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Two-hybrid analysis of human salivary mucin MUC7 interactions

Lucila S. Bruno [a], Xiaojing Li [c], Li Wang [b], Rodrigo V. Soares [a,1], Camille C. Siqueira [b], Frank G. Oppenheim [a,b], Robert F. Troxler [a,†], Gwynneth D. Offner [b,c,*]

[a] *Department of Biochemistry, Boston University Medical Center, Boston, MA 02118, USA*
[b] *Department of Periodontology and Oral Biology, Boston University Medical Center, Boston, MA 02118, USA*
[c] *Department of Medicine, Boston University Medical Center, Boston, MA 02118, USA*

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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

* Corresponding author. Department of Medicine, Boston University Medical Center, 650 Albany Street, X-510, Boston, MA 02118, USA. Tel.: +1 617 638 8221; fax: +1 617 638 8224.
E-mail address: goffner@bu.edu (G.D. Offner).
[1] Present address: Department of Periodontology, Pontificia Universidade Catolica, Belo Horizonte, MG, Brazil.
[†] Deceased December 18, 2004.

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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,