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Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice

(β-defensin/antibiotic/whey acidic protein)

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ABSTRACT **Tracheal antimicrobial peptide (TAP) is a member of the β-defensin family of antibiotic peptides found in the tracheal mucosa of the cow. TAP gene expression in the bovine airway is inducible by lipopolysaccharide and inflammatory mediators, suggesting that it functions to protect the upper airway from infection. Limited availability of bovine TAP (bTAP) has precluded investigation of its potential utility in agriculture and medicine. To overcome this problem, transgenic mice expressing bTAP using an expression vector driven by control sequences from the murine whey acidic protein (WAP) gene have been generated. The WAP/bTAP transcript was detected in RNA isolated from mammary tissue of transgenic females. bTAP was purified to homogeneity from milk via acid precipitation, reverse-phase HPLC, and ion-exchange chromatography. This milk-derived bTAP had antimicrobial activity against *Escherichia coli*. Amino-terminal peptide sequencing confirmed the identity of this material as a bTAP isoform. bTAP available from a mammary gland bioreactor will allow evaluation of bTAP for use as an antibiotic in agriculture and medicine.**

Peptide-based antimicrobial defense is an evolutionarily ancient mechanism of host defense found in both the animal and plant kingdoms (for reviews, see refs. 1 and 2). These peptides have been classified based on structural features and include the magainins, first isolated from amphibian skin secretions, as well as the defensins and β-defensins found in mammals. These last two analogous families are found in the neutrophils and in epithelial cells of mucosal tissues. They are predicted to function as a first line of host defense against microbial pathogenesis. Impairment of defensin activity has recently been implicated in chronic bacterial infections in cystic fibrosis patients (3).

In an attempt to understand the role of such molecules in mammalian host defense, a highly abundant cysteine-rich antimicrobial peptide was purified from the tracheal mucosa of the cow. This peptide, named tracheal antimicrobial peptide (TAP), exhibits broad-spectrum activity when assayed *in vitro* against several different strains of microbes, including some respiratory pathogens (4). The cDNA and gene encoding bovine TAP (bTAP) have been cloned, and it was found that the TAP gene is expressed exclusively in cells of the ciliated respiratory epithelium (5).

Soon after the discovery of TAP, the isolation and characterization of 13 cationic cysteine-rich antimicrobial peptides from bovine neutrophils was reported (6). These peptides form a class of highly conserved molecules, β-defensins, that share sequence similarity and include TAP as well as lingual antimicrobial peptide (LAP) (7). Expression of both TAP and LAP in tracheal epithelial cells has been shown to be modulated by the presence of bacteria and bacterial products as well as tumor necrosis factor α (8, 9). Recently, β-defensins have been discovered in humans (10), chickens, and turkeys (11).

Cysteine-rich antimicrobial peptides are cationic and interact with bacterial membranes to form pores (12). This property confers broad spectrum antimicrobial activity on defensins in general and bTAP in particular (4). Chemical synthesis of several defensins has been reported (12), but its cost is prohibitive, and improper folding of the protein may compromise activity. Limited characterization of antibacterial peptides has been accomplished beyond *in vitro* assays, largely due to the difficulties encountered in purifying these peptides from their natural sites of production. The production of cecropin B (a related antimicrobial peptide) in transgenic tobacco failed (13), and use of genetically engineered bacteria is problematic due to formation of inclusion bodies that are difficult to resolubilize and due to the inherent antibacterial activity of β-defensins.

The mammary gland has been used successfully as a bioreactor to produce a variety of biologically active proteins that require posttranslational modification, but their effect on the gland must be assessed empirically (14, 15). Preliminary studies indicate that the β-defensin bovine LAP (bLAP) may normally be expressed in mammary gland (C. L. Bevins, personal communication) and secreted in milk (S. Gorodetsky, personal communication). These studies suggested that the mammary gland might be able to produce bTAP. The transgenic mammary gland has the potential to produce large amounts of biologically active bTAP at relatively low cost. The yield of purified active protein is critical since the minimal effective dose of bTAP is in the range of 10 μg/ml. Once established, a transgenic line is portable, stable, and expandable. Availability of large amounts of bTAP will facilitate research in both agriculture and medicine as well as provide previously unavailable information about antibiotic peptides, their mechanism of action, and their range of activity. We, therefore, attempted to target the expression of a bTAP to the lactating mammary gland.

EXPERIMENTAL PROTOCOLS

Whey Acidic Protein (WAP)/bTAP Construct. bTAP genomic sequence (gb:L13373 bp 1420–3271) was excised as a *Bsa*HI–*Eam*1105I fragment and cloned into the pWe3′ vector, which contains 843 bp of rat WAP 3′ sequence from the middle of exon 3 to the polyadenylylation signal. The bTAP/WAP 3′ fragment was then excised as a *Kpn*I–*Spe*I fragment and cloned into the *Spe*I–*Sac*II sites of PBL103 so that it was situated 3′ to the rat WAP promoter in that vector. PBL103 contains rat WAP sequences from positions −949 to +33 with regard to transcription. The resultant transgene construct pWAP/bTAP

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Abbreviations: TAP, tracheal antimicrobial peptide; bTAP, bovine TAP; WAP, whey acidic protein; RP-HPLC, reverse-phase HPLC; IEC, ion exchange chromatography.

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contained the WAP promoter, the full genomic coding sequence of bTAP including both exons and the intron, part of WAP exon 3, all of WAP intron C, all of WAP exon 4, and 70 bp of the WAP 3′ untranslated region including the polyadenylylation signal. Cloning junctions and orientation of the fragments was verified by restriction enzyme digestion and DNA sequencing (Sequenase, United States Biochemical). The 4-kb transgene was excised as a *Bss*HII fragment and purified using QiaexII (Qiagen, Chatsworth, CA) prior to microinjection.

Transgenic Mice. Transgenic mice were prepared by microinjection using standard protocols (16). Genomic DNA was extracted from tails and analyzed by PCR (16) using a rat WAP +1 forward primer (5′-ATCAGTCATCACTTGCCTGCCG-CCG-3′) and a bovine β-defensin reverse primer (5′-AACAGGTGCCAATCTGT-3′). Lines were propagated in an ICR outbred background.

RNA. Mammary gland biopsies were taken from anesthetized mice at day 12–13 of lactation. Total RNA isolated using RNazol B (Cinna/Biotecx Laboratories, Friendswood, TX) was resolved on a formaldehyde/agarose gel in 1× Mops buffer (17) and transferred to Hybond N^+ nylon membrane (Amersham) in 10× standard saline citrate (SSC). A 1.9-kb bTAP genomic fragment was labeled with ^{32}P by random priming and used to detect the transgene transcript (17).

Milk. A constant pressure vacuum apparatus was employed to collect milk from anesthetized mice; 1.5 units of oxytocin was administered intramuscularly just prior to collection to ensure milk letdown. Milk was collected between days 8 and 15 of lactation, flash-frozen, and stored at −80°C until analysis.

Purification. Whole milk from mice was boiled in 10 vol of 10% acetic acid for 10 min and clarified by centrifugation at 10,000 rpm in a Beckman J.A. 17 rotor for 20 min. This enriched whey fraction was applied to a Sep-Pak (Waters) C_{18} cartridge and eluted with 60% acetonitrile/0.1% trifluoroacetic acid. The eluate was applied to a reverse-phase HPLC (RP-HPLC) column and fractionated with a linear gradient of 0–60% acetonitrile in H_2O/0.1% trifluoroacetic acid. Fractions were lyophilized, resuspended in 10 μl of H_2O, and assayed for antimicrobial activity. The fraction corresponding to the 32.5-min peak was applied to a sulfoethyl ion-exchange HPLC column (Poly LC, Columbia, MD). A 45-min linear gradient from 0 to 1.0 M NaCl in 25% acetonitrile/5 mM potassium phosphate, pH 5.3, was employed at a flow rate of 1 ml/min. The fraction eluting at 30 min was established to contain the antimicrobial activity.

Antimicrobial Assays. Antimicrobial activity was determined by the plate assay method (4). Briefly, a 1-μl aliquot of each fraction was spotted onto a lawn of *Escherichia coli* D31 on a Petri dish containing Bacto tryptone (10 g/liter), yeast extract (5 g/liter), 0.75% agarose, 25 mM Tris·HCl (pH 7.4), and 50 mM NaF and incubated for 2–4 hr at 37°C. Fractions with antimicrobial activity produce a cleared zone in the bacterial lawn, while those lacking activity do not.

Amino Acid Profile and Amino-Terminal Sequencing. These assays were performed in the protein chemistry core lab of Baylor College of Medicine. The amino acid profile was performed using an Applied Biosystems model 420A system (Perkin–Elmer) with phenylisothiocyanate derivatization. Amino-terminal sequencing was performed in an Applied Biosystems model 473A sequencer (Perkin–Elmer) in the liquid phase.

RESULTS AND DISCUSSION

Expression of bTAP mRNA. Several transgenic founders were generated using the WAP/bTAP genomic construct (Fig. 1*A*) and two lines were analyzed in detail. To demonstrate expression of the transgene, total RNA isolated from lactating mammary gland (day 12–13) was examined by Northern blot

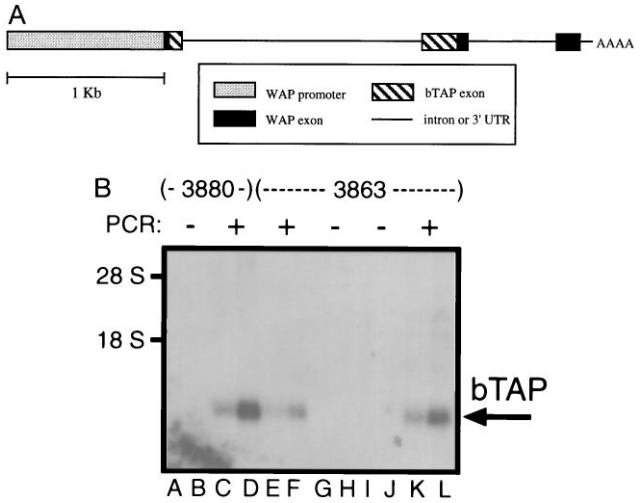

FIG. 1. WAP/bTAP construct and transcript in lactating mammary gland. (*A*) Schematic representation of the WAP/bTAP transgene. Shaded box, WAP promoter; solid box, WAP exons, hatched box, bTAP exons; line, introns and 3′ untranslated sequence. (*B*) Total RNA (1 μg, lanes A, C, E, G, I, and K; 5 μg, lanes B, D, F, H, J, and L) was loaded for each sample. Samples from the 3880 line (lanes A–D) founded by a female and from the 3863 line (lanes E–L) founded by a male are included. F_1 females judged to be transgenic (+) by PCR were assayed and contained the bTAP transcript (lanes C–F, K, and L). Nontransgenic siblings [(−) by PCR] from the F_1 generation of each line were included as negative controls (lanes A, B, and G–J). The blot was probed with a random primed bTAP genomic probe. A 5-hr exposure is shown. The bTAP transcript is indicated by arrow on right.

analysis. RNA from F_1 progeny of the 3880 line, founded by a female, contained a transcript corresponding to the predicted 750 bp size of bTAP (Fig. 1*B*, lanes C and D). RNA from a second line founded by the 3863 male contained the same sized transcript (Fig. 1*B*, lanes E, F, K, and L). Siblings identified as nontransgenic by PCR lacked this transcript (Fig. 1*B*, lanes A, B, and G–J). Variability in transcript intensity between siblings of the 3863 line may be an artifact of loading or a genuine variation within the line.

Benign Phenotype. Transgenic female mice gave birth to normal-sized litters and nursed pups to weaning without incident. Transgenic pups are indistinguishable from their nontransgenic siblings except by PCR. The transgene may be passed through male or female mice (Fig. 1*B*). Therefore, there is no apparent practical impediment to using a similar transgene to produce bTAP in milk of a livestock species.

Isolation of Recombinant bTAP from Milk. To demonstrate the presence of bTAP in milk, RP-HPLC was conducted on milk samples. After acid precipitation of caseins, an enriched whey fraction of nontransgenic murine milk was subjected to RP-HPLC. bTAP purified from bovine trachea was added to a second aliquot of the same material and subjected to the same analysis (Fig. 2*A*). A unique peak at 32 min was observed as a result of this added bTAP. Using relative peak areas from purified bTAP of tracheal origin and similar material added to milk and purified from an enriched whey fraction subjected to RP-HPLC, we conclude that efficiency of recovery is approximately 50%. A similar enriched whey fraction from a transgenic female and a nontransgenic sibling was subjected to the same analysis and a difference in the elution profile was again detected at 32.5 min (Fig. 2*B*). In this case the nontransgenic sample contained a smaller peak than the transgenic sample. This result could indicate a quantitative or a qualitative difference between the two samples. To demonstrate that this difference was qualitative, fractions corresponding to 32.5 min in the RP-HPLC elution profile were further purified using ion-exchange chromatography (IEC). Peaks from 32.5 min in

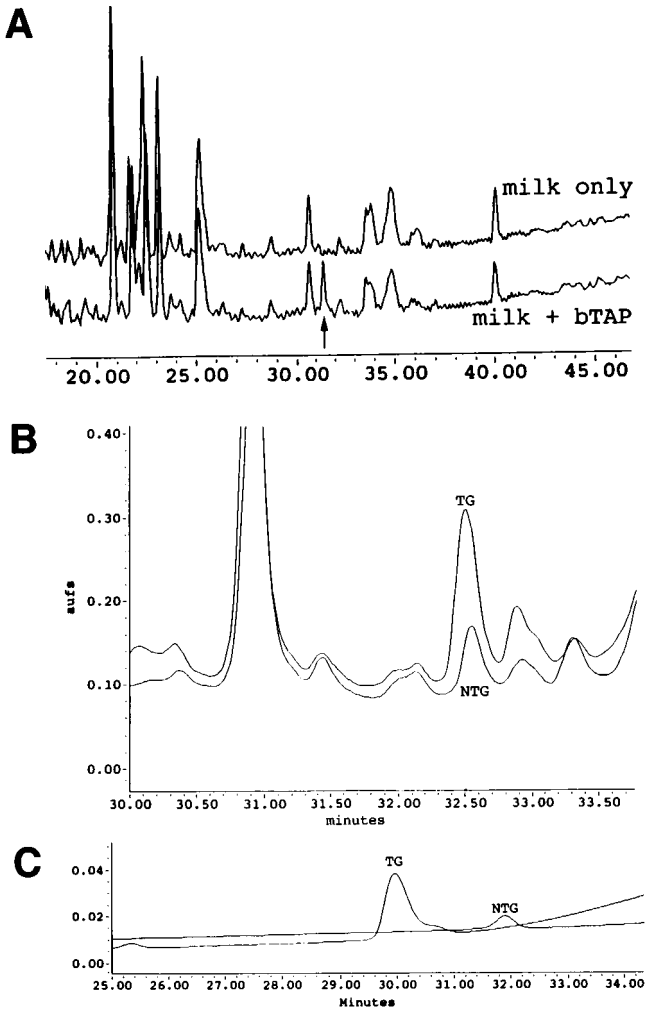

FIG. 2. Purification of bTAP from murine milk. (*A*) RP-HPLC of whey fraction of murine milk purified on a C_{18} SepPak column and eluted with 60% acetonitrile. The eluate was applied to a RP-HPLC column and fractionated with a linear gradient of 0–60% acetonitrile in H_2O/0.1% trifluoroacetic acid. The upper plot is nontransgenic milk alone, while the lower plot is similar material with bTAP isolated from tracheal mucosa added. The arrow indicates an additional peak from bTAP addition. (*B*) Transgenic (TG) versus nontransgenic (NTG) milk purified as in *A*. The peak of increased size at 32.5 min corresponds to antimicrobial activity in an *in vitro* bacterial lysis assay (data not shown). (*C*) IEC of material corresponding to 32.5-min fraction of HPLC shown in *B*. Antimicrobial activity in the *in vitro* assay is present in the 30-min fraction of the TG but not the NTG sample (data not shown).

Fig. 2*B* exhibited different behaviors in IEC (Fig. 2*C*) depending on whether their origin was in transgenic or nontransgenic milk. Our conclusion is that only the transgenic samples contained bTAP and that the observed difference in peak size in Fig. 2*B* corresponds to a qualitative difference in the eluted protein.

Antimicrobial Activity of Recombinant bTAP. To demonstrate the activity of the transgene product identified as bTAP in Fig. 2 *B* and *C*, fractions from RP-HPLC and IEC that corresponded to the unique peaks were assayed in a bacterial plate lysis assay (4). While fractions corresponding to the unique peaks in RP-HPLC and IEC histograms of transgenic milk exhibited antibacterial activity in this assay, corresponding fractions from nontransgenic samples did not (data not shown). This demonstrates that the purified peptide is functional bTAP and implies correct folding as well as correct proteolytic cleavage. This result also confirmed that the observed difference in peak size in Fig. 2*B* corresponds to a qualitative difference in the eluted protein.

Concentration of bTAP in Milk. Based on an amino acid profile, 300 pmol of purified bTAP were recovered from 500 μl of transgenic milk. If a recovery efficiency of 50% is assumed, this corresponds to a bTAP concentration in whole milk of 5 μg/ml. This is in the range of the minimal inhibitory concentration for *E. coli, Klebsiella pneumonia*, and *Candida albicans* (4). (Preliminary characterization of additional lines suggests expression levels that are severalfold higher.) Based on previous findings with protein C and insulin-like growth factor I, there may be additional bTAP secreted as an uncleaved proprotein in the milk (18–20).

Chemical Identity. To establish the chemical identity of bTAP purified from transgenic milk by RP-HPLC and IEC, it was subjected to amino-terminal protein sequencing. A unique amino terminus was observed and the sequence was N-Val-Gly-Asn-Pro-Val-Ser-Cys-Val-Arg-Asn-Lys-C. This corresponds to the published bTAP sequence (4) except that the Val and Gly residues apparent in positions 1 and 2 in milk-derived bTAP were absent from bTAP of tracheal origin. This amino-terminal extension represents an alternative processing product with antimicrobial activity, also purified from bovine tracheal mucosa (K. Cohen and C. Bevins, personal communication). Isoforms that are characterized by 2- or 4-amino acid extensions on the amino terminus were previously observed among 13 β-defensins from bovine neutrophils (6). All forms maintain similar activity *in vitro* and may represent differential processing on the part of the host.

Summary. Demonstration of bTAP activity in material purified from transgenic milk implies that the mammary gland is capable of correct folding of the molecule as well as cleavage of its amino-terminal propeptide. Recombinant bTAP isolated from milk in sufficient quantities may be evaluated for clinical use as an antibiotic. The frequent appearance of bacteria resistant to conventional antibiotics and the growing population of immunocompromised individuals create an increased demand for development of new antimicrobial drugs. This possibility will be contingent upon scale-up in livestock. Based upon previous results with protein C, the described WAP/bTAP construct should be suitable for use in swine (21). In addition, the transgenic mice described herein are important because they provide an experimental model for studying the *in vivo* effect of an antibacterial peptide without purification. Examination of the protective effect of bTAP on pups nursing from transgenic mothers in the face of an enteric *E. coli* challenge should be a good indication of the suitability of the WAP/bTAP transgene for use in protecting neonatal swine where diarrhea is of major economic concern. Generation of WAP/bTAP transgenic swine should facilitate large scale production of bTAP for evaluation as an antibiotic and examination of the effects of recombinant bTAP on enteric *E. coli* infection in baby pigs.

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