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Coordinate Induction of Two Antibiotic Genes in Tracheal Epithelial Cells Exposed to the Inflammatory Mediators Lipopolysaccharide and Tumor Necrosis Factor Alpha

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Peptides with potent broad-spectrum antibiotic activity have been identified in many animal species. Recent investigations have demonstrated that epithelial cells are a site of antibiotic peptide expression, suggesting that these peptides contribute to host defense at mucosal surfaces. Expression of tracheal antimicrobial peptide (TAP), a member of the β-defensin family of peptides, is inducible in cultured tracheal epithelial cells (TEC) upon challenge with bacterial lipopolysaccharide (LPS) (G. Diamond, J. P. Russell, and C. L. Bevins, Proc. Natl. Acad. Sci. USA, in press). In this study, an anchored reverse transcriptase PCR strategy was used to determine if TAP was the sole β-defensin isoform expressed upon stimulation of the cells with LPS. In addition to TAP, a second class of cDNA clones which encoded lingual antimicrobial peptide (LAP), a β-defensin peptide recently isolated from a different mucosal site, the bovine tongue, was identified (B. S. Schonwetter, E. D. Stolzenberg, and M. Zasloff, Science 267:1645–1648, 1995). Northern (RNA) blot analysis demonstrated in vivo expression of LAP mRNA in tracheal mucosa. Levels of LAP mRNA were higher in cultured TEC challenged with either LPS or tumor necrosis factor alpha than in control cells. Thus, a response of TEC exposed to inflammatory mediators is induction of antibiotic-encoding genes, including both *TAP* and *LAP*. This work complements the in vivo studies of Schonwetter et al. (cited above), which showed elevated levels of LAP mRNA in squamous epithelial cells of the tongue near sites of tissue injury and inflammation, by suggesting possible mediators of the in vivo observation. Together these lines of investigations support the hypothesis that inducible expression of endogenous antibiotic peptides by inflammatory mediators characterizes local defense of mammalian mucosal surfaces.

Antibiotic peptides are host defense molecules identified in many animal species (for reviews, see references 2 and 3). In mammals, they were initially identified in phagocytic leukocytes (13, 14), where these peptides contribute to the killing of engulfed microorganisms. The mechanism of antimicrobial activity of many of these peptides is via selective membrane disruption, leading to a characteristic broad spectrum of antibiotic activity which includes gram-negative and gram-positive bacteria, fungi, and certain membrane-enveloped viruses (2). More recent studies have found that similar antibiotic peptides are also made by certain epithelial cells (6, 8, 9, 12, 15, 18, 19), suggesting an additional role in defense of mucosal surfaces (1, 22). As mucosal surfaces are often the site where mammals initially encounter microorganisms, a more complete understanding of defense pathways at these sites may define important aspects of a first line of host defense. A current model proposes that antimicrobial peptides contribute to innate defenses of mucosal surfaces (1, 22).

We previously reported the isolation of tracheal antimicrobial peptide (TAP), a 38-amino-acid peptide from the bovine respiratory mucosa (8). TAP is expressed in columnar epithelial cells of the conducting airway (6). It was the first member of what is now recognized as a relatively large family of antimicrobial peptides, β-defensins, all of which have broad-spectrum microbicidal activity in vitro (8, 18, 20). Recently, a second β-defensin of epithelial origin, lingual antimicrobial peptide (LAP), was isolated, from bovine tongue, and its cDNA was cloned (18). In situ hybridization studies identified squamous epithelial cells as a site of LAP expression.

In the course of our studies on the regulated expression of TAP in tracheal epithelial cells (TEC), we observed dramatic up-regulation of TAP mRNA in cultured TEC stimulated with bacterial lipopolysaccharide (LPS) (5, 7). Since TAP is a member of a large gene family, it was important to determine if induction of β-defensin mRNA in these experiments reflected a collection of related transcripts from a group of β-defensin genes or was homogeneous. In addition to TAP, we report here the identification and characterization of the inducible expression of a second β-defensin gene in TEC.

MATERIALS AND METHODS

General methodology. Bovine TEC were grown in primary culture on a collagen matrix, where the cells maintain many epithelial characteristics (7, 11, 21). Human recombinant tumor necrosis factor alpha (TNF-α) was obtained from Boehringer Mannheim (Indianapolis, Ind.), and LPS was obtained from Sigma Chemical Co. (St. Louis, Mo.; catalog no. L-8643). Other methods and reagents used in this study were described previously (6, 7, 12). Purified plasmid DNA was sequenced by the Sanger method (17), and reported sequence was obtained from both strands of DNA. Sequence data were analyzed with MacVector software (IBI, New Haven, Conn.).

RNA hybridization analysis. Total RNA was isolated and analyzed as de-

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scribed previously (7). Ethidium bromide staining was used to initially document RNA integrity and uniform sample loading. The β-defensin probes were T2/253a (TCTCTGTCCAAGGGCACAGTTTCTGACTCCGCATCAG) and TAP/286a (GCTCTGTCAAAGGGCGCAGTTTCTGACTGGGCATTGA). Labeled probes were hybridized overnight to immobilized RNA in 37.5% (vol/vol) formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–5× Denhardt's solution–1% (wt/vol) sodium dodecyl sulfate (SDS) at 42°C and then washed at high stringency in 0.1× SSC–0.1% SDS at 57°C for 30 min (16). As a control for RNA integrity, the Northern (RNA) blots were hybridized with either an α-tubulin oligonucleotide probe, TUB632a (GTGGTGTGGGTGGTGAG GATAGAGTTGTAGGGCTCAAC), or an α-tubulin cDNA probe (6). The formamide concentration was increased to 50% in the hybridization solution when the α-tubulin cDNA probe was used, and the final stringency wash was changed to 0.1× SSC–0.1% SDS at 65°C for 30 min. All Northern blot filters were stripped of probe and checked by autoradiography before use in subsequent hybridizations.

For slot blot analysis, RNA samples were heat denatured for 10 min at 65°C, and two aliquots of each sample were applied to Zetabind membranes in 2× SSC, using a slot blot apparatus (Hybri-slot 24-well Filtration Manifold; Bethesda Research Laboratories) (4). Linearized plasmid DNAs containing equivalent amounts of β-defensin cDNA were applied as controls for hybridization conditions and relative hybridization signal strength. The samples were applied in duplicate, and the filter was cut to yield two filters with identical arrays of samples for hybridization with probe.

Signal intensities of Northern and slot blots were quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) analysis. The quantitated signal for β-defensin mRNA on Northern blots was normalized by using the tubulin mRNA signal in each sample lane. A Student *t* test (two tailed, $\alpha = 0.05$ [type I error rate]) was used to compare levels of LAP mRNA between two groups (LPS versus control or TNF-α versus control).

Anchored reverse transcriptase PCR analysis. The protocol of Frohman et al. (10) was modified. Poly(A)$^+$-enriched RNA from cultured TEC (Micro-fast Tract; Invitrogen, San Diego, Calif.) was reverse transcribed (Red Module; Invitrogen), using RACE-T17 (RACE stands for rapid amplication of cDNA ends) (GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT) as a primer. A portion of the resulting cDNA (approximately 10 ng) was used as a template in a PCR, using RACE-18 (GACTCGAGTCGACATCG) and TAP27s (ACGC CAGCATGAGGCTCCAT) as anchor and gene-specific primers, respectively. The reaction condition for PCR and the isolation and subcloning of the PCR products were as described previously (12).

Nucleotide sequence accession number. The GenBank accession number for the T2 cDNA sequence is U48357.

RESULTS AND DISCUSSION

Elsewhere we have reported that a response of TEC stimulated with bacterial LPS is a dramatic induction of TAP mRNA (5, 7). To address if additional β-defensin genes were coordinately induced in this system, we used an anchored reverse transcriptase PCR strategy to facilitate direct sequence analysis of individual cDNA clones. TAP and other β-defensin family members have significant sequence identity in the 5′ region of their mRNAs (6), and therefore we designed an upstream PCR primer capable of amplifying an array of TAP-related cDNA. RNA from LPS-stimulated TEC grown in primary culture was analyzed by using this approach. PCR products from these reactions were subcloned, and 20 independent clones were analyzed. Nineteen corresponded in sequence to TAP mRNA, whereas one clone, T2, was different from any that we had previously analyzed (Fig. 1).

To determine if T2 mRNA was expressed in tissues in addition to cultured epithelial cells, we chose a probe (T2/253a) for Northern blot analysis from a relatively unique sequence in the 3′ untranslated region. The Northern blot analysis of RNA from a collection of bovine tissues showed a strong hybridization signal for RNA from tracheal mucosa and trace hybridization with bone marrow and ileum samples (Fig. 2). To control for specific hybridization conditions, a dot blot containing 11 β-defensin clones (including TAP) was hybridized and washed under identical conditions. It showed hybridization of the probe only to the parent sequence on long exposure (data not shown; see Fig. 4).

The sequence of the T2 clone had an open reading frame which encoded a putative prepro-β-defensin peptide (Fig. 1).

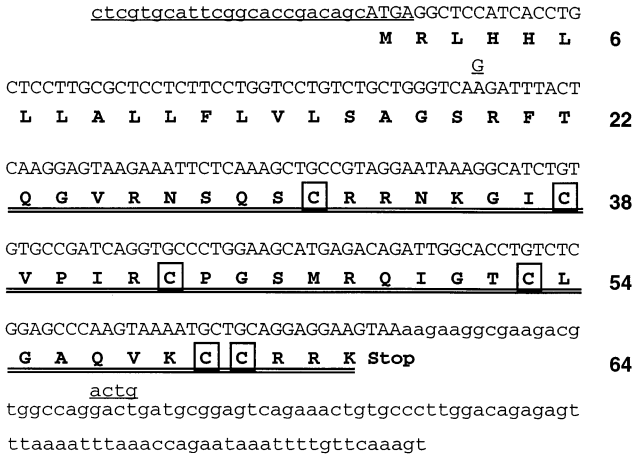

FIG. 1. Nucleotide sequence comparison of T2 and LAP cDNAs. Plasmid DNA from the RACE-PCR clone T2 was sequenced from both DNA strands in its entirety. The sequence shown is aligned with LAP cDNA, with additional sequence (nucleotides 1 to 27) and sequence differences (nucleotides 81 and 240 to 243) underlined (18). The deduced amino acid sequence of the open reading frame is indicated in single-letter code. The single-letter abbreviations for the amino acids are as follows: A, alanine; C, cysteine; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine. The mature peptide isolated by Schonwetter et al. (18) is double underlined. Conserved cysteine residues characteristic of β-defensins are boxed (8, 20).

Interestingly, the deduced sequence of the putative mature peptide corresponded exactly to LAP, a β-defensin peptide isolated from the bovine tongue by Schonwetter et al. (18). The cDNA sequence cloned from a lingual library was also identical except for two regions with nucleotide substitutions, one in the 3′ untranslated region and the other in the putative prepropeptide, encoding an arginine-for-glycine substitution. These differences likely represent alternative alleles of the same gene.

Schonwetter et al. showed that LAP was expressed in squamous epithelial cells of the dorsal aspect of the bovine tongue and that its mRNA was increased in traumatized tissue with an inflammatory infiltrate (18). We questioned if LAP mRNA was inducible in TEC. Northern blot analysis shows that LAP RNA levels are elevated in response to heat-killed *Pseudomonas aeruginosa*, a gram-negative bacterium, approximately 10-fold (Fig. 3; compare lanes 1 and 8 with lanes 4 to 7). Purified LPS

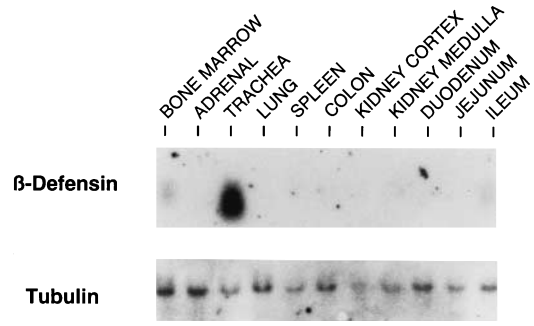

FIG. 2. Northern blot analysis of T2 mRNA in bovine tissues. Total RNA (20 μg) extracted from 11 different tissues was resolved by electrophoresis and transferred to a nylon membrane. The filter was sequentially hybridized under high-stringency conditions with oligonucleotide probe T2/253a (T2, β-defensin) and with an α-tubulin cDNA probe (Tubulin). For experimental details, see Materials and Methods.

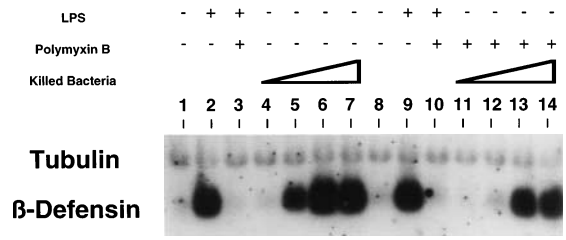

FIG. 3. Northern blot analysis of RNA from TEC challenged with heat-killed bacteria and bacterial LPS. Total RNA was isolated from the cultures of TEC (2×10^5 cells per culture) after incubation for 16 h without additions (lanes 1 and 8) or in the presence of purified LPS from *P. aeruginosa* (lanes 2, 3, 9, and 10; 100 ng/ml) or heat-killed *P. aeruginosa* (lanes 4 to 7 and 11 to 14, representing 10^4, 10^5, 10^6, and 10^7 bacteria per ml of tissue culture medium, respectively). Polymyxin B (1 μg/ml) was added to some of the cultures (lanes 3 and 10 to 14). The RNA was resolved by electrophoresis and transferred to a nylon filter. The filter was hybridized under high-stringency conditions with oligonucleotide probe T2/253a (T2, β-defensin) and with an α-tubulin cDNA probe (Tubulin).

also induced increased mRNA levels compared with the control cultures (compare lanes 2 and 9 with lanes 1 and 8). Comparable elevations in LAP mRNA levels were observed in each of several independent experiments with similar design (data not shown). Polymyxin B at a concentration of 1 μg/ml blocked the response to LPS (lanes 3 and 9) and the response to the heat-killed gram-negative bacteria (lanes 4 to 7 and 11 to 14). Previous studies determined that TAP mRNA was also inducible in TEC under these conditions (7).

LAP mRNA levels were also analyzed in TEC exposed to the inflammatory cytokine TNF-α (Fig. 4). LAP mRNA levels were 3.4-fold higher ($P = 0.02$, $n = 3$) in TEC exposed to 5 ng of TNF-α per ml for 16 h than in the control cells. This change is comparable to the 7.1-fold higher levels of LAP mRNA in TEC exposed to 100 ng of LPS per ml ($P = 0.01$, $n = 3$; Fig. 4). These values are comparable to those found when the same blot was similarly analyzed for TAP mRNA levels, where a 4.1-fold increase ($P = 0.04$) was seen with TNF-α stimulation, and a 8.7-fold increase ($P = 0.001$) was seen with LPS stimulation (autoradiography data not shown).

To estimate the relative levels of TAP and LAP mRNA in trachea and cultured TEC, a slot blot was used to compare hybridization signals in RNA samples, using specific oligonucleotide probes. Aliquots of TAP and LAP cDNAs (1.0 μg of each) were used to normalize for probe specific activity and

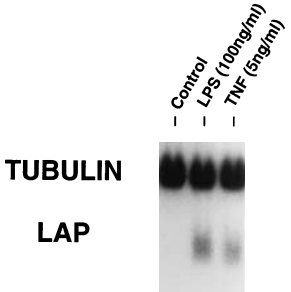

FIG. 4. Northern blot analysis of RNA from TEC challenged with LPS and TNF-α. Total RNA was isolated from the cultures of TEC (2×10^5 cells per culture) after incubation for 16 h without additions (lane 1) or in the presence of either purified LPS from *P. aeruginosa* or recombinant human TNF-α, as indicated. The RNA was analyzed as for Fig. 3. The blot shown is representative of a group of cultures ($n = 3$ for each condition). The relative intensities for LAP (T2) and tubulin signals were quantitated by PhosphorImager analysis. The values for normalized LAP mRNA signals were 156 ± 21 in control cultures, 529 ± 88 in cells exposed to TNF-α, and 1,110 ± 163 in cells exposed to LPS.

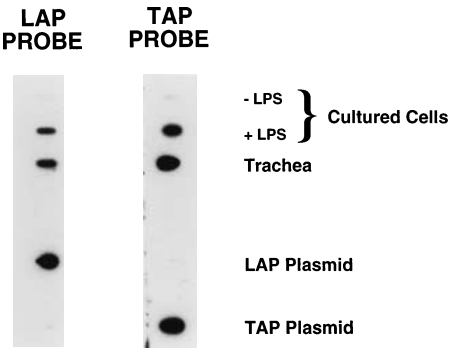

FIG. 5. Slot blot hybridization analysis of RNA from bovine tracheal mucosa and from TEC. Total RNA samples (5 μg) extracted from tracheal mucosa tissue and from cultures of tracheal epithelial cells (2×10^5 cells per culture) after incubation for 16 h without additions, or in the presence of purified LPS from *P. aeruginosa* (100 ng/ml), were denatured and applied in duplicate to a nylon filter by using a slot blot apparatus. For control of hybridization conditions, plasmid DNAs containing cDNA inserts encoding TAP (8) and LAP (T2) were linearized with *Hin*dIII, denatured with NaOH, neutralized with Tris buffer (pH 7.6), and applied to the same filter. The filters were hybridized under high-stringency conditions with the oligonucleotide probes T2/253a and TAP/286a. For experimental details, see Materials and Methods.

hybridization efficiency. The radioactive signals were quantitated by PhosphorImager analysis, and exposure times for autoradiography were adjusted to yield nearly equivalent signals for hybridization of the probes to the control plasmids. RNA samples from cultures of TEC in the absence and presence of LPS were compared with an RNA sample from the trachea (Fig. 5). This analysis demonstrated that LAP mRNA was increased 9-fold in this single sample in the presence of LPS, while TAP mRNA was increased nearly 15-fold. These values compare favorably with those presented above (Fig. 4) and also agree with other studies reported by our group on LPS-mediated TAP induction (7). Also, the ratio of TAP to LAP mRNA was comparable for RNA from tracheal tissue (3:1) and from the stimulated TEC cultures (4:1). (In addition to normalizing for the signal intensity, the hybridization to cDNAs for TAP and LAP [Fig. 5] demonstrate the specificity of the hybridization conditions used for this experiment as well as those used for Fig. 2 and 3. Also, the difference in the ratio of TAP to LAP mRNA assessed in these experiments, compared with the apparent ratio from anchored PCR experiments, probably reflects a bias resulting from the primer used in the PCR amplification. The primer sequenced matched the TAP sequence exactly, but there were two nucleotide differences from the LAP sequence [18]. This difference may have resulted in inefficient priming and subsequent skewing of the product ratios.)

In conclusion, the data indicate that one response of TEC to heat-killed gram-negative bacteria, purified LPS, and TNF-α is a coordinate induction of both TAP and LAP mRNA, albeit at different relative levels. The major β-defensin induced in this system is TAP, with LAP also inducible but at a reduced level. Although we have no evidence for the expression of other β-defensins in this in vitro system, we cannot exclude the possibility that additional β-defensins are also induced but at lower levels of expression. We anticipate that the observed increases in mRNA levels are accompanied by elevated antibiotic peptide levels, but this needs to be tested when antibodies become available. Second, the data demonstrate that LAP mRNA is expressed in TEC as well as in squamous epithelial cells of the tongue (18). To our knowledge, this is the first evidence supporting expression of a single antibiotic gene in more than one type of epithelial cell. Therefore, it would be of

interest to clone and characterize the promoter region of the LAP gene in order to characterize the *cis*-acting sequences governing its pattern of tissue expression. The cloning of the LAP gene may also help to clarify the possible existence of alternative alleles, as suggested by our sequence data. Third, Schonwetter et al. reported dramatic induction in vivo of LAP in lingual squamous cells with close proximity to inflammation and/or infection (18). Our in vitro data demonstrate a dramatic LAP mRNA induction in TEC exposed to either LPS or TNF-α. It is possible that bacterial endotoxin or inflammatory cytokines such as TNF-α are the mediators responsible for the induction previously observed in vivo. An interesting question remains as to the molecular details mediating the observed gene induction. The 5′-flanking region of the TAP gene contains several putative NF-IL6 recognition sequences and a single putative NF-κB binding site, offering attractive hypotheses of possible *cis*-acting sequences which may be important in inducible gene expression (6). Finally, the inducible expression of antibiotic-encoding genes following challenge of TEC with either heat-killed bacteria, LPS, or TNF-α supports the hypothesis that antimicrobial peptides contribute to a dynamic host defense system at mammalian mucosal surfaces. This observation strengthens and extends the current model proposed for antibiotic peptides in local host defense.

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