Plunc, a Member of the Secretory Gland Protein Family, Is Up-regulated in Nasal Respiratory Epithelium after Olfactory Bullectomy*

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Subtraction suppression hybridization was used with high throughput screening to identify transcripts of genes that are differentially expressed in nasal epithelium following lesioning of the olfactory bulb, termed bullectomy. We isolated the rat homologue of plunc, a murine gene highly expressed in lung and nasopharyngeal regions, by this method. Rat plunc encodes a 270-amino acid protein containing a putative signal peptide. plunc up-regulation in respiratory epithelium was confirmed by Northern blot and in situ hybridization. plunc mRNA was expressed in nasal epithelium, heart, lung, thymus, and salivary gland in adult rodent. plunc was expressed in nasal epithelium, thymus, and salivary gland during embryogenesis. Antibodies against Plunc detected a 31-kDa protein in lung, heart, and spleen. Rat nasal epithelium displayed robust immunoreactivity that was highly localized to the microvilli layer of respiratory epithelium. The expression of plunc was up-regulated after bullectomy in respiratory epithelium. We also detected secreted plunc in rat and human mucus. Sequence and homology analyses suggest that Plunc is a member of the secretory gland protein family with putative bactericidal/bacteriostatic function. This is the first protein found in respiratory epithelium whose expression is regulated by olfactory neuronal injury and may provide protection against infection subsequent to injury.

The olfactory epithelium, situated within the nasal cavity, is composed of olfactory receptor neurons (ORNs), 4 sustentacular supporting cells, and a mixed population of basal cells, some of which serve as ORN stem/progenitor cells for the generation of new olfactory sensory neurons throughout life (1–3). The initial events of odor detection occur in ORNs, making their survival vital to the survival of the animal (4). Interspersed among olfactory epithelium is respiratory epithelium, consisting of columnar epithelial cells and underlying basal cells. Any contribution of respiratory epithelium in regulating or responding to olfactory epithelial injury/physiological changes is unknown.

ORN axons project to the olfactory bulb (OB), which is their target. ORNs depend on the OB for their survival, and injury to the OB causes ORNs to degenerate, resulting in compromise of the olfactory epithelium (5). The degeneration of ORNs is followed by an increase in proliferation and maturation of basal cells to repopulate the olfactory epithelium. The OB can be lesioned experimentally, termed bullectomy. Bullectomy results in the hyper-induction of neurogenesis in the olfactory epithelium ipsilateral to the side of the lesion (6–8). These cellular changes in the damaged olfactory epithelium are accompanied by the changes in expression for many molecules that participate in olfactory neurogenesis (5, 10–12). These molecules may include neurotrophins, cytokines, growth factors, and, potentially, factors that serve a protective role during compromise of the olfactory epithelium.

Subtraction suppression hybridization (SSH) (13), like representational difference analysis (14) and differential display (15), is a PCR-based subtractive cDNA hybridization method used to identify and isolate cDNAs of differentially expressed genes. The method is based on suppression PCR and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of cDNAs within the target population, and the subtraction step excludes common sequences between the target and driver populations (13). The high level of enrichment, low background, and normalized abundance of cDNA in the subtracted mixture make this method ideal for rapid cloning of cDNAs from differentially expressed genes. By combining SSH with high throughput differential screening, 625 differentially expressed cDNAs from metastatic adenocarcinoma cell lines have been isolated with a true positive rate of 94%, when subtracted from its non-metastatic counterpart (16).

Using similar approaches in this study, clones of differentially expressed gene fragments were isolated in the adult rat nasal epithelium after olfactory bullectomy. We used this method to identify a number of differentially expressed genes and have characterized one of these clones in detail. A full-length clone was obtained from a rat olfactory cDNA library. This gene is highly homologous to a recently identified mouse gene, plunc (palate, lung, and nasal epithelium clone). Here, we provide data to indicate that Plunc is a secreted protein and
generate insight into one or more of the potential functions of Plunc by investigating its differential expression in our olfactory epithelium damage model as well as sequence analysis.

**Experimental Procedures**

**Experimental Animals and Tissue Preparation—**All experimental protocols were approved by The Johns Hopkins University Institutional Animal Care and Use Committee, and all applicable guidelines from the National Institutes of Health Guide for the care and use of laboratory animals were followed. Male adult Sprague-Dawley rats (100 g) were obtained from Harlan (Indianapolis, IN). For protein and RNA preparation, rats were decapitated and the olfactory epithelium was dissected and immediately processed. For immunohistochemistry and in situ hybridization, animals were anesthetized with Xylaklet and perfused with PBS followed by Bouin’s fixative. Tissue was dissected, post-fixed overnight in Bouin at 4 °C, washed in PBS, placed in 20% sucrose, and embedded in Tissue-Tek. Bouin’s fixed rat olfactory tissue was sectioned at 18 μm using an HM500M cryostat (Zeiss, Germany). Sections were affixed to Superfrost plus slides (Fisher Scientific), thaw-mounted for 2 h at room temperature, and stored at −80 °C until use.

**Bulbectomy—**Adult Sprague-Dawley rats were anesthetized with Xylaklet and fixed in a stereotaxic apparatus for surgery. The right olfactory bulb was exposed via a partial dorsal craniotomy and was ablated by suction. Care was taken to avoid damage to the contralateral (left) olfactory bulb. The ablation cavity was filled with Gelfoam to prevent retraction of the tissue. The rats were allowed to recover from anesthesia under a heat lamp. Following recovery from anesthesia, rats were returned to the animal colony and maintained on a normal diet until animals were killed at 3 day, 1 week, 2 weeks, or 3 weeks post-bulbectomy.

**Subtracted Library Construction by SSH—**Total RNA was purified from nasal epithelium tissues by the acid guanidinium thiocyanate/phenol/chloroform extraction method (17). Nasal epithelium as isolated contained both olfactory epithelium and respiratory epithelium. Polyadenylated RNA was further isolated using mRNA purification kit (Qiagen). Normal nasal epithelium (driver) and 3-day post-bullectomy (driver) were isolated with identical quantities of Sense cRNA as a control for specificity. The subtracted library cDNA (secondary PCR products) was cloned in Uni-ZAP XR (Stratagene) by using polyadenylated RNA isolated from normal olfactory epithelium and a ZAP-cDNA synthesis kit (Stratagene). Approximately 1 × 10^6 white colonies were screened under high stringency conditions using the 0.35-kb cDNA probe isolated from the subtracted cDNA fragment as above (rat plunc nucleotides 690–1040). Positive clones were sequenced by The Johns Hopkins University DNA Synthesis and Sequencing Facility.

**In Situ Hybridization—**The protocol was adapted from the Roche Molecular Biochemicals publication Nonradioactive In situ Hybridization. (Roche Molecular Biochemicals) for 30 min at 37 °C in TSB containing 2 mM CaCl₂. Slides were pre-fixed in Bouin’s fixative solution (4% formaldehyde, 2% glutaraldehyde (Sigma Chemical Co.) for 10 min at room temperature, washed twice with TBS, incubated with 0.1% SDS, 1% SDS, 0.01% sheared salmon sperm DNA) containing digoxigenin-labeled RNA. The following day, coverslips were removed by immersion in 2× SSC and slides were washed twice for 5 min in xylene to remove paraffin and rehydrated through a series of graded ethanol solutions. Sections were washed twice in TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) and treated for 10 min in 0.2 M HCl. Slides were washed twice in TBS and permeabilized with 1 μg/ml RNase-free Proteinase K (Roche Molecular Biochemicals) for 30 min at 37 °C in TBS containing 2 mM CaCl₂. Slides were pre-fixed in Bouin’s fixative solution (4% formaldehyde, 2% glutaraldehyde (Sigma Chemical Co.) for 10 min at room temperature, washed twice with TBS, incubated with 0.1% SDS, 1% SDS, 0.01% sheared salmon sperm DNA) containing digoxigenin-labeled RNA. The following day, coverslips were removed by immersion in 2× SSC, and slides were washed twice for 10 min each in 2× SSC and twice in 1× SSC. Single-stranded RNA probe was digested by RNase A (20 μg/ml) buffer (10 mM Tris, pH 7.5, 500 mM NaCl, and 5 mM EDTA) for 30 min at 37 °C and then washed twice for 20 min each in 0.1× SSC at 37 °C.

To visualize bound probe, sections were washed twice in TBS, and blocks with blocking buffer (10% fetal calf serum in TBS) for 15 min at room temperature. The slides were incubated for 1 h in 1:5000 dilution of anti-DIG Fab fragment (Roche Molecular Biochemicals) in blocking buffer. Sections were then washed twice in TBS and incubated for 5 min in AP buffer (0.1% Tris, pH 9.5, 0.1% NaCl, 50 mM MgCl₂). The color signal was developed in AP buffer containing 3.375 mg/ml nitro blue tetrazolium (Roche Molecular Biochemicals), 5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche Molecular Biochemicals), and 0.24 mg/ml levamisole. The color reaction was carried out at 4 °C and then terminated with a D₅₀.O.Wash. Coverslips were added to the developed slides with Aquapoly mount (Polysciences). Serial sections were hybridized with identical quantities of sense cRNA as a control for specificity. In all cases, no signal was observed using sense cRNAs as probes.

**Immunohistochemistry—**Immunohistochemistry was performed following the Vectastain ELITE protocol (Vector Laboratories). Tissue sections were permeabilized for 1 h in PBS containing 0.1% Triton X-100 and blocked for 1 h in PBS containing 1% bovine serum albumin and 4% normal serum. Section slides were incubated overnight at 4 °C in sections containing the affinity-purified Plunc antibody at a dilution of 1:50. Next day, slides were rinsed with PBS and incubated with Vectastain biotinylated secondary antibody (1:1000) for 30 min, followed by a 5 min rinse with PBS. Slides were incubated in the BC reagent for 30 min, rinsed in PBS, and developed using 25 mg/100 ml diaminobenzidine (Sigma Chemical Co.) in 50 mM Tris, pH 7.4.

**Generation of Antibodies to Plunc Peptide—**Rabbit polyclonal anti-serum to Plunc was generated against the peptide (CNITAElAVMKD-NQQGR, C for conjugation to KLH) consisting of amino acids 174–188 of the predicted rat Plunc sequence. The resulting immune sera (Zymed...
Laboratories Inc.) were affinity-purified against SulfoLink gel (Pierce)-bound peptide as per the manufacturer’s instructions.

Western Blot Analysis—Whole adult nasal epithelium and adult tissues were homogenized in radioimmunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) deoxycholic acid, and 0.1% SDS) and incubated on ice for 30 min. The extracts were cleared by centrifugation for 15 min in an Eppendorf microcentrifuge at 13,000 rpm at 4 °C. Supernatants (50 μg of protein per gel lane) were subjected to SDS-PAGE on a 4–15% gradient gel. Protein content was measured using a Bradford assay with bovine serum albumin as a standard. The separated proteins were transferred to nitrocellulose membrane, and the membranes were probed with rabbit anti-Plunc antiserum. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Molecular Biochemicals) was used as a secondary antibody at a 1:5000 dilution. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.). For mucus collection, anesthetized rats were injected with isoproterenol (30 mg/kg, intraperitoneal) to induce secretion. Secreted nasal mucus and tears were obtained and analyzed.

Human tears and mucus were from male lab volunteers (a Caucasian). Secreted nasal mucus and tears were obtained and analyzed. Isolated nasal mucus and tear samples were homogenized in radioimmune precipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) deoxycholic acid, and 0.1% SDS) and incubated on ice for 30 min. The extracts were cleared by centrifugation for 15 min in an Eppendorf microcentrifuge at 13,000 rpm at 4 °C. Supernatants (50 μg of protein per gel lane) were subjected to SDS-PAGE on a 4–15% gradient gel. Protein content was measured using a Bradford assay with bovine serum albumin as a standard. The separated proteins were transferred to nitrocellulose membrane, and the membranes were probed with rabbit anti-Plunc antiserum. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Molecular Biochemicals) was used as a secondary antibody at a 1:5000 dilution. Bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Inc.). For mucus collection, anesthetized rats were injected with isoproterenol (30 mg/kg, intraperitoneal) to induce secretion. Secreted nasal mucus and tears were obtained and analyzed.

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Results

plunc Is Identified as an RNA Transcript That Is Up-regulated after Bulbectomy—Total RNA isolated from normal nasal epithelium and from nasal epithelium of bulbectomized rats was used as the tester and driver for SSH, respectively. Both mRNA populations were reverse-transcribed and subjected to SSH as described previously (13). 960 cloned inserts were amplified by colony PCR, and the PCR products were dot-blotted to a Hybond N+ membrane. Duplicate filters were hybridized with restriction enzyme cleaved double-stranded cDNA of equal specific activities derived from driver and tester mRNA. The comparison of hybridization signals revealed differentially expressed genes. plunc (p for palate, l for lung, n for nose, and c for clone) was one of the clones identified as up-regulated after bulbectomy.

plunc showed high homology to a mouse gene sequence (Fig. 1) (18). The predicted amino acid sequence of rat plunc was aligned with the mouse sequence (278 amino acids; accession NP_055256), and the two proteins share 91% amino acid identity. More recently, a human plunc gene was identified by screening a human EST data base with a full-length mouse plunc cDNA sequence (19). Analysis of GenBankTM protein and nucleic acid data bases revealed that rat Plunc shared 73% amino acid identity with this human Plunc protein called Lunx (NP_057667). Based on its identities to rat and mouse Pluncs, human Lunx is orthologous to Plunc. The human lunx gene was assigned to chromosome 20. We predicted that our longest rat plunc clone (1079 bp) represented a near full-length cDNA sequence, because mouse plunc and human lunx are 1113 and 1020 bp, respectively, and of the mobility of our transcript was ~1.1k bp. The complete rat plunc sequence contained an open reading frame of 810 base pairs (270 codons) predicted to encode a protein with a molecular mass 27.8 kDa. There are 50 base pairs of 5’-untranslated sequence, and 219 base pairs of 3’-untranslated sequence.

To verify that plunc is expressed in nasal tissue and differentially expressed following bulbectomy, Northern blot analysis was performed (Fig. 2). When compared with its expression in normal nasal epithelium (Fig. 2A, lane 1, control), rat plunc was highly expressed in nasal epithelium ipsilateral to the side of the bulbectomy (Fig. 2A, lane 2, Bulbex). These results were quantified to reveal a 1.8-fold difference using glyceraldehyde-3-phosphate dehydrogenase as a standard. To examine the tissue expression profile of rat plunc, we performed RNA hybridization using a commercial tissue blot prepared from adult mouse (Fig. 2B). A partial rat plunc fragment (nucleotides 690–1040) was used as a probe. Both heart and lung expressed plunc (Fig. 2B). This observation contrasts with an earlier report that demonstrated that mouse plunc was detected only in lung tissue (18). Moreover, the signal in heart was much stronger than that of lung. No signal was detected in brain, spleen, liver, muscle, kidney, or testis.

Fig. 1. Multiple sequence alignment of rat, mouse, and human Pluncs. The alignment was performed using the PileUp program (see “Experimental Procedures”); final alignment was manual. Positions in which the three residues in rat, mouse, and human Plunc are identical are shaded gray. Signal peptide motif and G/C(1)nCPLPPL repeat sequence motifs are indicated by thin lines. Peptide sequence used for generation of anti-peptide antibody is indicated by the thick line.
Plunc is a Secreted Protein Expressed in the Nasal Respiratory Epithelium—Plunc expression in tissues was also examined by Western blotting using affinity-purified antibodies against a Plunc peptide (Fig. 1, dark line below amino acids 174–189 represents sequence used as the antigen for anti-peptide antibody preparation). Plunc immunoreactivity was present in lysates prepared from nasal epithelial tissue (Fig. 3A). The relative mobility of the single band identified corresponded to a molecular mass of 31 kDa (Fig. 3A, lane 1). The size of the protein is larger than the estimated size of 27.8 kDa, suggesting there may be post-translational modification of rat Plunc, such as protein glycosylation. When the antibodies were pre-absorbed with the peptide antigen, the immunoreactive band was no longer detected (Fig. 3A, lane 2).

To examine the patterns of tissue localization of Plunc protein in the adult, we performed immunoblot analysis on various tissue extracts from adult rats. As observed by Northern blot analysis, Plunc was present in lung and heart (Fig. 3B). In contrast to the results obtained by Northern blot analysis, Plunc immunoreactivity was abundant in spleen. Plunc was also detected in testis. However, the relative molecular mass of the band in testis was about 100 kDa, a mobility that was quite different from the 31-kDa band detected in other organs. This discrepancy between the results obtained by Western and Northern analyses of testis tissue may be attributed to the ability of the antisera to recognize a related but not identical protein in testis. Plunc mRNA was not detected in testis, suggesting that the message of the 100-kDa protein identified in testis may contain distinct mRNA sequences that prohibit its recognition using a cRNA probe to plunc by high stringency Northern analysis. Plunc protein was variably detected in kidney. There was no expression in liver, intestine, or brain (Fig. 3B). We also examined tissues from adult mice. As in rats, Plunc was robustly expressed in heart and lung (data not shown). However, in contrast to rats, Plunc expression was minimal in mouse spleen (data not shown). Interestingly, a considerable amount of Plunc immunoreactivity was observed in mouse kidney (data not shown). The significance of these findings is addressed in the discussion.

Plunc has a putative signal peptide sequence (amino acids 1–19), implying that it is a secretory molecule. To investigate whether Plunc might be a secretory protein, we induced mucus secretion in adult rats through the administration of isoprot- erenol and collected the mucus as previously described (20). The collected mucus was subjected to immunoblot analysis (Fig. 3C). Plunc was detected in the mucus from the external nares, indicating that Plunc is a secreted protein. We also demonstrated that human mucus contained Plunc. The relative mobility of human Plunc was smaller than that of rat Plunc, corresponding to the estimated size difference between these proteins. Rat Plunc immunoreactivity was visualized on Western blot as a diffuse band, a characteristic not seen when human mucus was examined, implying that rat Plunc may have post-translational modifications that are different from the human Plunc, such as the degree of glycosylation. We also tested tears and saliva from both rat and human subjects, but no significant amount of Plunc immunoreactivity was observed in these preparations (data not shown).

Plunc Is Highly Expressed in Adult Nasal Respiratory Epithelium—To investigate the cellular localization of plunc, in situ hybridization was performed. Serial coronal sections of adult rat nasal epithelial tissues were prepared and hybridized with a digoxigenin-labeled antisense RNA probe to plunc (Fig. 4). Nasal epithelium consists of stretches of respiratory epithelium that are dovetailed with stretches of olfactory epithelium, each with a distinctive cellular architecture (Fig. 4, diagram). Representative sections from anterior to posterior were examined (Fig. 4, A–D). plunc was highly expressed in the anterior and ventral regions of nasal epithelium, where respiratory...
epithelium is located. No signal was observed in regions containing olfactory epithelium. To confirm that plac is expressed exclusively in columnar epithelial cells in the respiratory epithelium, in situ hybridization was performed for olfactory marker protein (OMP) or plac on serial sections of nasal epithelium that contained both respiratory and olfactory epithelium (Fig. 4, E and F). OMP is expressed in mature ORNs and, therefore, identifies regions of olfactory epithelium that contain ORNs. OMP was expressed in olfactory epithelium and absent from respiratory epithelium as expected, whereas plac was expressed in the opposite distribution. In addition, plac expression was examined in sections containing transition zones between respiratory and olfactory epithelium (Fig. 4G, arrowhead). plac expression ceased at these transition zones (Fig. 4G, arrowhead), confirming its restriction to cells of the respiratory epithelium.

Plac is Up-regulated in Respiratory Epithelium Post-bulbectomy—plac was initially identified by screening for genes whose messages were up-regulated after olfactory bulbectomy. The up-regulation of plac message was confirmed by Northern blot analysis using RNA isolated from control and 3-day post-bulbectomy nasal epithelium (Fig. 2). This result indicated that SSH could identify differentially expressed messages and suggested one of two possibilities regarding plac expression post-lesioning of the olfactory bulb. One possibility is that plac expression is restricted to respiratory epithelium in normal animals but is induced in olfactory epithelium post-lesioning. Alternatively, plac is restricted exclusively to respiratory epithelium, and its expression is up-regulated in respiratory epithelium post-lesioning of the olfactory bulb. To examine these possibilities, in situ hybridization (Fig. 5, A–D) and immunohistochemistry (Fig. 5, E–H) for Plac were performed on nasal tissues isolated from animals that had received unilateral bulbectomies 3 days earlier. Sections containing nasal epithelium ipsilateral to the side of the lesion (Bulbectomy side) and contralateral to the side of the lesion (Control side) were examined. plac message was expressed in the normal respiratory epithelium of non-lesioned animals (Fig. 4) and was essentially unchanged on the side of the epithelium contralateral to olfactory bulbectomy (Fig. 5A). In contrast, the expression of plac increased dramatically in the respiratory epithelium ipsilateral to the bulbectomy (Fig. 5B). This up-regulation of plac expression persisted even after 2 weeks post-lesioning (data not shown). As seen in normal olfactory epithelium (Fig. 4), plac expression was not detected in olfactory epithelium on control or bulbectomized sides (Fig. 5, C and D, respectively).

Immunohistochemical analysis was performed on the same tissues using affinity-purified antibodies directed against a peptide representing amino acid 174–189 of Plac (Fig. 5E–H). Plac immunoreactivity was localized to the apical region of the cells of the respiratory epithelium on the control side contralateral to the olfactory bulbectomy (Fig. 5E), with a distribution and abundance similar that observed in non-lesioned animals (data not shown). Plac protein expression was increased in the respiratory epithelium ipsilateral to the lesion (Fig. 5F). There was no immunoreactivity observed in the olfactory epithelium on the control side (Fig. 5G). Interestingly,
To evaluate the relationships of the rat, mouse, and human Pluncs and to define the relationships of these proteins to other homologues, we performed multiple sequence alignment of the 11 related proteins. As expected from the pairwise sequence alignments, the three Plunc orthologues shared many identical amino acid residues (of 248 multiply aligned positions, 172 were identical). We observed several features of interest. Rat, mouse, and human Plunc are predicted to contain a signal peptide at residues 1–19, based upon the SignalP software package (www.cbs.dtu.dk/services/SignalP/) (24). We identified a motif, G(L/P)PLPL, which occurred three times near the amino terminus of rat and mouse (but not human) Plunc. A search for other proteins sharing this motif using pattern-hit initiated blast (www.ncbi.nlm.nih.gov/blast/) failed to identify other proteins sharing this motif. The multiple sequence alignment revealed only three positions (Cys-196, Cys-238, and Pro-239 of rat Plunc) that are identically conserved among all 11 proteins. However, conservative amino acid substitutions among all the proteins were apparent throughout the multiple sequence alignment and reached 85%. Another interesting domain is bactericidal permeability-increasing protein (BPI) domain.

A search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) of conserved domain data base with rat Plunc sequence revealed statistically significant relatedness to a family that includes bactericidal permeability-increasing protein (BPI)/lipopolysaccharide-binding protein/cholesteryl ester transfer protein amino-terminal domain (SMART data base accession number BPI1). The expected value is 0.005. This family includes murine von Ebner minor salivary gland protein, strengthening support for membership of Plunc in this group. Other members of the family are secreted from glands, such as a blood fluke developmentally regulated albumen gland gene (accession AAC00448) and several lipid-binding proteins. Additionally, two secreted proteins localized to nasal glands, rat RYA3 and rat RY2G5 (accession numbers CAA43065 and CAA43067, respectively) (25, 26) are members of the BPI family and share limited sequence identity with rat Plunc (26% amino acid identity over a span of 140 residues).

**DISCUSSION**

Here, we report the molecular cloning, expression profile study, and sequence analysis of a rat *plunc*. Rat *plunc* is highly homologous to mouse and human *plunc*, whose functions are still unclear. Based on the observation that Plunc is present in the nasal mucus and that its electrophoretic mobility in PAGE is higher than the estimated molecular weight, Plunc appears to be a secreted protein with post-translational modification. Rat Plunc was up-regulated in respiratory epithelium ipsilateral to the side of olfactory bulbectomy by both *in situ* hybridization and immunohistochemistry. In addition, Plunc appeared to be expressed in olfactory epithelium, not in respiratory epithelium. It is unlikely that Plunc is a stimulatory factor for neurogenesis in olfactory neuroepithelium, because...
Table I

Relationship of rat plunc to other proteins

Rat plunc sequence was analyzed by BLAST searching (BLAST 2.1.2, TBLASTN, and PSI-BLAST). Protein abbreviations: SP30, Bos taurus common salivary protein; SMGB1, neonatal submandibular gland proacin cell protein precursor; VEM, Von Ebner minor salivary gland protein. Organism abbreviations: cow, *Bos taurus*; human, *Homo sapiens*; mouse, *Mus musculus*; rat, *Rattus norvegicus*. Expressed sequence tags (ESTs) are listed from UniGene (see "Experimental Procedures"). Protein pairwise sequence comparisons were performed using the BESTFIT or GAP programs of GCG. Values in boxes indicate the percent amino acid identity between each two proteins, the number of gaps in the alignment (in parentheses), and the Z score using 100 random shufflings of a protein sequence in the pairwise alignment.

| Protein          | Organism | Accession | UniGene     | Length (aa) | Mouse plunc | Human lunx | Mouse VEM | Rat SMGB1 | Mouse PSP | Rat PSP | Cow bSP30a | Cow bSP30b | Mouse Protein 1 | Mouse Protein 2 |
|------------------|----------|-----------|-------------|-------------|-------------|------------|-----------|-----------|-----------|---------|-------------|-------------|-----------------|-----------------|
| Plunc            | Rat      | (none)    |             | 270         | 91% (1)     | 73% (4)    | 24% (7)   | 24% (7)   | 32% (13) | 30% (10) | 23% (9)    | 21% (7)     | 60% (2)         | 31% (8)         |
| Plunc            | Mouse    | NP_035256 | Mm.28430    | 278         | —           | 74% (5)    | 24% (6)   | 24% (8)   | 33% (14) | 31% (10) | 21% (6)    | 23% (7)     | 62% (3)        | 32% (8)        |
| Lunx             | Human    | NP_067667 | Hs.211092   | 258         | —           | 30% (10)   | 22% (8)   | 31% (12)  | 28% (11) | 23% (8) | 22% (9)    | 62% (8)     | 28% (5)        |                 |
| VEM              | Mouse    | NP_061205 | Mm.3783     | 474         | —           | 24% (5)    | 25% (9)   | 22% (6)   | 23% (8)   | 24% (10) | 24% (9)    | 20% (8)     |                 |                 |
| SMGB1            | Rat      | A42337    | Rn.9925     | 206         | —           | 40% (3)    | 40% (4)   | 27% (7)   | 29% (6)   | 21% (8) | 28% (16)   |            |                 |                 |
| Parotid secretory protein | Mouse    | NP_032979 | Mm.4538     | 235         | 70% (0)    | 34% (8)   | 31% (8)   | 30% (12)  | 18% (7)   |            |            |                 |                 |
| Parotid secretory protein | Rat      | AAC06334  | Rn.9766     | 235         | —           | 33% (6)   | 31% (7)   | 29% (11)  | 19% (10)  |            |            |                 |                 |
| bSP30s           | Cow      | AAB38282  | Bt575       | 243         | —           | 83% (2)   | 25% (9)   | 27% (10)  |            |            |            |                 |                 |
| bSP30b           | Cow      | AAB38283  | Bt576       | 240         | —           | 22% (8)   | 25% (9)   | 27% (10)  |            |            |            |                 |                 |
| Putative protein 1 | Mouse    | BAB26290  |             | 270         | —           |            |            |            |            |            |            |                 |                 |
| Putative protein 2 | Mouse    | BAB24670  |             | 232         | —           |            |            |            |            |            |            |                 |                 |
bPlunc is expressed exclusively in the respiratory epithelium and localized in the apical layer of the respiratory epithelium. One might suspect that Plunc would have difficulty coming in contact with olfactory stem/precursor cells located in basal layer of olfactory epithelium, although Plunc proteins are present in the ciliary layer of the olfactory epithelium after bulbectomy. Instead, plunc may be the target gene of cytokines or growth factors that are up-regulated upon olfactory bulbectomy to serve another function in response to injury. This is the first report of a protein expressed in respiratory epithelial cells whose expression is altered by injury to the olfactory epithelium or olfactory bulb.

Based on our data, Plunc is up-regulated after injury to the olfactory epithelium. It is not clear how Plunc is up-regulated in the respiratory epithelium after removal of olfactory bulb, which is the target for ORNs resident in olfactory epithelium. Our result implies that there may be some kind of communication between olfactory and respiratory epithelia. This communication might be caused by local fluctuation of cytokines, growth factors, or other factors after bulbectomy. Our results also demonstrate that Plunc is expressed in epithelial cells in segmental bronchi and in heart. By analogy to the olfactory system, Plunc may function in response to injury or insult in the respiratory epithelium. It is more difficult to hypothesize about the function of Plunc expression in heart. However, it would be very interesting to examine Plunc expression after injury to the heart and lung.

The NCI-H667 adeno-squamous carcinoma cell line and the PG-3 adenocarcinoma cell line are known to express Plunc (19, 27). It is interesting to note that Plunc expression in NCI-H667 is reduced by interferon γ, implying that Plunc is regulated either directly or indirectly by this cytokine. Examination of the 5’ regulatory region of plunc to determine whether there is any cytokine-responding element would be interesting. In the mouse 5’ regulatory region, there are, in fact, seven potential binding sites for STATs,2 which are known to regulate expression by various cytokines, including interferon γ (28–30).

By using peptide antibody to Plunc, we demonstrated that Plunc is secreted in the nasal mucus of rat and human. This finding, together with a conserved domain data base search that showed that Plunc has a bactericidal permeability-increasing protein (BPI) domain, suggest that Plunc might have secretory/antimicrobial functions. BPI is a cationic protein displaying a pivotal role in host defense against Gram-negative bacteria (31, 32). The spleen is known to be a critical organ to clear a Gram-negative bacteremia (33), and asplenia can cause sudden death of children as a result of infections of the respiratory systems (9). Interestingly, plunc message is expressed in the upper airways and Plunc protein (but not message) is abundant in rat spleen. These findings suggest that plunc may be a host defense molecule against inhaled Gram-negative bacterial infection.

Very recently, human Plunc (also called Luxn) was proposed as a marker of micrometastasis in non-small cell lung cancer (27). Luxn has been identified by two independent groups (19, 27). It is expressed around the developing palate and thymus, in nasal septum and nasal conchae, as well as in the trachea and main stem bronchi (18, 19). Luxn is found in all the lung carcinoma cell line, and it is differentially expressed in lymph nodes of cancer patients (27). Although Luxn was proposed as a marker of micrometastasis in non-small cell lung carcinoma (27), the nature and function of Plunc are largely unknown. Therefore, it would also be worth determining if there is a neutrophilicroliferative and/or survival role of Plunc.

There is a discrepancy between a previous report describing the expression patterns of mouse/human plunc (18, 19) and our rat plunc Northern result. Others did not detect mouse plunc expression in heart, but, in our study, rat plunc was highly expressed in heart by both Northern and Western analyses. Mouse Plunc UniGene has a few ESTs derived from heart, so a possible explanation is that, in mouse, plunc expression in the heart may not be as high as that in rat.

Thus, Plunc may protect against infection in epithelial tissues post-injury. Variations in the level of Plunc expression could well be correlated with differences in susceptibility to infections. There is precedent for this hypothesis. The relative abundance of a salivary protein, bSP30, is correlated with susceptibility to blot in cattle herds (21). A similar situation might exist for Plunc and respiratory infections. Alternatively, Plunc may also play a role in the etiology of blot. The specific tissue expression pattern of Plunc provides invaluable tools to researchers interested in these tissues.

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