Prostatic acid phosphatase is the main acid phosphatase with 5'-ectonucleotidase activity in the male mouse saliva and regulates salivation

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1Department of Clinical Chemistry, University of Helsinki and Helsinki University Hospital Laboratory, Helsinki, Finland; 2Research Programs Unit; Genome-scale Biology and Institute of Biomedicine, University of Helsinki, Helsinki, Finland; 3Finnish Centre for Laboratory Animal Pathology, Faculty of Veterinary Science, University of Helsinki, Helsinki, Finland; and 4Veterinary Pathology, School of Veterinary Science and Department of Infection Biology, Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom

Submitted 25 February 2014; accepted in final form 3 April 2014

Araujo CL, Quintero IB, Kipar A, Herrala AM, Pulkka AE, Saarinen L, Hautaniemi S, Vihko P. Prostatic acid phosphatase is the main acid phosphatase with 5'-ectonucleotidase activity in the male mouse saliva and regulates salivation. Am J Physiol Cell Physiol 306: C1017–C1027, 2014. First published April 9, 2014; doi:10.1152/ajpcell.00062.2014.—We have previously shown that in addition to the well-known secreted isoform of prostatic acid phosphatase (sPAP), a transmembrane isoform exists (TMPAP) that interacts with snapin (a SNARE-associated protein) and regulates the endo/exocytic pathways. We have also shown that PAP has 5'-ectonucleotidase and thiamine monophosphatase activity and elicits antinociceptive effects in mouse models of chronic inflammatory and neuropathic pain. Therefore, to determine the physiological role of PAP in a typical excocrine organ, we studied the submandibular salivary gland (SMG) of PAP-sufficient and wild-type C57BL/6J mice by microarray analyses, microRNA sequencing, activity tests, immunohistochemistry, and biochemical and physiological analyses of saliva. We show that PAP is the main acid phosphatase in the wild-type male mouse saliva, accounting for 50% of the total acid phosphatase activity, and that it is expressed only in the granular convoluted tubules of the SMGs, where it is the only 5'-ectonucleotidase. The lack of PAP in male PAP−/− mice was associated with a significant increase in the salivation volume under secretagogue stimulation, overexpression of genes related to cell proliferation (Irf7, Birc5) and immune response (Ccl3, Cxcl9, Cc3, Fpr2), and upregulation of miR-146a in SMGs. An increased and sustained acinar cell proliferation was detected without signs of glandular hyperplasia. Our results indicate that in PAP−/− mice, SMG homeostasis is maintained by an innate immune response. Additionally, we suggest that in male mice, PAP via its 5'-ectonucleotidase activity and production of adenosine can elicit analgesic effects when animals lick their wounds.

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addition to the acini and are fully developed at 4 wk when they comprise 19% of the gland volume in males, but only 8% in females (23). The saliva does not only contain digestive enzymes, but in particular the fraction secreted by the SMG is enriched in mucosal glycoproteins, antimicrobial components, immunoglobulins, growth factors (including epidermal and nerve growth factors), and other regulatory substances such as small peptides and hormones (3). Many of these factors are packed in exosomes, small vesicles (30–100 nm) generated by exocytosis of multivesicular endosomes (55) where TMPAP is also present (45, 46). Interestingly, the original description of exosomes identified the vesicles as carriers of 5′-eucnucleotide activity (56). Moreover, exosomes can also carry miRNA molecules (26, 41), which can contribute to the mechanism of paracrine regulation (19). Exosomes have been found in different body fluids including the saliva (29), and we have shown that TMPAP is present in exosomes derived from prostastic LNcap cells (46). As an exocrine secretion in the oral cavity, saliva is fundamental for the maintenance of oral and digestive tract homeostasis. In animals, it also plays an important role in wound healing, through licking. Additionally, the saliva in Mus musculus contains the androgen-binding protein (Abp), which is assumed to act as a pheromone that mediates mate recognition and is spread on pelage and the environment after grooming (31, 54). In this way, the animal can mark the territory with a biochemical signal (59). The reflexory salivary secretion is triggered protocol (67). Briefly, cryosections were dried for 15 min at 37°C, washed twice with 0.04 M Trizma-Maleate buffer (TMB) pH 5.6 and once with 8% (wt/vol) sucrose in TMB. The slides were then incubated for 2 h at 37°C in a solution containing 0.25% (wt/vol) substrate (TMP, AMP, ADP, or ATP), 8% (wt/vol) sucrose and 0.08% (wt/vol) Pb(NO3)2 in TMB. Subsequently, slides were washed for 1 min in 2% acetic acid, followed by three washes with TMB and development for 10 s in 1% Na2S in TMB. Sections were then washed in H2O, dehydrated in an alcohol gradient and xylene, and mounted with Pertex (HistoLab Products).

Measurement of total salivation volume under sympathetic/parasympathetic stimulation. Two-month-old wild-type (17 males, 9 females) and PAP−/− (15 males, 8 females) mice were weighed and then anesthetized by intraperitoneal injection of a mixture of 10 mg/kg xylazine (Rompun Vet 20 mg/ml, Bayer) and 36 mg/kg ketamine (Ketalar 50 mg/ml, Pfizer Oy) in sterile isotonic saline solution. After anesthetization, animals were intraperitoneally injected with a cocktail of secretagogues containing 2 mg/kg of (−)-isoproterenol hydrochloride (Sigma-Aldrich) and 0.5 mg/kg of pilocarpine hydrochloride (99% titration, Sigma-Aldrich). Five minutes after injection, the total saliva was collected in Eppendorf tubes on ice for 10 min, using a peristaltic pump at constant speed. The volume was determined with a micropipette. Samples were stored at −20°C for further analyses. The animals were anesthetized in small groups (4 to 5 animals) at the same time of the day to avoid differences in the salivation properties due to the circadian rhythm.

Determination of total protein concentration and α-amylase activity. Total protein concentrations were measured in the saliva samples collected from stimulated mice with the Pierce BCA Protein Assay kit (Thermo) following the manufacturer’s instructions. The enzymatic assay to evaluate the α-amylase activity was performed, following a modified Sigma A3176-SSSTAR01 protocol adjusted to microscale.

Microarray analyses of mouse SMG. Total RNA was isolated from SMG of 2-mo-old male PAP−/− and wild-type mice (8 per group), cut into four equal pieces and stored in RNA stabilization reagent (RNAlater, Qiagen). Each tissue fragment was lysed with Lysing Matrix D disposable heads in LT buffer (Qiagen) with 2-mercaptoethanol (Sigma), using a FastPrep FP120 homogenizer (Thermo Savant). The four lysates were pooled and total RNA was isolated with the RNeasy Midi kit (Qiagen) according to the manufacturer’s instructions. The microarray experiments were performed at the Functional Genomics Unit (FuGU), Biomedicum Helsinki by Two-Color Microarray-Based Gene Expression Analysis. Labeling and hybridization were done according to the manufacturer’s instructions. Combined experimental

MATERIALS AND METHODS

Ethics statement. The animal experimental protocols were approved by the Animal Experimentation Committee of The National Animal Experiment Board of Finland (ELLA). The work was undertaken under project license number EESL-2009-07019/Ym-23.

**Mice.** PAP−/− mice were generated by removing exon 3 (PAP3/V3A/S3A) of the prostatic acid phosphatase gene (Acpp), completely abolishing the expression of sPAP and TMPAP gene products (61). PAP−/− mice have been backcrossed to the C57BL/6j strain (Harlan Laboratories) for 16 generations. They were analyzed alongside age-matched C57BL/6j wild-type mice as controls. For tissue sampling, mice were killed by cervical dislocation and the SMG were immediately dissected unless otherwise specified.

**Determination of PAP activity in unstimulated total saliva.** Saliva samples (2.5 μl) from 2-mo-old male and female mice were assayed for phosphatase activity, using sodium p-nitrophenolphosphate substrate in citrate buffer pH 4.8 with and without 50 mM sodium tartrate, following the protocol described elsewhere (17). The activity was determined as the absorbance value at λ = 400 nm.

**TMP and AMP histochemistry of mouse SMG.** Wild-type and PAP−/− 2-mo-old mice (3 per group) were anesthetized with pentobarbital sodium (Mebunat Vet, 60 mg/ml, Orion Pharma) and intracardially perfused with phosphate buffer pH 7.4 (PB), followed by 4% paraformaldehyde (PFA) prepared in PB. SMG were fixed for 2 h in 4% PFA, then cryoprotected in 30% sucrose in PB. SMG were embedded in OCT (Tissue-Tek, Sakura). From these, 30-μm cryosections were prepared. Samples were assayed for PAP 5′-eucnucleotidase activity with thiamine monophosphate (TMP), AMP, ADP, and ATP substrates (Sigma-Alrich) according to Knystar-Csillika’s modified protocol (67). Briefly, cryosections were dried for 15 min at 37°C, washed twice with 0.04 M Trizma-Maleate buffer (TMB) pH 5.6 and once with 8% (wt/vol) sucrose in TMB. The slides were then incubated for 2 h at 37°C in a solution containing 0.25% (wt/vol) substrate (TMP, AMP, ADP, or ATP), 8% (wt/vol) sucrose and 0.08% (wt/vol) Pb(NO3)2 in TMB. Subsequently, slides were washed for 1 min in 2% acetic acid, followed by three washes with TMB and development for 10 s in 1% Na2S in TMB. Sections were then washed in H2O, dehydrated in an alcohol gradient and xylene, and mounted with Pertex (HistoLab Products).

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and reference samples were mixed with blocking agent, fragmentation and hybridization buffer, and hybridized to Agilent Mouse Genome Microarray 4×44K in dye-swap configuration, following the manufacturer’s instructions. Microarrays were washed and scanned with an Agilent Scanner, using protocols provided by the manufacturer. Feature Extraction software was used for image analysis. All control and saturated spots from the microarray data were excluded. The data were then subjected to linear normalization to allow comparison between arrays. Differentially expressed genes (DEGs) between PAP−/− and wild-type replicate samples were calculated using the median fold change (FC) value of the replicate pairs. DEGs with a Benjamini and Hochberg false discovery rate (FDR) corrected P value cutoff of 0.05 were selected for further analyses (13, 39). Ontological analyses were performed using GoMiner software (65), and ontological groups with “P value changed” < 0.05 were considered as significant. Genes were considered significantly downregulated or upregulated with an FC cutoff of 1.5. Network analyses were made with GeneMANIA software (62). The INTERFEROME database (48) was used to search for interferon-regulated genes in the DEGs list. The database was queried with parameters for in vivo experiments in *Mus musculus* with an FC cutoff of 1.5.

**Quantification of Cxcl9 and Irf7 in mouse SMG by qRT-PCR.** The cDNA was synthesized from 1,000 ng of total RNA derived from frozen SMG samples of 2-mo-old male mice of both genotypes (8 per group), using the DyNAmo cDNA Synthesis Kit (Finnzymes) as per the manufacturer’s instructions. Random hexamer primers were used in the RT-PCR, and cDNA was amplified using the DyNAmo Flash SYBR Green qPCR Kit (Finnzymes) according to the manufacturer’s procedure. All samples were run in duplicate, and the mean value of each duplicate was used for further calculations. A reference gene, *Hmbs*, was used to normalize for variation in sample quantity and quality. The ΔΔ- Ct method was used for relative quantification. Eight cDNA samples from the salivary gland of wild-type mice were pooled and used as a calibrator sample. Oligos used for qRT-PCR were as follows: mouse chemokine (c-x-c motif) ligand 9 NM_008599: 5′-GAG GCA TCC ACT ACA-3′ and 5′-TCT TCA CAT TTG CCG AGT-3′; mouse interferon regulatory factor 7 NM_016850: 5′-GAC TTC AGC ACT TTC TTC CGA G-3′ and 5′-TGG CTT GAC TAC CCA TGG-3′; mouse hydroxymethylbilane synthase NM_013551: 5′-ACT CTG CTT CGC TGG AT-3′ and 5′-AGT TGC CCA TCT TTC ATC ACT G-3′. The equipment used for cDNA synthesis was Perkin Elmer and for qPCR was Mx3005P instrument (Agilent Technologies).

**miRNA sequencing analyses of mouse SMG.** Total RNA was extracted from frozen 2-mo-old male mouse SMG samples (4 animals per genotype) stored in RNA later, using the mirNeasy kit (Qiagen) following the manufacturer’s protocol and stored at −70°C until further processing. Then, 1.0 µg of RNA was used for preparation of smallRNA libraries according to the user’s guide of the TruSeq Small RNA kit (Illumina). Library QC was evaluated with an Agilent Bioanalyzer (Agilent Technologies). Caliper LabChipXT (PerkinElmer) was used for enrichment of miRNA containing 147 bp ± 5% fraction from the smallRNA library amplification products. This fraction contains mature microRNAs generated from ~22 nt RNA fragments. The Illumina HiSeq platform was used for 50 bp PE sequencing of miRNA-enriched libraries. The miRseq data were analyzed by the Institute for Molecular Medicine Finland (FIMM) miRseq analysis pipeline with the main inputs being metric plots and expression data. The FIMM miRseq analysis pipeline is a modified version of the miRNA analysis and expression profiling pipeline, E-miR, originally developed by Buermans et al. (8). The Bowtie (28) mature tpm (transcripts per million) table was filtered for significantly (P < 0.05) dysregulated miRNAs carrying out a t-test comparison between PAP−/− and wild-type Bowtie tpm. Fold-changes were calculated from the ratio between PAP−/− and wild-type mean values.

Assessment of GCT and acinar cell proliferation and apoptosis, histopathological examination, and evaluation of lymphocyte infiltration in the SMG. SMG of male PAP−/− and wild-type mice aged 3.6, and 12 mo (4 mice per group) were fixed in formalin, and the specimens were embedded in paraffin. Sections (5 µm) were prepared to assess the degree of cell proliferation and apoptosis, deparaffinized, rehydrated, and stained. To detect proliferating cells, a rabbit polyclonal anti-Ki67 antibody (AB15580, Abcam) and the Vectastain Elite ABC Kit (Vector Laboratories) with hematoxylin counterstaining were used. To detect apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was conducted, using the FragEL DNA Fragmentation Detection Kit (Calbiochem) following the manufacturer’s instructions, and hematoxylin counterstaining. Ki67- and TUNEL-positive cells were quantified as follows: the sections were photographed with a Labovert FS microscope (magnification 40–400), 10 random fields of each section from noninflammatory areas. In each field, the total number of acinar and GCT cells as well as the number of Ki67- and TUNEL-positive acinar cells were counted. The acinar and GCT cells were discriminated on the basis of their morphological differences that were readily identifiable under the experimental conditions, without need for additional staining. The ratio of positive cells to the total number of cells per sample was compared between PAP−/− and wild-type groups for each marker, using two-sample t-test for equality of proportions with continuity correction for each age group, as implemented in the R statistical package version 2.10.1.

For the assessment of any histopathological changes and the degree of T and B cell infiltration, consecutive sections (3–5 µm) were prepared and routinely stained with hematoxylin and eosin and used for the immunohistological staining. Briefly, rabbit anti-human CD3 (Dako) and rat anti-CD45R (clone B220, Ly5; AbD Serotec) were used with the streptavidin peroxidase method, visualized with diaminobenzidine and Papanicolaou’s hematoxylin counterstain, following previously published protocols (10). The distribution and degree of interstitial lymphocyte infiltration was semiquantitatively assessed on the basis of the amount and distribution of T cells in the interstitium. The following score was applied: 0 (scattered individual T cells, no aggregates), 1 (scattered individual T cells, a few small aggregates), 2 (scattered individual T cells, several small aggregates), 3 (low number of individual T cells, several, partly larger aggregates), 4 (moderate number of disseminated T cells, and several, partly larger aggregates with slight atrophy of the surrounding glandular structures), 5 (numerous disseminated T cells, and several, relatively large aggregates with atrophy of the surrounding glandular structures), 6 (numerous disseminated T cells, and several large aggregates with atrophy of the surrounding glandular structures). The amount of B cells was not scored separately, since it followed the T cell distribution. U Mann-Whitney tests were performed to detect statistical differences in either score between the groups of mice, as implemented in R statistical package version 3.0.2.

**Accession codes.** Gene expression files containing microarray raw data can be accessed from ArrayExpress repository database (accession no. E-MTAB-2099).

**RESULTS**

PAP activity accounts for 50% of the total acid phosphatase activity in the saliva of wild-type male C57BL/6J mice. To evaluate whether there is detectable tartrate-sensitive acid phosphatase activity in mouse saliva, we performed activity tests and determined the amount of PAP activity in saliva measured as the tartrate-sensitive fraction of the total acid phosphatase activity. The total acid phosphatase activity against p-NPP in the saliva of male wild-type C57BL/6J mice was reduced ~50% in the presence of tartrate ion (Fig. 1A). We did not observe any tartrate-sensitive acid phosphatase
addition, body weight did not significantly differ between significant differences between the female genotypes either. In

The specificity of the TMP staining for the demonstration of performed lead (II) sulfur histochemical staining on SMG phatase activity in the SMG of wild-type and PAP ular salivary glands.

Histological examination of the SMG does not reveal structural differences between genotypes. Sexual dimorphism is a well-characterized feature of the mouse SMG (57). To determine whether the lack of PAP expression affects the structure of the SMG, we conducted histological analyses in both sexes to mine whether the lack of PAP expression affects the structure without significant structural differ-

Sexual dimorphism is a well-characterized feature of the mouse SMG (57). To determine the potential functional and morphological effects of a complete lack of PAP expression in the SMG. For an initial screening, we conducted microarray analyses of the SMG of 2-mo-old male PAP-/- and wild-type mice. Ontological analyses of significant DEGs revealed an upregulation in the expression of genes associated with cell proliferation (e.g., Mki67, AurkB, and Birc5) and immune response (e.g., Irf7, Cxcl9, Ccl3, Ccl4, Ccl8, Fpr2) mechanisms in PAP-/- mice (Fig. 4 and Table 1). These findings were subsequently confirmed by other means. For two selected genes, Cxcl9, a chemokine and T cell chemotactic (33), and Irf7, a player in the innate immune response (18), qRT-PCR revealed significantly higher transcription levels in PAP-/- mice, with fold changes of 4.9 (P = 0.001, n = 7) and 2.5 (P = 0.002, n = 7), respectively. Also, a query of the INTERFEROME database (48) with the list of DEGs in 2-mo-old PAP-/- mice showed that 52 of the 121 (over 40%) genes with altered transcription are interferon-regulated genes (IRGs) (data not shown).

In addition, we decided to explore the differential expression of miRNA in the SMG of PAP-/- and wild-type male mice.

activity in saliva samples of PAP-/- male mice or wild-type female mice (data not shown).

PAP-/- mice produce significantly more saliva than wild-type mice under stimulation with isoproterenol/pilocarpine secretagogues. To indirectly confirm the effect of PAP on salivation, we measured the total salivation volume in anesthe-

tized male and female mice under stimulation with secreta-
gogues. We found a significantly higher salivation volume (P = 0.03, n = 15) in male PAP-/- mice after normalization for the body weight (Fig. 1B). However, no difference was observed in total protein concentration or α-amylase activity (Fig. 1, C and D). In female PAP-/- mice we observed a small increase in the salivation volume after normalization, but the difference to the wild-type mice was not statistically significant. The protein concentration and α-amylase activity did not show significant differences between the female genotypes either. In addition, body weight did not significantly differ between wild-type and PAP-/- mice of each sex (data not shown).

Histological examination of the SMG does not reveal structural differences between genotypes. Sexual dimorphism is a well-characterized feature of the mouse SMG (57). To determine whether the lack of PAP expression affects the structure of the SMG, we conducted histological analyses in both sexes and genotypes. Figure 2 shows the presence of sexual dimorphism in both genotypes without significant structural differences in the distribution of acinar and GCT cells.

PAP exhibits 5’-ectonucleotidase activity against AMP, and TMP is a specific substrate for PAP in the mouse submandibular salivary glands. With the aim to evaluate the acid phosphatase activity in the SMG of wild-type and PAP-/- mice, we performed lead (II) sulfur histochemical staining on SMG cryosections, using TMP, AMP, ADP, and ATP as substrates. The specificity of the TMP staining for the demonstration of PAP activity was confirmed, since staining was seen in the SMG of wild-type mice (Fig. 3, A and C), but was completely absent in the PAP-/- mice samples (Fig. 3, B and D). Acid phosphatase activity against TMP was restricted to GCT cells (Fig. 3, A and C). In contrast, acid phosphatase activity against AMP was observed in both GCT and acinar cells of the wild-type mice (Fig. 3, E and G), but only in acinar cells of the PAP-/- mice (Fig. 3, F and H). Under experimental conditions, no activity was detected in wild-type nor PAP-/- SMG mice samples when either ADP or ATP was used as a sub-

Submandibular salivary glands of PAP-/- mice overexpress mir-146a and genes related to cell proliferation and immune response and show an increased proliferation rate and more intense inflammatory changes. We then decided to identify the potential functional and morphological effects of a complete lack of PAP expression in the SMG. For an initial screening, we conducted microarray analyses of the SMG of 2-mo-old male PAP-/- and wild-type mice. Ontological analyses of significant DEGs revealed an upregulation in the expression of genes associated with cell proliferation (e.g., Mki67, AurkB, and Birc5) and immune response (e.g., Irf7, Cxcl9, Ccl3, Ccl4, Ccl8, Fpr2) mechanisms in PAP-/- mice (Fig. 4 and Table 1). These findings were subsequently confirmed by other means. For two selected genes, Cxcl9, a chemokine and T cell chemotactic (33), and Irf7, a player in the innate immune response (18), qRT-PCR revealed significantly higher transcription levels in PAP-/- mice, with fold changes of 4.9 (P = 0.001, n = 7) and 2.5 (P = 0.002, n = 7), respectively. Also, a query of the INTERFEROME database (48) with the list of DEGs in 2-mo-old PAP-/- mice showed that 52 of the 121 (over 40%) genes with altered transcription are interferon-regulated genes (IRGs) (data not shown).

In addition, we decided to explore the differential expression of miRNA in the SMG of PAP-/- and wild-type male mice.

Fig. 1. The salivation volume is increased in the PAP-/- male mice stimulated with secretagogue drugs and the total acid phosphatase activity is reduced by 50% in the presence of tartrate ion in the saliva of male wild-type mice. A: total acid phosphatase activity in the absence and the presence of tartrate (specific inhibitor for PAP). WT, samples from wild-type mice; PAPKO, samples from PAP-/- mice; hPAP, human-purified PAP as positive control of the reaction. B: total salivation volume from stimulated mice. *P = 0.03, n = 15. C: total protein concentration in stimulated saliva. In all panels the error bars indicate SE values.

Fig. 2. The lack of PAP expression does not alter the morphology of the submandibular salivary gland (SMG). The sexual dimorphism present in SMG between male and female mice is evidenced by the clear presence of granular convoluted tubules (GCT) in male mice. Black arrows indicate acinar cells. White arrows indicate GCT cells that are clearly differentiated in male but not in female mice. The samples correspond to 12-mo-old mice.
The higher degree of GCT and acinar cell proliferation indicated by the microarray results was then confirmed in situ by a direct comparison of the amount of GCT and acinar cells that expressed the proliferation marker Ki67 in the SMG of male PAP<sup>−/−</sup> and wild-type mice at the age of 3, 6, and 12 mo. The degree of apoptosis in GCT and acinar cells was also determined on the basis of the number of TUNEL-positive cells. The comparative assessment revealed a significant increase in the degree of proliferation in the acinar cells in the SMG of the 6- and 12-mo-old PAP<sup>−/−</sup> mice (P < 0.001, n = 4) compared with the wild-type mice, whereas no difference was found in the degree of apoptotic cell death in both groups of mice at any age (Fig. 5, A and B). At the age of 6 mo, the GCT cells also showed a significantly higher degree of proliferation in the SMG of PAP<sup>−/−</sup> mice compared with wild-type mice (P < 0.001, n = 4). This was not observed anymore in the 12-mo-old mice (Fig. 5C); however, at this age, the proportion of acinar cells in relation to the total amount of acinar and GCT cells was significantly decreased (P < 0.001, n = 8) in PAP<sup>−/−</sup> mice, but remained constant in the wild-type mice (Fig. 6). In addition, no differences in the degree of apoptotic cell death were observed for GCT cells at any age (Fig. 5D).

DISCUSSION

In our previous work, we showed that PAP<sup>−/−</sup> mice developed prostate adenocarcinoma, and we proposed a model

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Fig. 3. The acid phosphatase activity against thiamine monophosphate (TMP) substrate was observed mainly in GCT cells of wild-type SMG (A and C) and is completely absent in PAP<sup>−/−</sup> samples (B and D). The acid phosphatase activity against AMP was observed mainly in GCT cells and to a lesser extent in acinar cells of wild-type SMG (E and G) and some activity remains in acinar cells of PAP<sup>−/−</sup> SMG (F and H). All of the pictures were taken with the same magnification.

For this purpose we performed miRNA sequencing using a next-generation sequencing platform. Our analysis indicated that miR-146a is the most significantly upregulated miRNA (FC = 1.84) in PAP<sup>−/−</sup> SMG.

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where TMPAP negatively regulates excocytosis by interacting with snapin, a SNARE-associated protein. This process in turn can be reinforced by both sPAP and TMPAP 5′-ectonucleotidase activity, via adenosine production and its cognate receptors (46). Having observed that TMPAP is present in exosomes (46) and that half of the total acid phosphatase activity in saliva of wild-type male mice is due to PAP, we decided to study the effects of the enzyme in mouse SMG as a model exocrine organ with high Acpp gene expression in male mice. The high PAP activity observed in the saliva of wild-type male mice, which is absent in female animals, suggests that PAP activity is required to support sex-specific requirements. High levels of PAP activity against the substrate TMP are detected in the GCT cells of SMG of wild-type male mice.

To interpret why PAP is present in the saliva of male mice only, we have to consider what is known about the regulation and physiological functions of PAP as well as the sexual dimorphism in the mouse SMG (Fig. 2). Considering the androgen dependence of the growth factor synthesis in the GCT (4, 34) and that the PAP expression is under androgen regulation in the rat prostate (42), we can expect a similar androgen dependence in mouse SMG on the basis of the lack of tartrate-sensitive acid phosphatase activity in wild-type female saliva. At present, we know that PAP 5′-mice display greater sensitivity in chronic inflammatory and neuropathic pain models mediated by adenosine (67). Moreover, recent studies indicate that intrathecal injected PAP effectively reduces pain in a dose-dependent manner in a rat model of cancer-induced bone pain. The analgesic effect of PAP was synergistically enhanced when a nucleoside transporter inhibitor was administered (11). Furthermore, the pain relief elicited by PAP injection in acupuncture points (20) supports the idea of a local analgesic effect in addition to the systemic effect observed by the intrathecal administration. Therefore, the 5′-ectonucleotidase activity of PAP could mediate pain relief via adenosine production, when male mice lick wounds inflicted on them in the wild through their fights for food and territory. This hypothesis is in line with the finding that P2X3 purinergic receptors, which are known to be nociceptive, are

### Table 1. Significant ontological terms obtained with GoMiner software using DEGs in SMG microarrays

<table>
<thead>
<tr>
<th>GO ID</th>
<th>P Value</th>
<th>Term</th>
<th>Genes</th>
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<td>Microtubule</td>
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<tr>
<td>5125</td>
<td>&lt; 10⁻⁴</td>
<td>Cytokine activity</td>
<td>Ebh1, Cx32, Lys6, Cxcl9, Lamd3, Psmb9, Lys1, Oas1a, Cbfb, Igx1, Tap7, Ccl2, Irf7, Oasl2, Mx1, Cbf3, Cxcr9, Ccl4, Cxcl10, Cxcl12, Cx32, Cx26, Cx36, Cx38, Cx43, Cx45, Cx46, Cx47, Cx48, Cx49.</td>
</tr>
<tr>
<td>2376</td>
<td>&lt; 10⁻⁴</td>
<td>Immune system process</td>
<td>Ulbp1, Lys6, Cxcl9, Lamd3, Psmb9, Lys1, Oas1a, Cbfb, Igx1, Tap7, Ccl2, Irf7, Oasl2, Mx1, Cbf3, Cxcr9, Ccl4, Cxcl10, Cxcl12, Cx32, Cx26, Cx36, Cx38, Cx43, Cx45, Cx46, Cx47, Cx48, Cx49.</td>
</tr>
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Superscript numbers in Genes column indicate fold-change value of differentially expressed genes (DEGs). GO, Gene Ontology; SMG, submandibular salivary gland.
present on sensory neurons in trigeminal, nodose, and dorsal root ganglia that innervate the skin (1). Hence, damage of cells in the skin via the release of cytosolic ATP leads to the excitation of nociceptive sensory nerves (27). However, extracellular ATP can be rapidly degraded by ecto-ATPases, ecto-ADPases, and 5'-ectonucleotidases to adenosine, which participates in physiological processes during wound healing (9). Moreover, Sowa et al. (49) showed that ecto-5'-nucleotidase (Nt5e) is coexpressed with PAP. This suggests that both enzymes can coordinately regulate nociception by metabolizing AMP to adenosine on free nerve endings in the epidermis and cells of the skin. The comparatively broad pH range (from 3 to 8) of PAP activity (58) versus the more narrow optimum pH for ecto-5'-nucleotidase [between 7 and 8 (66)] renders PAP a more effective 5'-ectonucleotidase under the acidic conditions of the “inflammatory soup” (25, 50). Therefore, we propose that only the presence of the PAP enzyme in saliva should be enough to exert its antinociceptive effect on the wounded skin of the mouse where AMP is already present. Our previous studies have shown the mechanistic effects of TMPAP in regulated secretion and exocytosis by interaction with snapin in prostate gland (46). Several studies revealed that the SNARE components are also present in the rat parotid and human submandibular glands, as part of the secretory machinery (22, 51, 53), in support of our hypothesis that PAP also regulates endo-/exocytosis in the mouse SMG. In addition, it has been reported that a PAP-like acid phosphatase activity is present in human endometrial glands during the secretory, but not the proliferative, phase (40). The increment in salivation volume of normalized total stimulated saliva observed in PAP+/- mice in comparison to wild-type mice suggests that TMPAP regulates the secretory process in GCT cells. Moreover, we did not observe differences in the total protein concentration or amylase activity between wild-type and PAP+/- mice. However, we cannot disregard potential differences in the particular composition of the saliva, considering that systemic diseases can induce alterations in the profile of components that are secreted into the saliva (15, 30).

In an effort to discover changes at the molecular level that result from the abolition of PAP in the male mouse SMG, we compared the gene expression profile of wild-type and PAP+/- mouse SMGs. Our analyses revealed that many genes related

**Fig. 5.** A significant increase in acinar cell proliferation was observed in SMG of 6- and 12-mo-old PAP+/- mice, but no changes in apoptosis were detected. **A:** bar plot showing the ratio (as percentage) between proliferative acinar cell counts and total amount of cells. **B:** bar plot showing the ratio (as percentage) between apoptotic acinar cell counts and total amount of cells. **C:** bar plot showing the ratio (as percentage) between proliferative GCT cell counts and total amount of cells. **D:** bar plot showing the ratio (as percentage) between apoptotic GCT cell counts and total amount of cells. **E:** table summarizing the total cell numbers per phenotype and age group in each experiment. In all bar plots the error bars indicate SE values. ***P < 0.001.

**Fig. 6.** The relative amount of acinar cells is significantly reduced and the relative amount of GCT cells is significantly increased in PAP+/- SMG at 12 mo of age. **A:** bar plot showing the ratio (as percentage) between the total count for acinar cells and the total amount of cells (acinar + GCT). **B:** bar plot showing the ratio (as percentage) between the total count for GCT cells and the total amount of cells (acinar + GCT). In both panels the error bars indicate SE values. ***P < 0.001. For total cell numbers, see Fig. 5E.
to inflammatory and immune response were upregulated in PAP\(^{-/-}\) mice. To further evaluate this finding, we undertook a histological evaluation of the SMG of both groups of mice at different ages. We generally observed a multifocal interstitial T and B cell infiltration of similar type and distribution in both groups of mice. Lymphocytic infiltrations in the salivary glands of mice are a common finding and increase in incidence and severity with age (16, 37). They have been considered as organ-specific autoimmune lesions (16), although if this were the case, a clear T cell dominance should be apparent (64), which was not observed in our study. At the end of the experiment, when mice were aged 12 mo, all animals exhibited a similar degree of lymphocyte infiltration. Interestingly, however, there was a quantitative difference at the earlier ages, when the infiltration was more intense in PAP\(^{-/-}\) mice. At the age of 3 mo, the difference was even significant. Data from our microarray and qRT-PCR experiments that were undertaken in animals aged 2 mo indicate that the observed infiltration is a consequence of increased lymphocyte recruitment into the salivary gland. This is in line with the observed upregulation of inflammatory chemokines (e.g., Ccl3, Ccl4, Ccl8, Cxcl9, and Cxcl10). Interestingly, we did not observe any further changes apart from focal replacement of acini by extensive focal lymphocyte infiltrates, despite the partly significant increase in acinar cell proliferation in the PAP\(^{-/-}\) mice. Since this was not associated with increased apoptotic death in this cell population, there is no evidence of an increased acinar cell turnover as a direct or indirect consequence of the lack of PAP.

Other authors showed that the formyl peptide receptor 2 (Fpr2), which we found significantly upregulated in our microarrays (Table 1), is expressed in acinar cells of mouse SMG (38). This receptor promotes intracellular calcium responses and...
found that over 40% of DEGs correspond to interferon-regulated genes (IRGs) (data not shown). Therefore, considering both the upregulation of genes related to the immunological response and the principally identical inflammatory response in the SMG of PAP−/− and wild-type mice, we concluded that the immunological function in PAP−/− mice remains responsive and hence we suggest that the immune system is responsible for keeping tissue homeostasis.

In summary, we propose that PAP is the main acid phosphatase in the SMG of male C57BL/6J mice and in saliva and elicits antinociceptive effects by wound lancing, via the generation of adenosine by its 5′-ectonucleotidase activity. PAP is only expressed in GCT cells in the SMG of male mice, and its lack of expression leads to dysregulation of the endo/exocytotic mechanisms, with a concomitant acinar cell proliferation effect. This proliferation is kept under control by the innate immune system by overexpression of Irf7, which induces the activation of immune effector cells and the upregulation of Fpr2, thereby contributing to the resolution of inflammation and the prevention of tissue damage.

ACKNOWLEDGMENTS

We acknowledge CSC (The Finnish IT Center for Science) for the allocation of computational resources.

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GRANTS

This work was supported by grants from The Sigrid Jusélius Foundation, the Academy of Finland (grant no. 132201) and Biocenter Finland (FinnMouse).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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