Crystal structure of the mouse innate immunity factor bacterial permeability-increasing family member A1

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Bacterial permeability-increasing family member A1 (BPIFA1) is an innate immunity factor and one of the most abundantly secreted proteins in the upper airways. BPIFA1 is multifunctional, with antimicrobial, surfactant and lipopolysaccharide-binding activities, as well as established roles in lung hydration. Here, the 2.5 Å resolution crystal structure of BPIFA1 from *Mus musculus* (mBPIFA1) is presented and compared with those of human BPIFA1 (hBPIFA1) and structural homologs. Structural distinctions between mBPIFA1 and hBPIFA1 suggest potential differences in biological function, including the regulation of a key pulmonary ion channel.

1. Introduction

Bacterial permeability-increasing family member A1 (BPIFA1) is a member of the bacterial permeability-increasing (BPI) superfamily of proteins (Bingle *et al.*, 2011), and has previously been known as short palate lung nasal epithelial clone 1 (SPLUNC1), secretory protein in upper respiratory tracts (SPURT), lung-specific X protein (LUNX) and nasopharyngeal carcinoma-related protein (NASG). BPIFA1 is one of the most abundantly secreted proteins in the upper airways of the lung, and demonstrates activities as a bacteriostatic agent, surfactant and lipopolysaccharide-binding factor (Liu *et al.*, 2013; Di, 2011; Wei *et al.*, 2014; Jiang *et al.*, 2013; Chu *et al.*, 2007; Walton *et al.*, 2016; Ahmad *et al.*, 2016). BPIFA1 employs these varied functions to maintain normal lung function by, for example, spreading fluid across the pulmonary epithelium and maintaining sterile airways (Tarran & Redinbo, 2014). Furthermore, BPIFA1 inhibits two different ion channels, the epithelial Na⁺ channel (ENaC) and the Ca²⁺-influx channel Orai1, using two distinct structural and functional motifs (Garland *et al.*, 2013; Wu *et al.*, 2017). The N-terminus of BPIFA1 impacts the function of ENaC, but not that of Orai1, while the C-terminal α -helix (α 6) of BPIFA1 is involved in controlling the activity of Orai1 but has no effect on ENaC. Owing to the important roles of BPIFA1 in lung health, we have sought to understand the structure of mammalian BPIFA1s, with the goal of pinpointing functional motifs and improving the development of BPIFA1-based therapeutics (LeMessurier *et al.*, 2016; Fellner *et al.*, 2016; Terryah *et al.*, 2017).

We reported the 2.8 Å resolution crystal structure of human BPIFA1 (hBPIFA1) in 2013 (Garland *et al.*, 2013), which was the first published BPIFA1 structure; additional structures of

wild-type and variant forms of hBPIFA1 followed in 2014 and 2016 (Walton *et al.*, 2016; Garland *et al.*, 2013; Ning *et al.*, 2014). hBPIFA1 was found to retain the conserved structural features of the BPI superfamily, which are defined by a central β -sheet core framed by α -helical motifs. The proline-rich 23-residue N-terminus of hBPIFA1, which has been shown to be essential for inhibiting ENaC to control lung fluid levels, has not been visualized in structures reported to date, likely due to structural mobility. Indeed, the inhibition of ENaC by the N-terminus of hBPIFA1 is pH-sensitive, and it has been demonstrated that salt bridges on the body of hBPIFA1 are important for ENaC inhibition, potentially by positioning the N-terminal motif of the protein (Garland *et al.*, 2013). Sequence analysis revealed that the surface salt bridges observed in hBPIFA1 are not conserved among mammalian BPIFA1 orthologs.

Given the importance of BPIFA1 in models of human physiology and disease (Britto & Cohn, 2015; Saferli *et al.*, 2015; Xue *et al.*, 2015; Seshadri *et al.*, 2012), we next focused on the BPIFA1 protein from *Mus musculus* (mBPIFA1). hBPIFA1 and mBPIFA1 share 66% sequence identity, and the regions where they vary in sequence suggest divergent mechanisms for biological function. For example, mBPIFA1 does not maintain the surface salt bridge that has been proven to be involved in hBPIFA1 function (Fig. 1). Furthermore, the proline-rich N-terminus of mBPIFA1 is 24 residues longer than the N-terminal region of hBPIFA1 (Fig. 1). As such, the mouse protein may extend its N-terminal motif further away from the surface of the protein, possibly allowing inhibition of ENaC in a manner independent of the surface charge on the

body of mBPIFA1. To understand the structural details of mBPIFA1 and how it differs from hBPIFA1, we determined the crystal structure of mBPIFA1 at 2.5 Å resolution and compare it with BPI-fold proteins of known structure.

2. Materials and methods

2.1. Cloning and expression

A construct for mBPIFA1 containing only amino acids 46–278, which removes the signal sequence, was synthesized by GenScript and cloned into a pLIC expression plasmid vector with an N-terminal hexahistidine tag and a *Tobacco etch virus* (TEV) protease cleavage site. The pLIC expression plasmid was transformed into *Escherichia coli* BL21-Gold competent cells (Agilent Technologies). The expression cells were cultured in Luria Broth (LB) medium in the presence of antifoam (50 μ l) and ampicillin (100 μ g ml⁻¹) with shaking at 275 rev min⁻¹ at 37°C. When an OD_{600 nm} of ~0.60 was reached, the temperature was reduced to 18°C and protein expression was induced with 0.10 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). The induced cells were allowed to grow overnight at 18°C and the culture pellets were collected the following morning by centrifugation at 4500g for 25 min at 4°C in a Sorvall (model RC-3B) swinging-bucket centrifuge. The cell pellets were stored at -80°C.

2.2. Protein purification

The cell pellets were resuspended in buffer A (20 mM potassium phosphate pH 7.4, 50 mM imidazole, 500 mM NaCl,

Figure 1

Sequence alignment of BPIFA1 from *M. musculus* (mouse; NCBI Reference Sequence NP_035256.2) and *Homo sapiens* (human; NCBI Reference Sequence NP_001230122.1), with the secondary structure of mouse BPIFA1 (mBPIFA1) shown above the alignment. The 19-amino-acid signal peptide of human and mouse BPIFA1 is boxed. The glutamine start position of the shortened mBPIFA1 construct employed in this study is highlighted (bold and underlined). The leucine and histidine residues important for surfactant activity and Orai1 binding, respectively, in hBPIFA1 and mBPIFA1 are shown in bold. Residues important for the pH-sensitive salt bridge in hBPIFA1 and the corresponding residues in mBPIFA1 are highlighted in gray.

1 mM 1,4-dithiothreitol (DTT), along with lysozyme, DNaseI and protease-inhibitor tablets. The cells were sonicated and the cell lysate was separated into insoluble and soluble fractions by centrifugation at 17 000g for 60 min in a Sorvall centrifuge (model RC-5B). The soluble fractions were syringe-filtered through a sterile 22 μ M polyethersulfone (PES) membrane, applied onto an Ni-NTA HisTrap gravity column (GE Healthcare Life Sciences) and washed with buffer A. Bound protein was eluted with buffer B (20 mM potassium phosphate pH 7.4, 500 mM imidazole, 500 mM NaCl, 1 mM DTT) and applied onto an S75 gel-filtration column in buffer C [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

(HEPES), 300 mM NaCl, 1 mM DTT] on an ÄKTAexpress (GE Healthcare Life Sciences). mBPIFA1 elutes from the S75 column as a single peak in buffer C. The resultant affinity-tagged mBPIFA1 protein was incubated overnight with TEV protease at 4°C to remove the hexahistidine tag, leaving only a serine, an asparagine and an alanine as non-native amino-acid residues at the N-terminus of the protein. This sample was again applied onto an Ni-NTA HisTrap gravity column, which retained the histidine tag, and the mBPIFA1 protein was collected in the flowthrough. The resultant mBPIFA1 protein was concentrated to a volume of <5 ml using 10 kDa molecular-weight cutoff centrifugal concentrators (EMD

Figure 2

Purification and crystallization of mBPIFA1. (a) UV absorbance trace for size-exclusion chromatography of a purified mBPIFA1 construct using an S75 column. Peak 4 was collected and identified as mBPIFA1. (b) SDS-PAGE gel of the purified mBPIFA1 construct. The far-left lane contains a Thermo Fisher Scientific PageRuler pre-stained protein ladder (labeled in kDa); the most concentrated sample of mBPIFA1 protein (10 mg ml⁻¹) is on the left and is serially diluted to the right by a factor of 0.5. (c) Photograph of mBPIFA1 crystals grown in 20% PEG 1000, 0.2 M lithium sulfate, 0.1 M phosphate-citrate pH 4.2 at 37°C. The distance between the numbered units on the scale bar is 100 μ m. (d) Ribbon representation of the arrangement of eight mBPIFA1 monomers, chains A–H (black, red, lime, blue, magenta, orange, green and cyan, respectively), in the asymmetric unit of the crystal structure.

Figure 3

Comparison of the eight monomer chains in the asymmetric unit of mBPIFA1. (a) Structural alignment of the eight monomer chains A–H of mBPIFA1 (black, red, lime, blue, magenta, orange, green and cyan, respectively). (b) A view of the structural variability between the eight monomers of mBPIFA1 in the $\beta 4$ – $\beta 5$ loop region. (c) A view of the structural variability between the eight monomers of mBPIFA1 in the $\beta 6$ – $\alpha 5$ loop and $\alpha 3$ – $\alpha 4$ regions. (d) Structural alignment plot of r.m.s.d. per residue for mBPIFA1 monomers A–H (black, red, lime, blue, magenta, orange, green and cyan, respectively).

Table 1

Data-collection and refinement parameters for mBPIFA1.

Values in parentheses are for the highest resolution shell.

Space group	C121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	132.2, 200.6, 89.5
α , β , γ (°)	90.0, 126.2, 90.0
Resolution range (Å)	41.8–2.50 (2.59–2.50)
Wavelength (Å)	1.0332
Unique reflections	64175 (6089)
Multiplicity	6.0 (5.9)
Completeness (%)	98.9 (94.8)
$\langle I/\sigma(I) \rangle$	24.8 (4.43)
Wilson <i>B</i> factor (Å ²)	31.8
<i>R</i> _{merge}	0.071 (0.416)
<i>R</i> _{work}	0.221 (0.271)
<i>R</i> _{free}	0.278 (0.351)
Molecules in asymmetric unit	8
Waters in asymmetric unit	325
Residues in asymmetric unit	1558
Average <i>B</i> factor (Å ²)	31.8
R.m.s.d., bonds (Å)	0.010
R.m.s.d., angles (°)	1.3
Ramachandran favored (%)	95
Ramachandran outliers (%)	1.0
Rotamer outliers (%)	0.33
PDB code	6baq

Millipore) and separated using a S75 gel-filtration column (Fig. 2*a*). Fractions were analyzed by SDS–PAGE and those with >95% purity were combined (Fig. 2*b*), concentrated, snap-frozen using liquid nitrogen and stored at –80°C in buffer C.

2.3. Crystallization

Initial crystallization conditions were identified in 96-well sitting-drop trays using a Rigaku Phoenix Liquid Handler with 10.5 mg ml^{–1} mBPIFA1 in buffer C. Crystallization trays were monitored and tracked using Rigaku Gallery 700 Plate Hotels held at 20°C. The initial crystal hit for mBPIFA1 was 20% PEG 1000, 0.2 *M* lithium sulfate, 0.1 *M* phosphate–citrate pH 4.2. Crystals in this initial hit condition were reproduced in the laboratory by the hanging-drop vapor-diffusion crystallization method at 37°C (Fig. 2*c*). Crystals were looped and soaked in the crystallization condition with 20% glycerol for cryoprotection. Data were collected from these crystals to 2.5 Å resolution at 100 K on the General Medical Sciences and National Cancer Institute Collaborative Access Team (GM/CA CAT) beamline 23-ID-B at the Advanced Photon Source (APS).

2.4. Structure determination

The 2.5 Å resolution data collected at an X-ray wavelength of 1.03 Å were manually processed by *HKL-2000* (Otwinowski & Minor, 1997; Table 1). The structure of mBPIFA1 was determined with the *Phaser* molecular-replacement tool in *PHENIX* using chain *B* of the hBPIFA1 structure with the α 4 helix (residues 89–100) deleted as a search model (PDB entry 4gkh); non-identical residues were trimmed to alanines using *CHAINS*AW (McCoy *et al.*, 2007; Stein, 2008). After phasing, initial model building was performed in *PHENIX* via the *AutoBuild* function. Subsequent refinements and manual

building were performed in *PHENIX* and *Coot*, respectively (Adams *et al.*, 2010; Emsley *et al.*, 2010; Afonine *et al.*, 2012). The final model consisted of 1558 amino acids with eight molecules in the asymmetric unit and was refined to an *R*_{work} and *R*_{free} of 0.221 and 0.278, respectively (Table 1 and Fig. 2*d*). Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 6baq.

3. Results and discussion

Mouse BPIFA1 crystals were grown in 20% PEG 1000, 0.2 *M* lithium sulfate, 0.1 *M* phosphate–citrate pH 4.2 at 37°C (Fig. 2*c*). The crystal structure of mBPIFA1 was determined by molecular replacement using a modified chain *B* from the hBPIFA1 structure as a search model (PDB entry 4gkh; Garland *et al.*, 2013) and was refined to a final resolution of 2.5 Å using data collected at a wavelength of 1.03 Å on

Figure 4
The crystal structures of mouse and human BPIFA1. (*a*) Ribbon representation of the 2.5 Å resolution crystal structure of the mBPIFA1 (teal; PDB entry 6baq) chain *F* monomer revealing residues 66–277 and lacking residues 108–113. The secondary structure of mBPIFA1 is composed of six central β -sheets framed by six α -helices. (*b*) Superposition of mBPIFA1 and hBPIFA1 monomers (teal and pink, respectively). The proteins share 66% sequence identity and exhibit an r.m.s.d. of 2.1 Å. mBPIFA1 has a short helix-like turn between β -sheets 3 and 4.

beamline 23-ID-B (GM/CA CAT) at the Advanced Photon Source (APS). The mBPIFA1 crystals belonged to space group $C2$, with a solvent content of 50% and an asymmetric unit containing eight monomers. The final PDB-deposited model has an R_{work} of 0.221 and an R_{free} of 0.278 (Table 1; PDB entry 6baq). The most complete monomer chain (chain *F*) is composed of 212 residues encompassing residues 66–278, with one gap between residues 107 and 113 where electron density is absent (Fig. 4a). Consequently, the mBPIFA1 chain *F* monomer was used for all figures and root-mean-square deviation (r.m.s.d.) analyses of mBPIFA1. The mBPIFA1 structure is comprised of six central β -strands framed by six

surrounding α -helices (Figs. 3a and 4a). The eight mBPIFA1 monomer chains share an average r.m.s.d. of 1.1 Å between $C\alpha$ positions, indicating a high degree of structural similarity (Fig. 3a). A plot of r.m.s.d. per residue reveals the following regions of structural variation between monomers: the loop connecting $\beta 6$ and $\alpha 5$, the loop connecting $\beta 3$ and $\beta 4$, and the loop connecting $\beta 4$ and $\beta 5$ (Figs. 3b, 3c and 3d). Furthermore, while the r.m.s.d. per residue plot identifies structural variability in the $\alpha 3$ – $\alpha 4$ region, note that sparse electron density in this region makes it difficult to appreciate the true level of structural flexibility that is present in this area (Figs. 3b, 3c and 3d). The r.m.s.d. values and r.m.s.d. per residue number plots

Figure 5

Structural analysis of mBPIFA1 and hBPIFA1. (a) Comparison of the $\alpha 6$ helices of mBPIFA1 and hBPIFA1. The side chains of the histidine residues important for Orai1 binding are displayed. (b) Comparison of equivalent residue positions in the $\alpha 4$ regions of mBPIFA1 and hBPIFA1. Distances between equivalent mBPIFA1 (teal) and hBPIFA1 (pink) positions (Leu116/91, Thr121/96 and Pro125/100, respectively) are provided. (c) Structural alignment plot of r.m.s.d. per residue for mBPIFA1 and hBPIFA1. The residue numbering has expanded due to gaps introduced during the alignment of these two proteins of differing lengths.

here were generated using the *Visual Molecular Dynamics* (VMD) plugin *MultiSeq* (Roberts *et al.*, 2006; Humphrey *et al.*, 1996).

The overall fold of the mouse BPIFA1 protein is similar to that of human BPIFA1 (Fig. 4*b*); the two structures superimpose with an r.m.s.d. of 2.1 Å (Fig. 4*b*). Similar to the r.m.s.d. per residue structural alignment plot of individual mBPIFA1 monomers, the r.m.s.d. per residue structural alignment plot of human and mouse BPIFA1 illustrates that the greatest structural variability is found in the following connecting loop regions: $\beta 6$ – $\alpha 5$, $\beta 3$ – $\beta 4$ and $\beta 4$ – $\beta 5$ (Fig. 5*c*). mBPIFA1 is

missing interpretable electron density for seven amino acids in the glycine-rich loop region between helices $\alpha 3$ and $\alpha 4$. This is a modest improvement in the number of residues observed in this region of the protein compared with the hBPIFA1 structure, which lacked the electron density to model 12 amino acids between $\alpha 3$ and $\alpha 4$. This region has been shown to be important for the surfactant and antimicrobial actions of hBPIFA1 (Walton *et al.*, 2016).

Intriguingly, despite the high amino-acid sequence similarity between human and mouse BPIFA1 in this region (Fig. 1), the orientations of the $\alpha 4$ structural motifs differ between mBPIFA1 and hBPIFA1 (Figs. 4*b* and 5*b*). We find that $\alpha 4$ in mBPIFA1 folds into two distinct helices, $\alpha 4a$ (residues 112–123) and $\alpha 4b$ (residues 124–129), whereas it is a single helix in hBPIFA1 (residues 90–101) (Fig. 5*b*). Furthermore, the $\alpha 4$ motif in mBPIFA1 is shifted in position by 10–13 Å between equivalent C α positions relative to that observed in hBPIFA1 (Fig. 5*b*). These results demonstrate that the $\alpha 4$ region of the mammalian BPIFA1 proteins is variable in structure. These observations suggest that distinctions in function between mBPIFA1 and hBPIFA1 could arise from structural differences in this area of the protein, which has been established to be involved in surfactant and antibacterial activities (Walton *et al.*, 2016).

mBPIFA1 is a structural homolog of the known BPI-fold proteins latherin (PDB entry 3zpm) and the dust mite allergen Der f 7 (PDB entry 3uv1). mBPIFA1 has r.m.s.d.s of 2.9 and 3.1 Å with latherin and Der f 7, respectively (Figs. 6*a* and 6*b*; Vance *et al.*, 2013; Tan *et al.*, 2012), with relatively low sequence identities of 23% and 13%, respectively. In spite of this, the three proteins exhibit a similar overall fold (Figs. 6*a* and 6*b*), with the noted exception of the extended $\alpha 4$ region in mBPIFA1 relative to latherin and Der f 7 (Fig. 6*c*). This structural observation supports the hypothesis that the $\alpha 4$ motif is involved in the unique antibacterial activities of the BPIFA1 proteins, which have not been reported for latherin or Der f 7 (Walton *et al.*, 2016; Ahmad *et al.*, 2016; Emsley & Cowtan, 2004; Tan *et al.*, 2012).

Finally, we compared the surface electrostatics exhibited by mBPIFA1 and hBPIFA1 in a region previously shown to be important for hBPIFA1 function. In hBPIFA1, the charged surface electrostatic patch involving Asp112, Lys138, Arg152 and Asp193 was found to be necessary for the inhibition of ENaC (Figs. 7*a* and 7*b*; Walton *et al.*, 2016). hBPIFA1 presents a largely positive electrostatic patch in this region (Fig. 7*a*) owing to the arrangement of the amino acids on this surface region of the human protein (Fig. 7*b*). By contrast, mBPIFA1 exhibits a negative surface charge in the corresponding region of the protein (Fig. 7*c*). The residues in this area of mBPIFA1 (Asn137, Thr163, Gln176 and Asn217) contain only a single charged residue at physiological pH: Asp137 (Fig. 7*d*). Thus, this functionally validated region of hBPIFA1 is distinct in mBPIFA1, suggesting that the mouse protein may function uniquely relative to the human factor.

The β -ENaC proteins from human and mouse share 84% sequence identity and are comparable in length at 640 and 638 residues, respectively. In spite of this, we note that the

Figure 6
Comparison of mBPIFA1 with structural homologs. (a) Superposition of mBPIFA1 and latherin monomers (teal and yellow, respectively). The proteins share 23% sequence identity and exhibit an r.m.s.d. of 2.9 Å. (b) Superposition of mBPIFA1 and Der f 7 monomers (teal and orange, respectively). The proteins share 13% sequence identity and exhibit an r.m.s.d. of 3.1 Å. (c) Close-up of the $\alpha 4$ regions of mBPIFA1, latherin and Der f 7 (teal, yellow and orange, respectively).

Figure 7

Electrostatic surface maps of mBPIFA1 and hBPIFA1. (a) Electrostatic surface representation of the hBPIFA1 structure showing positively and negatively charged regions. (b) Close-up of Asp112, Lys138, Arg152 and Asp193 on the surface of hBPIFA1. (c) Electrostatic surface representation of the mBPIFA1 structure showing positively (blue) and negatively (red) charged regions. Surface electrostatic potential maps were generated by the vacuum electrostatic assessment action in *PyMOL* and values are calculated according to Coulomb's law. (d) Close-up of Asp137, Thr163, Gln176 and Asn217 on the surface of mBPIFA1.

N-terminus of mBPIFA1 is 24 residues longer than the N-terminal region of hBPIFA1 (Fig. 1), which may allow the mouse protein to position its β -ENaC-regulating N-terminal motif further away from the body of the protein to engage this ion-channel target. We speculate that this would allow mBPIFA1 to act on β -ENaC in a manner that is independent of pH. In contrast, the $\alpha 6$ motif of hBPIFA1, a functionally important motif for Orai1 inhibition, is highly conserved both sequentially and structurally between the human and mouse proteins (Figs. 1 and 5a). Furthermore, the proposed critical histidine residues of the $\alpha 6$ motif are conserved at equivalent residue positions (Figs. 1 and 5a; Wu *et al.*, 2017), which suggests that a conserved mechanism of Orai1 inhibition is shared between mBPIFA1 and hBPIFA1. Taken together, the structural data reported here provide a basis for understanding the potential functional differences between the multifunctional proteins mBPIFA1 and hBPIFA1.

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