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Urinary lipocalins in *Mastomys coucha*

Kristina DANISZOVÁ[1], Kateřina JANOTOVÁ[1], Petr L. JEDELSKÝ[2] and Pavel STOPKA[1]*

[1] *Department of Zoology, Faculty of Science, Charles University in Prague, Viničná 7, Prague, 128 44, The Czech Republic; e-mail: pstopka@natur.cuni.cz*

[2] *Department of Cell Biology, Faculty of Science, Charles University in Prague, Viničná 7, Prague, 128 44, The Czech Republic*

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A b s t r a c t . The presence of large amounts of proteins in mammal urine is usually associated with a pathological condition and indicates serious renal lesions. However, there are few species with obligate proteinuria indicating that they must derive some benefit from this condition. Urinary proteins have been most extensively studied in the house mouse and the rat, and findings to date indicate that their function in intraspecific communication is complex and not yet fully understood. Other proteins of the same protein family as MUPs have been also found in urine of some other rodent species, and still less is known about these. In this study we demonstrate the existence of urinary lipocalins in *Mastomys coucha* for the first time. Our results support the hypothesis that urinary proteins may play an important role in chemical communication in species other than mice and rats. Information about the presence, concentration, and level of polymorphism of these proteins in different rodents may help us to understand their specific function.

Key words: Major Urinary Protein, chemical communication

Introduction

Mouse major urinary proteins (MUPs) were discovered in 1932 by Parfentjev who noticed a high normal proteinuria in laboratory mice. Urinary MUPs originate in the liver, from which they are released to the plasma and promptly excreted in the urine (Finlayson et al. 1965). The concentration of these proteins has been shown to reach several milligrams per millilitre of urine in male mice, although females often excrete MUPs in substantially lower concentrations. Thus MUP expression is sexually dimorphic as a consequence of the regulation by androgens especially testosterone (W i c k s 1941, T h u n g 1956). MUP expression has also been detected in several glandular tissues (S h a w 1983, S h a h a n et al. 1984, S h a h a n et al. 1987 a,b, U t s u m i 1999), although we focus here mainly on those excreted in the urine.

Major urinary proteins belong to the lipocalin protein superfamily. Members of this class are characterised by an eight-stranded β-barrel which incorporates a central hydrophobic cavity (B o c s k e i et al. 1992, Flower 1996, Z i d e k et al. 1999). This cavity allows binding and transport of different ligands, which is the general function of the lipocalins including MUPs (F l o w e r 1996). Ligands associated with urinary MUPs are hydrophobic endogenous semiochemicals, a number of which is known to have pheromonal effects (J e m i o l o et al. 1985, 1986, B a c c h i n i et al. 1992, R o b e r t s o n et al. 1993, N o v o t n y et al. 1999 a,b).

For a long time, MUPs were considered a metabolic curiosity (H o f f m a n 1970) and it took several decades from their discovery to proposing their possible function.

* Corresponding author

Vandenbergh et al. (1975) suggested for the first time that these proteins may play an important role in olfactory communication: namely, that they act either as pheromones influencing females' reproductive physiology or as carriers of the pheromones with this function (Robertson et al. 2001). Today, there is evidence supporting both these suggested functions. Firstly, the function of MUPs as carriers, which slow down release of pheromones and therefore increase the longevity of the signal, was confirmed (Hurst et al. 1998, Robertson et al. 2001). More recently, the hypothesis of direct pheromonal activity of MUPs has also been corroborated (More 2006, Chamero et al. 2007)

A lot of the information relating to MUPs was derived from studies of genetically homogenous mouse inbred strains, which demonstrate nearly no MUP heterogeneity within the strain (Clissold & Bishop 1982, Hainey & Bishop 1982, Robertson et al. 1996). However, it has been showed that different inbred lines exhibit distinct quantitative and qualitative patterns of MUPs, leading to the suggestion that in wild mice MUP heterogeneity should be even greater (Clissold & Bishop 1982, Hainey & Bishop 1982, Pes et al. 1999, Payne et al. 2001, Cheetham et al. 2009). Indeed, research on mice from free-living populations revealed considerable variation of MUP patterns between individuals mediated by expression of a specific subset of isoforms from the population pool (Robertson et al. 1997, Pes et al. 1999, Payne et al. 2001, Veggerby et al. 2001, Beynon et al. 2002).

The well-established role of MUPs consisting in providing protection and slow consecutive release of otherwise short-lived signals nonetheless does not sufficiently explain importance of their diversity (Hurst et al. 1998, Robertson et al. 2001). Thus, it was proposed that MUP variants transmit information about individual identity (Hurst et al. 2001). The fact that recognition among individuals depends on a difference in MUP type has been recently confirmed by the work of Cheetham and her collaborators (2007). It has been also demonstrated that the transmission of the identity signal requires direct contact with the scent mark, indicating that the information is contained in MUPs and not in the profile of their volatile ligands (Humphries et al. 1999, Nevison et al. 2003, Martinez-Ricos et al. 2007)

There is a growing body of evidence that pheromone carrier proteins are an important part of the chemosignalling system in many species (Bacchini et al. 1992, Marchese et al. 1998, Sandler et al. 2000). MUP-like lipocalins have been identified in the urine of other rodents (Robertson et al. 2007, Beynon et al. 2008), and *Mup* gene orthologues have been found in the genomes of several mammals from other families (Chamero et al. 2007, Logan et al. 2008).

The analysis of urinary lipocalins in rodents revealed substantial variation in their concentration as well as the level of polymorphism across species, however no tested species so far has revealed either the high concentration nor the level of polymorphism found in the house mouse. This indicates that the function of lipocalin pheromone carriers differs among rodent species reflecting their specific ecological requirements (Robertson et al. 2007, Beynon et al. 2008) or they could play the same role but with different importance. The level of diversity could, for example, depend on the number of individuals likely to be encountered according to social system (Dale et al. 2001, Thom & Hurst, 2004).

Despite of these findings the information we have about pheromone-carrying proteins in species other than *Mus musculus* remains very limited. In order to understand the details of the mechanisms of chemical communication in animals, it is vital to gather information about these proteins in a range of species and ascertain their precise roles in the different signalling systems.

In this study we investigate protein content in the urine of the rodent species *Mastomys coucha* (Smith 1936), which belongs to the so-called multimammate African rats. *Mastomys* spp. belongs to the diverse but entirely African tribe Praomyini, formerly referred to as Praomys group (Lecompte et al. 2002 a,b, 2005), which was identified as the sister lineage to the *Mus* (tribe Murini) (Steppan et al. 2005, Rowe et al. 2008, Lecompte et al. 2008). Our findings are compared to the results obtained by the corresponding analysis carried out on the house mouse and discussed with respect to ecology of the studied species.

Material and Methods

African multimammate rat, *Mastomys coucha* and inbred mice C57Bl/6 were used as the subjects in our experiment. Mice were purchased from AnLab s.r.o., the Czech Republic. Individuals of *Mastomys coucha* were obtained from private breed. Only few founders established the breed, thus tested animals were closely related. The animals were housed individually in plastic cages with food and water freely available and under the light schedule 12:12 h light cycle with lights off at 19.00 h in breeding facility at the Department of Zoology, Faculty of Science, Charles University, Prague, the Czech Republic. Nine adult males and nine adult females of each species were tested. Urine was collected in the first half of the light period. The animals were shifted into the clean plastic cage being gently held by the scruff of the neck and back. Usually, they urinated spontaneously within one or two minutes. Urine samples were then collected by pipetting into Eppendorf tubes, samples were frozen immediately and stored at -20°C until analysis.

The Bradford method (Bradford 1976) was used to estimate the concentration of the samples and the proper load needed for subsequent analysis. The samples were thawed, stirred and briefly centrifuged. Aliquots containing appropriate amount of proteins (max. 7 μl) were added to the sample buffer NuPAGE® LDS Sample Buffer (Novex, Invitrogen). No reducing agent was used. Then, the prepared samples were heated at 70°C for 10 minutes. Separation was performed by means of denaturing polyacrylamide gel electrophoresis (SDS-PAGE) using 10% NuPAGE® Novex Bis-Tris Midi Gels and NuPAGE® MES Running Buffer (Invitrogen Technologies, Paisley, UK).

Different concentrations of carbonic anhydrase (29 kDa), marker for SDS-PAGE (Sigma-Aldrich, St. Louis, MO, USA), were loaded onto the gels together with the samples to allow absolute quantification of separated proteins. The gels were stained with Coomassie® G-250 (SimplyBlue™ Safe Stain, Invitrogen Life Technologies, Paisley, UK) and their images were acquired using GS-800 calibrated densitometer. Protein bands of our interest (molecular weight about 19 kD) were quantified by Quantity One 1-D Analysis Software (BioRad Laboratories, Hercules, CA, USA), where the band volume is defined as the total intensity of all pixels in the volume boundary. Protein concentrations were then calculated from the calibration curve of carbonic anhydrase.

The proteins with molecular weight of nineteen kDa were analysed by mass spectrometry. Bands were cut from SDS-PAGE gel and covered with 100 μl 50 mM ammonium bicarbonate (ABC) buffer in 50% acetonitrile (ACN) containing 50 mM dithiotheritol (DTT) in the first step and with 50 mM iodoacetamide in the second step. After each step the samples were subjected to sonication in an ultrasonic cleaning bath for five minutes and then incubated for 15 minutes. The first step was then repeated to remove any excess iodoacetamide. Supernatant was discarded and the samples were sonicated five minutes in

100 μl of HPLC water and subsequently in 100 μl of ACN. Finally, ACN was discarded and the samples were let open for a couple of minutes to evaporate the rest of ACN. The samples were incubated at 37 °C overnight in 5 ng of trypsin (Promega) in 10 μl of 50 mM ABC and 10 μl of water containing 97% ^{18}O. Afterward, trifluoracetic acid (TFA) and ACN were added to reach final concentration 1% TFA, 30% ACN. The samples were sonicated for ten minutes and 0.5 μl was transferred onto MALDI target and allowed to dry. Dried droplets were covered with 0.5 μl drop of *alpha*-cyano-hydroxycinnamic acid solution (2 mg/ml in 80% ACN) and let dry.

Samples for *de novo* analysis were separated on Ultimate 3000 HPLC system (Dionex) coupled to Probot microfraction collector (Dionex). Tryptic peptides were loaded onto a PepMap 100 C18 RP column (3 μm particle size, 15 cm long, 75 μm internal diameter; Dionex) and separated with a gradient of 5% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid to 80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over a period of 45 minutes. Eluate was mixed 1:3 with matrix solution (20 mg/mL *alpha*-cyano-4-hydroxycinnamic acid in 80% ACN) prior to spotting onto a MALDI target.

Spectra were acquired on a 4800 Plus MALDI TOF/TOF analyser (Applied Biosystems/ MDS Sciex) equipped with a Nd:YAG laser (355 nm, firing rate 200 Hz). All spots were first measured in MS mode and then up to 10 of the strongest precursors were selected for MS/MS which was performed with 1 kV collision energy and operating pressure of the collision cell set to 10^{-6} Torr. Resulting MS/MS spectra were interpreted manually using double peaks formed by $^{16}O/^{18}O$ to distinguish y-ion series. The series of b-ions was matched to corresponding y-ion series by subtraction of y-ion mass from parent ion mass. Partial sequences were matched to known sequences from GenBank using BLAST (Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Separation of urine samples of *Mastomys coucha* on gel electrophoresis revealed a MUP-sized band (molecular mass about 19 kDa) which was detectable in both sexes. Subsequent measurement of the peptide fingerprint did not reveal any significant hits, thus *de novo* sequencing had to be performed. Partial sequence tags were manually deduced from spectra obtained with MALDI/TOF-TOF mass analyser. Fig. 1 shows an example of the spectrum and sequence deduced. BLAST found a clear homology with lipocalin family, when comparing partial sequences with other known sequences. The best similarity was found with alpha 2u-globulins, the well-known urinary lipocalins discovered in rat. In Fig. 2 the partial sequence derived from spectra is shown alongside those of rat and mouse urinary proteins.

Mean concentration of detected lipocalin in *Mastomys coucha* was 1,7 mg/ml in males and 0,5 mg/ml in females. The concentration of MUPs in C57/Bl house mice was approximately five times higher, with males reaching on average 8,6 mg/ml and females having 2,6 mg/ml. Difference in urinary lipocalins between these two species and sexes was tested using MANOVA; both species and sex significantly influenced MUP concentration (species: $F=138,03$; d.f.=1; $p< 0,000001$and sex: $F=74,66$; d.f.=1; $p< 0,000001$).

We also assessed the level of sexual dimorphism in MUP concentration: males of both tested species expressed approximately three times more protein than females, thus there was no significant difference in the sexual dimorphism between *Mastomys coucha* and inbred house mouse C57Bl/6 (MANOVA sex * species interaction: $F=0,04$, d.f.=1, $p= 0,846$).

```
                    *         20          *         40          *
Mastomys :  ----------------GDWFSIVLASDKREKIEENGSMR---------------- :  23
Rattus   :  EEASSTRGNLDVDKLNGDWFSIVVASDKREKIEENGSMRVFMQHIDVLENSLGF :  54
Mus      :  EEASSTGRNFNVEKINGEWHTIILASDKREKIEDNGNFRLFLEQIHVLENSLVL :  54
                           G W 3I66ASDKREKIE NG  R

                60          *         80          *         100
Mastomys :  ----------------------------EYLGYNTFTVL-------VLFHLINE :  42
Rattus   :  KFRIKENGECRELYLVAYKTPEDGEYFVEYDGGNTFTILKTDYDRYVMFHLINF : 108
Mus      :  KFHTVRDEECSELSMVADKTEKAGEYSVTYDGFNTFTIPKTDYDNFLMAHLINE : 108
                                        Y G NTFT6        66 HLIN

              *       120         *       140         *       160
Mastomys :  KNEETFQLMVLYGREPDLSSDIKEKFADLCEEHGLVRENVLDLTK--------- :  87
Rattus   :  KNGETFQAMVLYGRTKDLSSDIKEKFAKLCEAHGITRDNIIDLTKTDHCLQARG : 162
Mus      :  KDGETFQLMGLYGREPDLSSDIKERFAQLCEKHGILRENIIDLSNANRCLQARE : 162
              K1 ETFQ M LYGR  DLSSDIKE4FA LCE HG6 R N66DL3
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Fig. 1. Example of a peptide sequenced by tandem mass spectrometry. Fragment spectrum of a peptide (Mr=1192.6) was recorded. C-terminal fragments labeled by 50% H_2O^{18} are assigned to sequence EK(L/I)EENGSMR. Note that leucine and isoleucine cannot be distinguished by this method.

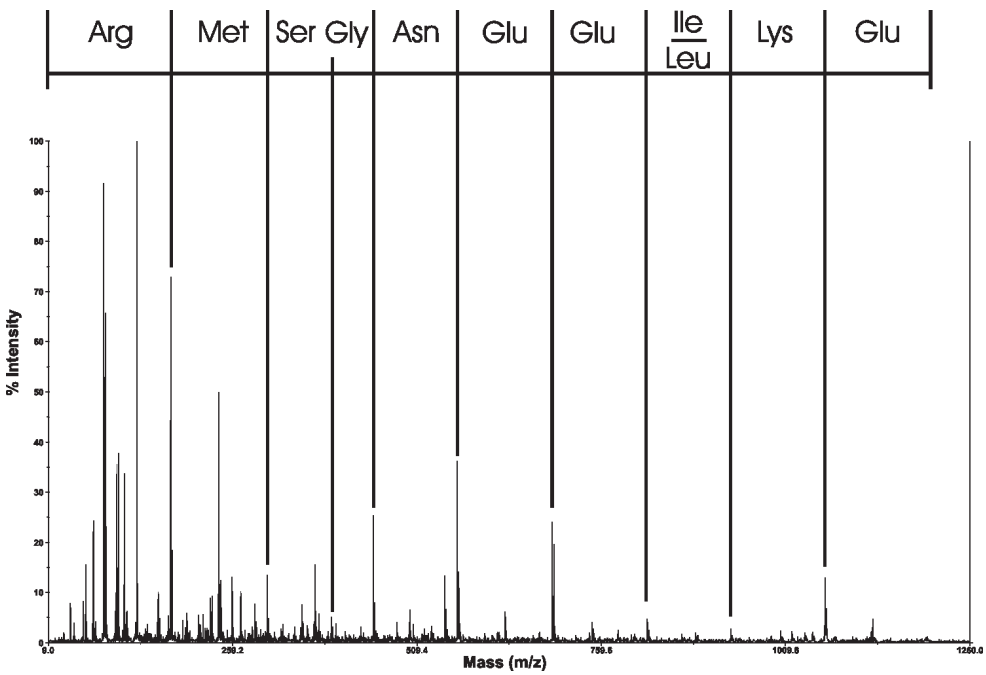

Fig. 2. Alignment of partial sequences obtained by *de novo* sequencing of *Mastomys coucha* urinary protein and known alpha$_{2u}$-globulin from *Rattus norvegicus* (gi|202610|gb|AAA40642.1|) and Major urinary protein 1 from *Mus musculus* (gi|15126575|gb|AAH12221.1|). Note that leucine and isoleucine cannot be distinguished by the method used.

Discussion

Results of recent surveys indicate that house mouse and rat are not exclusive possessors of MUP-like proteins. Similar lipocalins have also been found in the urine of other rodent species including *Mus macedonicus, Mus spretus, Phodopus roborovskii* and *Clethrionomys*

glareolus (Robertson et al. 2007, Beynon et al. 2008). On the basis of our study, the African multimammate rat *Mastomys coucha* can be included in the group of the rodent species in which urinary proteins occur. Our work has not yet assessed directly whether urinary proteins in this species bind pheromonally active volatile molecules, however it is seems plausible that they do so given the found sequence similarity with mouse and rat lipocalins that are known to perform this function (Flower 1996).

In general, sense of smell is essential for rodents which are primarily active at night, and which rely on urine scent marks as the main source of information about their conspecifics. Since *Mastomys coucha* is a nocturnal animal and occurs in very high densities (some populations have even become commensal with man (Skinner & Smithers 1990)), we assume that olfaction plays an important role in intraspecific communication in this rat. Thus although the concentration of urinary lipocalins in *Mastomys coucha* urine does not reach the values characteristic for mice, a role in chemical signalling is conceivable. The lack of data about concentration of urinary proteins in rodent species other than the house mouse makes interspecific comparison impossible; however such comparisons would help us to understand the role that MUP-like proteins play in communication. Moreover, even information concerning MUP concentration in wild mouse is not complete and the figures differ in the reports of the authors (Beynon & Hurst 2004, Stopkova et al. 2007).

Sampsell & Held (1985) aimed their attention at the differences in MUP mRNA expression. They found that levels of variation of liver MUP mRNA varies considerably among various species and subspecies of the genus *Mus*. They also observed sexual dimorphism in MUP expression, which differs substantially between species with male to female ratios ranging from one to several hundred. Specific levels of sexual dimorphism were also investigated by Stopkova et al. (2007), who analysed MUP liver expression and urinary concentration in two subspecies of house mouse *Mus m. domesticus* and *Mus m. musculus*. Differences in the level of sexual dimorphism in these two subspecies suggest that they could use MUPs for distinct purposes. This is very interesting in connection with observation that *M. m. musculus* females are choosier than *M. m. domesticus* because they tend to mate with a male of the same subspecies while *M. m. domesticus* does not display any preference (Smadja & Ganem 2002).

Our study revealed that both *Mastomys coucha* and inbred mice C57Bl/6 are sexually dimorphic in the concentration of urinary proteins they express. The level of this dimorphism is comparable between tested species, with males having three times more protein in urine than females. This indicates that males of both species invest relatively more energy into the production of these proteins, which suggests that they could be used to communicate male quality to females or rivals (Malone et al. 2005).

One of the possible explanations for variation in concentration, polymorphism and sexual dimorphism of these proteins observed among several more or less related rodents is the different ecology of the species involved. It has been suggested that MUP-like proteins could be expected to occur especially in rodents that live in dense, spatially overlapping social groups in close proximity to human settlements (Beynon et al. 2008). As mentioned above, *Mastomys* and house mice resemble each other considerably in some aspects of their biology. The several times lower concentration of urinary lipocalin in *Mastomys coucha* could be revealing in this case. However, there is as yet no information available about the minimum concentration of urinary protein required to ensure sufficient transfer of volatile molecules or to fulfil direct signalling role itself, so we cannot determine whether the quantity of protein in *Mastomys* urine is adequate for this purpose.

Until further evidence is collected, we assume that the MUP-like urinary protein we have discovered in *Mastomys coucha* is likely to act as a transporter in chemical communication in a similar manner to that observed in mice. Further investigation is necessary to improve our understanding of the function of these proteins. It is certainly surprising that even in the rat *Rattus norvegicus* whose biology is very similar to that of the house mouse and in whose genome 9 functional *Mup* orthologues were found, the protein pattern is very markedly consistent between individuals (B e y n o n et al. 2008, L o g a n et al. 2008). It is worth repeating that the number and the sample size of analysed rodent taxa are relatively low. Nevertheless, in the light of existing findings the concentration and diversity of urinary proteins in house mice remains unique.

A c k n o w l e d g e m e n t s

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