

Latherin and other biocompatible surfactant proteins

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Abstract

Horses and other equids are unusual in producing protein-rich sweat for thermoregulation, a major component of which is latherin, a highly surface-active, non-glycosylated protein that is a member of the PLUNC (palate, lung and nasal epithelium clone) family. Latherin produces a significant reduction in water surface tension at low concentrations (≤ 1 mg/ml), and probably acts as a wetting agent to facilitate evaporative cooling through a thick, waterproofed pelt. Latherin binds temporarily to hydrophobic surfaces, and so may also have a disruptive effect on microbial biofilms. It may consequently have a dual role in horse sweat in both evaporative cooling and controlling microbial growth in the pelt that would otherwise be resourced by nutrients in sweat. Latherin is also present at high levels in horse saliva, where its role could be to improve mastication of the fibrous diet of equids, and also to reduce microbial adherence to teeth and oral surfaces. Neutron reflection experiments indicate that latherin adsorbs to the air/water interface, and that the protein undergoes significant conformational change and/or partial unfolding during incorporation into the interfacial layer.

Introduction

Proteins with surfactant activity appear in a wide range of contexts in nature. Probably the best known are the surfactant proteins in the lungs of mammals, which are required to break the surface tension of the liquid lining the gas interchange surfaces, which will be particularly important given the small size of their alveoli [1]. The lung SPs (surfactant proteins) that are active in this regard, SP-B (primarily) and SP-C, operate in association with lipids (the other two, SP-A and SP-D do not show significant surfactance, and are innate immune defence lectins of the collectin family) [1]. Since the original descriptions of pulmonary surfactant proteins, those that are highly surface active in their native, folded state and in the absence of any association with lipids or carbohydrates, are receiving increasing interest. One of the first of these intrinsically surfactant proteins was latherin [2,3], which was originally described from horse sweat and much later established as a member of the PLUNC (palate, lung and nasal epithelium clone) family, the others including the hydrophobins of fungi [4]. A more recently described surfactant protein derives from the foam nests of a species of tropical frog [5–7], and this represents an informative case that is worth considering before moving on to latherin [8].

RSN (ranaspumin)-2: a small, membrane-compatible surfactant protein from the foam nests of frogs

One of the intriguing and potentially useful aspects of the protein surfactants that have been described from vertebrates is that they are compatible with cell membranes, whereas small-molecule surfactants such as soaps and detergents that are used in a wide range of cosmetic, food and industrial applications, such as SDS, would instantly penetrate and destroy cell membranes. A particularly dramatic illustration of membrane-compatible surfactant proteins is that involving the proteins present in the foam nests of some species of tropical and sub-tropical frog [5,6]. These proteins, the RSNs, are released by the female during mating and come into direct contact, at high concentrations, with naked eggs and sperm at the point of fertilization. The frogs then mix the secretions with water into a foam mass, with the eggs at its centre. The foam is highly stable, and can persist in conditions of direct tropical sunlight and exposure to microbe contamination for several days while the larvae develop. In the absence of eggs and larvae the foams can persist for at least 10 days. The foams contain no evidence of small-molecule or lipidic surfactants, but contain proteins and carbohydrates at similar concentrations, and collectively exhibit surfactance similar to SDS on a weight basis [5–7]. The mixture of proteins and other activities in the nests is now known to comprise several lectins (including three of a family only previously found in fish) and cystatin activity, all of which probably relate to antimicrobial defence. One of the proteins present is RSN-2, which is only ~ 12 kDa in molecular mass, and was suspected to have surface activity on the basis of a

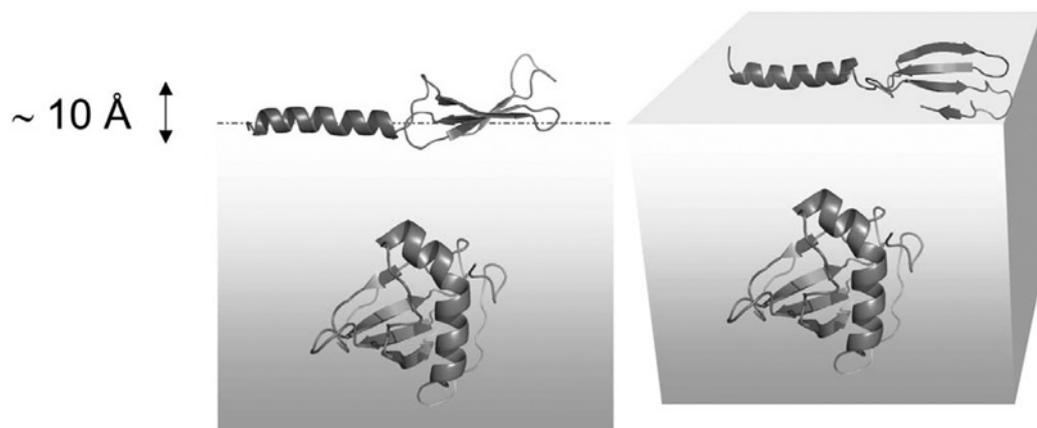
Key words: latherin, palate, lung and nasal epithelium clone (PLUNC), salivary protein, surfactant protein, sweat protein.

Abbreviations used: ANS, 8-anilino-naphthalene-1-sulfonic acid; BASE, breast cancer and salivary gland-expressed protein; BPI, bactericidal/permeability-increasing protein; LBP, lipopolysaccharide-binding protein; PLUNC, palate, lung and nasal epithelium clone; huPLUNC1, human PLUNC1; RSN, ranaspumin; SP, surfactant protein.

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Figure 1 | Model for conformational change in a surfactant protein at the air/water interface

A cartoon based on the known structure of RSN-2 [7], the egg and sperm membrane-compatible surfactant protein from the foam nests of a species of tropical frog, indicating how the compact closed form of the protein that exists in the bulk solution might open out at the air/water interface to expose the non-polar faces of the helix and sheet segments to the air while maintaining more polar contacts with the underlying water. Reprinted from [7], with permission.



highly charged, aspartic acid-rich C-terminal region [6,7]. RSN-2 has no equivalents in the protein databases, and so is either a type of protein that frogs evolved separately or has disappeared from land vertebrates.

RSN-2 expresses well in bacteria, and this allowed the solving of its structure by protein NMR [7]. This revealed a compact globular structure that, unexpectedly for a surfactant, showed no evidence of discrete hydrophobic and polar patches on the outside of the protein; such patches occur in fungal hydrophobins, which contribute to their amphiphilicity and self-assembly at the surface [9,10]. Moreover, neutron-scattering studies showed that RSN-2 associates with the air/water interface, and that the depth of the resultant protein layer is less than the minimal diameter of the protein in bulk solution [5]. It was consequently proposed that the protein unfolds at the air/water interface, exposing an internal apolar surface to the air, as illustrated in Figure 1. Such a mechanism may explain RSN-2's lack of damage to cell membranes if the opening of the protein only occurs at an air/water interface and not against a cell membrane; we are now testing this unfolding hypothesis by making direct modifications to the protein. Its membrane compatibility has already been exploited in the construction of an artificial photosynthesis system in which RSN-2 provided a stable foam into the walls of which were incorporated several elements of the plant photosynthesis system [11]. Experience with RSN-2 is now informing our approaches to understanding how latherin works as a surfactant, and hopefully also to explaining the biocompatibility of both proteins.

Latherin

Latherin was originally isolated from horse sweat in the 1980s, and shown to have strong surfactant properties and

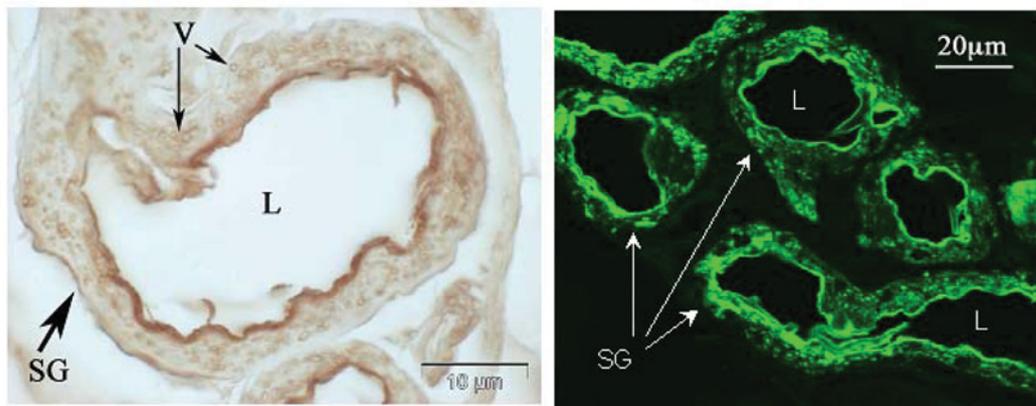
also to wet hydrophobic surfaces [2]. Because there was no sign of any association with lipid or glycosylation, latherin's surfactance must, like that of hydrophobins and RSN-2, be an intrinsic property of the protein alone. Latherin is considered to be the cause of the foaming of the sweat of exercising horses, which occurs predominantly where there is rubbing of harnesses, saddles, bridles, etc., and probably also the foaming of horse saliva. As part of our interest in biocompatible surfactant proteins, in parallel with working on the RSNs, we cloned cDNA-encoding latherin from horses and three other species of geographically dispersed equids: zebras, onagers and asses [3]. The amino acid sequences of the proteins from all four equids were very similar, with only slight divergences that are probably commensurate with the relatively recent divergence of extant equids from a common ancestor species (~2.3 million years) [12,13]. The latherin sequence from horses also contains stretches of amino acids that are identical with those of two allergens from their sweat that had been separately classified (Equ c 4 and Equ c 5 [14], now amalgamated with the former designation), and we found that some people that are allergic to horses have IgE (allergic-type antibody) reactivity to latherin [3]. Immunolocalization in horse skin revealed that latherin was present in the epithelial cells of the fundus region of sweat glands (Figure 2), localized to granules that are likely to be those that are lost in sweating [15]. RT (reverse transcription)-PCR confirmed latherin transcripts not only in the skin but also in a salivary gland, and in none of the other tissues that we sampled [3].

Latherin and PLUNCs

The amino acid sequence of latherin allies it to the PLUNC family, and more distantly to the larger BPI

Figure 2 | Localization of latherin in the sweat glands of horses

Immunoperoxidase labelling (left-hand panel) of a section of normal horse skin showing a dense dark band (brown) of staining proximal to the apical membrane of the sweat gland secretory epithelial cells. Cell-surface-associated latherin, or latherin originally occupying the lumen of the gland, is likely to have been lost during the preparation process. Staining is also apparent in intracellular vesicles. A similar distribution of latherin is visualized by immunofluorescence staining for latherin in horse skin (right-hand panel). Bright staining indicates the presence of latherin inside the secretory cells of the gland. SG, sweat gland; L, lumen; V, vesicles. Reprinted from [3], with permission.

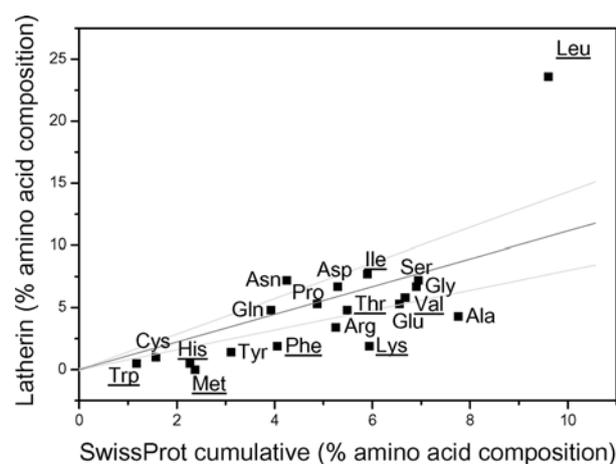


(bactericidal/permeability-increasing protein), CETP (cholesterol ester-transfer protein) and LBP (lipopolysaccharide-binding protein) proteins [8,16]. Its closest relative in humans is the apparently defective gene-encoded BASE (breast cancer and salivary gland-expressed protein) [8,16], for which homologues have been identified in rhesus macaques (AY913830) and as an allergen present in cats [17]. One curiosity is that the amino acid composition of latherin is enriched in apolar amino acids, as are other well-established surfactant proteins (with the curious exception of RSN-2), and leucine residues are in particular abundance (Table 1 and Figure 3). Amino acid composition and the predominance of one type is not a reliable predictor of biological function or biophysical activity, but it is perhaps significant that the other member of the PLUNC family from humans with a similar enrichment of leucine residues (huPLUNC1) has also been demonstrated to be surface-active [18]. Moreover, as has been postulated but as yet unproven for latherin, huPLUNC1 has been directly demonstrated to have antimicrobial biofilm activity [18]. If this is also confirmed for latherin, then this activity is likely to be due to latherin's biophysical activity, since we were unable to demonstrate lipopolysaccharide-binding by the protein (R.E. McDonald, A. Cooper and M.W. Kennedy, unpublished work), unlike its more distant cousin, LBP.

It could therefore be that latherin, like the hydrophobins, may have hydrophobic patches on its surface that allow it to act as a soap or detergent at the air/water interface by orienting such patches to the air, while its polar amino acid side chains orient into the aqueous phase. One test for exposed apolar patches in correctly folded or misfolded proteins, or exposed hydrophobic pockets, is binding by the environment-sensitive fluorophore [ANS (8-anilino-1-naphthalene-sulfonic acid)], but we found no

Figure 3 | Unusual amino acid composition of equine latherin

The amino acid composition of the mature form of horse latherin (i.e. after removal of its predicted N-terminal signal peptide), showing that the protein is unusually enriched in leucine residues. This plot also shows that the protein is slightly deficient in its content of essential amino acids (underlined) other than leucine itself. The percentage composition of each amino acid in latherin is plotted against the cumulative composition of all proteins entered into the Swiss-Prot/UniProtKB database (<http://www.expasy.ch/sprot/relnotes/relstat.html>). The dark line is a simple regression line set to pass through zero, and the lighter lines delimit the 95% CI. Adapted from [3] with permission.



evidence of ANS binding to latherin, whereas control proteins such as BSA or lipid-binding proteins bind strongly as visualized by a dramatic increase in ANS's

Table 1 | Amino acid compositions of latherin, its relatives, and other surfactant proteins

The number of amino acids and the percentage compositions of the proteins were calculated after the removal of their N-terminal presumptive secretory signal peptides, as predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>); in the case of the lung surfactants, SP-B and SP-C, only the surfactant-associated processed peptide is included. Their amino acid compositions, theoretical pI and the grand average of hydropathicity index (GRAVY) indices were all calculated with ProtParam (<http://www.expasy.ch/tools/protparam.html>). ProtParam defines apolar amino acids as any of isoleucine, leucine, valine, phenylalanine, cysteine, methionine and alanine residues, and polar amino acids as those with negatively (aspartic acid and glutamic acid residues) and positively (arginine and lysine residues) side chains. The sequences were from the following UniProtKB accession numbers: equine latherin, P82615; huBASE, Q86YQ2; huSPLUNC1, Q9NP55; huSPLUNC2, Q96DR5; huSPLUNC3, Q9BQP9; BPI, P17213; LBP, P18428; SP-B, P07988; SP-C, P11686; hydrophobins 1 and 2 of *Trichoderma reesei*, P52754 and P79073; RSN-2 of *Engystomops pustulosus*, B5DCK2. Because huBASE (human BASE) is incomplete, the apparent homologue in the rhesus macaque, *Macaca mulatta*, is also listed; MmBASE, Q2KKI5. The values in the SwissProt row are derived from statistical analysis of all of the proteins in the SwissProt database, see <http://www.expasy.ch/sprot/relnotes/relstat.html>.

Protein	Number of amino acids	L-Leucine (%)	Apolar (%)	Polar (%)	pI	GRAVY index
Latherin	208	23.6	44.2	17.3	4.2	+0.330
huBASE	159	15.7	40.3	15.1	5.33	+0.040
MmBASE	209	15.3	44.2	18.7	7.94	+0.123
huSPLUNC1	237	23.2	44.7	13.1	5.42	+0.563
huSPLUNC2	231	16.0	41.6	19.5	5.19	+0.113
huSPLUNC3	233	9.9	38.2	20.6	6.05	-0.308
BPI	456	10.3	39.0	18.0	9.35	-0.083
LBP	456	13.8	40.4	19.7	6.25	-0.002
SP-B peptide	79	17.7	60.8	13.9	9.44	+1.027
SP-C peptide	35	20.0	77.1	5.7	8.96	+2.466
Hydrophobin 1	75	9.3	45.3	8.0	5.74	+0.269
Hydrophobin 2	71	9.9	53.5	11.3	6.72	+0.694
RSN-2	96	11.5	32.3	47.9	5.16	-0.831
SwissProt		9.7	38.4	23.6		

fluorescence emission intensity [3]. We are currently close to solving latherin's structure by protein NMR, and our preliminary model of the protein in solution confirms that it comprises mixed α -helix and β -strands as in BPI and LBP, but that there is no evidence of discrete apolar or polar surface regions of any significance (S. Vance, A. Cooper, M.W. Kennedy and B.O. Smith, unpublished work).

In studying the disposition of latherin's single tryptophan residue, using intrinsic protein fluorescence and small-molecule quenching agents, we noted that the tryptophan residue appears to be fully exposed to solvent water, so presumably in an exposed position [3]. Tryptophan residues are not often found on the surface of proteins and are instead often deeply buried, but when surface-exposed they do seem to be involved in interaction with membranes (briefly reviewed in [19,20]), and conceivably also at an air/water interface. We did, however, note that only neutrally charged agents quenched the tryptophan residue's emission, but iodide, a charged and usually efficient quencher, failed to do so. This may have been due to nearby negatively charged amino acid side chains, which therefore may indicate that, despite its unusual disposition on the surface of the protein, it may not be involved in latherin's surfactance, unless, that is, the protein alters its conformation dramatically when it interacts with an air/water interface. In huPLUNC1, the

position occupied by the tryptophan residue in latherin is replaced by a threonine residue.

As with regard to RSN-2, neutron reflection experiments indicate that latherin absorbs into the air/water interface, and that the depth of this layer, estimated to be 10 Å (1 Å = 0.1 nm), is less than would be expected for a protein that by other measures behaves as a well-folded globular protein [3]. So, latherin may partially unfold or undergoes a significant conformational change on partitioning into the interfacial layer. The structures of BPI and LBP are known from X-ray crystallography, and both are long, narrow, slightly curved and cylindrical in shape [21–24]. These proteins are much larger than latherin, but even if latherin adopts a similar fold, this would still not be sufficient to reconcile the depth of the latherin surface layer unless its minimum diameter changes during absorption at the air/water interface. Whether similar conformational changes also occur when it adheres to and coats hydrophobic surfaces, either artificial [2,3] or the hairs of horses, remains an open question.

Biological functions of latherin

Only three species of mammal are known to sweat copiously to thermoregulate: humans, patas monkeys and horses; horses have high-protein low-salt sweat, whereas humans have

low-protein high-salt sweat. For a large-bodied flight animal, such as a horse, that has to accelerate quickly and maintain its speed for an endurance run, rapid and efficient heat dissipation is crucial. For such an animal that additionally has a thick, hairy pelt that is waterproofed and consequently will impede water flow, the rapid translocation of sweat water to the surface for evaporative cooling will present a particular problem. So, the action of latherin is probably to facilitate wetting of, and flow water quickly through, the pelt for evaporation at the surface of the pelt. The fact that latherin coated on to a hydrophobic surface can be subsequently washed off would permit the resumption of water resistance by an underlying oily layer once sweating has stopped.

It is less easy to postulate a function in relation to latherin in saliva. One possibility is that equids are adapted to a coarse, often dry, diet of plant materials with relatively low nutrient content, and, being hindgut fermenters with simple stomachs and no regurgitation and re-chewing of food, they need to chew and process large quantities of food through their foreguts rapidly. The function of latherin in saliva could therefore be to facilitate the wetting of their food, which will facilitate mastication and also increase the penetration into the food by salivary enzymes. In lubricating the food, latherin might also have the effect of conserving water in the processing and swallowing of food. If, like huPLUNC1 [18], latherin also has a disruptive effect on microbial biofilms, then this would be advantageous in the mouth by cleansing their teeth and mucosal surfaces. Such an antimicrobial activity would also, of course, be important in the skin and pelt, where an accumulation of microbes would be deleterious, especially as the sweat itself might provide a nutrient resource for microbes.

Latherin therefore presents a novel combination of biological and biophysical properties that are of interest in themselves for what they can teach us about intrinsically surfactant proteins. While the production of latherin in sweat glands appears to be a unique adaptation by horses to thermoregulation by sweating, the increasingly apparent overlap in its properties with the PLUNCs should be valuable in the elucidation of what PLUNCs do, and how. To this end, the forthcoming structure of latherin will be particularly informative.

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