ^{125}I-labelled APRP3 are shown in lane 1 of (*a*) and (*b*) respectively. After incubation with buccal epithelial cells, APRPs in reaction supernatants (lane 3) and epithelial cell extracts (lane 5) were examined by SDS/PAGE/autoradiography. ^{125}I-labelled APRPs in reaction supernatants (lane 2) and epithelial cell extracts (lane 4) containing methylamine as a transglutaminase inhibitor were examined by the same method. After incubation with buccal epithelial cells, ^{125}I-labelled APRP1 in the reaction supernatant (*a*, lane 3) and in the cell extracts (*a*, lane 5) appeared in higher-molecular-mass complexes including one which just entered the 3% stacking gel (arrow). In these same mixtures, ^{125}I-labelled APRP1 was also degraded into lower-molecular-mass components. Formation of the higher-molecular-mass complexes was almost completely inhibited by methylamine (*a*, lanes 2 and 4). ^{125}I-labelled APRP3 gave similar results (*b*, lanes 1–5).

obtained with a goat anti-APRP1 (1:500) obtained from Dr. A. Bennick (results not shown).

In experiments utilizing monoclonal antibodies, myeloma supernatants did not react with buccal epithelial cell extracts, and anti-mucin monoclonal antibodies did not react with callus extracts (Fig. 1, lanes e and g respectively). In addition, monoclonal antibodies to non-salivary antigens (neutrophil FMLP receptor) did not react with extracts of buccal epithelial cells (Fig. 1, lane f). In all experiments utilizing rabbit or goat sera, non-immune sera did not react with the buccal epithelial cell extracts (Fig. 2, lane e; Fig. 3, lane f). In the same experiments, immune sera failed to react with extracts of callus epithelial cells (Fig. 1, lane g; Fig. 2, lane f; Fig. 3, lane g).

Collectively, these results suggest that many salivary components adsorb to buccal epithelial cells and that some of these components may undergo enzyme-catalysed modification. These modifications appear to include cross-linking into higher-molecular-mass complexes as well as cleavage into lower-molecular-mass fragments.

Characterization of interactions between purified salivary components and buccal epithelial cells

Iodinated amylase, cystatin SN, APRP1 and APRP3 were incubated with buccal epithelial cells and were subsequently recovered from reaction supernatants or from epithelial extracts. Cross-linking of iodinated salivary components in both preparations was assessed by SDS/PAGE/autoradiography. The ^{125}I-labelled APRP1 and ^{125}I-labelled APRP3 recovered from experimental reaction supernatants (Fig. 4*a*, lane 3 and Fig. 4*b*, lane 3 respectively), and from experimental cell extracts (Fig. 4*a*, lane 5 and Fig. 4*b*, lane 5 respectively) appeared to be cross-linked into multiple components dispersed throughout the 14–98 kDa range as well as a single component which just enters the

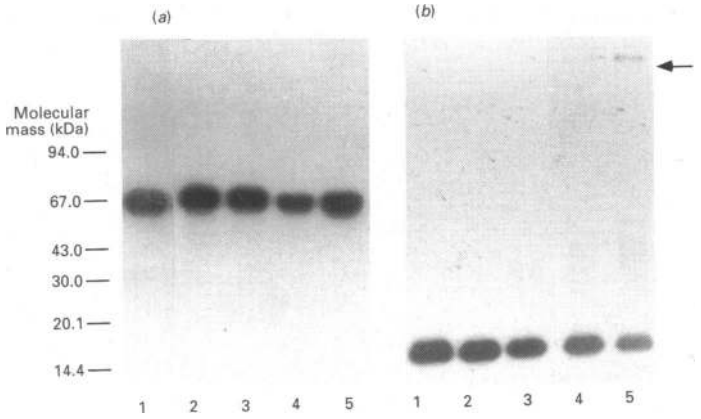

Fig 5. Incubation of salivary amylase (*a*) and cystatin SN (*b*) with buccal epithelial cells

Purified ^{125}I-labelled amylase and ^{125}I-labelled cystatin SN are shown in lane 1 of (*a*) and (*b*) respectively. ^{125}I-labelled amylase and cystatin SN were incubated with buccal epithelial cells by the same protocol used in Fig. 4. Amylase appeared to be unaltered by incubation with epithelial cells (*a*, lanes 2–5). A small amount of cystatin in epithelial cell extracts appeared to undergo high-molecular-mass complex-formation (arrow, *b*, lane 5).

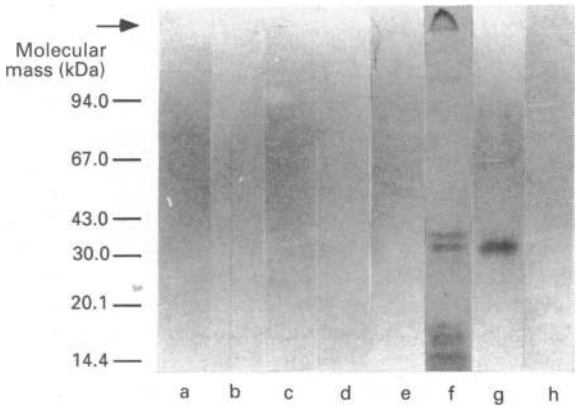

Fig 6. Reactivity of purified salivary molecules with liver transglutaminase

The cross-linking of [^{14}C]methylamine into salivary amylase (lane b), cystatin SN (lane d), partially purified APRPs (lane f) and APRP3 (lane g) by liver transglutaminase was assessed by SDS/PAGE/fluorography. As a negative control, 50 mM-EDTA was used to inhibit transglutaminase in reaction mixture containing amylase (lane a), cystatin SN (lane c) and partially purified APRPs as well as APRP3 (lane e, representative reaction). Reaction of the partially purified APRPs with transglutaminase produced two bands (just below 30 kDa) corresponding to APRP1 (upper) and APRP3 (lower) (lane f). Additionally, a high-molecular-mass complex was observed near the top of the stacking gel (designated by the arrow, left side) and lower-molecular-mass components were present in the 14–20 kDa molecular mass range (lane f). The reaction of purified APRP3 produced a single band at approx. 28 kDa (lane g). Cross-linking of [^{14}C]methylamine into partially purified APRPs and APRP3 was completely inhibited by excess EDTA (lane 3, representative reaction). Cystatin SN and amylase did not react with transglutaminase (lanes a–d). Liver transglutaminase showed negligible self-incorporation of [^{14}C]methylamine (lane h).

3% stacking gel (Fig. 4*a* and *b*, arrow). In contrast, the ^{125}I-labelled APRP1 and ^{125}I-labelled APRP3 starting material produced a single band on autoradiographs (Fig. 4*a* and *b*, lanes 1 respectively). Addition of a competitive transglutaminase inhibitor, methylamine hydrochloride, inhibited the formation of higher-molecular-mass components in both the reaction supernatant (Fig. 4*a* and *b*, lanes 2 respectively) and cell extract (Fig. 4*a* and *b*, lanes 4 respectively). In all of the inhibited reactions containing APRP3, predominant bands were present at relative mobility corresponding to APRP1. These findings were repeated with other transglutaminase inhibitors including iodoacetamide and EDTA (results not shown). Both APRPs also appeared to be cleaved into lower-molecular-mass fragments in all reaction mixtures, and this cleavage was not inhibited by methylamine hydrochloride. Some cross-linking of ^{125}I-labelled cystatin SN was observed in cell extracts, but it was significantly less than that found in the APRP samples (Fig. 5*b*, lane 5). ^{125}I-labelled cystatin SN in reaction supernatants appeared to be largely unaltered (Fig. 5*b*, lane 3). ^{125}I-labelled amylase appeared to be unaffected by incubation with buccal epithelial cells (Fig. 5*a*, lanes 2–5).

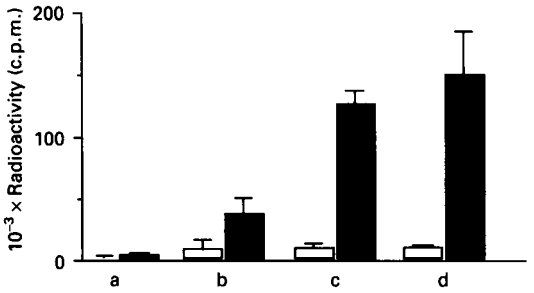

Fig 7. Cross-linking of salivary components into epithelial cell envelopes

Cross-linking of ^{125}I-labelled amylase (lane a, black), ^{125}I-labelled cystatin (lane b, black), ^{125}I-labelled APRP1 (lane c, black) and ^{125}I-labelled APRP3 (lane d, black) into buccal epithelial cell envelopes was assessed by γ counting. Amylase (lane 1) and cystatin (lane b) were not appreciably cross-linked into epithelial cell envelopes. Higher levels of both APRP1 and APRP3 (lanes c and d) were cross-linked to epithelial envelopes. Cross-linking of all salivary components into epithelial cell envelopes was inhibited by 50 mM-methylamine hydrochloride (lanes a–d, white). Error bars represent ±S.D.

Thus, within the group of salivary components examined, APRPs appear to be the most susceptible to cross-linking when incubated with buccal epithelial cells. Interestingly, APRPs also appeared to be cleaved into smaller fragments in reaction supernatants as well as extracts from buccal epithelial cells. These data suggest that enzymic modification of selected salivary components might occur during pellicle formation *in situ* and the mechanisms of modification include covalent cross-linking and proteolytic degradation.

Reaction of purified salivary components with liver transglutaminase

In these experiments, purified amylase and cystatin SN (Fig. 6, lanes b and d respectively), as well as a partially purified preparation of APRPs and APRP3 (Fig. 6, lanes f and g respectively) were allowed to react with purified transglutaminase in the presence of [^{14}C]methylamine. EDTA (50 mM) was added, as a transglutaminase inhibitor, to reactions containing amylase (lane a) and cystatin (lane c) as well as partially purified APRPs and APRP3 (lane e, representative reaction). In these reactions only the partially purified APRPs and APRP3 reacted with transglutaminase (Fig. 6, lanes f and g respectively). In the partially purified APRP preparation, two components (just below 30 kDa) corresponding to APRP1 (upper) and APRP3 (lower) reacted with transglutaminase (Fig. 6, lane 1). Additionally, a high-molecular-mass complex was observed near the top of the stacking gel (designated by the arrow, left side) and lower-molecular-mass components were present in the 14–20 kDa

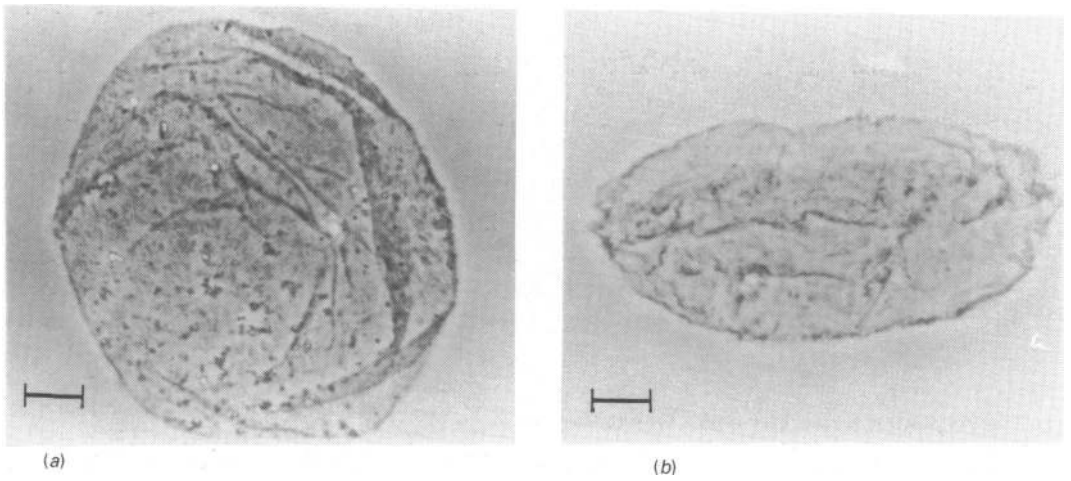

Fig 8. Recovery of epithelial cell envelopes from buccal epithelial cells

Buccal epithelial cells incubated with ^{125}I-labelled amylase, ^{125}I-labelled cystatin SN, ^{125}I-labelled APRP1 or ^{125}I-labelled APRP3 were repeatedly extracted with 4% SDS/10% 2-mercaptoethanol until no further radioactivity could be detected in the extraction buffer by γ counting (65 °C for 72 h). The resulting buccal epithelial cell envelope (*a*), as viewed at 100-fold magnification, appeared as a structure with the approximate peripheral shape of the buccal epithelial cell. Envelopes (*b*) were produced from dermal epithelium for comparison. Bar represents 10 μm.

molecular-mass range (Fig. 6, lane f). The reaction of purified APRP3 produced a single band at approx. 28 kDa (Fig. 6, lane g). The reaction of the APRP preparations was inhibited by addition of excess EDTA (Fig. 6, lane e, representative reaction). [^{14}C]Methylamine was not cross-linked to amylase or cystatin SN in the presence or absence of EDTA (Fig. 6, lanes a–d). Liver transglutaminase showed negligible self-incorporation of [^{14}C]-methylamine (Fig. 6, lane h). These data support our previous observations that the APRPs are better substrates for transglutaminase than the other salivary components tested.

Interactions of purified salivary components with epithelial cell envelopes

Previous reports from our laboratory suggested that salivary components were cross-linked to the epithelial cell envelope (Bradway *et al.*, 1989). Similar experiments were performed to determine if the purified salivary components were cross-linked to epithelial cell envelopes. After 72 h of extraction, the amount of ^{125}I-labelled salivary components cross-linked to epithelial cell envelopes was determined by γ counting. The amount of ^{125}I-labelled amylase cross-linked to cell envelopes was negligible (Fig. 7, lane a, black). Although a small amount of ^{125}I-labelled cystatin SN was recovered with envelopes (Fig. 7, lane b, black), it was significantly less than either ^{125}I-labelled APRP1 or ^{125}I-labelled APRP3 (Fig. 7, lanes c and d, black respectively). The addition of methylamine to all reaction mixtures inhibited the recovery of salivary components with cell envelopes (Fig. 7, lanes a–d, white). The resulting epithelial cell envelopes, as viewed at 1000-fold magnification, appeared as a structure with the approximate peripheral shape of the epithelial cell (Fig. 8*a*). The buccal epithelial cell envelopes appeared similar to those recovered by the same method from dermal epithelium (Fig. 8*b*). These combined data suggest that cross-linking of salivary components to the envelopes is a transglutaminase-catalysed event. The selective pattern of cross-linking also corresponds to the cross-linking of salivary components in buccal epithelial cell extracts.

DISCUSSION

The cellular location of the transglutaminase activity described in this study is not known. Transglutaminase in other epithelial systems can exist as both cytosolic and membrane-bound isoenzymes (Simon & Green, 1984, 1985; Thacher & Rice, 1985; Martinet *et al.*, 1988). Previously, we proposed that recovery of transglutaminase activity from an aqueous buffer incubated with buccal epithelial cells suggested that soluble cytosolic isoenzymes of transglutaminase were released from cells made 'leaky' by the partial degradation of the plasma membrane (Bradway *et al.*, 1989). Cross-linking of salivary components to the epithelial cell envelope suggests that the underlying envelope is indeed exposed to saliva possibly by membrane degradation (Bradway *et al.*, 1989). However, recent characterization of buccal epithelial cell transglutaminase suggests that in the upper layers of buccal epithelium the membrane-bound isoenzyme of transglutaminase predominates (Ta *et al.*, 1990). Several reports indicate that membrane-bound transglutaminases can catalyse interactions between the cell surface and extracellular matrix proteins (Murtaugh & Davies, 1984; Birckbichler *et al.*, 1985; Barsigian *et al.*, 1988). It is possible that transglutaminase from both soluble and membrane-bound sources contributed to the observed cross-linking. In a previous study (Bradway *et al.*, 1989), we have noted that some inhibitors produced only partial inhibition of transglutaminase in whole epithelial cells. This suggests that inhibitors were not used in high enough concentration to eliminate transglutaminase activity, or possibly that domains within salivary molecules may have access to transglutaminase enzymes sheltered from inhibition in a membrane-protected environment.

In the present study, a prominent band which corresponds to APRP1 (approx. 30 kDa) was noted in methylamine-inhibited buccal epithelial cell extracts. Since we have previously had difficulty in completely inhibiting transglutaminase activity in whole buccal epithelial cell preparations (Bradway *et al.*, 1989), we feel that this band may represent a complex of APRP3 cross-linked to small peptides associated with the epithelial cell surface by an incompletely inhibited transglutaminase. Small transglutaminase-reactive glutamine-rich peptides may arise from cleavage of both the epithelial cell envelope precursor involucrin, proline-rich salivary proteins or possibly other peptides of unidentified origin (Simon & Green, 1988; Minaguchi *et al.*, 1988). In our laboratory, we have shown that [^{14}C]putrescine is cross-linked into buccal epithelial cell-associated peptides (14 kDa and 20 kDa), in a reaction that is partially or completely abrogated by transglutaminase inhibitors (Bradway *et al.*, 1989).

It is possible that these peptides may be cross-linked to APRP3 through lysine residues, however, transglutaminase has been reported to catalyse the formation of bis γ-glutamyl putrescine cross-links between glutamine-containing proteins (Schrode & Folk, 1978; Piacentini *et al.*, 1990). Thus it is possible that these peptides may also be cross-linked to APRP3 through bis γ-glutamyl cross-links utilizing putrescine which is synthesized in abundance as a by-product of oral microbial metabolism (Kleinburg *et al.*, 1978). Alternatively, formation of this 30 kDa band may be mediated by a mechanism other than transglutaminase catalysis. Interestingly, in the presence of methylamine, further cross-linking of APRP1 is not seen. It is possible that the APRP3 molecules are more susceptible than APRP1 to reaction with peptides available in the epithelial surface environment.

In a previous report of salivary cross-linking to buccal epithelial surfaces, it was not determined which salivary components were involved in cross-linking (Bradway *et al.*, 1989). Although many salivary components contain the peptide-bound glutamine residues required for the reaction of proteins with transglutaminase, reports from other laboratories indicate that proteins containing glutamine express variable reactivity with transglutaminase (Gorman & Folk, 1981; Jelenska *et al.*, 1981; Simon & Green, 1988). In the present study, the reactivity of anti-APRP with high-molecular-mass components in buccal epithelial cell extracts suggested that native APRPs may be good substrates for transglutaminase *in vivo*. The apparent absence of cross-linked cystatin and amylase from immunoblots of buccal epithelial cell extracts indicated that they may not be readily cross-linked *in vivo*. With purified salivary molecules, the reactivity of ^{125}I-labelled APRP with epithelial cell extracts or cell envelopes was greater than either ^{125}I-labelled cystatin SN or ^{125}I-labelled amylase. Although the amount of ^{125}I-labelled cystatin SN cross-linked in epithelial cell extracts appeared to be relatively small, cross-linking was demonstrated to epithelial cell envelopes. In contrast, ^{125}I-labelled amylase did not appear to be cross-linked in epithelial cell extracts or cell envelopes. The relatively poor reactivity of both cystatin SN and amylase with transglutaminase was underscored in experiments where they apparently did not react with liver transglutaminase. Collectively, these observations indicate a relative selectivity in the reaction of these molecules with buccal epithelial cell transglutaminase.

Given the high glutamine content in APRPs (32–34 mol of glutamine of APRP), it was not surprising that they reacted with transglutaminases. In the reactions of partially purified APRPs with purified transglutaminase, APRP1 and APRP3 as well as high- and low-molecular-mass components reacted. The low-molecular-mass components have the relative mobility of basic proline-rich peptides or statherin, both of which may be present in the APRP preparation at this stage of purification. Since the basic proline-rich proteins share sequence homology with the glutamine-rich regions of the APRPs, and statherin *C*-terminal peptides have previously been shown to react with transglutaminase, it is not unlikely that the observed low-molecular-mass components represent the reaction of these salivary components with transglutaminase (Bennick, 1987; Bradway *et al.*, 1988). Additionally, the formation of the high-molecular-mass complex in the stacking gel may be the result of cross-linking between APRPs and basic proline-rich proteins which contain as many as 57 residues of lysine/1000 by composition (Saitoh *et al.*, 1982).

It was surprising that amylase (14 mol of glutamine/mol) and cystatin SN (8 mol of glutamine/mol) proved to be relatively poor substrates for transglutaminase. Conversely, lysines (25/mol and 6/mol of amylase and cystatin SN respectively) might have served as primary amine donors for transglutaminase-mediated cross-linking. Of these two proteins, only cystatin SN showed any evidence of cross-linking. In other cell systems, it appears that conformational constraints may play a role in the reactivity of proteins with transglutaminase (Bowness *et al.*, 1987; Simon & Green, 1988). Amylase and cystatin SN have secondary conformational characteristics (Payan *et al.*, 1980; Barrett, 1987) that may constrain glutamine and lysine residues from reacting with transglutaminase. APRPs, on the other hand, do not have any known secondary structural characteristics, suggesting that their higher degree of 'flexibility' may allow their glutamines more freedom to react with transglutaminase (Bennick, 1975; Braunlin *et al.*, 1986).

The gel profiles obtained by the reaction of epithelial cell extracts with anti-APRP (Fig. 3) suggested that APRP may undergo multiple interactions at the buccal epithelial cell surface *in vivo*. First, intact APRPs may undergo homotypic complexing with proline-rich proteins in saliva or heterotypic complexing with different salivary components or non-salivary molecules of epithelial cell origin. Second, proteolytic fragments of APRPs may be involved in similar interactions. This latter possibility is supported by the susceptibility of ^{125}I-labelled APRP to apparent proteolytic cleavage *in vivo* as observed in this and other studies (Bennick *et al.*, 1983; Minaguchi *et al.*, 1988). It is also possible that proteolytic cleavage of the APRPs may increase their reactivity with transglutaminase. Indeed, proteolytic cleavage has been shown to increase the number of reactive glutamine residues in involucrin, a substrate of epithelial transglutaminase (Simon & Green, 1988).

Data from the present study indicate that salivary mucins, amylase, cystatin and APRPs are all adsorbed to the surface of buccal epithelial cells. In addition, other salivary components such as lysozyme and secretory IgA may be involved (Bradway *et al.*, 1985). Interestingly, several of these molecules have undergone proteolytic cleavage either before or after adsorption. A comparison of mucosal pellicle constituents with those previously reported for enamel pellicle (Hay, 1973; Kraus *et al.*, 1973; Orstravik & Kraus, 1973; Al Hashimi & Levine, 1989) suggests that pellicles on these two surfaces may be qualitatively similar. It has been proposed that differential adsorption of salivary molecules on to oral surfaces results in the formation of pellicles with unique functional characteristics (Hillman *et al.*, 1970; Gibbons & van Houte, 1971; Kashket & Donaldson, 1972; Ericson & Magnusson, 1976). Furthermore, it is postulated that structural modification of salivary components before or after adsorption may be an important determinant in the functional characteristics of these pellicles. For example, modifications of enamel pellicles through the action of proteinases (Reinholdt & Kilian, 1987) and glycosidases (Stinson *et al.*, 1982; Gibbons *et al.*, 1983; Morris & McBride, 1984; Murray *et al.*, 1984) may affect subsequent microbial attachment. The data from the present study suggest that similar events may occur on mucosal surfaces. In addition, differential cross-linking of salivary components or their degradation products by a buccal epithelial transglutaminase may represent an additional element of structural modification which contributes to the unique functional nature of mucosal pellicles.

The release of transglutaminase from buccal epithelial cells suggests that transglutaminases may catalyse cross-linking reactions either at the tissue–environment interface or in saliva *per se*. Only trace amounts of transglutaminase activity have been detected in whole or mixed saliva, suggesting that the enzyme may be inhibited in whole saliva; possibly through oxidation of the thiol active site by oxidative enzymes such as peroxidases. The apparent release of transglutaminase activity into reaction buffers *in vitro* suggests that transglutaminase is not inactivated by its release from epithelial cells. The possibility that transglutaminase at the tissue–environment interface may be protected

by cellular reducing agents such as glutathione should be further investigated.

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