Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*

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Abstract

The urine of the house mouse, *Mus domesticus*, contains large amounts of proteins that are specifically synthesized in the liver to be secreted in the urine. These proteins, termed major urinary proteins (MUPs), have multiple roles in the communication of information in urine-derived scent marks. They bind low-molecular-mass volatile pheromones, and effect their delivery to the scent mark, followed by a slow release that is controlled by the rate of dissociation from the MUPs. However, this family of proteins is extremely polymorphic, more than might be expected for a simple role of ligand binding and release. We have analysed the polymorphism in wild mice, and have now shown that the pattern of MUPs in the urine acts as a type of individuality 'bar code' that signals the identity of the owner of the scent mark. This multiplicity of function, from a generic ligand-binding property to an extremely specific individuality, sets the MUPs apart from other lipocalin family proteins that are involved in chemical signalling.

Introduction

The 'classical' view of semiochemistry of land mammals ascribes the role of signal mediators to low-molecular-mass volatile compounds that transmit information by virtue of their volatility. However, it is becoming increasingly clear that other classes of molecule, particularly proteins, also have a key role in scent signals and in the mediation of chemical messages between individuals. There is growing evidence for the role of relatively small proteins, all of the lipocalin family, that are secreted into scent sources in a number of species [1], although, in other contexts, other proteins might also act as pheromone-binding moieties [2]. In this brief review, we discuss the structure and function of one such group of proteins, the major urinary proteins (MUPs) that are found in the secretions of the house mouse, *Mus domesticus*. In particular, we summarize recent data that clarify their roles in chemical communication. For other recent reviews of this class of proteins, which provide additional background, see [3–5]. Although the MUPs have been detected in many tissues and secretions [6–9], we will concentrate on those MUPs that are present in the urine of rodents, specifically from the house mouse. These MUPs are termed uMUPs [10] to indicate that they are secreted in urine and to discriminate them from MUPs that are expressed in other secretions, including saliva and milk.

General characteristics of expression of uMUPs

Many of the early studies on uMUPs and their mRNA were conducted with inbred mouse strains, such as Balb/c and C57BL/6 [7,11], although there were some early studies on wild-derived mice [12]. We have extended these studies to wild-caught or wild-derived mice bred in captivity, and a substantially more complex picture of uMUP expression has developed. It is now clear that output of MUPs in urine is substantial, and in some animals, we have noted urinary protein concentrations as high as 70 mg/ml, although values of 10–30 mg/ml are more typical (J. Hurst, R. Beynon and C.E. Payne, unpublished work). This might amount to a daily production of between 20 and 40 mg of protein/day. This is a substantial loss of protein and the evolutionary persistence of this protein loss in wild mice implies that it has a highly selected biological function, rather than being an artefact of production of inbred mice.

It has been stated repeatedly that the production of uMUPs is specific to male mice [5,13]. In part, this conclusion may have originated from the early studies on inbred strains, although even some of the early cDNA cloning studies were completed on female mouse liver [14]. In wild-caught populations, the production of MUPs by female mice is quite marked, at approx. 30% of the male output. Of course, urinary protein concentration is inadequate as a parameter to express urinary output, as this varies with the concentration of urine. Therefore, we routinely measure the ratio of protein to creatinine in urine. Creatinine is produced from creatine in muscle by non-enzymic processes and is eliminated exclusively in the urine. For mice of similar body mass, the protein/creatinine ratio provides a simple correction for urine dilution. Using this measure, the output of MUPs by female wild mice remains at approx. 30% of that of male mice.

Complexity of uMUP expression

The early studies on inbred mouse strains (especially Balb/c and C57BL/6) clarified that each inbred strain expressed

Key words: behaviour, communication, MUP, rodent, semiochemistry.

Abbreviations used: MHC, major histocompatibility complex; MUP, major urinary protein; uMUP, urinary MUP.

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a complex pattern of uMUPs, and that these two strains expressed different patterns of distinct uMUP variants [7,8]. Many uMUPs have pI values that span a relatively narrow range from 4.2 to 4.9 and these can be resolved by isoelectric focusing, yielding a pattern of approx. four to six individual protein bands. Because these animals were genetically identical, all individuals from any one strain express the same pattern, and a relatively small number of uMUP bands. Analysis of liver cDNA sequences and of the MUP genomic region confirmed that many of these proteins were the products of different genes. The MUP gene cluster on chromosome 4 may contain as many as 35 MUP genes, split roughly equally into Group I genes (actively transcribed and translated) and Group II pseudogenes [15].

We have conducted detailed analysis of uMUPs to establish which of the published liver cDNA sequences correlate with protein products [10,16]. The desalted mixture of uMUPs was resolved by high-resolution ion-exchange chromatography and their masses were determined by electrospray ionization mass spectrometry, which typically yields a mass to an accuracy of $\pm 0.01\%$, or ± 2 Da for a 20 kDa protein. At this level, it is feasible to match the observed mass with the mass predicted from cDNA-inferred protein sequences, making appropriate mass corrections for the loss of signal peptide and formation of a disulphide bond. In some cases, the purified uMUP was digested with endopeptidase LysC which cleaves after lysine residues, and which fragments uMUPs into, typically, 10 or 11 fragments. The masses of most of these fragments can be measured by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS, and to a typical mass accuracy of 100 mDa. At this level of mass accuracy, single amino acid substitutions are readily observable, which can then be confirmed, if required, by tandem MS.

The complexity of the uMUP profiles in inbred mouse strains led us to determine the expression of uMUPs in wild-caught mice [17,18]. On isoelectric focusing, the banding patterns covered roughly the same pI range, but were substantially more complex, both within and between individuals. Many of the bands were uMUPs that had not previously been observed in the inbred populations. Mass spectrometric analysis confirmed that the new protein species observed were the products of new alleles or genes and, in some instances, the new variants could be pinpointed to a single amino acid change [19]. These genetically heterozygous wild mice each express between four and fifteen (or more) bands on isoelectric focusing. Moreover, the pattern of bands produced by females is as complex as that produced by males, which supports a role of MUPs in chemical signalling in both sexes, rather than being associated exclusively with the generation of male-specific cues directed at females or used in intrasexual male competition. In this respect, our data are consistent with early studies on mRNA and genomic analysis [12].

Parenthetically, we note that the nucleic acid and protein database entries for MUPs are a source of some confusion. The process of curation and regularization of the sequence databases means that, in some instances, different MUP accession numbers (originally separate entries) have been subsumed into a single entry. However, in some instances, this curated entry implies a protein, the mass of which has never been observed in electrospray ionization mass spectra of urinary proteins from the same strain of mouse. There is a need for a rationalization of the sequences and of a clearer definition of the relationship between those proteins that are predicted and those that are actually observed.

Roles of uMUPS

The MUPs are members of the lipocalin family of proteins (http://www.jenner.ac.uk/lipocalin.htm). The generic features of this family include a conserved folding pattern (the lipocalin fold) that comprises an eight stranded β-pleated sheet that is circularized to create a β-barrel that is then 'sheared' to generate a flattened or ellipsoid shape [13,20–22]. This β-barrel encloses an internal cavity that is the binding site for a range of predominantly hydrophobic ligands [23–29]. The idea that uMUPs bind semiochemically active molecules in urine was therefore a logical development of the growth in knowledge about the nature of this family of proteins. The first confirmation of this proposition came from studies that showed that pregnancy block [30] and puberty acceleration [31] were associated with high-molecular-mass fractions of mouse urine. This might be attributable to the bound ligands, and a role for proteins in isolation remains unclear. A recent report suggested that uMUPs, stripped of natural ligands by dialysis and solvent extraction, retained some effectiveness in pregnancy block ([32], but see also [33]).

The main source of MUPs in the mouse is urine. The proteins that are secreted from the liver seem to be quantitatively excreted, and there is no evidence for renal uptake of uMUPs after glomerular filtration [34]. Urine is the primary source of semiochemicals in mice and they deliberately deposit numerous small scent marks. As such, investigation of a role for uMUPs in chemical communication should focus on those chemosignals that are perceived in these urine scent marks. Our strategy has been to combine detailed biochemical characterization of uMUPs and their ligands with manipulation of urine-derived scent signals in complex behavioural assays to clarify the role of uMUPs in scent-mark signalling.

A 'naturalistic' experimental approach

Many studies on odour recognition in rodents use tests of discrimination, such as Y-maze or habituation/dishabituation tests [35–38]. These tests are predominantly a measure of the discriminatory ability of the chemical senses, but to understand the functional significance, there is a need to link such olfactory capability to appropriate behavioural responses. Our approach to the analysis of uMUP function is based on measures of investigatory and competitive countermarking responses to male urinary scent marks. Dominant

mice use scent marks to advertise territory ownership and competitive ability [39–41], and will deposit numerous scent marks around their defended territory [42,43]. Moreover, a urine mark from an intruding male will elicit a strong counter-marking response from the territory owner, such that increased numbers of scent marks are deposited in the immediate vicinity of the intruder's mark [43,44]. These scent marks are used by females to assess the relative competitive ability of different males and, hence, their potential quality as mates [45,46]. In our experimental model, we manipulate the chemical composition of the scent marks that are introduced into the environment of a territory owner and assess investigation of the introduced stimulus and deposition of counter-marks. A combination of investigation and counter-marking thus assesses detection and response.

Most of our studies are conducted with wild or wild-derived mice. Although inbred mouse strains offer potent control of genomic differences in chemical communication, there some complications with their use in naturalistic behavioural experiments [47]. The housing of multiple individuals in small cages may have selected for phenotypes that have suppressed aggressive or competitive behavioural responses. Moreover, the process of inbreeding requires several tens to hundreds of generations of brother–sister mating, a situation that never pertains in the natural environment. It is possible that the normal mate selection mechanisms that maintain genetic heterozygosity have been selected against in such a breeding programme, and that these mechanisms are in part related to chemosignal perception. Finally, since mice of the same strain are genetically identical, they have no experience of differences in individual scent signatures that mice would usually employ to recognize individuals. This lack of experience may influence the development of the olfactory process and may therefore diminish or modify the responses of inbred mice.

Implicit in our experimental system is the assumption of the ability of a mouse to determine that the scent stimulus differs from its own scent marks. There are two types of ownership assessment that might operate. First, an animal might assess a scent source as 'non-self', but fail to associate the scent with a specific individual. Alternatively, an animal might be able to learn the chemical profile of a scent mark and then identify the individual donor of that mark. In neither case is there any requirement to invoke a genetically encoded template of 'self' or 'kin'; a learned association is an acceptable alternative. Indeed, studies using cross-fostered offspring provide good evidence for the lack of any 'hard-coded' self image [48].

Slow release of volatiles

Unlike auditory or visual modes of communication, scent marks have the potential to remain in the environment when the source animal is no longer present. Furthermore, communication over a distance requires that the semiochemicals are volatile (dispersal as dusts or aerosols would only take place under restricted environmental conditions, and might not be reliable). The physicochemical properties of a volatile molecule can conflict with its use as an airborne semiochemical. A high volatility would be commensurate with a rapidly decaying signal, in which case, a high intensity/long range is obtained at the expense of longevity of signal. However, a relatively involatile semiochemical might elicit an extended response, but at the expense of range or intensity. Reversible binding of a volatile molecule to a less volatile component would have the effect of extending the time course of release, provided that the rate of dissociation was slower than the rate of evaporation. It has been suspected that, because MUPs bind semiochemicals, they act in a slow-release process. We proved this in a series of experiments in which the behavioural response (investigation) was correlated with a loss of volatile ligands. Fresh urine marks from male mice are usually approached with caution by other male mice, but as the scent mark ages, this latency to approach declines. Addition of a competitive 'displacer' molecule that bound tightly to the MUPs [49] eliminated any latency to visit, commensurate with a virtually instantaneous loss of bound ligands [50]. Competitive displacement of the highly volatile natural ligands created an extremely short-lived signal.

Thus uMUPs act as a releasing device for volatile ligands. Less clear is the relationship between this role and the extreme polymorphism that is a feature of this class of proteins. One possibility is that each individual uMUP is specific for a subclass of volatile ligands. However, studies using fluorescent reporter molecules [28] or natural ligands [29] or studies of the kinetics or release of ligands from strains that express different populations of uMUPs [51] suggest that any such effects are minor. Indeed, the only difference that was observed was between uMUPs that exhibit sequence polymorphisms in the ligand binding cavity, specifically at position 56 in the mature sequence. This can be a phenylalanine, valine or leucine/isoleucine residue. This is the only polymorphism thus far identified that is located in the cavity; all other polymorphisms seem to be located at the surface of the protein structure [19,52].

Communication of individuality signals

It is well established that, in mice, urine-derived scent marks are a key medium for delivery of semiochemicals. Following from the premise that a scent mark persists when the donor is absent, it follows that other mice must be able match the scent mark to the donor's scent by chemical composition alone. This 'ownership signature' needs to be consistent, even as the scent mark ages. It should not, for example, be modulated by diet, by infection, or by social or reproductive status. Both ourselves and others [3,10,52] have proposed that the pattern of MUPs in urine has the potential to provide such an individuality signal. Recently, we have found strong evidence to support this hypothesis [53]. The bioassay in these experiments was based on competitive counter-marking, a good measure of the ability of a territory owner to recognize

scent marks from other individuals. In the first series of experiments, genetically heterogeneous brothers from wild-derived litters were selected on the basis of their uMUP type alone, such that the uMUP type of the scent-mark donor was either the same as that of the territory owner or different from it. Although all urine samples (except their own) aroused an investigatory response (J. Hurst, unpublished work), only the MUP-different marks stimulated enhanced counter-marking. In the second series of experiments, the territory owner's urine was adulterated by the addition of a highly purified recombinant MUP. After this adulteration, the urine elicited a strong counter-marking response, consistent with it being recognized as being derived from a different animal.

At present, we do not know whether uMUPs alone, or uMUPs with bound ligands, are responsible for such effects. Of course, a specific role for uMUPs as direct-signalling molecules in chemical communication implies that there will be receptor(s) for these proteins. Evidence is accumulating for a role for uMUPs in chemoreception in the vomeronasal organ [32,54], but whether there are multiple receptors, each specific for a single uMUP polymorphic variant, remains to be seen.

Other roles of uMUPs

Other roles in chemical signalling have been proposed for uMUPs. The binding of the volatile ligands in the calyx might not only extend longevity of the signal, but could also protect the ligands from chemical degradation, such as oxidation [26]. Secondly, it is feasible that it is a uMUP–ligand complex that elicits an effect, and there is a need for further work in which either recombinant proteins, or proteins that have been demonstrated to be fully depleted of natural ligands, are assessed thoroughly for pheromonal properties. Finally, in terms of territorial marking, a signal mediated by a single volatile molecule is not 'cheat-proof', as a receiver cannot discriminate between a large signal that was deposited some time ago and a small signal that was deposited recently. Clearly, these two extreme states could have critical consequences for advertising territory ownership. For the signal to be cheat-proof, it is necessary to assess the concentration of one semiochemical relative to some 'time-base' reporter that decays at a different rate. Provided that the two components decay at different rates, the ratio between them is changing continuously over time, and is independent of the size of the scent mark that is deposited. uMUPs are remarkably stable in the environment, and their decay rate is very low. They might well serve this additional function of a time base, although with such a stable reference molecule, the receiver animal is really assessing the concentration of the volatile component.

Finally, uMUPs might also act as vehicles to deliver low-molecular-mass semiochemicals to the vomeronasal organ or to the olfactory epithelium. Effects mediated this way would, of course, require contact between the scent source and the nose of the animal, and experimental designs based on detection of gas-phase chemicals might not be able to define the role of the uMUPs. With respect to a role in ligand binding, the discovery of MUP expression in the nasal system of the mouse [29,55] suggests that at least some variants have a role similar to that reported for odorant binding protein (OBP) [56,57].

Integrated view of MUPs in chemical communication in mice

Mouse urine is a rich source of semiochemicals, both volatile and involatile. Any integrated model of chemical communication in mice must provide a role for each class of component. A key issue relating to the role of odour in chemical signalling is whether or not animals use scent sources to discriminate between self and non-self, or whether they are able to associate an odour profile with a single individual. The ability of subordinate mice to specifically evade the dominant mouse, even in a complex population [39], implies an ability to assess individual ownership of scent marks. In this respect, at least one additional system has to be built into the model. It has been known for some time that mice can be trained to discriminate between urine odours of pairs of animals that are major histocompatibility complex (MHC) congenic [58]. This has led to the concept of an MHC-encoded odourtype, which elicits a chemical cue that is specific to the individual and which has been suggested to drive individuality recognition and mate choice, hence maximizing allelic heterogeneity in the MHC [36,59–61]. However, MHC-encoded odourtypes can be disrupted by food [62], or by manipulation of the bacterial flora [63], which would render a putative individual-specific odourtype susceptible to rapid change.

uMUPs are present in urine at concentrations as much as 10^6 times greater than soluble MHC fragments, exhibit a high degree of polymorphic variation, and are able to associate with low-molecular-mass semiochemicals. They act as slow releasers and as individuality-coding molecules in their own right. Our current view is that a scent mark deposited in the environment requires a stable component for two reasons. First, to provide a reference against which all other volatiles can be measured, and secondly, to provide a stable, persistent 'label' to advertise ownership of the scent mark.

It is clear that within the context of a carefully controlled and largely homogeneous genetic background, MHC is an important part of the odourtype. Whether or not it has the same role in the wild counterparts of laboratory mice remains to be seen, although studies with wild-derived mice are encouraging [35,64–66]. We believe that both systems will make a contribution, but that the relative contribution of each, in a range of behavioural systems, requires investigation.

This work has been supported by research grants from the Biotechnology and Biological Sciences Research Council (BBSRC). It is a great pleasure to acknowledge the many contributions of our research groups.

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Received 6 September 2002