The major urinary protein system in the rat

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Abstract

The genomes of rats and mice both contain a cluster of multiple genes that encode small (18–20 kDa) eight-stranded β-barrel lipocalins that are expressed in multiple secretory tissues, some of which enter urine via hepatic biosynthesis. These proteins have been given different names, but are mostly generically referred to as MUPs (major urinary proteins). The mouse MUP cluster is increasingly well understood, and, in particular, a number of roles for MUPs in chemical communication between conspecifics have been established. By contrast, the literature on the rat orthologues is much less well developed and is fragmented. In the present review, we summarize current knowledge on the MUPs from the Norway (or brown) rat, *Rattus norvegicus*.

Introduction

Rodents, especially rats and mice of the family Muridae, have long been known to excrete physiologically large amounts of protein in urine that consists predominantly of a mixture of small (18–20 kDa) proteins known as the MUPs (major urinary proteins) (previously known as α_{2u}-globulins in rats) [1,2]. Despite some obvious similarities between the urinary proteins from laboratory rats (*Rattus norvegicus*) and mice (*Mus musculus domesticus*), studies on the characterization and regulation of these proteins has continued largely independently. In both species, homologous proteins are synthesized in the liver [3–5] as precursors [5,6], and, after excision of the signal peptide and formation of disulfide bonds, the small proteins are secreted into the bloodstream to be finally excreted into urine [1,7]. MUPs are also expressed in other tissues, including salivary glands, mammary glands, nasal tissue and respiratory epithelia [8–13]. Despite the specificity of the source implicit in the name MUP, this term is well established and its use should continue. However, naming practice for the rat proteins has been informal, and the proteins have also variously been referenced by the terms 'α2u globulin', '$\alpha 2\mu$ globulin' and '$\alpha_{2\mu}$ globulin'; in particular, the term '$\alpha_{2\mu}$ globulin' seems to have arisen from a misunderstanding [14,15]. As urinary MUPs are potent allergens, an additional naming convention is used, such that the allergenic house mouse MUPs are referred to as Mus m1 (or Ag1, or MA1) and Rat n1 respectively [16]. In the present review, we refer to 'mouse MUPs' and 'rat MUPs' as terms that encompass these similar groups of eight-stranded β-barrel lipocalin proteins [15].

Key words: α_{2u}-globulin, major urinary protein (MUP), rodent, scent marking, semiochemistry, urine.

Abbreviations: MUP, major urinary protein; RGSC, Rat Genome Sequence Consortium.

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The *Mup* gene cluster

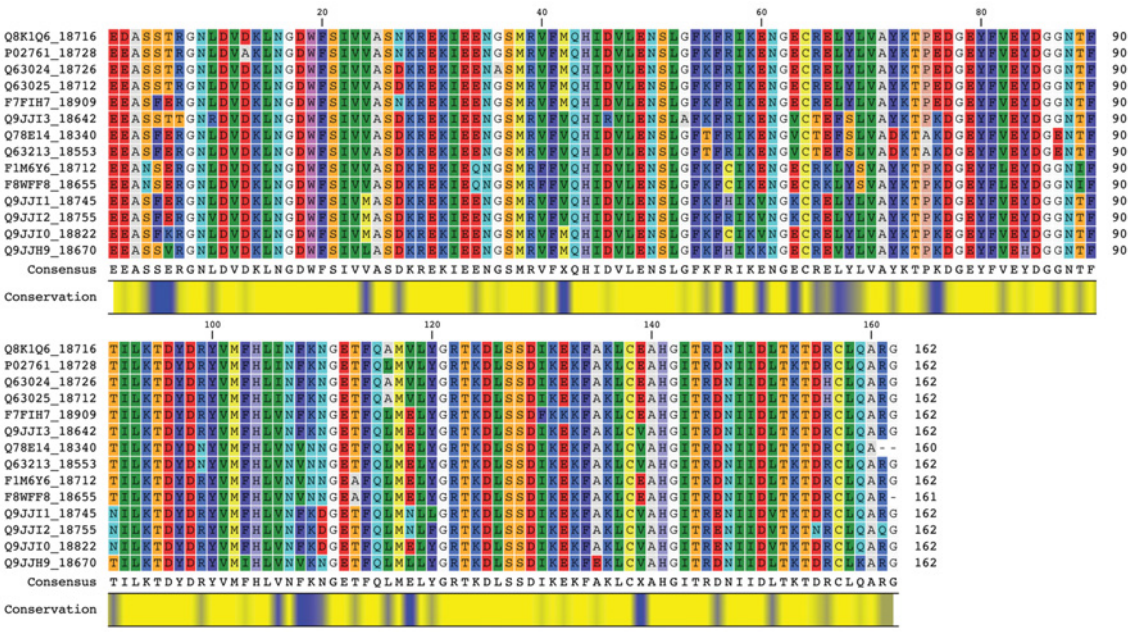
Rat and mouse MUPs are both encoded by multigene families clustered in the *Mup* locus on chromosome 4 in the mouse and chromosome 5 in the rat. On the basis of the current release of the C57BL/6 genome sequence (GRCm38), there are 21 protein-coding genes [17,18]. These mouse *Mup* genes can be grouped, on the basis of sequence relatedness, into a central region, where genes are of recent evolution and display high similarity (97 %), and two peripheral regions of older and significantly more divergent genes (82–94 % similarity). This classification is sustained at the level of the protein products of these genes [19].

Acquisition and annotation of the rat genome sequence is less developed than that of the mouse. The reference genome for *Rattus norvegicus* (brown Norway strain BN/SsNHsd) is provided by the RGSC (Rat Genome Sequence Consortium). The rat genome sequencing is not yet finished; there are gaps in the genome sequence and the sequencing of the Y chromosome is still in progress. The latest version of the reference genome assembly is the RGSC version 5.0 released in March 2012. Annotation of the genome can be accessed through different genome browsers: Rat Genome Database (http://rgd.mcw.edu), Ensembl (http://www.ensembl.org/Rattus_norvegicus/Info/Index), NCBI (http://www.ncbi.nlm.nih.gov/genome/73) and UCSC (http://genome-euro.ucsc.edu/).

Manual annotation performed by Logan and colleagues on the previous release (Rnor_3.4) shows that there are a minimum of nine genes and 13 pseudogenes [17], in good agreement with Southern blot [20] and fluorescence *in situ* hybridization [21] analysis. The rat genes do not seem to be grouped into different regions, and all genes have sequence similarity of approximately 90 % (Figure 1). However, an unsequenced gap in this region of rat chromosome 5 also compromises characterization of the *Mup* locus in the rat [17]. In the most recent genome release, some *Mup* genes have been removed despite direct evidence for the protein product

Figure 1 | **Primary sequence alignment of rat MUPs**

Near full-length rat MUP sequences were aligned using CLC Sequence Viewer, version 7.02 (http://www.clcbio.com). Regions of maximal variation are highlighted in blue in the conservation trace. A second entry (Q9JJI4) has the same mature sequence as Q8K1Q6, and has been omitted from the alignment.



(Table 1) and new gene candidates have been identified, reflecting the difficulty in assembly and annotation of these repetitive multigene DNA tracts. As an example, the gene encoding the most frequently observed and abundant MUP isoform in rat urine (mature mass 18728 Da; UniProt ID P02761) was previously annotated in version 3.4, but has been removed in version 5.0. Clarity of gene–protein relationships must await complete genome data.

Mature protein products of the rat *Mup* gene cluster

Although there is growing information about the genome structure and rat *Mup* structural genes, information on expression of specific proteins is less well advanced. The considerable sequence similarity between the individual proteins (Figures 1 and 2) impairs precise discrimination and quantification of the gene products, and will require in-depth analysis of MUPs through MS or MS/MS; it is unlikely, for example, that sets of antibodies will be generated to discriminate between each isoform. Provided the MUP mixture is relatively simple, ESI–MS can be used to profile the mixture [22,23]. With a mass accuracy of 1 Da in 10000 Da, this is usually adequate to relate a mature protein product to a known gene. The rat MUP nascent polypeptide chain undergoes minimal post-translational processing, and is usually restricted to excision of the signal peptide and formation of a disulfide bond (Cys^{64}–Cys^{156}). In the absence of variable or more complex modification, it is feasible to acquire the average mass of the MUPs directly from urine samples and these can often be directly reconciled with a putative encoding gene. The urine from inbred laboratory strains of rat contains multiple MUPs of different masses. Although the sequence similarity of rat MUPs is very high (Figure 1), small numbers of amino acid substitutions often lead to readily discriminable masses (Table 1).

Because of multiple generations of inbreeding of laboratory rats in pursuit of genetic homogeneity, the MUP pattern is very similar in each individual of the same sex. Experience with the mouse MUPs has revealed an extraordinary degree of phenotypic variation in urinary MUPs in wild caught populations, which typically have more complex MUP patterns than the simple homozygous pattern expressed in C57BL/6 mice. Multiple new mature protein masses and sequences, of which only some are represented in the C57BL/6 genome, can be readily observed in wild-caught individuals [24–26]. Early investigations suggest that the genotypic and phenotypic plasticity in rat urinary MUPs is more muted (S.D. Armstrong, S.A. Roberts, A. MacNicoll, J.L. Hurst and R.J. Beynon, unpublished work), but this awaits confirmation with larger sample sets.

Structural studies

Rat MUPs, in common with their mouse counterparts, belong to the lipocalin family [15]. Tertiary structure is highly conserved among lipocalins, and is characterized by eight antiparallel β-sheets organized in a β-barrel that defines a hydrophobic central cavity that forms the binding site (Figure 3). This organization confers the capacity to carry

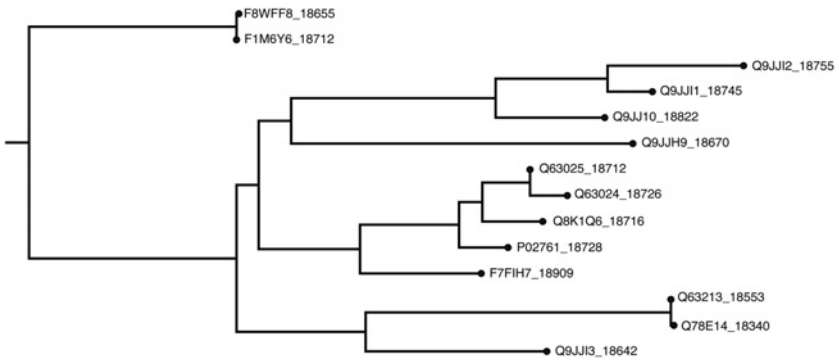
Table 1 | **Current knowledge of rat MUP genes, transcript expression and protein products**

This Table is a compilation of data from two releases of the rat genome sequence (Baylor 3.4/rn4, Nov 2004 and RGSC Rnor 5.0, March 2012) using the annotations compiled in the Rat Genome Database (http://rgd.mcw.edu/) and UniProt (http://www.uniprot.org/). Where possible, data relating to predicted mature protein product is cross-correlated with experimental data that confirm true protein products. In addition, proteins of other mature masses are not represented in the genome sequences to date, whether because of missing genes in the assembly or small sequencing errors [58].

UniProt accession number	Predicted mature mass (Da)	UniProt name	RGD genome annotation	RGD identifier	RGD gene symbol	mRNA tissue expression
Q78E14	18340	Obp3; rat salivary gland α_{2u}-globulin, type 1	Version 3.4 and version 5.0	708508	*Obp3*	Liver (BC086942); salivary gland (X14552); submaxillary gland (J00738)
Q9JJI3	18642	α_{2u}-Globulin; protein LOC259244; RCG31988; PGCL3	Version 3.4 and version 5.0	708501	*LOC259244*	Preputial gland (AB039824)
Q9JJH9	18670	α_{2u}-Globulin; Mup4; RCG31930; α_{2u}-globulin PGCL8	Version 3.4 and two loci in version 5.0	735211/6503461	*Mup4/LOC100909412*	Preputial gland (AB039829)
Q9JJI1	18745	α_{2u}-Globulin; PGCL6	Version 3.4 and version 5.0	735107	*Mup5*	Preputial gland (AB039827)
Q8K1Q6	18716	α_{2u}-Globulin; protein LOC298109; RCG32004	Version 3.4	1593271	*LOC298116*	Liver (BC086943)
Q9JJI4	18716	α_{2u}-Globulin PGCL2	Version 3.4	1593273	*LOC298109*	Preputial gland (AB039823)
P02761	18728	MUP; PGCL1; allergen rat n1; α_{2u}-globulin PGCL1	Version 3.4	708506	*LOC259246*	Liver (M26835); liver (M26837); preputial gland (AB039822); liver (BC088109); liver (BC098654); spleen (BC105816); U31287; liver (V01220); liver (J00737)
F7FIH7	18909	Protein LOC100909412	Version 3.4 and two loci in version 5.0	1587665/1583727	*LOC688457/LOC500473*	
F1M6Y6	18712	Protein LOC259245 (transcript 1)	One transcript in version 3.4 and two transcripts in version 5.0	1593272	*LOC298111*	
F8WFF8	18655	Protein LOC298111 (transcript 2)	One transcript in version 3.4 and two transcripts in version 5.0	1593272	*LOC298111*	
Q9JJI2	18755	α_{2u}-Globulin; PGCL5	Version 3.4	708503	*LOC259245*	Preputial gland (AB039826)
Q63213	18553	α_{2u}-Globulin; PGCL4		Submaxillary gland (J00738); preputial gland (AB039825)		
Q9JJI0	18822	α_{2u}-Globulin; PGCL7		Preputial gland (AB039828)		
Q63024	18726	Rat α_{2u}-globulin (L type)		Liver (M26836)		
Q63025	18712	Rat α_{2u}-globulin (S type)		Liver (M26838)		

Figure 2 | **Phylogenetic tree of primary sequences of rat MUPs**

The phylogenetic tree was based on the alignment in Figure 1, using CLC Sequence Viewer, version 7.02 (http://www.clcbio.com).



small hydrophobic molecules. This attribute is of functional significance: MUPs excreted into urine are involved in olfactory communication either by binding semiochemical molecules or acting as pheromones in their own right, whereas circulating MUPs have been ascribed a role in lipid transport and xenobiotic detoxification [27,28]. In rats, MUPs are involved in the development of an inducible pathology: hyaline droplet nephropathy in male rats (discussed below). The X-ray crystallographic structures of a mouse and a rat MUP were published in 1992 [29], although there is no active PDB file that refers to the rat MUP structure discussed in that publication. More recent structures of rat MUPs have become available either in unliganded form or as a complex with the hyaline droplet inducer D-limonene 1,2-epoxide [30]. These structures were obtained from MUP purified from rat urine and, unsurprisingly, the structure refers to the most abundant form (UniProt ID P02761). The sequence of this rat protein, P02761, was compared with the 21 *M. musculus* MUPs deposited in the MGI (Mouse Genome Informatics) database (http://www.informatics.jax.org). A BLAST comparison yields 71 % identity and 84 % homology (positives) with no gaps between Glu^{20} and Gly^{181} and the mature mouse MGI_MUP4 sequence with structural features identical in both proteins. Conserved features between MUPs of the two species extend to the binding calyx, lined with 20 amino acid side chains (Phelan et al. [30a] in this issue of *Biochemical Society Transactions*) that differs at only four positions from mouse MGI_MUP4 ($Leu^{24} \rightarrow Val^{24}$, $Ile^{101} \rightarrow Val^{101}$, $Glu^{118} \rightarrow Val^{118}$ and $Val^{40} \rightarrow Met^{40}$). The most striking difference in the binding calyx in the rat is an overall increase in hydrophobicity and the addition of a bulky methionine residue in the centre of the rat MUP calyx, indicating the potential for different molecular specificity between mouse and rat MUPs.

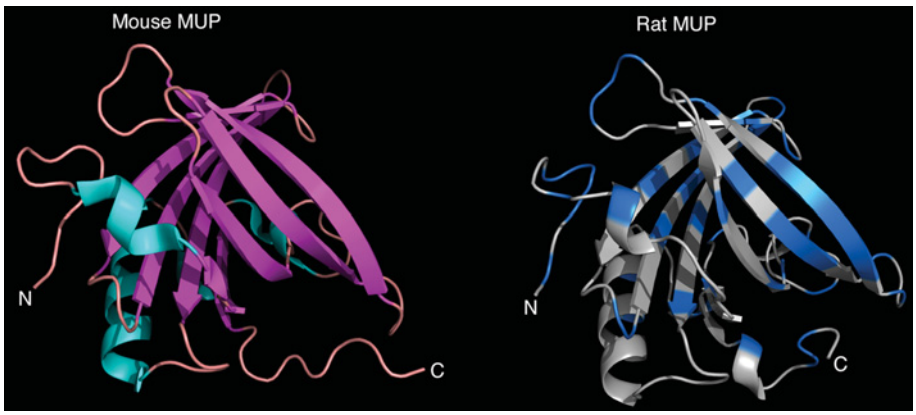
Tissue specificity of rat MUP expression

MUP expression has been described in liver, but also in other tissues, including salivary, lachrymal, meibomian, mammary, preputial and perianal glands, nasal tissue and respiratory epithelia [12,13,31–33]. The hepatic expression of rat MUPs is under complex multihormonal control [34,35] giving some clues about their potential function. Testosterone, growth hormone, thyroid hormones, glucocorticoids and insulin all regulate gene transcription. Moreover, hepatic expression is sex-dependent and under developmental control: in males, MUP expression is undetectable in immature male rats, but increases to a maximal level at 9–12 weeks (coinciding with sexual maturation), is sustained during adulthood and declines gradually during senescence [2,32,36,37]. Female rats have very low levels of MUP expression, but treatment of ovariectomized females with androgens confers a male-like MUP phenotype, whereas administration of 17β-oestradiol to mature male rats supresses MUP synthesis [38]. By contrast, expression in submaxillary glands is constitutive, does not exhibit sexual dimorphism, increases after weaning and decreases before puberty [12,32]. In lachrymal glands, MUP expression is 3–5-fold higher in males than females. Hypophysectomy does not affect expression in submaxillary glands, but decreases expression in lachrymal glands [13].

One key difference between rats and mice is the expression of MUPs in preputial glands: rats express and produce a significant amount of MUPs in their preputial glands, whereas no MUP mRNA has been detected so far in mouse preputial glands [33]. Preputial glands play an important role in semiochemical communication in rodents and are one of the principal sources of volatiles that evince multiple behavioural responses [39–41]. In the female rat, the equivalent gland to the preputial gland is referred to as the clitoral gland. Studies thus far indicate that expression of MUPs in preputial and clitoral glands are equivalent in males and females, expression peaking at 30–40 days and decreasing slightly in adults [32]. MUP expression in rat mammary gland is evident during mid-gestation and is not detectable in nursing animals or after litters are weaned [32]. Finally, the nasally expressed isoform in rats has also been named OBP3 (odorant-binding protein 3) due to the localization and predicted function [42], although

Figure 3 | Three-dimensional structures of mouse and rat MUPs

Backbone cartoons were generated from 1I04.PDB and 2A2U.PDB for mouse and rat respectively. For the rat structure, blue is used to highlight those amino acid positions that differ between the gene products listed in Table 1.



from sequence data, it is clear that this protein belongs to the MUP family.

Expression of rat MUPs in urine

The expression of MUPs in the urine of rats and mice exhibits sexual dimorphism. However, whereas in the house mouse MUP urinary output is typically 3–4-fold higher in males than equivalent females [43], the dichotomy is much more pronounced in rats; in one study using Wistar rats housed under standard conditions and urine collected using metabolic cages, MUP concentration was approximately 120-fold higher in adult males (1.09 ± 0.07 mg/ml) than in females (0.009 ± 0.001 mg/ml) [44].

Another key difference in metabolism of MUPs between the rat and the mouse relates to the behaviour of the proteins as they pass through the glomerular filter into the urine. Whereas mouse MUPs bypass any resorption, in the rat approximately 60 % of the MUP that passes through the glomerular filter is reabsorbed in the proximal tubule [45,46]. Once absorbed, the MUP is degraded in the lysosomal compartment; however, a part of this fraction is partially proteolysed by removal of eight amino acids from the N-terminus and four amino acids from the C-terminus, generating a truncated protein of ~15.5 kDa described as A2-f (α_{2u} fragment) that accumulates in the cytosol [47]. The accumulation of this truncated form in the proximal tubule, together with other fatty-acid-binding proteins, supports a possible role of the proteins as binding proteins that facilitate fatty acid transport and oxidation in the proximal tubule of the kidneys [27,48]. A lysosomal cysteine protease seems to be responsible for the partial degradation, although the specific enzyme and the mechanism by which the protein escapes further lysosomal proteolytic degradation, accumulating in the cytosol, has yet to be identified. However, the sexual dimorphism in MUP expression resulting in extremely low levels in females suggests that there cannot be a critical role for these proteins in fatty acid metabolism.

The uptake of rat MUPs by the kidney has also attracted attention because of a role in xenobiotic-induced hyaline nephropathy, caused by accumulation of protein droplets in the kidney. One of the consequences of toxicological tests of hydrophobic hydrocarbons is renal damage elicited by lysosomal protein accumulation (for a review, see [28]). This pathology is exclusive to the male rat, reflecting the much higher level of MUP expression in the male. The mechanism proposed for the accumulation is that when the xenobiotic ligand is bound to the protein, lysosomal proteolysis is inhibited, leading to accumulation of the protein and consequent renal damage [49]. However, this is likely to be a by-product of an abnormal toxic condition rather than an evolved function directly related to the role in natural populations.

Role of rat MUPs in semiochemistry

As animals that are largely nocturnal or active under cover, olfactory signals in rats are important. Rats live in colonies, but the mating system and the social organization of males depend of the population density in the colony. At low density, rats are territorial and polygynous (one male dominates a territory with access to a number of females within a family group). At high density, rats become polygynandrous (several males cohabit with several females consisting of both dominant and subordinate individuals) [50]. The role of chemical signalling in the maintenance of a similarly complex, but different, social structure has been well studied in the mouse, but information about parallel systems in the rat is lacking. Urine has the potential to play an important role in communication between rats, but information about the role of rat MUPs is scarce. Early studies showed that urine contains information about the individual, including sex, age, social status, reproductive status and

individuality [51–53]. Adult male and female rats mark using urine to advertise their sexual availability. Urine marking in males is influenced by testosterone: marking behaviour increases when there is a receptive female around and decreases in castrated males, although this behaviour can be restored by administration of testosterone [54]. Females also mark more when they can smell other rats nearby, and females prefer urine marks of high-testosterone males [51]. Although the role of MUPs in rat communication is uncertain, several studies hint at such a role, for example, MUP binding properties [55] and similarity to mouse MUPs as well as the ability of rat MUPs to stimulate neurotransmitter release in the female amygdala, leading to a change in locomotor activity [56] and the capacity to activate neurons in the VNO (vomeronasal organ) [57]. As our understanding of the chemical complexity of rat scent marks (and scent marking behaviour) becomes more detailed, it is likely that a complexity as subtle as that in the house mouse will emerge. This is likely to reflect species-specific differences in social structure and the communication required to sustain this structure.

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