REVIEW ARTICLE

The lipocalin protein family: structure and function

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The lipocalin protein family is a large group of small extracellular proteins. The family demonstrates great diversity at the sequence level; however, most lipocalins share three characteristic conserved sequence motifs, the kernel lipocalins, while a group of more divergent family members, the outlier lipocalins, share only one. Belying this sequence dissimilarity, lipocalin crystal structures are highly conserved and comprise a single eight-stranded continuously hydrogen-bonded antiparallel β -barrel, which encloses an internal ligand-binding site. Together with two other families of ligand-binding proteins, the fatty-acid-binding proteins (FABPs) and the avidins, the lipocalins form part of an overall structural superfamily: the calycins. Members of the lipocalin family are characterized by several common molecular-

recognition properties: the ability to bind a range of small hydrophobic molecules, binding to specific cell-surface receptors and the formation of complexes with soluble macromolecules. The varied biological functions of the lipocalins are mediated by one or more of these properties. In the past, the lipocalins have been classified as transport proteins; however, it is now clear that the lipocalins exhibit great functional diversity, with roles in retinol transport, invertebrate cryptic coloration, olfaction and pheromone transport, and prostaglandin synthesis. The lipocalins have also been implicated in the regulation of cell homeostasis and the modulation of the immune response, and, as carrier proteins, to act in the general clearance of endogenous and exogenous compounds.

INTRODUCTION

Members of the lipocalin protein family are typically small secreted proteins which are characterized by a range of different molecular-recognition properties: their ability to bind small, principally hydrophobic molecules (such as retinol), their binding to specific cell-surface receptors and their formation of macromolecular complexes. The lipocalins are a large and ever-expanding group of proteins exhibiting great structural and functional diversity, both within and between species. Although they have, in the past, been classified primarily as transport proteins, it is now clear that members of the lipocalin family fulfill a variety of different functions. These include roles in retinol transport, cryptic coloration, olfaction, pheromone transport, and the enzymic synthesis of prostaglandins; the lipocalins have also been implicated in the regulation of the immune response and the mediation of cell homeostasis.

Despite common characteristics and common functions, the lipocalin family has been defined largely on the basis of sequence similarity; the constitution of the family is surveyed in Table 1: its principal members are listed together with a summary of their biochemical properties. Already the lipocalin protein family is too large a subject to be reviewed exhaustively; instead we focus, in turn, on three key aspects: sequence and structure relationships of the lipocalins, their capacity for molecular recognition, and our burgeoning understanding of function within the family.

SEQUENCE AND STRUCTURE RELATIONSHIPS

From its initial identification [1], the apparent size of the lipocalin family has grown significantly to encompass a large corpus of protein sequences. Within this the lipocalins display unusually low levels of overall sequence conservation, with pairwise se-

quence identity often falling below 20%, the threshold for reliable alignment. However, all lipocalins share sufficient similarity, in the form of short characteristic conserved sequence motifs, for a useful definition of family membership to be made [2,3]. This analysis shows the lipocalin protein family to be composed of a core set of quite closely related proteins, the kernel lipocalins, and a smaller number of more divergent sequences, the outlier lipocalins (see Table 1). Kernel lipocalins, which form by far the largest self-consistent subset within the whole set of related sequences, share three conserved sequence motifs (see Figure 1) [2,3], which correspond to the three main structurally conserved regions of the lipocalin fold and have proved useful in the design of primers for cloning studies [4]. The first of these three characteristic motifs is shared by all lipocalins and can be used as a diagnostic of family membership. The outlier lipocalins match no more than two of these three motifs and are more diverse, forming distinct groups at the sequence level: the α_1 -acid glycoproteins (AGPs), odorant-binding proteins, and Von Ebner's-gland proteins.

Hitherto, lipocalins had only been found in eukaryotic organisms, mostly in vertebrates, although some have been identified in other phyla. This includes several examples from arthropods: butterfly insecticyanin, grasshopper lazaro [5], cockroach Bla g 4 protein [6] and lobster crustacyanin [7]; and there is evidence to suggest that carotenoprotein lipocalins may also be present in species from the phylum Coelenterata [8]. Other calycins, FABPs and avidins are also present in both vertebrates and invertebrates, including Arthropoda and Coelenterata, but thus far there is only one example from prokaryotes, namely streptavidin. Recent reports have, for the first time, identified bacterial lipocalins [9,10]. The existence of prokaryotic lipocalins has profound implications for our under-

Abbreviations used: FABP, fatty-acid-binding protein; RBP, plasma retinol-binding protein; Blg, β -lactoglobulin; BBP, bilin-binding protein; MUP, major urinary protein; OBP, odorant-binding protein; NGAL, neutrophil lipocalin; VEGP, von Ebner's-gland protein; apo, apolipoprotein; PGD₂, prostaglandin D₂; APP, acute-phase protein; PP14, pregnancy protein 14; A1M, α_1 -microglobulin; AGP, α_1 -acid glycoprotein; MAC, membrane-attack complex; QSP, quiescence-specific protein; CHM, chicken heart mesenchymal cells; SCR, structurally conserved region.

Table 1 Properties of members of the lipocalin protein family

Members of the lipocalin protein family listed as a Table with a summary of their physical and chemical properties; the proteins listed are divided between kernel and outlier lipocalins [2,3]. Molecular masses are given in kDa. Where a property, such as glycosylation, has been shown to be present experimentally this is indicated by a +, shown to be absent by a —; otherwise, where this is unknown the value is left blank. Data are taken from references cited in the text. Well-established abbreviations of particular lipocalins, as used in the text, are given in the last column.

Protein	Subunit molecular mass	pI	No. of residues	Oligomeric state	Glycosylation	Number of disulphides	Abbreviation
Kernel lipocalins							
Retinol-binding protein	21.0	5.5	183	Monomer	—	3	RBP
Purpurin	20.0		175				PURP
Retinoic acid-binding protein	18.5	5.2	166	Monomer	—	1	RABP
α_{2u} -Globulin	18.7	5.7–6.7	162	Dimer	—	1	A2U
Major urinary protein	17.8	5.5–5.7	161	Dimer	—	1	MUP
Bilin-binding protein	19.6		173	Tetramer	—	2	BBP
α -Crustacyanin	350.0	4.3–4.7	174/181	Octamer of heterodimers	—	2/2	
Pregnancy protein 14	56.0		162	Homodimer	+		PP14
β -Lactoglobulin	18.0	5.2	162	Dimer/monomer	—	2	Blg
α_1 -Microglobulin	31.0	4.3–4.8	188	Monomer + complexes	+	1	ATM
C8 γ	22.0		182	Part of complex	—	1	C8 γ
Apolipoprotein D	29.0–32.0	4.7–5.2	169	Dimer + complexes	+	2	ApoD
Lazarillo	45.0		183	Monomer	+	+	LAZ
Prostaglandin D synthase	27.0	4.6	168	Monomer	+	1	PGDS
Quiescence-specific protein	21.0	6.3	158			1	QSP
Neutrophil lipocalin	25.0		179	Monomer/dimer + complexes			NGAL
Choroid plexus protein	20.0		183	Monomer	—		
Outlier lipocalins							
Odorant-binding protein	37.0–40.0	4.7	159	Dimer		0	OBP
von Ebner's-gland protein	18.0	4.8–5.2	170	Dimer		1	VEGP
α_1 -Acid glycoprotein	40.0	3.2	183	Monomer	+	2	AGP
Probasin	20.0	11.5	160				PBAS
Aphrodisin	17.0		151		+	2	

standing of how the family has evolved, suggesting that it is very much older and more widespread than has been supposed.

In contrast with their low conservation at the sequence level, analysis of available lipocalin crystal structures, which include plasma retinol-binding protein (RBP) [11], β -lactoglobulin (Blg) [12], insecticyanin [13], bilin-binding protein (BBP) [14,15], major urinary protein (MUP) and α_{2u} -globulin [16], odorant-binding protein (OBP) [17] and epididymal retinoic acid-binding protein [18], shows that the overall folding pattern common to the lipocalins is highly conserved. The nature of this common structure is now well described (see Figures 1 and 2) [2,11]. The lipocalin fold is a highly symmetrical all- β protein dominated by a single eight-stranded antiparallel β -sheet closed back on itself to form a continuously hydrogen-bonded β -barrel, which, in cross-section, has a flattened or elliptical shape and encloses an internal ligand-binding site. The eight β -strands of the barrel, labelled A–H (see Figure 1), are linked by a succession of +1 connections. These seven loops, labelled L1 to L7 (see Figure 1), are all typical of short β -hairpins, except loop L1; this is a large

Ω loop which forms a lid folded back to close partially the internal ligand-binding site found at this end of the barrel.

Together with two other distinct families of ligand-binding proteins, the FABPs and the avidins, the lipocalins form part of the calycin protein superfamily [2,19]; see Figures 2 and 3. Like the lipocalins, both families have β -barrel structures. The FABP barrel is ten-stranded and discontinuous and that of the avidins, although eight-stranded, is less elliptical in cross-section than that of the lipocalins. Beyond an obvious functional similarity (the binding of hydrophobic ligands) these families are characterized by a similar folding pattern (an antiparallel β -barrel, with a repeated +1 topology, possessed of an internal ligand-binding site), within which large parts of their structures can be equivalenced. They are also characterized by a conserved sequence motif which corresponds to an unusual structural feature (a short 3_{10} -like helix leading into a β -strand) conserved in conformation and its location within the fold [2,19]; other than the conservation of this motif, the three families share no discernible global sequence similarity. Members of the calycin triumvirate

Figure 1 Structure of the lipocalin fold

(a) Characteristic features of the lipocalin fold. An unwound view of the lipocalin fold orthogonal to the axis of the barrel [2]. The nine β -strands of the antiparallel β -sheet are shown as arrows and labelled A–I. The N-terminal 3_{10} -like helix and C-terminal α -helix (labelled A1) are also marked. The hydrogen-bonded connection of two strands is indicated by a pair of dotted lines between them. Connecting loops are shown as solid lines and labelled L1–L7. The two ends of the β -barrel are topologically distinct [21]. One end has four β -hairpins (L1, L3, L5 and L7); the opening of the internal ligand-binding site is here and so is called the Open end of the molecule. The other has three β -hairpin loops (L2, L4 and L6); the N-terminal polypeptide chain crosses this end of the barrel to enter strand A via a conserved 3_{10} helix affecting closure of this end of the barrel: the Closed end of the molecule. Those parts which form the three main structurally conserved regions (SCRs) of the fold, SCR1, SCR2 and SCR3, are marked as boxes. Three sequence motifs which correspond to these SCRs are shown (MOTIF 1, MOTIF 2 and MOTIF 3). The first three sequences are from kernel lipocalins and the second three from outlier lipocalins. Note that MOTIF 1 is well conserved in all sequences, whereas the other two, particularly MOTIF 2, are only well conserved in kernel lipocalin sequences [2,3]. (b) The lipocalin β -barrel in cross-section β -strands are shown as triangles. Triangles pointing downwards indicate a strand direction into the plane of the paper and those pointing upwards indicate a strand direction out of the plane of the paper. The view shown, down the axis of the barrel, is orthogonal to that in (a). Connecting loops are shown as continuous lines. Labelling and features shown are as in (a). The closure of the sheet to form the lipocalin β -barrel breaks the symmetry of its elliptical cross-section, distinguishing between its two foci and suggesting a sidedness to the barrel also apparent in the location of the marked SCRs.

Figure 3 Relationship between avidin, lipocalin and FABP folds expressed as a structural transformation

If we imagine the removal of N- and C-terminal segments from a lipocalin structure (by cutting it at the start of the 3_{10} helix prior to strand A and at the end of strand H) and then make the necessary insertion of strands, then the resulting structure would be essentially that of an FABP. The transformation between avidin and FABP structures requires insertion of two strands into the centre of its β -barrel core and the dilation of loop L1, and from lipocalin to avidin necessitates the loss of both N- and C-terminal peptides and the truncation of loop L1. In no case is any change in topology or organization of the fold required, only the loss or gain of features and such minor structural adjustments as is normal between members of homologous families. Transforming a protein structure from any other family into either the avidin, lipocalin or FABP folds requires changes of gross conformation and topology more severe than any of these interconversions. The structural representations follow the conventions of Figure 1; β -strands are shown as arrows and labelled by letter. The N-terminal 3_{10} -like helix and C-terminal lipocalin α -helix (labelled A1) are marked. The hydrogen-bonded connection of two strands is indicated by a pair of dotted lines between them. Connecting loops are shown as continuous lines; loop L1 is labelled for each fold.

also share a distinct structural signature: an arginine or lysine residue (from the last strand of the β -barrel) which forms several hydrogen bonds with the main-chain carbonyl groups of the N-terminal 3_{10} -like helix and packs across a conserved tryptophan (from the first strand of the β -barrel) [20].

COMMON MOLECULAR-RECOGNITION PROPERTIES

Ligand binding

The lipocalins are, perhaps, best known for their binding of a remarkable array of small hydrophobic ligands. The structural features of the lipocalin fold, a large cup-shaped cavity, within

the β -barrel, and a loop scaffold at its entrance, are well adapted to the task of ligand binding: the amino acid composition of the pocket and loop scaffold, as well as its overall size and conformation, determining selectivity [21]. To accommodate ligands of different size and shape, the binding sites of different lipocalins can be quite different. For example, compare the binding mode exhibited by MUP (Figure 2a), which binds its small ligand deep within its pocket entirely enclosed by side chains, with that of BBP (Figure 2b), which binds its large and relatively hydrophilic ligand in a solvent-exposed site predominantly formed from the loop scaffold; see also Figure 4. Table 2 summarizes much of the available data concerning the binding of endogenous and exogenous ligands by members of the family. These include

Figure 2 Schematic ribbon drawings of protein structures

(a) MUP; (b) BBP; (c) FABP; and (d) avidin. Ray-traced schematic ribbon drawings of protein structures; following convention [148], β -strands are shown as smoothly curving arrows, α -helices as spiral ribbons, and loops not in secondary structures are displayed as a smoothed coil [149]. Ligand molecules are shown using a coloured all-atom representation.

Figure 4 Polarity of the lipocalin fold and its multiple molecular recognition properties

The Figure shows a schematic representation of the lipocalin fold and visualization of the multiple interactions underlying its molecular-recognition properties. **(a)** Simplified schematic of the three-dimensional structure of the lipocalin fold; the β -strands of the lipocalin barrel are shown as arrows; the loops of the Open end of the fold are also shown, as is the N-terminal 3_{10} helix. The location of the internal ligand-binding pocket is shown as a highlighted semicircle. **(b)** Highly schematic cartoon summarizing the key structural features of the lipocalin fold and emphasizing its structural polarity; the ligand-binding pocket and hypothetical receptor-binding surface patch are highlighted. **(c)** Complexation with soluble macromolecules. The two modes exhibited by the lipocalins are shown; non-covalent association, such as between RBP and transthyretin, and covalently linked, either by disulphides (C8 γ and C8 α , apoD and apolipoproteins, or NGAL and gelatinase) or other groups (A1M and IgA). **(d)** Cell membrane receptor binding. Interaction with membrane proteins either via the lipocalin surface patch (Purpurin, C8 γ , etc) or the loop scaffold (RBP). **(e)** Ligand-binding modes. Lipocalins bind hydrophobic ligands with a range of size and shape; extreme examples are shown, namely the binding of a small ligand, entirely enclosed within the internal pocket, and the binding of a large steroid, bound partly in the pocket and partly by the loop scaffold in an altogether more exposed manner.

molecules with critical biological functions: retinoids (retinol and retinoic acid), arachidonic acid, and various steroids. Binding of such substances may have functional significance for some of the lipocalins; however, it is important to draw a distinction between the demonstration of binding *in vitro* and the identification of endogenous ligands. The retinol binding exhibited by Blg, for example, may only reflect its general affinity for a range of different small hydrophobic molecules; retinol in milk is associated with fat globules, and retinol bound to Blg has yet to be detected. Blg also binds long-chain fatty acids and triacylglycerols. It has been shown that careful extraction procedures, which do not reduce pH or increase the ionic strength of whey, allow the isolation of Blg from milk with bound endogenous fatty acids [22,23]. The composition of these bound fatty acids, mostly palmitate and oleate, resembles that of fatty acids extracted from milk, indicating that there is no appreciable selectivity of individual fatty acids. Thus the broad selectivity of binding exhibited by some lipocalins, such as Blg or AGP, may reflect a general transport role, such as the clearance of unwanted endogenous, or exogenous, compounds. Thus the binding of many of the molecules listed in Table 2 may not have any

physiological relevance and may even give rise to pathological conditions. For example, acute exposure to many important industrial and environmental chemicals, including components of unleaded petrol, causes a toxic syndrome, known as α_{2u} -globulin nephropathy, in the kidney of adult male rats. This syndrome is characterized by an excessive accumulation, in proximal-tubule epithelial cells, of lysosomal protein droplets composed of large amounts of α_{2u} -globulin and the degeneration and necrosis of cells lining the proximal tubule [24]. Chronic exposure leads to an escalating progression of symptoms often resulting in kidney failure and death.

Receptor binding

There is experimental evidence to show that a number of lipocalins are bound by specific cell-surface receptors and may be internalized by receptor-mediated endocytosis; for example, it has been shown that, in the liver, the retinol-RBP complex is taken up by receptor-mediated endocytosis in parenchymal and stellate cells [25], probably involving potocytosis [26], whereas

Table 2 Ligand binding by members of lipocalin protein family

Ligand-binding properties of the lipocalin protein family are summarized; where the ability to bind retinol or retinoic acid has been demonstrated experimentally this is indicated by a +, or shown not to bind by a —; where this is unknown, or is uncertain, by a ?. Known, or suspected, endogenous ligands are shown, as are compounds known to bind *in vitro*. Where these lists are long, for example AGP or crustacyanin, this is abbreviated; the interested reader is referred to the given references.

Protein	Retinol	Retinoic acid	Endogenous ligand	Other binding	References
RBP	+	+	Retinol	Retinal (+), retinyl acetate (+), β -ionone (+), <i>cis</i> -retinoids (—), β -carotene (—), cholesterol (—), terpenoids (—), β -lonylideneacetate (—), long-chain esters of retinol and retinoic acid (—)	[138,139]
RABP	—	+	Retinoic acid	Synthetic retinoids (—), retinal (—)	[126]
Purpurin	+	?	?		[132]
MUP	?	?	2-(s-Butyl)thiazoline, 2,3-dehydroexobrevicomin, 4-(ethyl)phenol	All (+): 2-isobutyl-3-methoxypyrazine, methyl dihydrojasmonate, (+)-methylfenchol, methyl dihydrojasmonate, thymol, 2-nonenal, β -ionone	[56,58]
A2U	+	?	Pheromones?	All (+): 2,2,4-trimethylpentane, decalin, JP-5, isophorone, 1,4-dichlorobenzene, α -limonene, dimethyl methylphosphonate, chloroethene, pentachloroethane, hexachloroethane, cyneole, 2-isobutyl-3-methoxypyrazine, thymol, methyl dihydrojasmonate, (—)-methylfenchol, α -limonene oxide, 2,4,4-trimethylpentan-1-ol, 2,4,4-trimethylpentan-2-ol, 2,2,4-trimethylpentan-1-ol, α -tetralone, isophorone, α -tetralol, 2-hexanone, 2,5-dichlorophenol, phenylethylalcohol, nonen-2-al	[24,58,140]
Apo D	?	?	Progesterone/pregnenolone	Bilin (+), cholesterol (—), arachidonic acid (+)	[111,141,142]
PP14	—	—	?		[92]
Blg	+	?	Fatty acids?	All (+): stearate, palmitate, laurate, oleate, heptane, butane, pentane, iodobutane, SDS, Methyl Orange, toluene, n-octylbenzene- <i>p</i> -sulphonate, <i>p</i> -nitrophenol, <i>p</i> -nitrophenyl acetate, <i>p</i> -nitrophenyl β -glucuronide, <i>p</i> -nitrophenyl sulphate, <i>p</i> -nitrophenyl pyridoxal phosphate, heptan-2-one, octan-2-one, nonan-2-one, trifluorotoluene, hexafluorobenzene, β -ionone, haemin, protoporphyrin IX, ellipticine	[31,143,144]
BBP	?	?	Biliverdin IX γ		[82]
Crustacyanin	?	?	Astaxanthin (3,3-dihydroxy- β , β -carotene-4,4'-dione)	Range of carotenoids (+), and other molecules	[145,146]
C8 γ	+	+	?		[44]
A1M	+	?	?		[147]
VEGP	+	?	?	Denatonium benzoate (+), cholesterol (—), stearate (+) palmitate (+), fatty alcohols (+), phospholipids (+), glycolipids (+)	[76,77,78,79]
A1AG	?	?	Histamine?	Extensive range of drugs and other compounds	[105]
PGDS	?	?	Prostaglandin H ₂		[86]

AGP is endocytosed via clathrin-coated pits [26]. There is increasing evidence, from a wide variety of different tissues, that RBP binding to its target cells occurs via specific surface receptors [27,28]. A cell-surface receptor for α_1 -microglobulin (A1M) has also been identified [29,30], and there is additional evidence to suggest the existence of receptors for MUP [16], Blg [12,31], and OBP [32]. Epididymal secretory protein has been shown to bind to the plasma membrane of spermatozoa [33], and may be another lipocalin to act via a specific surface receptor. It has been hypothesized that the three conserved sequence motifs characteristic of the family, which lie next to each other forming a surface patch at the Closed end of the lipocalin fold, constitute a common cell-surface receptor binding site (see Figure 4) [2,34]. However, Sivaprasadarao and colleagues showed, using mutagenesis, that specific amino acids within the Open end loop scaffold are responsible for binding to the RBP receptor [32]. Although the mechanism of retinol transfer from RBP to the cell interior remains controversial [35], it seems that retinol is taken up by target cells through a specific cell-surface receptor that recognizes RBP. Sivaprasadarao and Findlay propose that RBP remains external to the cell, with only retinol being internalized by the RBP receptor in a manner not involving receptor-mediated endocytosis [36]. In contrast, Noy and co-workers have suggested that retinol dissociates spontaneously from RBP outside the cell

and undergoes passive transfer through the plasma membrane to be bound by high-affinity sites in the cytoplasm [37,38]. Thus the structural determinants of binding to different membrane-bound receptors, although still poorly understood in themselves, clearly reside in different parts of the lipocalin fold for different family members.

Macromolecular complexation

The ability of lipocalins to form complexes with soluble macromolecules is arguably their least well known molecular-recognition property [21]. In plasma, RBP is usually complexed to the protein transthyretin: RBP binds transthyretin with an association constant of 1×10^7 M; only about 4% of total plasma RBP is free, the rest being part of a transthyretin complex [39]. Transthyretin has a higher affinity for halo-RBP than apo-RBP, and its interaction with RBP is also sensitive to both ionic strength and pH; the complex dissociates at low ionic strength and is only stable between pH 5.0 and 9.0. Purpurin is the component of adherons (large extracellular multi-component macromolecular complexes present in cultured chick retina growth medium) which mediates cell–cell and cell–substratum adhesion through its interaction with a specific cell-surface receptor [40]. About 80% of all plasma apolipoprotein (apo) D

exists as disulphide-linked complexes: predominantly with apoA-II in high-density lipoproteins and plasma, and mainly with apoB-100 in low-density lipoproteins and very-low-density lipoproteins [41].

More recently, it has been shown that neutrophil lipocalin (NGAL) is covalently attached to human neutrophil gelatinase (type IV collagenase) via an intermolecular disulphide [42,43], although most of the protein is secreted in an uncomplexed form. These authors propose a regulatory role for NGAL on the action of gelatinase. The lipocalin C8 γ , one of three subunits of C8 [the penultimate component of the membrane attack complex (MAC) of complement], is covalently linked to C8 α by an inter-molecular disulphide bridge [44]. C8 γ may have a regulatory function helping to protect host cells from lysis [45]; there is evidence that C8 γ is the site of interaction with the C8 binding protein, also known as homologous restriction factor, found on the cell surface of erythrocytes and other cells [46]. Half of plasma A1M exists in a free form, the rest in 1:1 covalent complexes with other macromolecules: albumin [47] and immunoglobulin A in humans [48]; fibronectin [49], via an intermolecular disulphide bridge and α_1 -inhibitor-3 in the rat [50]. The components of the A1M-IgA complex are linked covalently between A1M and the C-terminal nine residues of IgA [48]. The charge heterogeneity of free A1M is due to a tightly associated chromophore also responsible for its characteristic yellow-brown fluorescence [30]. There may be more than one chromophoric group per protein [51], one of which is covalently bound to cysteine-34 [52]. The complex does not exhibit the charge heterogeneity and fluorescence of free A1M, implying that the chromophore at cysteine-34 is involved in the link. Formation of the A1M- α_1 -inhibitor-3 complex abrogates the inhibitory action of the inhibitor, presumably preventing it from forming cross-links to proteinases [49,50]. It is not known whether A1M can affect its other macromolecular partners in a similar way, although its immunoglobulin complex exhibits both antibody activity and many of the biological actions of free A1M. It is tempting to suggest that A1M, and other lipocalins such as NGAL and C8 γ , may down-regulate plasma protein activity by complex-formation.

The protein-protein interactions which underlie complex-formation by the lipocalins are mediated by the loop scaffold forming the Open end of the molecule (see Figure 4); the variability of loop length and conformation and the different amino acid composition shown by these loops may be the principal means by which different lipocalins are able to form macromolecular complexes with high affinity and selectivity; for example, the correct positioning of free cysteine residues enables a number of lipocalins to form disulphide-cross-linked complexes. The recently published crystal structure of the RBP-transthyretin complex [53] suggests that a more complete understanding of these processes will follow as more crystal structures, and results of mutagenesis studies, become available.

FUNCTION

The lipocalins have, in general, been classified as extracellular transport proteins. In this regard they are typified by RBP [11,35], virtually the sole retinol transporter in plasma, which binds a single all-*trans*-retinol molecule as its physiological ligand. RBP is synthesized in hepatic parenchymal cells, where the apoprotein is saturated with retinol, triggering its secretion into general circulation. 85–90% of plasma RBP carries bound retinol, while about 96% is complexed to another plasma protein: transthyretin. The RBP-transthyretin complex is much larger than RBP alone, preventing its loss by filtration through the

kidney glomeruli. RBP-mediated retinol transport fulfills several physiological functions [54]. First, it facilitates the transfer transport of insoluble retinol from storage sites in the liver to peripheral tissues. Secondly, RBP protects bound retinol from oxidation. Thirdly, the synthesis of RBP regulates retinol release from the liver and mediates the specificity of its uptake by target cells. Interaction of RBP with target cells is crucial to its biological function: RBP is recognized by a specific cell-surface receptor, releasing retinol, and losing its affinity for transthyretin. The resulting apo-RBP is filtered by the kidney, reabsorbed and catabolized; thus RBP carries only one retinol molecule before being degraded.

Although few members of the lipocalin family are as well characterized functionally as RBP, as we shall see there is mounting evidence that lipocalins fulfil many different and potentially significant biological functions, including roles in mediating pheromone activity, olfaction, cryptic coloration, enzymatic synthesis, immunomodulation, and the regulation of cell homeostasis.

Pheromone activity

Rodent urine contains an unusually large amount of protein, and this phenomenon has been studied extensively in both rats and mice. MUP is the major protein component of mouse urine [55] and is expressed in several different secretory tissues of the mouse [56]. The major site of MUP synthesis is the liver; the protein is secreted by the liver into serum, where it circulates at relatively low levels before being rapidly filtered by the kidney and excreted.

Expression of MUP mRNA is under different developmental and hormonal control in different tissues. In the liver, expression of MUP is stimulated by androgens, principally testosterone, and is modulated by thyroxine and peptide growth hormones: addition of thyroxine or growth factors in the presence of testosterone increases MUP expression 150-fold [57]. However, constitutive expression of MUP has been demonstrated in the salivary and lachrymal glands [56]. The sex-dependent expression of MUP (adult male mice secrete 5–20 times as much MUP as do females) and its ability to bind a number of odorant molecules [57] is consistent with the suggestion that MUP acts as a pheromone transporter [59]; the protein may be excreted into the urine carrying a bound pheromone which is released as the urine dries and the protein denatures. This proposal is strongly supported by the work of Bacchini and colleagues, who have successfully purified MUP from mouse urine with bound ligands [60]. They identified three components from the total ligand extracted from the purified protein: the largest proportion (around 70%) was 2-(*s*-butyl)thiazoline, with 2,3-dehydroexobrevicomin and 4-(ethyl)phenol comprising minor fractions of about 15% each. However, only about 40% of protein contained bound ligand. The first two of these compounds are known to have pheromone activity in male rat urine, eliciting many sexually related responses in female rats. A recent report has shown that MUP, acting via the vomeronasal organ after appropriate physical contact with male mouse urine in their environment, can accelerate the onset of puberty in female mice [61]. Interestingly, this seems to be a function of the protein itself; MUP devoid of ligands, either by extraction or competitive displacement, is still active, while an organic extract containing these volatile ligands shows no activity. Moreover, a peptide corresponding to the N-terminus of MUP is also active. These results suggest that MUP is not only a carrier of pheromones, but also a pheromone in its own right.

α_{2u} -Globulin, a close homologue of MUP, is the major protein component of adult male rat urine, accounting for 30–50% of

total excreted protein [62]. As its electrophoretic mobility is similar to that of serum α_2 -globulin, it was named ' α_{2u} -globulin' with the subscript 'u' denoting its origin in urine. This choice of nomenclature has proved confusing; the protein is often wrongly called α_2 -microglobulin. α_{2u} -Globulin is secreted into the plasma by a number of tissues, where it circulates before being filtered through the kidney; between 20 and 50 % is reabsorbed by the proximal tubule of the nephron, the rest being excreted. Hepatic parenchymal cells are the principal sites of α_{2u} -globulin synthesis in mature male rats [63], although it is also expressed, at much lower levels, in many other tissues. The physiological regulation of α_{2u} -globulin expression is under multi-hormonal control [62]: treatment with androgenic steroid hormones stimulates α_{2u} -globulin expression, with 5 α -dihydrotestosterone being the most potent, while steroidal and non-steroidal oestrogens are strongly inhibitory. Combination of androgens, glucocorticoid, thyroxine, and growth hormone are required to maintain or restore normal levels of protein secretion. Although the exact physiological role of α_{2u} -globulin remains unclear, there is good circumstantial evidence that it functions in pheromone transport. This is consistent with its observed binding properties [58], its close similarity with MUP, its sex-dependent expression and the known properties of male rat urine.

Aphrodisin, another lipocalin, is the major macromolecular component of hamster vaginal discharge [64], and is secreted by vaginal tissue and the Bartholin's gland [65]. These secretions, acting via the vomeronasal organ, are known to elicit a copulatory response in male hamsters: if applied to the hind parts of an unconscious male hamster and another male hamster is encouraged to nuzzle the treated area with its snout then this hamster will reproducibly attempt an abortive copulation. By depleting these discharges of volatile components and by fractionation, it can be shown that aphrodisin is responsible for these effects, suggesting it is, like MUP, a mammalian proteinaceous pheromone [66]. Cloned aphrodisin expressed in *Escherichia coli* shows only modest activity compared with the isolated hamster protein, but is fully active when combined with organic extracts of vaginal discharge [67], suggesting that it is the protein-ligand complex which is fully functional.

Olfactory and gustatory proteins

The molecular basis of both olfaction and gustation is rather less well understood than the mechanisms underlying the other physical senses. Thus the discovery of specific proteins, usually referred to as OBPs, associated with olfactory tissue, which seem able to bind odorant molecules with high specificity, has generated considerable interest [68]; several of these proteins are lipocalins [68–70]. Two independent studies identified a soluble protein from bovine nasal mucosa which constituted about 1 % of total isolated protein [71,72]. This protein binds the bell-pepper odorant 3-isobutyl-3-methoxypyrazine, and became known as pyrazine-binding protein; it is most abundant in the tubulo-acinar cells of the respiratory epithelium of nasal mucosa, but is also present in olfactory mucosa, olfactory neurons and nasal secretions [73]. Pevsner et al. cloned and sequenced a protein, homologous with bovine pyrazine-binding protein, from rat nasal mucosa, which they called rat OBP [71]. Rat OBP is localized to the lateral nasal, or Sterno's, gland, the largest of the 20 discrete rat nasal glands of the rat. Lee et al. identified, cloned and sequenced a similar protein from the olfactory tissue of the frog *Rana pipiens* which they named protein BG (Bowman's gland) [69]. Analysis of mRNA distributions showed that this protein was specific to frog olfactory tissue. It is thought that the

OBPs may function by concentrating and delivering odorant molecules to their receptors.

Recently, two lipocalins, specifically expressed in the posterior and vomeronasal glands of the mouse nasal septum, have been identified and were suggested to act in the chemoreception of, as-yet-unidentified, small lipophilic pheromones [74]. One of these proteins was immunolocalized on the vomeronasal sensory epithelium, the site of primary pheromone reception, and the immunoreactivity was greatest during periods when contact between animals plays an important role in modulating behaviour.

It has been suggested that another lipocalin, highly expressed by the small acinar von Ebner's salivary glands of the tongue, but not in the secretory duct, fulfills a similar function, in salivary secretions, to that of OBPs in olfactory mucosa: the selective binding of sapid chemicals and their transport to taste receptors [75]. There is evidence that von Ebner's-gland protein (VEGP) can help to clear the bitter-tasting compound denatonium benzoate *in vivo* [76], suggesting a possible clearance function for the protein in taste reception, although it fails to bind other bitter compounds [77]. VEGP is also secreted by the lachrymal gland into tear fluid, where it has been known historically as 'tear prealbumin' [78]. Together with lysozyme and lactoferrin, VEGP forms 70–80 % of total tear protein, although this decreases in diseases affecting the lachrymal gland. Tear VEGP has been suggested to enhance the bactericidal activity of lysozyme and to have an inherent antimicrobial function, perhaps through the transport of compounds with antibacterial properties [79]. VEGP has been shown to bind retinol [78], and can be co-extracted with fatty acids, particularly stearate and palmitate, fatty alcohols (including cholesterol), phospholipids and glycolipids [79]. It has been postulated that VEGP may act as a transporter of lipids, synthesized in the dorsal, or meibomian, glands of the eyelid, to the thin film they form at the air/tear-fluid interface. Interestingly, a recent report indicates that the lipocalin apoD is secreted, as a disulphide-linked dimer, by the lachrymal glands and might also be a carrier of meibomian lipids in tear fluid [80].

Coloration

A number of lipocalins act in invertebrate coloration: bilin-binding protein from the cabbage white butterfly (*Pieris brassicae*), the closely related protein insecticyanin from *Manduca sexta* (tobacco hornworm) and the lobster protein crustacyanin. Like other members of the family they bind small molecules, and gain their colorant properties from interaction with their ligands.

A variety of different bilin pigments, derived from haem breakdown products, are distributed widely in insects. Biliverdin IX γ is amongst the most common, especially in butterflies and moths [81]. These pigments are usually associated with proteins, and they contribute significantly to coloration in the epidermis or interlamellar space of the wing. Two insect bilin-binding lipocalins have been studied in great detail: insecticyanin from the tobacco hornworm [82] and BBP from *Pieris brassicae* [13,14,83]. Both are blue pigments in their complexes with biliverdin IX γ . The precise functions of these proteins is unclear; apart from their role as pigments, it has been suggested that they may function in photoreception and protection from photo-induced free radicals. Many of these pigment complexes are remarkably stable, and persist, during insect development, from their synthesis during the larval stage, through the pupal stage into adulthood.

Crustacyanin (meaning 'shell blue') is the general name given to the carotenoprotein complex found in the epicuticle, or

calcified outer layer, of the lobster carapace [7,84]. It acts as the dominant pigment of the lobster shell, giving rise to its characteristic blue colour. In solution, crustacyanin exists as an equilibrium mixture between several distinct forms, differing in their physical and spectral properties [84,85]. The native, blue form (α -crustacyanin), which predominates *in vivo*, will, at low ionic strength, form α' -crustacyanin; this in turn changes to purple β -crustacyanin on standing. The $\alpha \rightleftharpoons \alpha'$ transition is favoured by low ionic strength and is reversible, while conversion into β -crustacyanin is irreversible. Native α -crustacyanin is an octamer of hetero-dimers, totalling 16 separate polypeptide chains, each dimer binding two molecules of astaxanthin, β -crustacyanin corresponding to the free heterodimer.

Prostaglandin D (PGD₂) synthesis

Glutathione-independent PGD₂ synthase (EC 5.3.99.2) is the main factor involved in synthesis of PGD₂ in the brain, accounting for over 90 % of activity in the rat; it is responsible for catalysing the conversion of PGH₂ into PGD₂ in the presence of various thiol compounds [86,87]. PGD₂ is a major prostaglandin in mammalian brains, functioning in the central nervous system as both a neuromodulator and a trophic factor. The enzymic activity of PGD synthase makes it unique among the lipocalins. It is localized in the choroid plexus, meninges and oligodendrocytes, but is also a major component of cerebrospinal fluid. Immunocytochemistry indicates that the protein is associated with the rough endoplasmic reticulum and the outer nuclear membranes of rat oligodendrocytes and seems to be a peripheral membrane protein easily dissociated by detergents.

A near homologue of PGD synthase has been identified in cane-toad (*Bufo marinus*) choroid plexus, where it is the most abundant protein secreted into the cerebrospinal fluid [88]. It is also found in other areas of the brain, albeit at much lower levels, and is expressed throughout amphibian metamorphosis. The choroid plexus helps form the barrier between blood and cerebrospinal fluid, and it may be that this member of the lipocalin family helps transport lipophilic molecules across the blood/brain barrier.

Immune modulation

The plasma levels of many proteins change during the acute-phase response, a complex physiological reaction to stress and inflammatory stimulation which plays an important role in many disease states. Proteins demonstrating elevated concentrations are called positive acute-phase proteins (APPs). These include the lipocalins AGP, NGAL, pregnancy protein 14 (PP14) and A1M. Negative APPs include the lipocalin RBP. APPs are thought to have an anti-inflammatory function, preventing ongoing tissue damage, as well as other roles well-suited to lipocalins, such as the transport of factors. Another lipocalin, C8 γ , is part of the membrane-attack complex of human complement.

The major protein product expressed between the late luteal phase of the menstrual cycle and the first trimester of pregnancy is a progesterone-regulated lipocalin called PP14 [89,90]; although first isolated from placenta, its principal site of synthesis is the secretory glandular epithelium of the endometrium and the decidua [91]. PP14 is also present in high concentrations in amniotic fluid, uterine luminal fluid, and at lower levels in plasma, but is not expressed by other tissues [89]. Variation in PP14 levels in these fluids reflects changes in endometrial PP14 secretion. Recently, Morrow et al. have shown that PP14 is expressed by haematopoietic cells of the megakaryocytic lineage and demonstrates potent immunosuppressive properties which

can be blocked by PP14-specific antibodies [92]. PP14 has also been reported to suppress the activity of natural killer cells and T-cells [93]. Seppala et al. recently reviewed other immunoregulatory properties of PP14 [94]. In the presence of PP14, interleukin-2 loses the ability to increase the mitogen-induced T-cell proliferation. PP14 inhibits the reactivity of mitogenic lymphocytes to phytohaemagglutinin in a dose dependent manner. The immunosuppressive effects of decidua extracts are greatly inhibited by anti-PP14 antibodies [95].

Blg, a close homologue of PP14, is the major protein component of whey from the milk of many mammals, synthesised by epithelial cells of the mammary gland regulated by prolactin and secreted into the lumen to accumulate in milk. Although an enormous amount is known about the physical and biochemical properties of Blg [31], its physiological function remains unclear. Blg may act in the delivery of free retinol to the absorptive cells of the intestine, an idea given support by evidence that Blg can enhance retinol uptake in rat intestine [96]. Thus it may be that Blg is a general carrier of nutritionally important, but insoluble, molecules between mother and child. It is also tempting to suggest some immunoregulatory function for Blg, analogous to that of PP14, but as yet there is no evidence to support this conjecture.

The lipocalin A1M is a well-studied plasma protein which has inhibitory effects on the immune system [30,97]. It is a positive APP, demonstrating enhanced hepatic expression, although plasma levels do not change significantly during inflammation. A1M suppresses antigen-induced polyclonal proliferation of cultured lymphocytes; this inhibition is partial at normal plasma concentrations and complete at 10–20 fold higher levels [98]. A1M is glycosylated by three separate chains (22 % of its overall mass is carbohydrate); corresponding glycopeptides can account for most or all of the immunosuppressive activity of A1M [99]. The formation of complexes, with IgA, fibronectin and α_1 -inhibitor-3, for example, may also play a role in regulating the immune system [48–50]. The increased secretion of the protein after stimulation by interleukin-6 (released by macrophages and T-lymphocytes) may be part of a negative-feedback system. A1M also inhibits the spontaneous migration of neutrophil granulocytes *in vitro* and suppresses the chemotactic attraction of granulocytes by a cytokine concentration gradient released by triggered monocytes, macrophages, and B- and T-lymphocytes [100].

Murine NGAL exhibits a 7–10-fold increase in expression in cultured mouse kidney cells infected by simian-virus 40 or other viruses [101]. In another study [102], NGAL was identified as a major secretory product of lipopolysaccharide-stimulated cultured mouse macrophages, suggesting that the protein might function in the defence against infection. Recently, NGAL has been shown to be identical with SIP24, a previously identified secretory product of quiescent mouse fibroblasts induced by serum, dexamethasone, basic fibroblast growth factor, phorbol ester, and PGF_{2x} [103]. Mouse plasma levels of NGAL rise, as a result of increased expression levels in the liver, in response to intramuscular turpentine injection. Tumour necrosis factor- α can regulate NGAL expression in cultured liver cells. These findings indicate that NGAL is a positive APP and may possess immunosuppressive or anti-inflammatory properties, possibly linked to its regulation of neutrophil gelatinase or other plasma protein [42]. The uterus is also a major site of NGAL synthesis, especially at parturition, when expression increases significantly, suggesting a physiological role for the protein in uterine secretions [104].

The lipocalin AGP (or orosomucoid) is an abundant plasma protein [105,106]. It is a positive APP; after induction by

turpentine injection, AGP becomes one of the dominant proteins expressed by the liver and is known to accumulate at sites of inflammation. AGP has shown many immunoregulatory properties: the ability to inhibit platelet aggregation [107], an involvement in wound healing (possibly through its interaction with collagen), the inhibition of neutrophil activation, inhibition of phagocytosis, and it possesses non-specific immunosuppressive properties [105,108]. AGP has an unusually high carbohydrate content of 40 % and is consequently unusually acidic and soluble [106]; several studies show that the deglycosylated protein demonstrates little or none of AGP's immunosuppressive properties. The characteristic glycosylation of AGP changes during the acute-phase response, with concomitant effects on its immunomodulatory properties: for example acute inflammation leads to a large increase in sialyl-Lewis-X-substituted AGP, which may inhibit selectin-mediated granulocyte invasion of inflamed endothelium [109]. However, the physiological function of AGP remains unclear; it binds a very wide variety of diverse ligands; rather too diverse, in fact, for any inferences to be drawn regarding transport functions of the protein [105].

Although several lipocalins have been shown to possess immunosuppressive properties *in vitro*, it is not yet clear whether this is a property of the protein itself, perhaps mediated by complex-formation or receptor activation, results from the transport of a bound ligand, or, as most are highly glycosylated, is mediated by attached carbohydrate, as is the case for A1M and AGP. The relevance of such results to the physiological functioning of these lipocalins *in vivo* is not clear. The plasma level of AGP is monitored during pregnancy and can be used as a diagnostic and prognostic marker in a variety of disease states [105], including cancer chemotherapy, renal dysfunction, myocardial infarction, arthritis, and multiple sclerosis; AGP levels also rise during infection or after surgery. Other lipocalins have also been used as biochemical markers in disease: RBP (used clinically as a marker of tubular reabsorption in the kidney) [35], A1M (the most accurate marker of tubular proteinuria) [110], PP14 (a clinical marker of endometrial status) [94], and apoD (a marker in gross cystic breast disease) [111]. However, evidence linking the observation of immunosuppression in the test tube and effects in the normal and pathological functioning of the immune system is lacking for the lipocalins; further work is needed to establish this link and demonstrate their role *in vivo*. In passing, it is worth noting that several lipocalins have been shown to give rise to inflammation [6]: bovine β -lactoglobulin, rodent urinary proteins and cockroach Bla g 4 protein are all allergens, the latter specifically associated with bronchial asthma.

One of the main functions of complement, the lysis of foreign cells, is mediated by the end product of the complement cascade; the MAC, a large pore-forming multimeric protein complex which induces rapid osmotic cell lysis. Complement component C8, the penultimate component of MAC, is a large multimeric protein composed of three subunits [45], two large glycosylated proteins, C8 α and C8 β , and a smaller non-glycosylated protein, C8 γ . The lipocalin C8 γ contains three cysteine residues, one of which (cysteine-40) forms an intermolecular disulphide to C8 α [44,112]. C8 β is responsible for binding to the evolving MAC complex, while C8 α is responsible for both membrane insertion and the C9 binding capacity of the complex. C8 γ is not obligatory for C8 to function within MAC, but may be part of a regulation mechanism protecting host cells from MAC-induced lysis [45,46].

Cell regulation

Several lipocalins have been implicated in the mediation of cell regulation [113]. Among the first to be so implicated was

quiescence-specific protein (QSP). Subconfluent, actively dividing or transformed cells do not synthesize QSP. Confluent cells, including those stimulated by hormones or growth factors, express the protein in a density-dependent manner. Bedard et al. showed that QSP is among the most prevalent proteins secreted by quiescent chicken heart mesenchymal cells (CHM) cells [114]. QSP is virtually absent from CHM cells transformed by Rous-sarcoma virus or sparse cells stimulated by hormones or growth factors. However, hormone-stimulated cells grown to confluence produce significant quantities of the protein, albeit at much reduced levels compared with quiescent cells; levels of QSP expression increase with cell density. Even very dense transformed cells do not express the protein.

Chick embryo fibroblasts give similar results: a density-dependent expression of QSP at confluence but negligible protein production when sparse. Likewise, QSP secretion begins as cultured chondrocytes enter the hypertrophic phase, the last of three stages of chondrocyte differentiation [115]; its level of expression increases as the cell population matures, becoming a major secretory product with a time-dependent distribution keyed to developmental status of mesenchymal cells [115,116]. QSP expression is a specific consequence of the dense confluent state rather than of growth arrest in general, such as results from cell starvation. These properties are consistent with a protein stabilizing mature cell populations. However, the work of Nakano and Graf shows that rapidly growing v-Myb-transformed promyelocytes express high QSP [117]. However, v-Myb induces an abnormal pattern of gene expression and may abolish an inhibitory mechanism present in untransformed cells.

The lipocalin apoD is a mammalian plasma lipoprotein that has long been thought to function in cholesterol metabolism [118], although its precise physiological role remains uncertain. Most apoD is localized in some form of high-density-lipoprotein particle, representing about 5 % of high-density-lipoprotein protein, where it may help enhance lecithin:cholesterol acyltransferase activity [119]; ApoD is identical with gross-cystic-disease-fluid protein (GCDFFP)-24 [111], a known progesterone/pregnenolone-binding protein, which constitutes over half the protein component of breast cyst fluid. Simard et al. [120] observed a correspondence between the steroid-induced secretion of apoD by cultured human prostate cancer cells and the inhibition of cell growth and also a higher concentration of apoD in well differentiated cells. In a related study, Provost et al. found little apoD expression by human diploid fibroblasts cells in sparse culture, while confluent quiescent cells did express the protein [121]. Cells whose growth is arrested by serum starvation also expressed the protein. Provost et al. also noted that expression levels rise as cells reach confluence. A more recent report has shown that expression of the apoD gene is induced by all-*trans*-retinoic acid in certain human breast-cancer cell lines [122]. This was accompanied by secretion of apoD, progression of cells to a more differentiated phenotype and the inhibition of cell proliferation.

Boyles et al. [123] and Spreyer et al. [124] both report a correlation between increased apoD expression and nerve regeneration consistent with the protein acting in the repair process. Boyles et al. report a 500-fold increase in apoD expression in regenerating rat, rabbit and marmoset peripheral nerves. They find apolipoprotein D is produced by astrocytes and oligodendrocytes in the central nervous system, and by neurolemmal cells and fibroblasts in the peripheral nervous system. Spreyer et al. report a 40-fold increase in apoD levels in the extracellular space of regenerating crushed rat neurons, although its expression was restricted to endoneurial fibroblasts. In light of these observations, the identification of a close homologue of apoD,

the grasshopper lipocalin Lazarillo, is of particular interest [5]. Lazarillo is expressed by a subset of developing neurons and is anchored to the cell surface by a glycosyl-phosphatidylinositol group. The protein is necessary for the correct navigation of growing axons in the grasshopper embryo and suggests that Lazarillo may function as a signalling molecule mediating axonal pathfinding in the developing nervous system [125].

Homologous lipocalins have been identified as composing 15–20% of the complex mixture of proteins secreted into the lumen of the epididymis from rat and lizard (*Lacerta vivipara*) [33,126]. They are known as epididymal secretory proteins and are able to bind retinoic acid, a known morphogen and regulator of gene expression, with high affinity and specificity [18]. Their synthesis is under strict androgen control and they are secreted from the principal cells of the proximal portion of the epididymis. The proteins are believed to have a significant role in the process of spermatogenesis and sperm maturation. It may be that this function is related to their ability to bind retinoic acid [126,127] and to bind to the plasma membrane of the head region of the spermatozoa [33].

Probasin, originally isolated from the nuclei of rat dorsolateral prostate epithelial cells [128], is another lipocalin implicated in cell regulation. Probasin mRNA expression, which is regulated by androgens, gives rise to both a secreted and a nuclear form of probasin [129], the relative abundance of the two forms being correlated with cell type. Probasin concentration also seems to be closely linked with cell age and state of differentiation, consistent with a role in cell regulation [128].

Purpurin, a lipocalin localized almost exclusively in neural cells of the retina [40,130], binds both glycosaminoglycan [130] and retinol [131], and is believed to function in the control of cell differentiation, adhesion, and survival [132]; experiments have shown the protein to promote the survival of cultured chick retinal nerve cells [131]. AGP has growth-promoting effects on cultured cells and has been shown to be involved in the process of nerve regeneration [133]. Apart from its inhibitory effects on the immune system, AIM also exhibits mitogenic properties [30]. Studies on mouse lymphocytes have shown an increase in thymidine uptake by B- and T-cells caused by AIM [134,135]. The protein also induces proliferation of B-lymphocytes in the absence of serum, a mitogenic effect which can be either enhanced or inhibited by other plasma components [136]. This effect seems to be a function of the C-terminus of the protein rather than its glycosylation.

The need to transfer retinoid or steroid molecules between cells in a controlled manner is a powerful argument for the involvement of extracellular carrier proteins, such as lipocalins, in mediating their effects *in vivo*, and the delivery, via endocytosis, of a lipocalin to nuclear receptors suggests a plausible mechanism of action. However, the role of lipocalins in mediating cell homeostasis remains open to question; changes in expression correlated with changes in morphology or growth arrest may be coincidental rather than causative, and, despite good circumstantial evidence, clearly further work is required to prove their mechanistic involvement in cell regulation.

CONCLUSION

The lipocalins are a structurally and functionally diverse family of proteins; this, at least, is clear. We have seen how structural studies of the family and an increasing understanding of their molecular-recognition properties illuminates insights into their biological function. Only a few years ago, the family remained poorly understood; now many studies are showing how much more interesting and important the lipocalins are than had been

supposed. It has been the purpose of this review to highlight this change of emphasis, and so raise awareness of the lipocalin family. Roles in transport, enzymic synthesis, immunomodulation, olfaction, pheromone signalling and cell regulation have been demonstrated or inferred. Moreover, as we have seen, clinical studies have shown the practical importance of many lipocalins as biochemical markers in health and disease. For example, drug binding to AGP is important pharmacologically; with serum albumin, it represents the major site of drug binding in plasma, and affects bioavailability, particularly as AGP levels vary during different disease states [105].

However, many aspects of the family provide scope for further investigation. Greater understanding of the precise relationship between kernel and outlier lipocalins will come, particularly, from the crystal structures of outliers, as well as from analysis of protein sequences and the structure of lipocalin genes. Genomic sequencing and cloning studies will further explore the species distribution and evolutionary relationship of the lipocalins, both to each other and to other members of the calycin superfamily. Many aspects of the multiple molecular-recognition properties of the lipocalins are now increasingly well understood. However, for many members of the family the nature and extent of ligand binding is still unclear; for some their natural ligand, or ligands, remain unknown, while for others binding is, as yet, wholly uncharacterized. Likewise, the question of how widespread, among the family, is the formation of complexes, with either soluble macromolecules or membrane-bound receptors, has yet to be answered fully. The nature of both the protein–protein and protein–small molecule interactions exhibited by the family will be illuminated both by crystal structures of lipocalins complexed with macromolecules and small molecule ligands and by directly probing function through mutagenesis studies.

Future studies of the lipocalin family will aim to understand both the particular functional specialization of individual lipocalins and the possible general physiological roles of the family as a whole in the context of a growing appreciation of the general principles which underlie their common behaviour. For example, the very broad tissue distribution of many lipocalins suggests that they may fulfill either a number of tissue-specific functions or alternatively have a single, but fundamental, role, perhaps some general clearance function preventing the harmful local accumulation of exogenous, or endogenous, hydrophobic molecules [78,122]. Moreover, is the apparent multifunctionalism displayed by many members of the family real or ‘artificial’? Does it result solely from their multiple molecular-recognition properties, or does it reflect true functional adaptation? To what extent are the apparent biological properties of individual lipocalins mediated directly by the protein structure itself (ligand binding [35,83,84], complexation [35,44,50] and receptor interaction [61,136]), by post-translational modifications (glycosylation state [99,109], chromophores [51], or membrane anchors [5]), or by combination of the two, such as the formation of disulphide linked complexes [41,44,49]? Are the allergenic properties of several, evolutionarily distinct lipocalins [6] merely a co-incidental property of their individual structures, or do they result from conserved structural features, such as the ability to interact with cellular receptors, common to the family? Can the immunosuppressive and cell-regulation functions of the family be linked to macromolecular complexation, perhaps through the modulation of proteinase-mediated cell signalling [137], either by the down-regulation of protease inhibitors [50] or by the direct inhibition of proteases themselves [42]? The immunosuppressive and cell-regulatory properties of the lipocalins suggests that they might be examined as candidates for protein therapeutics. Lipocalin research has entered an exciting era; our understanding

of the family has broadened and deepened in recent years; the answers to many intriguing questions about this fascinating family of proteins are now within reach.

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