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Two-hybrid analysis of human salivary mucin MUC7 interactions

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Abstract

MUC7 is a low molecular weight monomeric mucin secreted by submandibular, sublingual and minor salivary glands. This mucin has been implicated in the non-immune host defense system in the oral cavity since it binds and agglutinates a variety of oral microbes. To investigate interactions between this mucin and other secretory salivary proteins, a submandibular gland prey library was screened with baits encoding the N- and C-terminal regions of MUC7 in the yeast two-hybrid system. The N-terminal region interacted with several secretory salivary proteins, whereas the C-terminal region did not. Interacting proteins included amylase, acidic proline-rich protein 2, basic proline-rich protein 3, lacrimal proline-rich protein 4, statherin and histatin 1. Formation of complexes between these proteins and the N-terminal region of MUC7 was confirmed in Far Western blotting experiments. Interactions between mucin and non-mucin proteins in saliva could protect complex partners from proteolysis, modulate the biological activity of complexed proteins or serve as a delivery system for distribution of secretory salivary proteins throughout the oral cavity.

Keywords: Mucin; Protein–protein interaction; MG2; Salivary protein

1. Introduction

Saliva is essential in the maintenance of oral health. The biological functions of this fluid can be mostly attributable to mucin and non-mucin proteins that are secreted by major and minor salivary glands. Among other important functions, these proteins are known to maintain the integrity of soft and hard tissues [1,2], to modulate the oral microflora [3] and to provide lubrication for mastication, speech and swallowing [4].

Mucins are the principal protein components of the mucous layer which coats epithelial surfaces in the gastrointestinal, respiratory and reproductive tracts as well as in the oral cavity [5]. Mucins are thought to have a major role in protection of oral epithelial surfaces from chemical and mechanical injury as well as in the non-immune host defense system [4]. Two distinct mucins, MUC5B (MG1) and MUC7 (MG2) are synthesized and secreted by submandibular, sublingual and minor salivary glands [6–9]. MUC5B is a high molecular weight gel forming mucin that contributes to the viscoelastic properties of saliva [10], exhibits a high affinity for hydroxyapatite [11], is a component of the acquired enamel pellicle [12] and binds to certain strains of bacteria [13]. MUC7 is a low molecular weight monomeric mucin that exhibits affinity for cementum [14] but not for hydroxyapatite surfaces [11]. This mucin also binds to several strains of bacteria including oral *Streptococci* [15], the periodontal pathogen *Actinobacillus actinomycetemcomitans* [16,17] and *Pseudomonas aeruginosa* [18]. A recombinant polypeptide containing the N-terminal 144 amino acid residues of MUC7 (rNMUC7 [19]) as well as a derived peptide [20] have also been shown to exhibit fungicidal activity against the opportunistic yeast *Candida albicans*.

Abbreviations: SMSL, submandibular/sublingual secretion; BD, binding domain; AD, activation domain; X-α-Gal, 5-bromo-4 chloro-3-indoyl-α-D-galactopyranoside; IPTG, isopropyl β-D-1-thiogalactopyranoside; TBST, 10 mM Tris–HCl, pH 7.5 containing 150 mM NaCl and 0.05% Tween 20; BCIP, 5-bromo-4-chloro-3-indoyl-phosphate; NBT, nitro blue tetrazolium; RIPA, phosphate buffered saline containing 1% Nonidet P-40, 0.1% SDS and 0.5% sodium deoxycholate

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The polypeptide backbone of MUC7 is organized into 5 domains [21]. Domain 1 (residue 1–51) contains a histatin-like region and a leucine zipper segment, domain 2 (residues 52–144) is enriched with respect to serine and threonine residues, domain 3 (residues 145–282) consists of six heavily O-glycosylated 23 residue tandem repeat (TR) sequences rich in serine, threonine and proline, domain 4 (residues 283–334) contains degenerate repeats, and domain 5 (residues 335–357) contains a second leucine zipper segment. The recombinant protein rNMUC7 consists of domains 1 and 2 of MUC7 and previous work has shown that it binds several strains of *S. mutans* [19] and *A. actinomycetemcomitans* [17]. More recently, we screened a random peptide display library with native MUC7 isolated from submandibular/sublingual secretion (SMSL) and identified a peptide containing a sequence found in lactoferrin [22]. A lactoferrin–MUC7 complex was detected in SMSL and this interaction was confirmed on Far Western blots. This study raised the possibility that MUC7 could form complexes with other proteins in salivary secretions.

The present investigation was undertaken to examine interactions between MUC7 and non-mucin secretory salivary proteins using the yeast two-hybrid system. Domains 1 and 2 (Bait-N) and domains 4 and 5 (Bait-C) of MUC7 were used to screen a submandibular gland prey library. Protein–protein interactions were observed between Bait-N and a subset of secretory salivary proteins, whereas no interactions were observed between Bait-C and any protein in the submandibular gland prey library. Far Western blotting experiments confirmed interactions detected in yeast two-hybrid screens suggesting that MUC7 may participate in physiologically relevant complexes in salivary secretions.

2. Materials and methods

2.1. Preparation of bait constructs and prey library

Poly A+ RNA was isolated from human submandibular gland using the Fast Track isolation kit (Invitrogen, Carlsbad, CA). Bait-N (domains 1 and 2) and Bait-C (domains 4 and 5) fragments were generated by RT-PCR using sense and antisense primers with *Nde*I and *Pst*I sites at the 5′ ends, respectively. The Bait-N primers were: sense, 5′ ATCACGCTACATATGGAAGGTCGA-GAAAGGGATCAT; antisense, 5′ GATGTACTGCAGGTCTTGTGGA-GCTGGGGAATT. The Bait-C primers were: sense, 5′ ATCACGCTACA-TATGACCACAGCTGCCCCAATTACC; antisense, 5′ GATGTACTG-CAGTTGCTCCACCATGTCGTCAA. Bait-N primers amplified a 432-bp fragment encoding residues 1–144 of MUC7 and Bait-C primers amplified a 225-bp fragment encoding residues 283–357 of MUC7. Bait fragments were cloned into the yeast binding domain (BD) vector pGBKT7 that carries the Trp gene (Clontech, Palo Alto, CA).

A library of submandibular gland PCR products was prepared from poly A+ RNA according to manufacturer's protocols (Clontech). The submandibular gland PCR products, linearized activation domain (AD) vector pGADT7-Rec (carrying the Leu gene) and Bait-N or Bait-C were cotransformed into competent yeast cells (strain AH109). In this system, PCR products are integrated into the AD vector by homologous recombination. Transformed cells were spread on series of plates containing –Trp, –Leu, –Trp–Leu, –Trp–Leu–His (triple dropout) and –Trp–Leu–His–Ade (quadruple dropout) medium and incubated 4–6 days at 30 °C. Quadruple dropout plates contained the chromogenic substrate, X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside). Transformants in which there is an interaction between bait and prey protein express the enzyme α-galactosidase which converts X-α-Gal to a blue pigment resulting in the appearance of blue colonies on the plate. Transformations were also carried out with positive control (pGADT7-RecT+ pGBKT7-53) and negative control (pGADT7-RecT+ pGBKT7-Lam) plasmids provided by the manufacturer. Positive control plasmids encode SV40 T antigen and p53 protein that are known to interact and negative control plasmids encode SV40 T antigen and lamin C which do not interact.

2.2. Selection and analysis of positive interactions

Blue colonies were transferred to fresh quadruple dropout plates containing X-α-Gal and incubated for 4–6 days at 30 °C. This process was repeated and colonies that maintained their phenotype and survived stringent nutritional selection were considered positive clones. Plasmid DNA was then isolated from the remaining blue colonies and inserts were amplified by PCR using AD vector specific primers. The obtained PCR products were sequenced and sequences were analyzed by a BLAST search of GenBank.

2.3. Liquid α-galactosidase assay

Putative positive interactions between bait and prey proteins were analyzed using an α-galactosidase assay which measures the quantity of enzyme secreted into the culture medium. Quadruple dropout liquid medium (lacking X-α-Gal) was inoculated with positive colonies and incubated at 30 °C with shaking (250 rpm) until the absorbance at 600 nm reached 0.5–1.0 (~16–18 h). Yeast cultures (1 ml) were centrifuged at 14,000 rpm for 2 min and 8 μl of the supernatant was mixed with 24 μl of assay buffer (2 volumes of 0.5 M sodium acetate, pH 4.5, 1 volume of 100 mM *p*-nitrophenol-α-Gal). The reaction was incubated at 30 °C for 1 h and terminated by addition of 960 μl of 0.1 M Na_2CO_3. The absorbance at 410 nm was measured in a Hitachi U-3010 spectrophotometer and α-galactosidase units were determined and compared to positive and negative controls.

2.4. Preparation of rNMUC7

The recombinant protein, rNMUC7, contains domains 1 and 2 (the N-terminal 144 residues) of MUC7 and has an apparent molecular weight of 24 kDa on SDS-PAGE [19]. To prepare recombinant protein, *E. coli* cells harboring the expression vector pNMUC7 were induced with IPTG (1 mM) for 1 h. Cells were collected, resuspended in ice-cold lysis buffer (20 mM Tris–HCl, pH 7.9, containing 500 mM NaCl and 5 mM imidazole), disrupted by sonication, centrifuged and rNMUC7 was isolated from the supernatant by affinity chromatography on a nickel column (Novagen, Madison, WI). Further purification of rNMUC7 was achieved by chromatography on Superose 12 as described [19].

2.5. Western blots

Amylase, acidic proline-rich protein 2 (PRP 2), statherin and histatin 1 and 3 were isolated from parotid secretion in our laboratory. A synthetic peptide corresponding to histatin 5 was synthesized commercially (American Peptide Company, Sunnyvale, CA). Purified proteins and synthetic histatin 5 (5 μg) were electrophoresed on 10% or 15% polyacrylamide gels under denaturing conditions and transferred electrophoretically to nitrocellulose membranes (Protran, Schleicher and Schuell, Keene, NH) in buffer containing 192 mM glycine, 25 mM Tris-base, 20% methanol at 100 V for 1 h at room temperature. Blots were equilibrated in 10 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween 20 (TBST) for 5 min and blocked with 5% milk/TBST at room temperature for 1 h. Blots were then washed with TBST (3 times for 10 min) and incubated with a primary antibody diluted in 1% milk/TBST at room temperature for 1 h. For probing Western blots, rabbit anti-amylase (Accurate Chemical and Scientific Corp., Westbury, NY) was diluted 1:300, goat anti-PRP 1 was diluted 1:1000, rabbit anti-statherin was diluted 1:500 and rabbit anti-histatin 5 was diluted 1:500. Antibodies against PRP1, statherin and histatin 5 were prepared in our laboratory. Blots were washed with TBST (3 times, 10 min) and incubated with the appropriate species-specific second antibody coupled to alkaline phosphatase. Goat anti-rabbit (Promega, Madison,

WI) and rabbit anti-goat secondary antibody (Sigma, St. Louis, MO) were diluted 1:7500 in 1% milk/TBST and blots were incubated at room temperature for 1 h. Membranes were washed and color development was obtained by addition of BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) according to the manufacturer's instructions (Promega).

2.6. Far Western blots

Far Western blots were performed using a modification of the method described previously [23]. Briefly, blots containing purified rNMUC7 were washed 10 min with 20 mM HEPES, pH 7.5, containing 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol and 0.1% Nonidet P-40 (buffer A) at 4 °C and blocked with 5% milk in buffer A with agitation for 5 h at 4 °C. Blots were washed (10 min) with buffer A and incubated with 100 μg of purified amylase, PRP 2, statherin, histatin 1, histatin 3 or histatin 5 dissolved in buffer A containing 1% milk overnight at 4 °C. Blots were washed (4 times, 10 min) with PBS containing 0.2% Triton X-100 and subsequently were washed (2 times, 10 min) with the same solution containing 100 mM KCl. Immunodetection of proteins that bound to rNMUC7 under these conditions was carried out using the antibodies described above.

3. Results

3.1. Two-hybrid screen of a submandibular gland prey library

3.1.1. Bait-N

Screening of the submandibular gland cDNA library with Bait-N encoding domains 1 and 2 comprising the N-terminal 144 residues of MUC7 resulted in growth of 63 colonies on quadruple dropout plates. Of these, 58 colonies turned blue and 5 colonies did not. The latter were considered to be false positives and were not examined further. Yeast cells transformed with positive control plasmids all turned blue whereas those transformed with negative control plasmids did not grow on quadruple dropout plates.

3.1.2. Bait-C

When the submandibular gland prey library was screened with Bait-C, no colonies grew on triple or quadruple dropout plates (Table 1). To rule out that this was due to failure of yeast cells to take up one or both plasmids, a series of control experiments was performed by plating cells on −Trp, −Leu and−Leu−Trp medium. Growth on −Trp medium indicated that the cells had taken up the Bait-C vector while growth of cells on −Leu medium indicated that they had taken up the submandibular gland prey PCR fragments and the linearized AD vector and that prey fragments had become integrated into the vector. Growth on −Trp−Leu medium indicated that cells had taken up both Bait-C and prey constructs. This screening procedure was repeated a second time and again, no colonies grew on triple or quadruple dropout plates. These results show that interactions between domains 4 and 5 (comprising the C-terminal 75 residues) of MUC7 and proteins in the submandibular gland prey library did not occur.

3.2. Identification of proteins interacting with Bait-N

Plasmid DNA from selected blue colonies exhibiting α-galactosidase activity was isolated and sequenced. Of these sequences, a majority contained open reading frames (ORFs) encoding proteins present in the GenBank database (Table 2). Among these, six were secretory salivary proteins known to be expressed in submandibular gland including amylase, acidic PRP 2, basic PRB 3, lacrimal PRP 4, statherin and histatin 1. All of these proteins have the potential to interact with MUC7 in salivary secretions. Three additional sequences were identified as proteins that are unlikely to interact with MUC7. One of these was profilin 2, a cytoplasmic protein involved in actin polymerization [24]. A second was peroxiredoxin 3, one of a family of proteins that promote anti-oxidant reactions and occur in the cell cytosol, mitochondria and plasma [25] and a third was laminin α5, a component of the basement membrane [26].

DNA sequencing yielded interpretable results for 23 of the selected blue colonies exhibiting putative protein–protein interactions on quadruple dropout plates. The sequences of other prey plasmids from blue colonies lacked primer sequences, were out of frame, contained multiple stop codons or contained ORFs that could not be identified in GenBank. These likely represent cloning artifacts or false positives.

3.3. Analysis of protein–protein interactions

Yeast colonies listed in Table 2 were cultured and the relative strength of protein–protein interactions was estimated in α-galactosidase assays. All 23 colonies exhibited α-galactosidase activity greater than that of the positive control (SV40 T antigen and p53) (Fig. 1). The activity of clones encoding amylase was 3.4, acidic PRP 2 was 3.3, lacrimal PRP 4 was 2.8, the group of basic PRB 3 clones was 1.5–5.0, statherin was 3.5 and histatin 1 was 1.2 times greater than that of the positive control. Interestingly, the α-galactosidase activity of profilin 2 was 2.7 and of peroxiredoxin 3 was 2.5 times greater than the positive control. The activity of laminin α5 was not tested. In all experiments, the negative control (SV40 T antigen and lamin C) exhibited enzymatic activity equivalent to one fifth of that observed in the positive control.

3.4. Confirmation of interactions on Far Western blots

It is recognized that even after screening under highly stringent conditions that putative interactions detected in yeast two-hybrid screens can in fact be false positives. Therefore, we were interested to determine whether interactions between domains 1 and 2 of MUC7 (Bait-N) seen in our two-hybrid

Table 1
Growth of colonies on selective medium after cotransformation of Bait-C and a submandibular gland prey library

Selective Medium	Number of Colonies from Bait-C cotransformation
−Trp	>8000
−Leu	>5000
−Trp/−Leu	>800
−Trp/−Leu/−His	0
−Ade/−Trp/−Leu/−His	0

Table 2
Proteins interacting with Bait-N

Protein	Number of clones	Genbank accession numbers
α-Amylase	1	1351933
PRP 2 (acidic)	1	131008
PRB 3 (basic)	14	6679187
PRP 4 (lacrimal)	1	6005802
Statherin	1	4507261
Histatin 1	1	4504529
Peroxiredoxin 3	1	18203831
Profilin 2	2	16753215
Laminin α5	1	21264601

screens could be confirmed by additional methods. This was accomplished by examining whether selected secretory salivary proteins could bind to a recombinant protein (rNMUC7) comprising domains 1 and 2 of MUC7 in Far Western blotting experiments.

Salivary amylase, PRP 2, statherin and histatins 1, 3 and 5 were electrophoresed and proteins were transferred to nitrocellulose membranes. Western blots probed with antibodies against these proteins contained immunoreactive bands with the expected molecular weights (Fig. 2A). It should be noted that while the PRP 2 clone from the yeast two-hybrid system screen specifically encoded PRP 2, the blots were probed with antibodies against PRP 1 since these two proteins are immunologically indistinguishable. Also, while the histatin clone from the yeast two-hybrid screen specifically encoded histatin 1, we included histatin 3 and 5 in the Far Western blotting experiments because the three major histatins are highly homologous [27,28] and antibodies against histatin 5 cross-react with histatin 1 and 3.

Blots of rNMUC7 were separately incubated with amylase, PRP 2, statherin and histatins 1, 3 and 5 and probed with the corresponding antibodies. As a control, one blot was probed directly with anti-MG2 antibodies revealing an immunoreactive band of 24 kDa corresponding to rNMUC7 as well as an immunoreactive band of 48 kDa corresponding to rNMUC7 dimers ([19]; Fig. 2B). Lighter immunoreactive bands representing rNMUC7 multimers were also observed. Far Western blots of rNMUC7 incubated with each of the purified proteins followed by the appropriate antibody revealed a similar pattern (Fig. 2C) although the blot probed with anti-PRP 1 antibodies contained only a very weak immunoreactive band (data not shown). We were unable to carry out Far Western blotting experiments to confirm interactions observed in yeast two-hybrid screens with basic PRB 3 and lacrimal PRP 4 because, to our knowledge, antibodies against these proteins are not available. No attempt was made to examine profilin 2, peroxiredoxin 3 or laminin α5 on Far Western blots since these are not bona fide secretory salivary proteins and interactions between them and MUC7 are likely to be physiologically irrelevant. Collectively, these results provide strong evidence that domains 1 and 2 of MUC7 can form complexes with a variety of secretory salivary proteins.

4. Discussion

The present work has shown that domains 1 and 2 of MUC7 can participate in protein–protein interactions with several structurally diverse proteins occurring in salivary secretions. The function of these complexes is unknown at the present time but could involve modulation of the biological properties of complex partners. For example, formation of a complex between two (or more) proteins could either enhance or diminish the intrinsic activity of either one. More specifically,

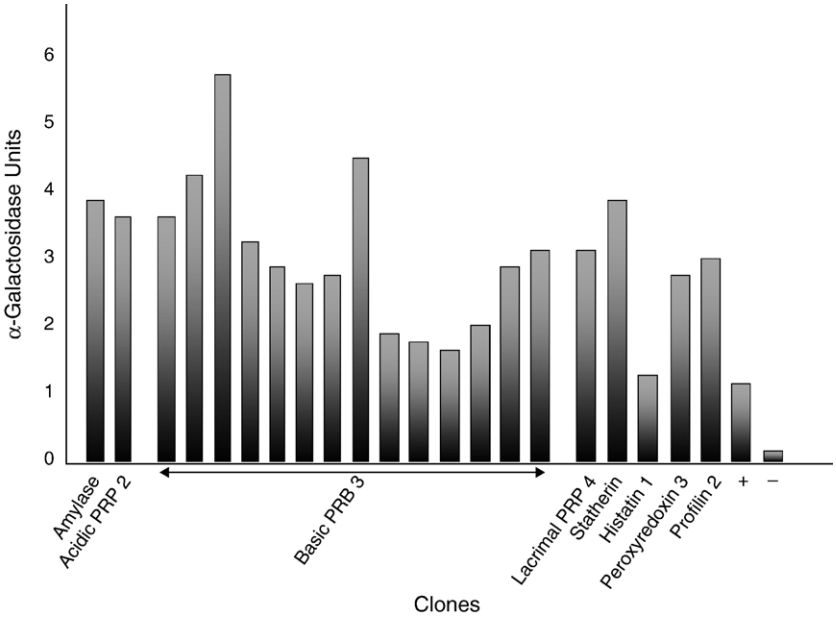

Fig. 1. Qualitative estimation of interaction strength using the α-galactosidase assay. Yeast were grown in quadruple dropout medium, centrifuged and the supernatant was assayed for enzymatic activity according to the manufacturers protocol. The symbols + and − represent α-galactosidase activity in positive and negative controls, respectively.

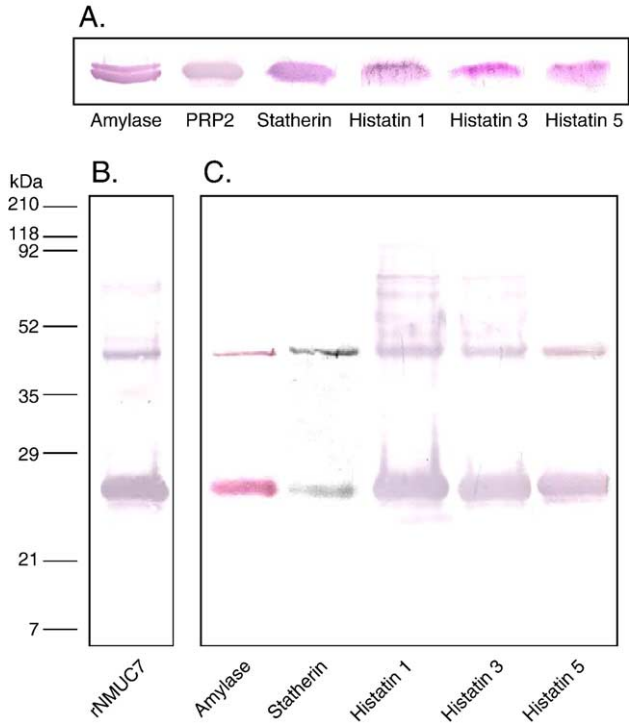

Fig. 2. Confirmation of putative protein–protein interactions between the N-terminal region of MUC7 and amylase, PRP 2, statherin and histatin 1, 3 and 5. (A) Western blots of purified salivary proteins probed with their cognate antibodies. (B) Western blot of rNMUC7 probed with anti-MG2 antibodies. (C) Far Western blots showing interactions between rNMUC7 and salivary proteins. Blots containing rNMUC7 (24 kDa) were separately incubated with purified salivary proteins and after washing, were probed with antibodies directed against these proteins.

such complexes could affect the activity of an enzyme such as amylase or could alter the binding affinity of proteins such as PRP 2 or statherin for the tooth surface or could inhibit or promote the microbicidal activity of a protein such as histatin 1. Furthermore, complexes could protect one or both proteins from proteolysis in the harsh environment of the oral cavity. In addition, the formation of complexes between a mucin and smaller non-mucin proteins could serve as a safe delivery system for distribution throughout the oral cavity.

Yeast two-hybrid screens of a human submandibular gland cDNA library have shown that domains 1 and 2 of salivary mucin MUC7 (Fig. 3A) participate in putative protein–protein interactions with a subset of secretory salivary proteins including amylase, acidic and basic proline-rich proteins, statherin and histatin 1. These putative interactions were verified in α-galactosidase assays and in Far Western blotting studies where purified amylase, statherin and histatin 1 were shown to bind to rNMUC7 immobilized on nitrocellulose membrane. Since rNMUC7 was expressed in bacteria and lacked N- or O-linked carbohydrates, the identified interactions between rNMUC7 and purified salivary proteins represent bona fide protein–protein interactions.

Two points related to the confirmation of interactions deserve further comment. First, while a strong immunoreactive band was observed on Western blots of purified PRP 2 probed with the anti-PRP 1 antibody (Fig. 2A), only a weak immunosignal was observed when the antibody was used to probe Far-Western blots of rNMUC7 incubated with PRP 2 (data not shown). There are several possible explanations for this result. First, while PRP 2 and MUC7 may interact in the environment of the yeast cell, it is possible that the PRP 2-binding domain on MUC7 is masked by immobilization on nitrocellulose. Alternatively, while the anti-PRP 1 antibody is polyclonal, there may be dominant epitopes related to the proline-rich repeating nature of the sequence and it is possible that such an epitope is involved in binding to MUC7 and is therefore unavailable to react with the antibody.

Second, as shown in Fig. 1, there is a near 3-fold difference in the α-galactosidase activity of the 14 identified PRB 3 clones. All of the clones contained the C-terminal 39 amino acids of PRB 3 (Fig. 3B), while some contained varying numbers of additional residues at the N-terminal end. However, there was no apparent correlation between α-galactosidase activity and the lengths of the sequences of individual clones. While it is possible that the differences in activity reflect subtle variations in protein folding within the yeast cell, we conclude that this assay provides qualitative, rather than quantitative, estimates of the strengths of observed protein–protein interactions.

It is also of interest that interactions were detected between prey proteins in the library and Bait-N, but not Bait-C. Bait-N encodes the N-terminal region of MUC7 in which domain 1 (residues 1–51) contains a histatin-like subdomain and a leucine zipper-like segment and domain 2 (residues 52–144) is enriched with respect to serine, threonine and proline ([21]; Fig. 3A). A search of the NCBI conserved domain database did not identify any recognizable protein motifs in domains 1 and 2 of MUC7. A structural analysis of domain 1 predicts two short α-helical regions (residues 5–11, 38–46) and no other ordered structure whereas an analysis of domain 2 predicts several short regions of β sheet and no other ordered structure (SOPMA, ExPASy Molecular Biology Server; http://www.expasy.ch). Failure to detect interactions with Bait-C was somewhat surprising in view of the fact that domain 4 (residues 283–334) resembles domain 2 with respect to amino acid composition and predicted secondary structure. Similarly, domain 5 (residues 335–357) resembles domain 1 with respect to predicted secondary structure and the presence of a leucine zipper-like segment (Fig. 3A). Based on predictive structural analysis, it is not immediately apparent why secretory salivary proteins interacted with domains 1 and 2 (N-terminal 144 residues) but not with domains 4 and 5 (C-terminal 75 residues) of MUC7. In the secretory apparatus of human submandibular/sublingual and minor salivary glands, serine and threonine residues in domains 2, 3 and 4 of MUC7 become decorated with N- and O-linked glycans although the precise sites of glycosylation are unknown due to microheterogeneity. It is unlikely that MUC7 bait proteins synthesized in yeast are glycosylated to any appreciable extent because they do not enter the secretory apparatus but are redirected to the nucleus by virtue of the nuclear localization signal present in the GAL-4 BD fusion partner. The finding that screens with Bait-N revealed putative interactions whereas Bait-C did not, suggests

A.

Bait-N

EG**RERDHELRHRRHHHQ**SPKSHFE***LPHYPGLL***AHQKPFIRKSYKCLHKRCRPKLPPSPNKPPKFPNPHQPPK

HPDKNSSVVNPTLVATTQIPSVTFPSASTKITTLPNVTFLPQNATTISSRENVNTSSSVATLAPVNSPAPQD

Bait-C

TTAAPITTPNSSPTTLAPDTSETSAAPTHQTTTSVTTQTTTTKQPTSAPGQNKISRF***LLYMKNLL***NRIIDDM

VEQ

B.

```
PRP 2  QDLNEDVSQEDVPLVISDGGDSEQFIDEERQGPPLGGQQSQPSAGDGNQDDGPQQGPPQQGGQQQQGPPPPQGKP
PRB 3  --------------------------QRGPRGPYPPGPLA-PPQPFGPGFVPPP--PPPPYGPGRIPPPPP--AP
PRP 4  QSTDNDVNYEDFTFTIPDVEDSSQRPDQGPQRPPPEGLLPRPPGDSG NQDDGPQQRPPKPGGHHRHPPPPPFQN

PRP 2  QGPPQQGGHPPPPQGRPQGPPQQGGHPRPPRGRPQGPPQQGGHQQGPPPPPGKPQGPPPQGGRPQGPPQGQSPQ
PRB 3  YGPGIFP--PPPPQP----------------------------------------------------------
PRP 4  QQRPPQRG-HRQLSLPRFPSVSLQEASSFFRRDRPARHPQE---Q--PLW-------------------------
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Fig. 3. Sequences of MUC7 bait plasmids and alignment of PRPs found in two-hybrid screens. (A) Amino acid sequences of inserts in MUC7 Bait-N and Bait-C plasmids. Domain structures indicated are those described by Gururaja et al., [21]. Solid underlining indicates domain 1 (Bait-N) and domain 4 (Bait C) and dashed underlining indicates domain 2 (Bait-N) and domain 5 (Bait-C). The histatin-like domain in Bait-N is indicated by bold-faced type and the leucine zippers in Bait-N and Bait-C are shown in bold, italic type. (B) PRP 2, PRB 3 and lacrimal PRP 4 were aligned to maximize homology by introducing gaps using ClustalW (www.ch.embnet.org/software/ClustalW.html). Proline residues are shown in bold face type. Dashes represent gaps.

that glycosylation in yeast cells is not a factor in these experiments.

Domains 1 and 2 of MUC7 interacted with three salivary proteins that are proline rich (PRP 2, PRB 3, PRP 4) and three salivary proteins that are not (amylase, statherin, histatin 1). This would suggest that there might be two (or more) distinct regions in the N-terminal region of MUC7 where these interactions can occur. The finding that acidic and basic proline-rich proteins participated in these putative interactions is not surprising since the amino acid proline is a key residue for ligand binding to other proteins [29]. Proline-rich ligands have been shown to interact with a variety of signaling proteins containing SH3 domains such as Src [30], Abl [31] and Amphiphysin I [32] or those containing WW domains such as YAP [33], Dystrophin [34] and FE65 [35]. Ligands that bind to SH3 and WW domains have core consensus sequences such as PPVPPR, PLPXLP, PXXP and PPPPP where X is any residue [29] and very similar sequences are present in the acidic and basic proline-rich proteins that interacted with MUC7 (Fig. 3B). In addition, proline-rich ligands of signaling proteins frequently adopt a polyproline type II helix [29] and this structural motif can be predicted to occur in salivary proline-rich proteins [36].

The three non-proline-rich proteins that interacted with domains 1 and 2 of MUC7 are quite different from one another and from the proline-rich proteins discussed above. Salivary amylase is a ~60 kDa glycoside hydrolase containing 496 amino acids that catalyzes hydrolysis of glycosidic bonds in dietary starch and is the most abundant protein in parotid secretion [37,38]. This enzyme is one of a family of hydrolysases containing an $(\alpha/\beta)_8$ barrel domain as well as other globular domains. Statherin is a 43-amino acid tyrosine-rich peptide that occurs in both parotid and SMSL secretions [39]. The N-terminal 11 residues are predicted to form an α-helix and phosphoserines at residues 2 and 3 result in statherin having a high affinity for hydroxyapatite surfaces and thus a crucial role in demineralization and remineralization of tooth surfaces [40]. The C-terminal region of statherin containing 7 tyrosine and 7 glutamine residues is hydrophobic and is predicted to have a random coil conformation. Histatin 1 is a 38 residue histidine-rich peptide that exhibits very little secondary structure and exhibits potent killing activity against the opportunistic yeast, *C. albicans* [27,41,42]. Amylase is very different from statherin and histatin 1 with respect to size and statherin and histatin 1 are very different from amylase with respect to overall structure. Nevertheless, all three proteins were found to interact with domains 1 and 2 of MUC7 in yeast two-hybrid screens. Based on structural considerations, each would be predicted to bind to a different region of the polypeptide backbone of MUC7. In addition, amylase, statherin and histatin 1, or portions within these molecules, are not predicted to form polyproline type II helices and, on this basis, may interact with yet another class of binding sites on MUC7. A more refined map of interactions between the N-terminal region of MUC7 and secretory salivary proteins could be obtained using deletion constructs in two-hybrid screens or identification of putative binding domains by screening phage display libraries.

Finally, high throughput yeast two-hybrid screens of the entire proteomes of *Saccharomyces cerevisiae* [43], *Drosophila melanogaster* [44] and *Caenorhabditis elegans* [45] have identified interactions between proteins involved in replication, transcription, translation, cell cycle regulation, metabolism and many other cellular processes. This has provided a wealth of new information on proteins known only as ORFs in entire genomes, on proteins with no known function and on proteins without orthologues in other organisms. The situation with proteins in salivary secretions or whole saliva is quite different. The oral cavity is exposed directly to the external environment and saliva provides a protective barrier covering both hard and soft tissues. In contrast to the intricate interactomes within cells of living organisms involving thousands of proteins, the

"complexome" of saliva contains a more limited group of secretory components. While proteins in cells are involved in a host of different biological processes, proteins in saliva carry out three principal broadly defined functions. First, mucins and non-mucin proteins provide lubrication for mastication, speech and swallowing [4]. Second, phosphoproteins such as acidic proline-rich proteins and statherin are involved in regulation of calcium phosphate homeostasis essential for mineralization and demineralization of teeth [40]. Third, MUC7 and other non-mucin proteins are components of the innate host defense system, where they mediate clearance of microbes from the oral cavity, modulate the proteolytic potential of oral fluids and in some cases, exert a direct microbicidal effect [2,42,46]. The present investigation represents a first step towards elucidating the interactions between secretory salivary proteins, presents evidence that complexing can occur in secretions of exocrine glands and has shown that such interactions are not limited to proteins occurring within cells.

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