Coordination of humoral immune factors dictates compatibility between

*Schistosoma mansoni* and *Biomphalaria glabrata*

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Abstract

Immune factors in snails of the genus *Biomphalaria* are critical for combating

*Schistosoma mansoni*, the predominant cause of human intestinal schistosomiasis.

Independently, many of these factors are known to play an important role in, but not fully define, the compatibility between the model snail *B. glabrata*, and *S. mansoni*. Here, we demonstrate association between four, previously characterized humoral immune molecules; *BgFREP3, BgTEP1, BgFREP2* and *Biomphalysin*. We also identify unique immune determinants in the plasma of *S. mansoni*-resistant *B. glabrata* that explain the incompatible phenotype. These factors coordinate to initiate haemocyte-mediated destruction of *S. mansoni* sporocysts via production of reactive oxygen species. The inclusion of *BgFREP2* in a *BgFREP3*-initiated complex that also includes *BgTEP1* almost completely explains resistance to *S. mansoni* in this model. Our study unifies many
independent lines of investigation to provide a more comprehensive understanding of the
snail immune system in the context of infection by this important human parasite.

Introduction

Schistosomiasis, a disease caused by parasitic trematodes of the genus *Schistosoma*,
is the second-most socioeconomically devastating parasitic disease, with an estimated
252 million people infected worldwide in 2015 (Disease, Injury, & Prevalence, 2016;
Mortality & Causes of Death, 2016). Larval digenean trematodes share a common feature
in the use of snails (gastropod mollusks) as intermediate hosts for transmission to a
vertebrate host (Esch, Barger, & Fellis, 2002). The snail *Biomphalaria glabrata* transmits
several species of trematode, including *Schistosoma mansoni*, the predominant causal
species of intestinal schistosomiasis (Pila, Gordy, et al., 2016). The molecular
interactions between *B. glabrata* and *S. mansoni* have been studied extensively towards
better understanding this essential life cycle stage of an important human parasite
(Adema, 2015).

Various genetically determined resistance phenotypes exist with respect to *S.
mansoni* infection in *B. glabrata* (Richards, 1975a, 1975b). Some *B. glabrata* snails (such
as BS-90 strain) naturally resist *S. mansoni* infection. Susceptible *B. glabrata* (such as M-
line strain) can develop transient levels of acquired resistance following previous
exposure to incompatible or radiation-attenuated parasites with modest cross protection to
related digenean species (Lie & Heyneman, 1975; Lie, Heyneman, & Lim, 1975;
Sullivan, Richards, Joe, & Heyneman, 1982). Previous research has focused on
identifying the mechanisms responsible for determining resistance profiles. Using in vivo
and in vitro models of *B. glabrata* snails, it has been demonstrated that the killing of *S. mansoni* larvae is associated with a haemocyte-mediated cytotoxic mechanism, and passive transfer of natural resistance to *S. mansoni* has been successfully accomplished when haemocytes from a susceptible *B. glabrata* strain are incubated in cell-free hemolymph (plasma) from a resistant strain (Bayne, Buckley, & DeWan, 1980a, 1980b; Granath & Yoshino, 1984; Loker & Bayne, 1982). Thus, haemocytes from resistant or susceptible *B. glabrata* strains do not appear to differ a priori in their cytotoxic capabilities, but their response requires activation by some humoral factor(s) (Bayne et al., 1980b; Granath & Yoshino, 1984; Vasquez & Sullivan, 2001), for proper recognition of *S. mansoni* and enhancing haemocyte cytotoxicity (Hahn, Bender, & Bayne, 2001).

Researchers have long sought immune determinants present in resistant *B. glabrata* plasma that specifically activate haemocytes to encapsulate and destroy *S. mansoni* sporocysts. Identified in *B. glabrata*, a large family of soluble lectins termed fibrinogen-related proteins (*BgFREPs*) attracted widespread interest because of their unique structure (Adema, Hertel, Miller, & Loker, 1997; Gordy, Pila, & Hanington, 2015), capacity for somatic diversification (Zhang, Adema, Kepler, & Loker, 2004), and immune function (Hanington et al., 2010; Hanington & Zhang, 2011). *BgFREPs* are composed of a C-terminal fibrinogen (FBG) domain and either one or two N-terminal immunoglobulin superfamily (IgSF) domains linked to the FBG domain by an interceding region (ICR) (Hanington et al., 2010; Pila, Li, Hambrook, Wu, & Hanington, 2017). Numerous studies have implicated *BgFREP3* as playing a central role in *B. glabrata* resistance to digenetic trematodes (Hanington et al., 2010; Hanington, Forys, & Loker, 2012; Hanington &
Zhang, 2011; Lockyer et al., 2012; Lockyer et al., 2008; Pila, Li, et al., 2017). **BgFREP3** contains two tandem IgSF domains whereas **BgFREP2** has one (Leonard, Adema, Zhang, & Loker, 2001). The expression of **BgFREP2** in BS-90 snails is dramatically up-regulated (57-fold) after exposure to *S. mansoni*, whereas M-line snails do not feature such a drastic upregulation, thereby suggesting a role for **BgFREP2** in resistance (Hertel, Adema, & Loker, 2005). Interestingly, the time course for heightened **BgFREP2** expression overlaps the interval within which sporocysts are encapsulated and killed, and only occurs in BS-90 haemocytes (Dinguirard et al., 2018; Hertel et al., 2005). Furthermore it was reported that **BgFREP2** is reactive with polymorphic glycoproteins found on the tegumental surface of larval *S. mansoni* (Mone et al., 2010).

In addition to **BgFREP**s, other soluble immune effector factors which are involved in both the direct killing of sporocysts and preparation of haemocytes to mount a cell-mediated response have been characterized (Pila, Li, et al., 2017). These factors include *B. glabrata* thioester-containing proteins (**BgTEP**s) (Bender, Fryer, & Bayne, 1992; Mone et al., 2010; Portet et al., 2018) and Biomphalysin (Galinier et al., 2013). **TEP**s are key components of invertebrate and vertebrat immune responses, aiding in melanization, opsonization, and killing of invading pathogens (Baxter et al., 2007; Blandin, Marois, & Levashina, 2008; Levashina et al., 2001; Pila, Li, et al., 2017; Portet et al., 2018; Povelones et al., 2013; Yassine & Osta, 2010). **BgTEP** was first characterized as an alpha macroglobulin proteinase inhibitor, and has recently re-immersed as a molecule of interest due to its association with **BgFREP2** and *S. mansoni* polymorphic mucins (*SmPoMucs*), and the capacity to bind to various invading pathogens (Bender et al., 1992;
Mone et al., 2010; Portet et al., 2018). Biomphalysin is a cytolytic protein in *B. glabrata* belonging to the β pore-forming toxin (β-PFT) superfamily (Galinier et al., 2013). Biomphalysin binds to the surface of *S. mansoni* sporocysts in the absence of plasma, while its cytolytic activity is drastically increased when plasma is present, suggesting that other factor(s) within the plasma may mediate the conversion of the oligomeric pre-pore to a functional pore (Galinier et al., 2013). Although the functional mechanisms of these factors are not thoroughly understood, studies suggest that these factors function as key determinants in the final outcome of *S. mansoni* challenge of *B. glabrata* (Galinier et al., 2013; Mone et al., 2010).

While studies have implicated *BgFREP3* in resistance to *S. mansoni*, the underlying mechanism of *BgFREP3* function; how it binds to *S. mansoni* sporocyst surfaces, and then how recognition is translated into haemocyte engagement, activation, and ultimately parasite encapsulation, is still unknown (Hanington et al., 2010). Here, we report an association between *BgFREP3*, *BgTEP1*, Biomphalysin (UniProtKB/TrEMBL: A0A182YTN9 and A0A182YTZ4) and *BgFREP2* (UniProtKB/TrEMBL: A0A2C9L9F5). *BgFREP3* associates with *BgTEP1* and Biomphalysin in both M-line and BS-90 strains, but only in BS-90 strain uniquely interacts with *BgFREP2* and other versions of *BgFREP3* (a variant of *BgFREP3.3*), providing us with an important insight into why BS-90 strain is refractory to *S. mansoni* infection. In this study, we demonstrate that *BgFREP3* binds to *S. mansoni* sporocysts without any other soluble plasma factors, yet *BgFREP2* relies on *BgTEP1*. However, *BgFREP3* still interacts with *BgTEP1* to form unique immune complexes, which significantly enhance the ability of haemocytes and
plasma from *S. mansoni*-susceptible *B. glabrata* (M-line) to kill *S. mansoni* sporocysts. A more striking finding is that the combination of *BgFREP3*, *BgTEP1* and *BgFREP2* renders M-line haemocytes capable of killing *S. mansoni* sporocysts at nearly the same level as *S. mansoni*-resistant BS-90 haemocytes. Reactive oxygen species (ROS) is shown to play an important role during this haemocyte-mediated killing of *S. mansoni* sporocysts. These results provide insight into how the numerous previously characterized immune factors known to be important in the anti-*S. mansoni* immune response to *B. glabrata* are acting in concert to defend the snail host.

**Results**

*BgFREP3* interacts with *BgTEP1*, Biomphalysin, *BgFREP2* and other *BgFREP3* variants in snail plasma.

To identify the factors in snail plasma which interact with *BgFREP3*, we produced recombinant *BgFREP3* (r*BgFREP3*, GenBank: AAK28656.1) using an insect expression system (Fig. 1A and Figure 1 – figure supplement 1) and performed a series of pull-down experiments. We initiated our investigations with *BgFREP3* because we observed that it is expressed at greater abundance in BS-90 compared with M-line snails. This observation was confirmed using Western-blot that showed that the hemolymph of BS-90 snails contain more *BgFREP3* than does M-line snail hemolymph (Fig. 1B). Among the proteins identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the eluent of these pull-down experiments was *BgTEP1* (Fig. 1C). We performed four r*BgFREP3* pull-down experiments, each yielded a band at approximately 200-kDa, which corresponded to the full-length *BgTEP1*, which was identified from both M-line and BS-
90 strains (Fig. 1C). In three instances, we cut the bands and sent for mass spectrometry identification, two of which detected the \textit{B}g\textit{TEP}1 pulled down by \textit{rB}g\textit{FREP}3 in \textit{M}-line plasma, while the third detected \textit{B}g\textit{TEP}1 in both \textit{M}-line and BS-90 strains (Fig. 1D). From the LC-MS/MS analysis we obtained a total of 17 unique peptides, 16 peptides were detected from \textit{M}-line strain, 4 peptides were from BS-90 strain, and 3 peptides were present in both BS-90 and \textit{M}-line strains (Table. S2 and Fig. 1E). While all peptides mapped to \textit{B}g\textit{TEP}, there is uncertainty as to which specific \textit{B}g\textit{TEP} proteins are present. Twelve peptides mapped to \textit{B}g\textit{TEP}-ACL00841.1; 15 peptides mapped to \textit{B}g\textit{TEP}1.1-AD\textit{E}45332.1, \textit{B}g\textit{TEP}1.2-AD\textit{E}45339.1, \textit{B}g\textit{TEP}1.3-AD\textit{E}45340.1, \textit{B}g\textit{TEP}1 1.4-AD\textit{E}45341.1 and \textit{B}g\textit{TEP}1.5-AD\textit{E}45333.1, however, there was overlap between some of these peptides, which mapped to all known \textit{B}g\textit{TEP} sequences (Figure 1 – figure supplement 2). The identified \textit{B}g\textit{TEP}1 showed a relatively large difference from \textit{B}g\textit{TEP} (ACL00841.1), but could not be distinguished from other \textit{B}g\textit{TEP}1 sequences (Figure 1 – figure supplement 2), so we termed it \textit{B}g\textit{TEP}1.

The full-length sequence of \textit{B}g\textit{TEP}1 generally contains 1445 or 1446 amino acids (Figure 1 – figure supplement 2). The peptides identified by LC-MS/MS cover 16.3% of the full-length sequence (Figure 1 – figure supplement 2). The identified peptides are distributed over the full-length \textit{B}g\textit{TEP}1, with the peptides identified from the BS-90 strain being concentrated in the N-terminal region (Fig. 1D). Background transcript abundance of \textit{B}g\textit{TEP}1 detected by qRT-PCR in \textit{M}-line and BS-90 snails, suggest that there is no significant difference (\( P > 0.05 \)) in baseline \textit{B}g\textit{TEP}1 expression between the two strains (Figure 1 – figure supplement 3). These results indicate that the interaction of \textit{B}g\textit{FREP}3 with \textit{B}g\textit{TEP}1 doesn’t require the involvement of any parasite components.
Recombinant BgTEP1 (rBgTEP1, GenBank: ADE45332.1) was also used to perform a series of pull-down experiments (Fig. 1A and E). Biomphalysin (UniProtKB/TrEMBL: A0A182YTN9 and A0A182YTZ4) was identified in both M-line and BS-90 plasma from rBgTEP1 pull-down experiments and was also found to be present in the pull-down studies using BgFREP3 (Fig. 1C and E). Analysis suggests that this Biomphalysin (A0A182YTZ4) was most similar (97% amino acid identity) to the Biomphalysin previously reported by Galinier and Portela et al. (Galinier et al., 2013) (GenBank: KC012466) (Figure 1 – figure supplement 4). The 13 peptides identified by LC-MS/MS covered 28.3% of Biomphalysin (A0A182YTN9) amino acid sequence (Fig. 1F, Table S2 and Figure 1 – figure supplement 4). These peptides were evenly distributed throughout the Biomphalysin protein (Fig. 1F). The theoretical molecular weight of Biomphalysin (A0A182YTN9 and A0A182YTZ4) is ~64-kDa. The Biomphalysin we identified via protein pull-down emerged at three different molecular weights, one at > 400-kDa (Fig. 1C), which likely represents the heptamer formed by Biomphalysin (Galinier et al., 2013), the second site at roughly 60-kDa (Fig. 1C and E), which is in agreement with the theoretical molecular weight, and the third at around 55-kDa (Fig. 1C), which may represent the size after proteolysis (Galinier et al., 2013).

Comparing rBgFREP3 pull-down results between M-line and BS-90 *B. glabrata* identified 2 unique proteins in the BS-90 lane. One of these proteins is BgFREP2 (UniProtKB/TrEMBL: A0A2C9L9F5) (Fig. 1C and E). Five peptides were identified by LC-MS/MS analysis and covered 16.6% of BgFREP2 (GenBank: AAK13550.1) amino acid sequence and were located throughout the IgSF and FBG of the protein (Fig. 1G and Table S2 and Figure 1 – figure supplement 5). Three of the five identified peptides
specifically belonged to *BgFREP2* (AAK13550.1), so the identified *BgFREP2* appears to represent a specific *BgFREP2* variant that is distinguished from other *BgFREP* members that have been published (Figure 1 – figure supplement 5).

Only *BgFREP2* was identified, at ~55-kDa, from the BS-90 strain plasma when *rBgFREP3* was used as the bait protein in pull-down experiments (Fig. 1C). This contrasts with our other data in which *BgFREP2* was identified from plasma of both M-line and BS-90 strains when *rBgTEP1* was used as bait in pull-down experiments (Fig. 1E). *BgFREP2* was identified by LC-MS/MS from two distinct molecular weight bands in the *rBgTEP1* study, one band, at ~60 kDa also included peptides that mapped to Biomphalysin, the other band, identified at ~55-kDa was contained peptides for only *BgFREP2* (Fig. 1C and E). Thus, all of the *BgFREP2* peptides were identified from protein bands that were of greater molecular weight than the predicted *BgFREP2*, which is 43.8-kDa. However, this is consistent with the observed molecular weight of *BgFREP2* by Moné *et al.* (Mone et al., 2010), and could be explained by post-translational modifications such as glycosylation (Adema et al., 1997; Mone et al., 2010; Zhang, Zeng, & Loker, 2008).

Another unique protein identified from the BS-90 lane was a variant of *BgFREP3*. Of the 9 peptides identified by LC-MS/MS, 7 specifically matched *BgFREP3.3* (Genbank: AEO50747.1), the other two matched other members of *BgFREP3* family (*BgFREP3.3-AAO59915.1, BgFREP3.2-AEO50746.1, BgFREP3.2-AAK28656.1* and *BgMFREP3-AAK13548.1*), suggesting that this protein is likely a FREP3 variant (Figure 1 – figure supplement 6 and Table S2). The 7 peptides that specifically belonged to *BgFREP3.3* (AEO50747.1) covered 15.6% of the amino acid sequence length and mainly...
concentrated in the interceding region (ICR) (5 peptides), one peptide in each of the IgSF2 domain and FBG domain of $Bg$FREP3.3 were also identified (Figure 1 – figure supplement 6).

**$Bg$FREP3 independently recognizes and binds to primary sporocysts of *S. mansoni.***

Our data suggests a close and complex interaction between $Bg$FREP3, $Bg$TEP1, Biomphalysin and $Bg$FREP2. These factors have each independently been reported to be involved in the recognition of *S. mansoni* sporocysts (Adema et al., 1997; Galinier et al., 2013; Hanington et al., 2010; Hanington et al., 2012; Mone et al., 2010; Portet et al., 2018; X. J. Wu et al., 2017). However, only Biomphalysin is known to directly bind to the surface of *S. mansoni* sporocysts without the aid of any other plasma factors (Galinier et al., 2013). The mechanism by which $Bg$FREP3, $Bg$TEP1 and $Bg$FREP2 bind to primary sporocysts of *S. mansoni* is still not clear. To explore this issue, we produced r$Bg$FREP3, r$Bg$TEP1 and r$Bg$FREP2 (Fig. 1A) to observe whether they associate with primary sporocysts of *S. mansoni*. Immunocytochemistry clearly shows that r$Bg$FREP3 is able to interact with *S. mansoni* sporocysts independently, while r$Bg$TEP1 and r$Bg$FREP2 alone do not have such capabilities (Fig. 2). However, incubation of sporocysts with pre-combined r$Bg$FREP2 and r$Bg$TEP1 yields a signal, suggesting that these two factors require complex formation prior to being able to associate with *S. mansoni* sporocysts (Fig. 2).

**$Bg$FREP3 and $Bg$TEP1 form an immune complex.**

To further investigate whether $Bg$FREP3 and $Bg$TEP1 form a complex, we carried out a set of immunoblotting experiments with recombinant proteins. As expected, r$Bg$FREP3 did form a complex with r$Bg$TEP1 (Fig. 3A). The complex appeared in a
region of high molecular weight (> 460-kDa), and the signal decreased gradually with the decrease of rBgTEP1:rBgFREP3 ratio in a dose-dependent manner (Fig. 3A). As shown in Fig. 3A, rBgFREP3 exists as a high molecular weight multimer in a non-denatured state. Since the molecular weights of the rBgFREP3 multimer and the rBgFREP3-rBgTEP1 complex were both > 460-kDa, it was difficult to distinguish them using Western bolt. To confirm the interaction between rBgFREP3 and rBgTEP1, a far-Western blotting approach, in which decreasing amount of rBgTEP1 was imprinted on the membrane, and then detected by using biotinylated rBgFREP3 as primary recognition agent was performed. A band was detected in the lane with the largest amount of rBgTEP1, confirming the interaction between rBgFREP3 and rBgTEP1 (Fig. 3B).

BgFREP2 and BgTEP1 have been shown to form immune complexes with SmPoMucs derived from S. mansoni (Mone et al., 2010), suggesting that BgFREP2 and BgTEP1 associate with each other in snail plasma. To test this assumption, we performed a set of immunoblot assays using rBgFREP2 and rBgTEP1. The results of Western blot under non-denaturing conditions showed that a protein at > 460 kDa after mixing rBgFREP2 and rBgTEP1. This protein decreased in intensity on the Western blot in a dose dependent manner along with the decrease of the rBgTEP1:rBgFREP2 ratio (Fig. 3C upper panel). However, rBgFREP2 does not exist as a multimer under non-denaturing conditions (Fig. 3C upper panel), which is consistent with the findings of Zhang et al. that BgFREP2 did not form a multimer in snail plasma (Zhang et al., 2008). In addition, we further verified the interaction between rBgFREP2 and rBgTEP1 by Far-Western blot (Fig. 3C lower panel).
Association of BgFREP3 and BgTEP1 facilitates recognition and killing of *S. mansoni* sporocysts.

Since BgFREP3 does not rely on BgTEP1 to recognize pathogens, but does associate with BgTEP1, we hypothesize that BgTEP1 plays a role in the downstream immune response triggered by BgFREP3 recognition of *S. mansoni* sporocysts. We treated primary sporocysts with haemocytes or plasma from BS-90 and M-line *B. glabrata* snails with rBgFREP3, rBgTEP1 and a combination of the two in vitro.

Plasma (hemoglobin-low/free) from the resistant BS-90 strain of *B. glabrata* snails is naturally able to kill 67% (SEM 3.7%; n=10) of *S. mansoni* sporocysts by 48 h post incubation, while plasma from the susceptible M-line strain, which kills 20% (SEM 4.5%; n=10), is not significantly more effective than the medium control in which 13% (SEM 2.1%; n=10) were killed at 48 h (Fig. 4A). The addition of rBgFREP3 (36% [SEM 5.0%; n=10]) and rBgTEP1 (32% [SEM 3.3%; n=10]) independently did not significantly enhanced the ability of M-line plasma to kill *S. mansoni* sporocysts by 48 h (Fig. 4A).

However, the combined addition of rBgFREP3 and rBgTEP1 did significant increase M-line-plasma-mediated killing of *S. mansoni* sporocysts by 48 h post incubation (56% [SEM 4.8%; n=10]) compared to the application of either recombinant protein alone. The combined addition of rBgFREP3 and rBgTEP1 rendered the capacity of M-line plasma able to kill *S. mansoni* sporocysts statistically insignificant from BS-90 plasma (P > 0.05) (Fig. 4A). Recombinant proteins, in the absence of *B. glabrata* plasma, did not display any capacity to kill *S. mansoni* sporocysts (Figure 4 – figure supplement 1A). This suggests that rBgFREP3 and rBgTEP1 activate a factor in plasma to confer the ability to kill sporocysts.
Haemocytes derived from BS-90 strain *B. glabrata* are innately capable of destroying *S. mansoni* sporocysts, killing 88% (SEM 3.6%; n=10) of sporocysts after 48 h of incubation in vitro (Fig. 4B). The basal ability of M-line haemocytes to kill sporocysts (13% [SEM 3%; n=10]) is not significantly different from the medium-alone control (13% [SEM 2.1%; n=10]), and is only slightly increased by adding r*Bg*FREP3 (24% [SEM 3.4%; n=10]) at 48 h (Fig. 4B). Addition of r*Bg*TEP1 did significantly increase sporocyst killing by M-line haemocytes to 40% (SEM 3.9%; n=10) (Fig. 4B), however, this killing response was significantly enhanced to 61% (SEM 5.5%; n=10) when r*Bg*FREP3 and r*Bg*TEP1 were incubated with M-line haemocytes together (Fig. 4B). However, the combination of r*Bg*FREP3 and r*Bg*TEP1 did not convey a killing capacity to M-line snails that paralleled BS-90 haemocytes (Fig. 4B). This suggests that there is yet another factor(s) affecting this process, causing haemocytes from BS-90 and M-line to differ in their ability to destroy sporocysts. In our r*Bg*FREP3 pull-down experiments, *Bg*FREP2 was one of the two proteins uniquely identified as associating with r*Bg*FREP3 in the plasma of BS-90 *B. glabrata* (Fig. 1C). Incubation of r*Bg*FREP2 in combination with r*Bg*FREP3 and r*Bg*TEP1 enabled M-line haemocytes to kill *S. mansoni* sporocysts (83% [SEM 4.8%; n=6]) to statistically indistinguishable (p > 0.05) levels as BS-90 haemocytes (88% [SEM 4.7%; n=6]) (Fig. 4C). To verify whether the r*Bg*FREP2-r*Bg*TEP1 complex dramatically enhanced the ability of M-line haemocytes to killing *S. mansoni* sporocysts above either factor independently, as does the r*Bg*FREP3-r*Bg*TEP1 complex, a similar experiment was performed, showing that r*Bg*FREP2-r*Bg*TEP1 did not play such a role (Fig. 4D). Independent treatments of r*Bg*FREP2 (21% [SEM 3.1%; n=10]) and r*Bg*TEP1 served controls (Figure 4 – figure supplement 1B),
demonstrating that BgFREP2 on alone does not facilitate sporocyst killing above control, whereas rBgTEP1 killing (50% [SEM 3.9%; n=10]) is indistinguishable (P > 0.05) from rBgFREP2 and rBgTEP1 combined (55% [SEM 3.7%; n=10]) (Fig. 4D).

These results made us speculate that BgFREP3 was the determining factor controlling sporocyst engagement and killing. Addition of three anti-rBgFREP3 antibodies to the mixture of rBgFREP3, rBgTEP1 and rBgFREP2 significantly abrogates the increase in the ability of M-line haemocytes to kill sporocysts (51% [SEM 3.1%; n=6] compared to 83% [SEM 4.8%; n=6]) (Fig. 4C). Pre-incubation of BS-90 haemocytes with the anti-rBgFREP3 antibodies also significantly reduced their ability to destroy sporocysts (68% [SEM 3.1%; n=6] compared to 88% [SEM 4.7%; n=6]) (Fig. 4C).

Combined pre-immunized IgG purified from serum from the same rabbits from which the antibodies were produced was used as a negative control, demonstrating that it did not significantly affect the killing S. mansoni sporocysts by M-line (51% [SEM 3%; n=6]) and BS-90 haemocytes (90% [SEM 3.7%; n=6]) (Figure 4 – figure supplement 1C).

These results suggest that rBgFREP3 plays a central role in initiating snail haemocyte-mediated anti-S. mansoni immune responses.

Finally, we hypothesize that haemocyte-mediated killing likely involves the production of ROS, which have been shown to be crucial to the haemocyte-mediated clearance of S. mansoni sporocysts (Hahn, Bender, & Bayne, 2000; Hahn et al., 2001). To investigate the role of ROS in the combined ability of rBgFREP3 and rBgTEP1 to enhance the ability of M-line haemocytes to kill S. mansoni sporocysts, ROS inhibitors N-acetyl-L-cysteine (NAC) and Diphenyleneiodonium (DPI) were applied to the sporocyst killing assays. Co-incubation of BS-90 haemocytes with NAC and DPI
significantly reduced their ability to kill *S. mansoni* sporocysts (45% [SEM 5.6%; n=6] and 57% [SEM 4.2%; n=6] respectively) compared to BS-90 haemocytes in the vehicle control (89% [SEM 3.1%; n=6]) by 48 h post incubation (Fig. 4E). The ability of M-line haemocytes primed by the r*BgFREP3-r*BgTEP1* complex to destroy sporocysts (67% [SEM 4.9%; n=6]) was also significantly abrogated by the addition of NAC (40% [SEM 3.7%; n=6]) or DPI (52% [SEM 3.1%; n=6]) (Fig. 4F).

**Discussion**

In 1984, Yoshino *et al.* successfully achieved passive transfer of resistance to *S. mansoni* from a refractory *B. glabrata* strain to susceptible snails via injection of cell-free hemolymph (plasma) (Granath & Yoshino, 1984). Similar passive transfer of resistance had previously been shown using an in vitro model (Bayne et al., 1980a, 1980b; Loker & Bayne, 1982). Although the oxidation of haemoglobin, which is the main protein component (97% of total protein) in *B. glabrata* plasma was later found to be toxic to *S. mansoni* sporocysts in long-term cultures (Bender, Bixler, Lerner, & Bayne, 2002), and thus likely explained the passive transfer study results, further studies suggest that specific anti-parasitic factors must be present at or above a certain threshold level in *S. mansoni*-resistant snail plasma to stimulate a haemocyte-mediated destruction of sporocysts within 24–48 h post-infection (Dinguirard et al., 2018). Although many important immune factors have been identified from *B. glabrata* plasma/haemocytes, such as *BgFREPs* (Adema et al., 1997), *BgTEP1* (Mone et al., 2010), Biomphalysin (Galinier et al., 2013), Toll-like receptors (*BgTLRs*) (Pila, Tarrabain, Kabore, & Hanington, 2016), granulin (*BgGRN*) (Pila, Gordy, et al., 2016) and macrophage...
migration inhibitory factor (BgMIF) (Baeza Garcia et al., 2010), it is still not known which factors ultimately underpin Biomphalaria immunity *S. mansoni*. Here, we provide insight into the broader interactions that take place between *B. glabrata* immune factors that leads to development of an *S. mansoni*-resistant phenotype.

Previous studies have shown that BgFREPs are capable of binding to digenean trematode sporocysts and are up-regulated in response to larval infection (Adema et al., 1997; X. J. Wu et al., 2017; Zhang, Leonard, Adema, & Loker, 2001). BgFREP3 has been shown to be a central part of the anti-*S. mansoni* immune response (Hanington et al., 2010). BgFREP3 is up-regulated based on three characteristics (size, strain, and acquired resistance) of resistance of *B. glabrata* to infection with *S. mansoni* or *Echinostoma paraensei* (Hanington et al., 2010). Knock-down of BgFREP3 in resistant snails using siRNA-mediated interference altered the phenotype of resistant *B. glabrata* by increasing susceptibility to *E. paraensei* infection (Adema, 2015; Hanington et al., 2010).

Nevertheless, we have little knowledge of the underlying mechanism by which BgFREP3 interacts with other snail plasma factors in order to signal and trigger an immune response after recognizing the pathogen. In order to explore this mechanism, we conducted a series of pull-down experiments that allowed us to discover the interaction between BgTEP1, Biomphalysin and BgFREP2 with BgFREP3 (Fig. 1).

Proteomic analysis of *B. glabrata* plasma revealed that BgFREP3, BgTEP1 and BgFREP2 display affinity for *S. mansoni* sporocyst tegumental membrane proteins (Portet et al., 2018; X. J. Wu et al., 2017). BgTEP1 was previously reported to play a role in BgFREP2 recognition of *S. mansoni* sporocyst SmPoMucs, (Mone et al., 2010; Portet et al., 2018). Our immunofluorescence results illustrate that BgFREP3 independently
recognizes *S. mansoni* sporocysts, whereas *BgFREP2* must be involved with the participation of *BgTEP1* (Fig. 2). We speculate that this may be due to their structural differences: *BgFREP3* possesses two IgSF domains, yet *BgFREP2* has only one; *BgFREP3* exists as a multimer in its natural state (Fig. 3), whereas *BgFREP2* does not (Zhang et al., 2008). Alternatively, it is possible that the targets they recognize are different, so the immunoaffinity reactions that occur are also fundamentally different. A recent report suggests that *BgFREPs* are likely to form multimers through the coiled-coil region of the ICR. However, this has not been supported by experimental evidence and does not completely rule out the possibility that *BgFREP* multimer formation is mediated by the FBG or IgSF domain (Gorbushin, 2019).

*BgTEP1* and *BgFREP3* form a complex without any *S. mansoni* molecules present (Fig. 3). Since *BgFREP3* does not require *BgTEP1* to recognize pathogens, *BgTEP1* is more likely to play a role in the downstream immune response initiated by *BgFREP3*. Following the addition of *rBgFREP3* and *rBgTEP1*, plasma demonstrated an increased capacity to kill sporocysts compared to controls (Fig. 4A). Because the *B. glabrata* plasma used in these tests had haemoglobin removed, the toxicity of hemoglobin oxidation to *S. mansoni* sporocysts was ruled out. These studies also demonstrated that recombinant *BgFREP3* and *BgTEP1* alone were not able to increase sporocyst killing, but showed that the addition of cell-free plasma with both recombinants was required, thereby implying the necessity of another factor. Based on our pull-down experiments, we think that this factor could be a Biomphalysin. Biomphalysin displays similarities to members of the β-PFT superfamily (Howard & Buckley, 1985; MacKenzie, Hirama, & Buckley, 1999; Wilmsen, Leonard, Tichelaar, Buckley, & Pattus, 1992; Xu, Wang, Yu, &
Sun, 2014), and has cytolytic activity mediated by a plasma factor(s) that remains to be identified (Galinier et al., 2013). Our results suggest a scenario in which BgFREP3 and/or BgTEP1 could be the factors associating with Biomphalysin to promote plasma-mediated sporocyst killing. BgFREP3 and/or BgTEP1 might activate Biomphalysin in an unknown manner, assist in the oligomerization of Biomphalysin while it forms its heptameric channel, or mediate the conversion of the oligomeric prepore to a functional pore. Further investigation is warranted to illustrate the role of BgFREP3 and BgTEP1 in the activation of Biomphalysin.

Within 2–3 h of S. mansoni entry into B. glabrata, haemocytes surround the developing sporocysts forming multi-layered cellular capsules that kill encapsulated larvae within 24–48 h post-infection (Dinguirard et al., 2018; Loker & Bayne, 1982). This occurs in both susceptible and resistant snail strains and has been replicated in our in vitro functional studies, where only resistant snails can eventually destroy the sporocysts without external assistance. Dinguirard et al. conducted a comparative proteomic analysis on the responses of haemocytes participating in S. mansoni sporocyst encapsulation reactions from susceptible and resistant B. glabrata strains, showing that striking differences in proteins expressed, with susceptible snail haemocytes exhibiting extensive downregulation of protein expression and a lower level of constitutively expressed proteins involved immunity (e.g., BgFREP2) and oxidation-reduction compared to resistant snails (Dinguirard et al., 2018). This could explain why BS-90 haemocytes alone can kill S. mansoni sporocysts. After challenge, the transcript abundance of numerous BgFREPs increases by 3-fold or greater in BS-90 when compared to M-line B. glabrata (Adema et al., 1997). Noteworthy in the context of our current study is that following
challenge BS-90 snails with *S. mansoni*, transcript abundance for *BgFREP2* increases over 50-fold by 1 day post-exposure (Dinguirard et al., 2018; Hertel et al., 2005), *BgFREP3* increases around 4-fold by 1 day post-exposure (Hanington et al., 2010), and *BgTEP1* which increases by 2- to 3.5-fold from 6 to 24 h post-exposure (Portet et al., 2018). These dramatic increases in the abundance of immune-relevant transcripts occurring over the first 12-24 h post exposure to *S. mansoni* sporocysts in BS-90 snails aligns closely with our results that demonstrate sporocyst killing by BS-90 haemocytes rapidly increases after 12 h post-exposure (Fig. 4).

Numerous studies have demonstrated the production of ROS by haemocytes in *B. glabrata*, especially H2O2, which plays a vital role in anti-schistosome defense (Galinier et al., 2013; Hahn et al., 2000, 2001). Haemocytes from *S. mansoni*-resistant snails produce significantly more ROS than susceptible snails (Bender, Broderick, Goodall, & Bayne, 2005; Bender, Goodall, Blouin, & Bayne, 2007; Goodall, Bender, Broderick, & Bayne, 2004). In our study, ROS was found to be critical in BS-90 haemocyte-mediated *S. mansoni* sporocyst killing, as this ability was significantly reduced after pre-incubation with the ROS inhibitors NAC and DPI (Fig. 4E). However, according to the phenomenon of passively transferred resistance, the cytotoxic potential of susceptible haemocytes may not fundamentally different from resistant snails (Granath & Yoshino, 1984). We infer that M-line plasma may lack some factors or the levels of these factors do not reach a certain threshold and fail to stimulate haemocytes to produce ROS, or M-line haemocytes are short of some receptors to receive signals that up-regulate ROS. Previously, our team identified a toll-like receptor (*BgTLR*) from haemocyte surface of *B. glabrata* snails, which differed in abundance between M-line and BS-90 strains (Pila, Tarrabain, et al., 19...
2016). It has been repeatedly demonstrated in other organisms that activation of Toll-like receptors is associated with increased intracellular ROS (Asehnoune, Strassheim, Mitra, Kim, & Abraham, 2004; Tsung et al., 2007; Wong et al., 2009).

Our results indicate that the \( Bg\)FREP3-\( Bg\)TEP1 complex plays a role in raising ROS levels in M-line haemocytes (Fig. 4F). We found that BS-90 haemolymph contains more \( Bg\)FREP3 protein than M-line (Fig. 1B). Moreover, the BS-90 haemocytes significantly increased the expression level of \( Bg\)FREP2 during encapsulating \( S. \) mansoni sporocysts compared with susceptible snail haemocytes (Dinguirard et al., 2018). These suggest that \( Bg\)FREP3 and \( Bg\)FREP2 may play a crucial role in inducing haemocytes which take part in encapsulating sporocysts to generate ROS. We suppose that \( Bg\)TEP1 also plays an opsonin role in the \( B. \) glabrata snail haemolymph, which interacts with molecules on the surface of haemocytes, thereby promoting the phagocytosis of pathogens.

Finally, our data hints at fundamental differences in the \( Bg\)FREP3-mediated immune response between BS-90 and M-line \( B. \) glabrata. Protein Pull-down experiments yielded two obvious and unique protein bands that were present in BS-90 but not M-line pull-downs. We identified the bands to be a variant of \( Bg\)FREP3.3 and \( Bg\)FREP2 (Fig. 1C), showing different immune factor interactomes between different strains. This suggests that \( Bg\)FREP3 variants and \( Bg\)FREP2 in BS-90 plasma are more prone to \( rBg\)FREP3 binding, and the combination of different versions of \( Bg\)FREPs seems to play an important and unknown role in snail resistance. Both \( Bg\)FREP2 and \( Bg\)FREP3 are characterized by high diversity, our results imply that the versions of \( Bg\)FREP2 and \( Bg\)FREP3 in the two snail strains are different. After exposure to \( S. \) mansoni, the expression of \( Bg\)FREP2 in resistant snails is superior to that of susceptible snails.
(Dinguirard et al., 2018; Gordy et al., 2015; Hertel et al., 2005). This again suggests that

BgFREP2 is important in determining the outcome of the compatibility of B. glabrata
against S. mansoni. Our study implies that BgFREP3 seems to play an "all or none"
switching effect in determining B. glabrata-S. mansoni compatibility, while BgFREP2
seems to play an additive role.

It has been decades since the discovery and initial characterization of BgFREPs in
the B. glabrata immune response to S. mansoni. While numerous studies since have
implicated both BgFREPs (BgFREP3 and BgFREP2) along with a suite of other immune
factors, as being important elements of the snail immune response to S. mansoni, how the
response is coordinated has remained elusive. Our study presents a model in which many
of the known determinants of snail resistance to S. mansoni are found to work in concert
to protect the snail host against infection. We map a mechanism for the interaction of
BgFREP3, BgTEP1, BgFREP2 and Biomphalysin with S. mansoni sporocysts in B.
glabrata snail haemolymph that ultimately leads to sporocyst killing (Fig. 5). Central to
this response is BgFREP3, which is able to recruit unique representatives of both
BgFREP2 and BgFREP3 in S. mansoni-resistant BS-90 B. glabrata. With this new-found
understanding of the B. glabrata immune response comes the ability to now undertake
comprehensive assessments of how modifications to both the snail host and the parasite
influence the underpinning immunological drivers of compatibility in this important
model for studying human schistosomes.

Materials and Methods

Live material and Experimental Treatments.
M-line and BS-90 strain *B. glabrata* snails were used in this study. The M-line strain is highly susceptible to the PR-1/ NMRI strain *S. mansoni* infection (Newton, 1955), while the BS-90 strain is resistant. M-line and BS-90 strain *B. glabrata* snails, and *S. mansoni* were maintained at the University of Alberta as described previously (Pila, Gordy, et al., 2016). NMRI strain of *S. mansoni* was obtained from infected Swiss-Webster mice provided by the NIH/NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (Cody et al., 2016). All animal work observed ethical requirements and was approved by the Canadian Council of Animal Care and Use Committee (Biosciences) for the University of Alberta (AUP00000057).

**Recombinant BgFREP3, BgTEP1 and BgFREP2 Synthesis and Purification.**

Recombinant *BgFREP3* (r*BgFREP3*, derived from GenBank: AY028461.1), *BgTEP1* (r*BgTEP1*, GenBank: HM003907.1) and *BgFREP2* (r*BgFREP2*, GenBank: AY012700.1) were generated by using the Gateway cloning system according to the manufacturer’s instructions (Life Technologies) as previously described (Hambrook, Kabore, Pila, & Hanington, 2018; Pila, Gordy, et al., 2016; Pila, Peck, & Hanington, 2017; Pila, Tarrabain, et al., 2016), with primer information as shown in Table. S1 and Sf9 cell codon-optimized *BgFREP3* Genscript as shown in Fig. S1. Briefly, the coding regions were amplified with Phusion high-fidelity DNA polymerase from targeted DNA templates in pUC57 plasmids (synthesized by GenScript). Blunt-end PCR products were cloned into the pENTR/D-TOPO vectors to generate entry clones. Plasmids from these entry clones were extracted and cloned into pIB/V5-His-DEST vectors in a Clonase recombination reaction to produce the expression clones. Recombinant plasmids were extracted from the expression clones and then transfected into Sf9 insect cells using...
Cellfectin reagent (Life Technologies). Cells that survived the screening of the antibiotic blasticidin (Thermo Fisher Scientific) were collected and lysed. Protein expression of \(rBG\)FREP3, \(rBG\)TEP1 and \(rBG\)FREP2 were detected by Western blot using monoclonal antibody against the V5 tag (Thermo Fisher Scientific) on the recombinant proteins.

Sf9 cells stably expressing recombinant \(rBG\)FREP3, \(rBG\)TEP1 and \(rBG\)FREP2 were selected, some of them were frozen as a seed stock and the rest were maintained in a medium containing blasticidin. Cultures of Sf9 cells expressing recombinant proteins were scaled up, and the pellet (containing \(rBG\)FREP3) or the medium (containing secreted \(rBG\)TEP1 and \(rBG\)FREP2) was collected. Recombinant proteins were purified from the cell pellet lysate or the culture medium with HisTrap FF (GE Healthcare) columns using fast protein liquid chromatography (ÄKTA Pure, GE Healthcare). Purified \(rBG\)FREP3, \(rBG\)TEP1 and \(rBG\)FREP2 were dialyzed against PBS buffer twice for 2 h each and then once overnight using Slide-A-Lyzer dialysis kit (Thermo Scientific).

**Preparation of *B. glabrata* plasma and pull-down experiment.**

Hemolymph was obtained from M-line and BS-90 strain *B. glabrata* snails by the head-foot retraction method (Pila, Gordy, et al., 2016). Upon collection, hemolymph from ~5 snails of each strain (8–12 mm in diameter) was immediately placed in 1.5 mL tubes on ice, EDTA was added to a final concentration of 150 μg/mL. Samples were centrifuged twice at 20,000 \(\times\) g for 15 min and the cell-free fraction was collected. The 4 M Imidazole Stock Solution was added to a final concentration of 10 mM imidazole. Plasma samples were used immediately for subsequent pull-down experiment.

Pull-down assays were conducted by using Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific) according to the manufacturer’s instructions with...
some modifications. Briefly, first we prepared the “bait” proteins, rBgfREP3 and rBgTEP1, from previously purified proteins or Sf9 cell lysate expressing rBgfREP3 or concentrated medium containing rBgTEP1. We then loaded the HisPur Cobalt Resin equilibrated for at least 30 minutes at room temperature onto the column according to the kit instructions. The prepared polyhistidine-tagged rBgfREP3 and rBgTEP1 was immobilized on the columns after five times washing with wash solution [1:1 of Tris-buffered saline (TBS) solution:Pierce Lysis Buffer containing 10 mM imidazole]. For cell lysates or concentrated medium containing rBgfREP3 and rBgTEP1, protein expression was previously analyzed by SDS-PAGE and Western blot, and 800 µL was added; for purified rBgfREP3, at least 150 µg (about 500 µL) was added. After incubation for 2 h at room temperature or overnight at 4°C, the columns were washed 5-8 times with wash solution and then 800 µL of prepared plasma (prey) containing 10 mM imidazole was added to the columns. The columns were incubated at 4°C for overnight with gentle rocking motion every few h. After that, the columns were washed 5-8 times with the wash solution, and then eluent was added and incubated at room temperature for 30 min with gentle rocking on a rotating platform.

The eluent collected by centrifugation was used for SDS-PAGE analysis and silver staining (Pierce™ Silver Stain for Mass Spectrometry, Thermo Scientific) was performed according to the manufacturer's instructions. After silver staining, we excised the desired bands for LC-MS/MS to identify the proteins.

**Mass spectrometry analysis.**

Mass spectrometry and protein identification was completed by Alberta Proteomics and Mass Spectrometry Facility at University of Alberta. In-gel trypsin digestion was
performed on the samples. Briefly, the excised gel bands were reduced (10 mM mercaptoethanol in 100 mM bicarbonate) and alkylated (55 mM iodoacetamide in 100 mM bicarbonate). After dehydration enough trypsin (6 ng/μL, Promega Sequencing grade) was added to just cover the gel pieces and the digestion was allowed to proceed overnight (~16 h) at room temperature. Tryptic peptides were first extracted from the gel using 97% water/2% acetonitrile/1% formic acid followed by a second extraction using 50% of the first extraction buffer and 50% acetonitrile.

The digested samples containing tryptic peptides were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 μm inner diameter (300 Å, 5 μm, New Objective). The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 30,000 and m/z range of 400–2,000. The fourteen most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and a B. glabrata proteome database (UniProt) was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8-Da. Peptides were searched with carbamidomethyl
cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

**Sporocyst transformations of *S. mansoni* and immunofluorescence staining.**

Miracidia were obtained from eggs isolated from the livers of mice infected with *S. mansoni* (NMRI) as described previously (Hambrook et al., 2018). Briefly, newly hatched miracidia were collected for cultivation in Chernin’s Balanced Salt Solution (CBSS) (Chernin, 1963) containing 1 g/L each of glucose and trehalose as well as 1% pen-strep antibiotics (Life Technologies). After cultivation for 24 h at 27°C under normoxic conditions, most miracidia transformed to primary sporocysts. Primary sporocysts were washed five times with snail PBS (sPBS) (Yoshino & Laursen, 1995) and transferred to 15 mL centrifuge tubes (Corning). All in-tube washes were performed at 4°C and sporocysts were pelleted by centrifugation for 2 min at 300 × g.

After overnight 2% paraformaldehyde/sPBS (pH 7.2) fixation, sporocysts were washed five times with sPBS followed by 1 h incubation in 5% BSA/0.02% azide/sPBS (blocking buffer) at room temperature. Sporocysts was grouped and incubated with rBgFREP3 (500 ng/μL), rBgTEP1 (50 ng/μL) and rBgFREP2 (150 ng/μL) in blocking buffer overnight at 4°C; control group were incubated in blocking buffer without recombinant proteins. Following five washes with 1% BSA/sPBS, sporocysts were treated with anti-V5 antibody (Life Technologies) diluted 1:400 in blocking buffer.

Following primary antibody treatment, sporocysts were washed five times with 1% BSA/sPBS and incubated with a solution containing 7.5 U/mL Alexa Fluor 488 phalloidin (Invitrogen) and 4 μg/mL Alexa Fluor 633 goat anti-mouse secondary antibody (Invitrogen) in blocking buffer overnight at 4°C. Finally, sporocysts were
washed five times with 1% BSA/sPBS, dropped into a drop of DAPI staining solution,
incubated for 5 min at room temperature, and transferred onto microscope slides.
Preparations were then covered with a cover glass and sealed with nail polish to prevent
desiccation. Slides were imaged using an Axio imager A2 microscope (Zeiss), and
analyzed using Zen 2011 software (Zeiss) and Photoshop CS4 (Adobe Systems Inc.,
USA).

**Anti- BgFREP3 polyclonal antibody generation and validation.**

The IgSF1 and FBG domains of BgFREP3 were screened for regions of presumed
antigenicity using the OptimumAntigen™ design tool (GenScript). Three polyclonal
antibodies were generated specific for different peptides (14-15 amino acids long, ~ 4.4-
kDa) targeting each domain. The information of these peptides is: anti-IgSF #1:
CSFKKDDLSDSKQRS; anti-IgSF #2: CHNKYSEGRIDKSSN; anti-IgSF #3:
CNINKDLDFKEQNIT; anti-FBG #1: TTFDRDNDEYSYNC; anti-FBG #2:
GWKEYRDGFGDYNIC and anti-FBG #3: CLNGKWGSSDFAKGV. Peptides were
synthesized by GenScript and used to immunize rabbits. Rabbits received a primary
immunization and two secondary boosts at 2 and 5 weeks. One week after the second
boost, rabbits were exsanguinated and the serum IgG was affinity-purified using protein
A/G affinity column. These were further purified using affinity resin conjugated with the
immunization peptides. The anti-BgFREP3 antibodies used in this study were effective
for Western blot detections at a concentration of 1:1,000.

The purified antibodies were supplied by GenScript along with synthesized peptides
and pre-immune serum. Specificity of the antibodies was tested against the respective
peptides as well as snail plasma and haemocyte lysates. Dot blots were initially used to
determine whether the antibodies recognized their peptides as well as the specificity of
the recognition. Briefly, 2 µL of each supplied peptide was slowly pipetted in one spot on
a nitrocellulose membrane that was divided into a grid with a pencil. The deposited
peptides were dried at room temperature for about 10 min, creating a spot of
approximately 2–3 mm. The membrane was then incubated in blocking buffer and
processed using the same procedure described for Western blot following sample transfer
to nitrocellulose membranes. In order to determine that the peptides were not being
recognized by any pre-immune serum components, the above procedure was followed
exactly with the exception that the respective pre-immune sera were used for the primary
incubations in place of antibodies.

**Western and far-Western blot analysis.**

To test whether rBgTEP1 complexes with rBgFREP3 and rBgFREP2, purified
rBgTEP1 was incubated with rBgFREP3 or rBgFREP2 in the following ratios (wt/wt in
µg): 1:1, 1:0.75, 1:0.5, and 1:0.25 in 1.5 mL tubes for 2 h at room temperature on a
Labquake shaker (ThermoFisher Scientific) to allow potential complexes to form. After
the incubation, samples were subjected to western blot detection under denaturing
(Laemmli protein loading buffer with β-mercaptoethanol and heated at 95°C for 10 min)
and non-denaturing (no β-mercaptoethanol nor heating) conditions, respectively.

The Western blot experiment process was as described previously (Pila, Peck, et al.,
2017). Samples were loaded on 6-14% (vol/vol) SDS/PAGE gels and run on the Mini
PROTEAN Tetra system (Bio-Rad) at 150 V for 1.5-2 h. Samples were then transferred
for 1.5 h onto 0.45 µm supported nitrocellulose membranes (Bio-Rad). Blocking was
done for 1 h at room temperature in 5% (wt/vol) skimmed milk or bovine serum albumin
(BSA) prepared in TBS solution plus 0.1% Tween-20 (TBS-T buffer) before staining for 1 h in anti-V5 mouse primary antibody at a dilution of 1:5,000 in blocking buffer.

Membranes were washed in TBS-T buffer for 10 min, then twice for 5 min each and once in TBS solution for 5 min. Membranes were then incubated for 1 h in HRP-conjugated rabbit anti-mouse antibody diluted 1:5,000 in blocking buffer followed by a wash step as described above. Finally, the blot was developed by incubating the membranes in SuperSignal West Dura Extended Duration substrate (Thermo Scientific).

Chemiluminescent signals were acquired on the ImageQuant LAS 4000 machine (GE Healthcare).

To detect the BgFREP3 background expression in the B. glabrata snail hemolymph, the hemolymph of four snails was obtained from each of M-line and BS-90 strain B. glabrata snails. The hemolymph from each snail was diluted twice with lysis buffer (50 mM Tris, pH 7.8; 150 mM NaCl; 1% Nonidet P-40 and 150 μg/mL EDTA). After incubation for 30 minutes on ice, samples were centrifuged at 20,000 × g for 15 min and the supernatant was collected. After quantification, samples (160 μg/well) were used for subsequent Western blot analysis. The primary antibodies were polyclonal antibodies raised in rabbits against the IgSF and FBG domains of rBgFREP3 (1:500 dilution) and the secondary antibody was a HRP-conjugated goat anti-rabbit IgG (1:5000 dilution).

Far-Western blotting is used to probe a membrane containing transferred protein with another protein to detect specific protein-protein interactions (Y. Wu, Li, & Chen, 2007). In our study, we detected rBgTEP1 (“prey” protein) separated by SDS-PAGE with the biotinylated rBgFREP3 and rBgFREP2 (“bait” or “probe” proteins) and a streptavidin-HRP chemiluminescent detection system. If rBgTEP1 forms a complex with
rBgFREP3 or rBgFREP2, rBgFREP3 would be detected on spots in the membrane where rBgTEP1 was located. First, rBgFREP3 and rBgFREP2 was biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the manufacturer's instructions. Recombinant BgTEP1 was separated by SDS-PAGE, and transferred to a membrane, as in a standard western blot. Recombinant BgTEP1 in the membrane was then denatured and renatured as described previously (Y. Wu et al., 2007). The membrane was then blocked and probed with biotinylated rBgFREP3 or rBgFREP2. After washing as described above in a standard western blot, the membrane was incubated with streptavidin-HRP diluted 1:100,000 in blocking buffer at room temperature for 1 h. Chemiluminescent signals were detected using the ImageQuant LAS 4000 machine (GE Healthcare).

**Quantitative real-time PCR analysis of BgTEP1 expression.**

A TRIzol reagent was utilized to extract total RNA from 5 whole unchallenged snails from each strain independently, after which RNA was purified using a PureLink RNA mini kit (Life Technologies). A NanoVue spectrophotometer (ThermoFisher Scientific) was used to determine RNA concentration, and first-strand cDNA synthesis using the qScript cDNA synthesis kit (Quanta Biosciences) was performed using 1 μg of RNA template. The cDNA was diluted fivefold, and 5 μL was used as template in RT-PCR using primers (Table S1) specific for BgTEP1 and B. glabrata β-actin (BgActin) in a SYBR Green detection system (PerfeCTa SYBR Green FastMix; Quanta Biosciences). All RT-PCRs were performed on a QuantStudio 3 PCR system (Applied Biosystems) using the following thermocycling conditions: initial hold at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with data collection every cycle.
Specificity for the gene specific primers RT-PCR was confirmed by continuous melt curve analysis.

**In vitro *S. mansoni* sporocyst killing assays.**

Haemolymph from 25 M-line or BS-90 strain *B. glabrata*, sterilized as reported in Hahn *et al.*, 2001 (Hahn *et al.*, 2001), was pooled into 1.5 mL tubes and kept on ice. Haemocytes were isolated from plasma by centrifugation at 70 × g for 10 min at 4°C followed by aspiration of the cell free plasma, which was kept for assessment of plasma-mediated killing of sporocysts. Haemocytes were resuspended in modified Bge-cell medium (mBge) medium [22% Schneider’s Drosophila medium (Thermo Scientific), 7 mM D-glucose, 24 mM NaCl, and 20 mg/mL gentamicin at pH 7.4] and washed three times following the same centrifugation protocol. Haemocytes were then resuspended to a final concentration of 50 cells/µL in mBge medium and 200 µL of this suspension was added to the wells of a 96-well tissue culture plate pre-treated with poly-L-lysine that contained 10 *S. mansoni* sporocysts that were transformed using previously published protocols (Hambrook *et al.*, 2018). Treatment consisted of 200 pM of each recombinant protein (*BgFREP3, BgTEP1, BgFREP2*). Three anti-*BgFREP3* antibodies (anti-IgSF #1 and #3, anti-FBG #1) that were generated to peptides within the IgSF and FBG domains were used as a cocktail (combined concentration of 2.5 mg/mL) were used the antibody blocking assays. Sporocyst viability was assessed using the protocol published by Hahn *et al.*, 2001 (Hahn *et al.*, 2001). Briefly, Propidium iodide (Millipore Sigma), was added to each well to a final concentration of 10 µg/mL. Sporocyst viability was assessed using a fluorescent inverted microscope (Zeiss) starting at 3 h post incubation with haemocytes/plasma and then measured again at 6, 12, 24, 36 and 48 h post incubation.
Sporocyst death was determined by propidium iodide-staining of the nuclei of the sporocyst. Any sporocyst deemed dead, was left in the well until the end of the time-course so that the experimental conditions were not disturbed differentially in wells where sporocyst mortality was more frequent.

The pooled plasma from M-line and BS-90 snails. Cell-free plasma was ultracentrifuged at 30,000 rpm for 3 h at 4°C to remove haemoglobin, which is known to have a toxic effect on sporocysts (Bender et al., 2002). Ultracentrifuged plasma was mixed 50:50 with mBge medium at room temperature and then incubated with 10 S. mansoni sporocysts according to the protocol outlined above.

Assessment of reactive oxygen species production by haemocytes in vitro.

To assess the role that ROS had on sporocyst killing by haemocytes the above haemocyte in vitro killing assays were repeated with addition of two well characterized inhibitors of ROS; diphenyleneiodonium chloride (DPI) and N-acetyl-L-cysteine (NAC). Both ROS inhibitors (DPI at a final concentration of 150 nM and NAC at a final concentration of 1 mM) were incubated along with haemocytes and S. mansoni sporocysts as described above in the haemocyte-based sporocyst killing assay.

Bioinformatic and Statistical analysis

We used Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to align the amino acid sequences for BgTEPs, Biomphalysins and BgFREPs which acquired from GenBank [National Center for Biotechnology Information (NCBI)]. The alignment files were downloaded and visually inspected for obvious inaccuracies, modified if need be, and then annotated based on information provided by NCBI. To show the distribution of the identified peptides over a full length sequence, the amino acid sequences of
BgTEP1.5 (ADE45333.1), Biomphalysin (A0A182YTN9) and BgMFREP2 (AAK13550.1) were imported into Vector NTI Advance 11.5 (Thermo Fisher Scientific) and the identified peptides were annotated. Images produced by Vector NTI software were saved by FSCapture and further processed with FSCapture and Adobe Photoshop CS4. To determine significant differences in sporocysts killing assays, one-way ANOVA with Tukey’s post hoc tests were performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad; www.graphpad.com). Statistical significance threshold was set at $P \leq 0.05$.

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**Author Contributions**

H.L. and P.C.H. designed research; H.L., J.R.H., E.A.P., A.A.G., and J.F. performed research; H.L., J.R.H., E.A.P., X.W. and P.C.H. analyzed data; and H.L., J.R.H., E.A.P., A.A.G., and P.C.H. wrote the paper.

**Declaration of interests:** The authors declare no competing interests.
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Figure titles and legends

Figure 1
Figure 1. Pull-down experiments of \( rBgFREP3 \) and \( rBgTEP1 \). A: Schematic diagram and Western blot analysis of recombinant proteins, the ends of which are coupled to the tags V5 and ploy-His for protein immunoassay and purification operations. B: Western blot analysis of \( BgFREP3 \) background expression in hemolymph of M-line and BS-90 snails. The hemolymph of four snails from each M-line and BS-90 strain was used for detection, and anti-IgSF #1 and anti-FBG #1 antibodies were used as primary antibodies. C: Pull-down experiments with \( rBgFREP3 \). The eluate was collected and separated on 6% (upper panel) and 10% (lower panel) SDS-PAGE before silver staining. E: Pull-down experiments with \( rBgTEP1 \). The eluate was separated on 10% SDS-PAGE. In the pull-down experiment, cell-free plasma from susceptible (green) or resistant (red) \( B.\ glabrata \) snails was incubated with Sf9 cell lysates expressing \( rBgFREP3 \) or concentrated medium containing \( rBgTEP1 \) together (interactome experiments, last two lanes) or alone (controls, first three lanes). Bands that differ between control and interactome experiments were cut, proteins submitted to tryptic digest and analyzed by LC-MS/MS for identification. In interactome experiments, bands that differ between M-line and BS-90 were marked with red arrows. The results of mass spectrometry analysis are shown in the figure. D, F and G: Distribution of peptides identified by LC-MS/MS on \( BgTEP1.5 \) (ADE45333.1), Biomphalysin (A0A182YTN9) and \( BgFREP2 \) (AAK13550.1). Green background: from M-line strain in the \( rBgFREP3 \)-pull-down; red background: from BS-90 strain in the \( rBgFREP3 \)-pull-down; green box: from M-line strain in the \( rBgTEP1 \)-pull-down; red box: from BS-90 strain in the \( rBgTEP1 \)-pull-down.
Figure 2. Immunofluorescence analysis of interaction between rBgFREP3, rBgFREP2, and rBgTEP1 with *S. mansoni* sporocysts. Sporocysts were incubated with vehicle or recombinant proteins (rBgFREP3, rBgFREP2, and rBgTEP1 alone) and
combination (rBgFREP2 plus rBgTEP1) and immunostained using anti-V5 primary IgG and Alexa Fluor 633 goat anti-mouse secondary antibody. The sporocysts were stained with DAPI (for nucleus, blue), phalloidin (for F-actin labeling, green) and fluorescent secondary antibody (for recombinant proteins, red).
Figure 3. Immunoblot analysis of the interaction between rBgTEP1 and rBgFREP3 and rBgFREP2. A: Purified rBgTEP1 was incubated with rBgFREP3 in the indicated ratios (wt/wt in µg) for 2 h at room temperature. After the incubation, Western blot analysis was carried out under denaturing (heating with reducing agent, the right) and non-denaturing (no heating, no reducing agent, the left) conditions, respectively. B: Decreasing amount of rBgTEP1 was blotted onto the membrane and then subjected to standard Western blot (the left) and far-Western blot (the right) analysis. C: The interaction between rBgFREP2 and rBgTEP1. Western blot analysis under non-denaturing (the upper) and far-Western blot analysis (the below) was carried out,
respectively. In the far-Western blot, the biotinylated $rBgFREP3$ and $rBgFREP2$ acts as a primary antibody and the secondary antibody is a streptavidin-HRP; the red arrow indicates the band that symbolizes the interaction between $BgTEP1$ and $rBgFREP3$ or $rBgFREP2$. 

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Figure 4. Association of rBgFREP3, rBgTEP1 and rBgFREP2 facilitates M-line snails to kill S. mansoni sporocytes. Effects of rBgFREP3, rBgTEP1 and rBgFREP3-
rBgTEP1 complex on killing of *S. mansoni* sporocysts by M-line plasma (A) and haemocytes (B). C: Before being exposed to sporocysts, M-line haemocytes were pre-incubated with rBgFREP3-rBgTEP1-rBgFREP2 combination in presence or in absence of anti- *B*gFREP3 antibodies (abrogation treatment). BS-90 haemocytes were incubated with anti-*B*gFREP3 antibodies. D: The effect of rBgFREP2, rBgTEP1 and rBgFREP2-rBgTEP1 complex on the destruction of sporocysts by M-line haemocytes. E and F: The role of ROS in killing of *S. mansoni* sporocysts by haemocytes from *B. glabrata* snails. E: The addition of ROS inhibitors NAC and DPI abolished the ability of BS-90 haemocytes to destroy sporocysts. F: Killing sporocysts of M-line haemocytes rendered by rBgFREP3-rBgTEP1 complex was annulled by pre-incubation with ROS inhibitors NAC and DPI. Statistically significant difference symbols in A, B and D: asterisk (*) represents comparison with M-line haemocytes or plasma, * P < 0.05, ** P < 0.01; a represents comparison with BS-90 haemocytes or plasma, P < 0.05; b represents comparison with both single treatment (rBgTEP1/rBgFREP3 or rBgFREP2), P < 0.05; c represents comparison with only *B*gFREP3 treatment, P < 0.05; bars represent SEM, n=10. Statistically significant difference symbols in C: # represents comparison BS-90 haemocytes with BS-90 haemocytes+anti-rBgFREP3 antibodies, P < 0.05; $ represents comparison M-line haemocytes+rBgFREP3+rBgFREP2+rBgTEP1 with M-line haemocytes+rBgFREP3+rBgFREP2+rBgTEP1+anti-rBgFREP3 antibodies, P < 0.05; bars represent SEM, n=10. Statistically significant difference symbols in E and F: e represents comparison NAC treatment with BS-90 haemocytes or M-line haemocytes+rBgFREP3+rBgTEP1, P < 0.05; f represents comparison DPI treatment with...
BS-90 haemocytes or M-line haemocytes+rBgFREP3+rBgTEP1, P < 0.05; bars represent SEM, n=6.
Figure 5. Interaction of BgFREP3, BgFREP2, BgTEP1 and Biomphalysin in B. glabrata snail hemolymph and possible functional mechanisms against S. mansoni infection. This figure is a schematic drawing based on the results of previous studies and our work.
Supplemental figure titles and legends

>Derived from GenBank AY028461.1 *Biomphalaria glabrata* fibrinogen-related protein 3-2 precursor, gene, complete cds.

This sequence is derived from GenBank AY028461.1. There are differences in nucleotide sequence but the amino acids match 100%. The differences are due to codon-optimization for expression in insect Sf9 cells. GenBank annotations are used to locate the individual BgFREP3.2 domains: underline, signal peptide; gray shading, IgSF1 domain; double underlining, transmembrane domain.

Figure 1—figure supplement 1. Nucleotide sequence for cloning rBgFREP3. This sequence is derived from GenBank AY028461.1. There are differences in nucleotide sequence but the amino acids match 100%. The differences are due to codon-optimization for expression in insect Sf9 cells. GenBank annotations are used to locate the individual BgFREP3.2 domains: underline, signal peptide; gray shading, IgSF1 domain; double underlining, transmembrane domain.
underline, small connecting region; gray shading plus frame, IgSF2 domain; wavy underline, ICR; bold underline, FBG domain; delete line, termination codon.
CLUSTAL O (1.2.4) multiple sequence alignment

BgTEP           KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

BgTEP1.1        KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

BgTEP1.2        KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

BgTEP1.3        KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

BgTEP1.4        KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

BgTEP1.5        KITSTEAIDSLAYEIRSRSDHVKGVSLELSGQREFNATFKVEPSWAPIAQLLMYYIRRDS 540

************************:***********************************

BgTEP1.5        GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

BgTEP1.4        GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

BgTEP1.3        GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

BgTEP1.2        GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

BgTEP1.1        GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

BgTEP           GILSIDIDIPLNATSIDIKVSLNKQTTAEKRISKSYSMSNNYLQLSLLSKLVKAESDVLI 480

***************:********************************************

BgTEP1.5        TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

BgTEP1.4        TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

BgTEP1.3        TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

BgTEP1.2        TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

BgTEP1.1        TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

BgTEP           TAYVQVTTPDGLPPTDSNLSLSVYTSVTYQMTVPDQELYSPSSFSGSYPLPGQNMSLPAN 420

******************** **************************************

BgTEP1.5        DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

BgTEP1.4        DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

BgTEP1.3        DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

BgTEP1.2        DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

BgTEP1.1        DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

BgTEP           DIRRSVHLNEKKKIKITAFVIEASTGIKLNGSSVITYYGNRYQIKFLEMTPAVFKPGLQY 360

**************************************************** ******

BgTEP1.5        DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

BgTEP1.4        DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

BgTEP1.3        DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

BgTEP1.2        DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

BgTEP1.1        DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

BgTEP           DTTLSGSVEAKYTFGQPVYGLVLLQIGENVDTIDKCNVNRKVTEISFEIKGKGNFSVPLE 300

******************************* ***:************************

BgTEP1.4        TFGVVEGFFDLSDQPSFGTWKINVRTETYSGAEQLFVEAEYDPLRFQDVGLPPFALLS 240

BgTEP1.3        TFGVVEGFFDLSDQPSFGTWKINVRTETYSGAEQLFVEAEYDPLRFQDVGLPPFALLS 240

BgTEP1.2        TFGVVEGFFDLSDQPSFGTWKINVRTETYSGAEQLFVEAEYDPLRFQDVGLPPFALLS 240

BgTEP1.1        TFGVVEGFFDLSDQPSFGTWKINVRTETYSGAEQLFVEAEYDPLRFQDVGLPPFALLS 240

BgTEP           TFGVVEGFFDLSDQPSFGTWKINVRTETYSGAEQLFVEAEYDPLRFQDVGLPPFALLS 240

****************************.******************************

BgTEP1.5        SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

BgTEP1.4        SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

BgTEP1.3        SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

BgTEP1.2        SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

BgTEP1.1        SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

BgTEP           SSDILSILIQTDKAIYKPKERVNFRILAVYYNLQLYTGTFHYEILDPYDNKINVLSGVSG 180

*********:**************************************************

BgTEP1.5        YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

BgTEP1.4        YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

BgTEP1.3        YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

BgTEP1.2        YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

BgTEP1.1        YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

BgTEP           YSFSIPEGPKSLLTANGTFSPGVRGTLSPIDFNHLCSYCRILLKYNPLQIFEQDFIQI 120

*************************************************.**** ****

BgTEP1.5        MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

BgTEP1.4        MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

BgTEP1.3        MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

BgTEP1.2        MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

BgTEP1.1        MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

BgTEP           MRMKLNLILFVFYLVFQECQGGKYFISAPRNVGTAYDIYSVDILKQIDGNVTVEA1LQD 60

CLUSTAL O (1.2.4) multiple sequence alignment
BghiP          FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGT-TAGPALTAFVLVALLKAKE 1079
BghiP1.1       FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGT-TAGPALTAFVLVALLKAKE 1079
BghiP1.2       FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGT-TAGPALTAFVLVALLKAKE 1079
BghiP1.3       FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGT-TAGPALTAFVLVALLKAKE 1079
BghiP1.4       FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGT-TAGPALTAFVLVALLKAKE 1079
BghiP1.5       FIFVDKEIILKASMLLIDRQNLDGSFNEFGKVLDRNTQGTTTAGPALTAFVLVALLKAKE 1080

BghiP          LADVQYCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVTSYALAEAKSQLAQ 1139
BghiP1.1       LADVQDCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVASYAFAEAKSQLAQ 1139
BghiP1.2       LADVQDCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVASYAFAEAKSQLAQ 1139
BghiP1.3       LADVQDCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVASYAFAEAKSQLAQ 1139
BghiP1.4       LADVQDCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVASYAFAEAKSQLAQ 1139
BghiP1.5       LADVQDCKNNNKCRYYLLGNATLNATRNLERLMLADSIDDQFSLAVASYALAEAKSQLAQ 1140

BghiP          STFEKLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1199
BghiP1.1       STFEKLLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1199
BghiP1.2       STFEKLLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1199
BghiP1.3       STFEKLLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1199
BghiP1.4       STFEKLLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1199
BghiP1.5       STFEKLLTVFQKQGGLEYWSANSTVNNEELNRFINWRPPQRPARDPIDILLTSYAITYSS 1200

BghiP          LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSTYGSKSFRPDTNITIYVSDMNT 1259
BghiP1.1       LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSSYGSKSFRPDTNITIYVSDMNT 1259
BghiP1.2       LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSSYGSKSFRPDTNITIYVSDMNT 1259
BghiP1.3       LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSSYGSKSFRPDTNITIYVSDMNT 1259
BghiP1.4       LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSSYGSKSFRPDTNITIYVSDMNT 1259
BghiP1.5       LGRLDEALPSVRWLTLQKNAQGGFVSTQDTVVGLQALSFYGSKSFRPDTNITIYVSDMNT 1260

BghiP          HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319
BghiP1.1       HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319
BghiP1.2       HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319
BghiP1.3       HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319
BghiP1.4       HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319
BghiP1.5       HLTMNVKSNALSLQIQEIQSNSQDFSITASGSGLALLDIEYSFNVLKELSKPVFDVNTV 1319

BghiP          LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1379
BghiP1.1       LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1379
BghiP1.2       LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1379
BghiP1.3       LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1379
BghiP1.4       LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1379
BghiP1.5       LLDDKLDSFNIMVCTKFLLKHDTGMVVQEVSIPSGFVPDLSTLGQVAGVKRSERKGSIVA 1380

BghiP          CLNCCP 1445
BghiP1.1       CLNCCP 1445
BghiP1.2       CLNCCP 1445
BghiP1.3       CLNCCP 1445
BghiP1.4       CLNCCP 1445
BghiP1.5       CLNCCP 1446

IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1439
IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1439
IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1439
IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1439
IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1439
IYFDK1GSSSLCS1 VMTEAKYAKSQKYVRTDYDYYEPANQATYFYQPRRTLRDDTCDV 1440

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Figure 1—figure supplement 2. Alignment of multiple BgTEP amino acid sequences and distribution of identified peptides. Peptides identified by LC-MS/MS from rBgFREP3 pull-down experiments are highlighted in gray. The GenBank accession numbers of each entry are: BgTEP, ACL00841.1; BgTEP1.1, ADE45332.1; BgTEP1.2, ADE45339.1; BgTEP1.3, ADE45340.1; BgTEP1.4, ADE45341.1 and BgTEP1.5, ADE45333.1.
Figure 1—figure supplement 3. Normalized transcript abundance of \( Bg \)TEP1 in unchallenged M-line (black bar, \( n=5 \)) and BS-90 (grey bar, \( n=5 \)) snails indicating a lack of significant difference (\( p > 0.05 \)) between the two strains.
| Score | Expect | Method                        | Identities | Positives | Gaps |
|-------|--------|-------------------------------|------------|-----------|------|
| 1143 bits(2957) | 0.0 | Compositional matrix adjust. | 538/572(94%) | 555/572(97%) | 0/572(0%) |

**Figure 1—figure supplement 4. The alignments of two Biomphalysin variants**

( UniProtKB/TrEMBL: A0A182YTN9 and A0A182YTZ4) identified by LC-MS/MS.

Peptides identified from A0A182YTN9 and A0A182YTZ4 are highlighted in gray and underline, respectively. Two red oval boxes representing differences in peptide sequences that distinguish two Biomphalysin variants.
Figure 1—figure supplement 5. Alignment of multiple BgFREP amino acid sequences and distribution of identified peptides. Peptides identified by LC-MS/MS are highlighted in gray. The GenBank accession numbers of each entry are: BgMFREP1 partial, AAK13549; BgMFREP2 precursor, AAK13550; BgFREP3-2 precursor, AAK28656; BgMFREP4 precursor, AAK13551; BgMFREP5 partial, AAK13546; BgMFREP6 partial, AAK13552; BgFREP7.1 precursor, AAK28657; BgMFREP8 partial, AAK13553; BgMFREP9 partial, AAK13554; BgMFREP10 partial, AAK13555; BgMFREP11 partial, AAK13556; BgFREP12.1 precursor, AAO59918; BgFREP12-FBG2, AAT58639; BgFREP13.1 precursor, AAO59922 and BgFREP14, ABO61860.
**Figure 1—figure supplement 6. Alignment of multiple BgFREP3 amino acid sequences and distribution of identified peptides.** Peptides identified by LC-MS/MS are highlighted in gray, the overlapping region of peptides are bold underline. GenBank annotations of BgFREP3.2 (AAK28656.1) are used for locate the individual BgFREP3 domains: orange, signal peptide; green, IgSF1 domain; blue, small connecting region; pink, IgSF2 domain; black, ICR; red, FBG domain.
Figure 4—figure supplement 1. Controls of functional verification experiments. A:

Only the media and recombinant proteins rBgFREP3 and rBgTEP1 were present, without
snail plasma or haemocytes, to control the sporocyst killing assay of rBgFREP3, rBgTEP1 and the combination of both. **B:** The controls of the sporocyst killing assay of rBgFREP2, rBgTEP1 and rBgFREP2-rBgTEP1 complex. **C:** Pre-immune serum from the same rabbit which raised the anti-rBgFREP3 antibodies were incubated with BS-90 and M-line haemocytes for control.
### Table S1 Primer list for cloning and quantitative RT-PCR.

| Name            | Protein                        | Primer sequence                                      |
|-----------------|--------------------------------|------------------------------------------------------|
| **For cloning:**|                                |                                                      |
| *BgMFREP2 precursor (AY012700.1)* | AAK13550.1                      | Forward: 5'-CAC CAG GAG GAT CTT AGT AAT GGC GTC GC -3'<br>Reverse: 5'-GAC CCT TGG CGC GTT TAG CTC TAT TTT TCT -3' |
| *BgFREP3.2 precursor (Derived from AY028461.1)* | AAK28656.1                      | Forward: 5'-CAC CAG GAG AAG AAA CGA AAT GGC TCG TCT CTT CTT GC -3'<br>Reverse: 5'-CAT CTC AGT GAA CGA CAC GGA -3' |
| *BgTEP1.1 (HM003907.1)* | ADE45332.1                      | Forward: 5'-CAC CAT GAG AAT GAA GCT GAA T -3'<br>Reverse: 5'-TGG GCA ACA GTT GAG GCA -3' |
| **For q-PCR:** |                                |                                                      |
| *BgTEP1.1*      |                                | Forward: 5'-CACCAATGGCGATGGTATTAG-3'<br>Reverse: 5'-CTGACTCTCTCAACAGGCTTAG-3' |
| *BgActin*       |                                | Forward: 5'-GCTTCCACCTCTTTGCTTCTTTGAGCCTCTG-3'<br>Reverse: 5'-GAACGTAAGCTTTGGGACATCTG-3' |
Table S2: The identified peptides of BgTEP1, Biomphalysin, BgFREP2 and BgFREP3.3 by LC-MS/MS.

| Sequence | # PSMs | # Protein groups | Protein Group Accessions | # times identified |
|----------|--------|------------------|--------------------------|-------------------|
| **BgTEP1:** | | | | |
| P1       | FQVDVLPPALLSGLTSGVEAK | 2 | 1 | A0A2C9M761 | 1 |
| P2       | YTFGQVYGVLQVQLQENGTVTDIDK | 4 | 2 | A0A2C9L049; A0A2C9M761 | 2 |
| P3       | KVTEISFEIK | 1 | 1 | A0A2C9M761 | 1 |
| P4       | ITAVFETASTGIK | 1 | 1 | A0A2C9L049 | 1 |
| P5       | SYMSNNYQSLLSKL | 3 | 1 | A0A2C9LWE8 | 3 |
| P6       | ITSTEAASLEYR | 3 | 1 | A0A2C9LWE8 | 3 |
| P7       | SGVQELNQQR | 7 | 2 | A0A2C9LWE8; D7QYI1 | 4 |
| P8       | QGGMGAPMAMSFR | 1 | 1 | D7QYI1 | 1 |
| P9       | VFRPPFVSLTYPR | 1 | 1 | D7QYI1 | 1 |
| P10      | ENFPLTHTRPGQNASQIQR | 1 | 1 | D7QYI1 | 1 |
| P11      | SNMADAVJR | 2 | 1 | D7QYI1 | 2 |
| P12      | ATNLQSEELNK | 2 | 1 | D7QYI1 | 2 |
| P13      | IIDLNNQQR | 11 | 2 | A0A2C9KIQ1; D7QYI1 | 3 |
| P14      | QNIDSFGNEFGK | 3 | 2 | A0A2C9KIQ1; D7QYI1 | 3 |
| P15      | SQLAOSTFEK | 2 | 1 | D7QYI1 | 1 |
| P16      | LDEALPSVR | 3 | 1 | D7QYI1 | 2 |
| P17      | KGSIAVIAFDK | 1 | 1 | D7QYI1 | 1 |
| **Biomphalysin:** | | | | |
| P1       | CNEINSYINALDR | 6 | 2 | A0A182YTQ9; A0A182YTQ4 | 5 |
| P2       | VGYFQLOGLR | 9 | 2 | A0A182YTQ9; A0A182YTQ4 | 5 |
| P3       | GYLFNLESAR | 2 | 2 | A0A182YTQ9; A0A182YTQ4 | 2 |
| P4       | GCSCPGPGYFTGLYR | 5 | 2 | A0A182YTQ9; A0A182YTQ4 | 4 |
| P5       | ADGDYLFKL | 2 | 2 | A0A182YTQ9; A0A182YTQ4 | 2 |
| P6       | NGFTWADTR | 7 | 2 | A0A182YTQ9; A0A182YTQ4 | 4 |
| P7       | WCEGDKNGER | 1 | 2 | A0A182YTQ9; A0A182YTQ4 | 1 |
| P8       | LNLVFGWGFAVK | 2 | 2 | A0A182YTQ9; A0A182YTQ4 | 2 |
| P9       | SVIEDLQAEVDSSGLYVR | 9 | 2 | A0A182YTQ9; A0A182YTQ4 | 6 |
| P10      | SSITLGMAGAK | 1 | 2 | A0A182YTQ9; A0A182YTQ4 | 1 |
| P10/2    | QTQSSITLGMAGAK | 1 | 1 | A0A182YTQ9; A0A182YTQ4 | 1 |
| P11      | FGDSVPPYK | 3 | 2 | A0A182YTQ9; A0A182YTQ4 | 3 |
| P12      | LTDETQFQFTLTGK | 7 | 1 | A0A182YTQ9; A0A182YTQ4 | 6 |
| P13      | LEKVEGTSVNVK | 2 | 2 | A0A182YTQ9; A0A182YTQ4 | 1 |
| **BgFREP2:** | | | | |
| P1       | YOPVATLPSVTVT | 16 | 1 | A0A2C9L9F5 | 9 |
| P2       | SSTDIAVSAFEYQDR | 5 | 1 | A0A2C9L9F5 | 5 |
| P3       | LDKGVDVDIQSR | 22 | 2 | A0A2C9L9F5 | 9 |
| P4       | TDGGWIFQOR | 13 | 1 | A0A2C9L9F5 | 9 |
| P5       | DCYDSN LNQK | 2 | 1 | A0A2C9L9F5 | 1 |
| **BgFREP3.3:** | | | | |
| P1       | SIQDELSNLHNMNISSISWKLNSFSTAVMDKKDDIK | 2 | 1 | AEO50747.1 | 1 |
| P2       | DIDTMEIVIRHELSSNK | 1 | 1 | AEO50747.1 | 1 |
| P3       | NNSFAQYQSSFKISEKEK | 3 | 1 | AEO50747.1 | 1 |
| P4       | NEHWNCNLTVRTLFQFDIDIK | 1 | 1 | AEO50747.1 | 1 |
| P5       | KDINTQODSR | 2 | 1 | AEO50747.1 | 1 |
| P6       | QNIVNNKR | 2 | 1 | AEO50747.1; XP_013062145.1 | 1 |
| P7       | QOFDDIVK | 1 | 2 | AEO50747.1; XP_013062145.1 | 1 |
| P8       | ESLKAINQNIKNK | 8 | 8 | | 1 |
| P9  | AINQNIKNINKDLDFK | 2   | 8   | AAO59915.1; AAK28656.1; AEO59746.1; AAK13548.1; AAC47701.1; AQX34544.1; AQX34587.1; AQX34558.1. |

**PSMs**: peptide-to-spectrum matches.
Figure 1
### A

| Reducing | Non-reducing |
|----------|--------------|
| $rBg$TEP (µg): | 0 1 1 1 1 | $rBg$TEP (µg): | 0 1 1 1 1 |
| $rBgFREP3$ (µg): | 1 1 0.75 0.5 0.25 | $rBgFREP3$ (µg): | 1 1 0.75 0.5 0.25 |

![Western blot images](image1.png)

### B

| Western blot | Far-western blot |
|--------------|------------------|
| $rBg$TEP (µg): | 2.0 1.3 0.6 0 | $rBg$TEP (µg): | 2.0 1.3 0.6 0 |

- Anti-V5 antibody
- Biotinylated $rBgFREP3$

### C

| Non-reducing |
|--------------|
| $rBg$TEP (µg): | 0 1 1 1 1 |
| $rBgFREP3$ (µg): | 1 1 0.75 0.5 0.25 0 |

![Far-western blot images](image2.png)

- Biotinylated $rBgFREP2$

---

**Figure 3**
Figure 4
Figure 1—figure supplement 1. Nucleotide sequence for cloning rBgFREP3. This sequence is derived from GenBank AY028461.1. There are differences in nucleotide sequence but the amino acids match 100%. The differences are due to codon-optimization for expression in insect Sf9 cells. GenBank annotations are used to locate the individual BgFREP3.2 domains: underline, signal peptide; gray shading, IgSF1 domain; double underline, small connecting region; gray shading plus frame, IgSF2 domain; wavy underline, ICR; bold underline, FBG domain; delete line, termination codon.
Peptides identified by LC-MS/MS from 203
and distribution of identified peptides. Peptides identified by LC-MS/MS from

Figure 1—figure supplement 2. Alignment of multiple BgTEP amino acid sequences and distribution of identified peptides. Peptides identified by LC-MS/MS from
rBgFREP3 pull-down experiments are highlighted in gray. The GenBank accession numbers of each entry are: BgTEP, ACL00841.1; BgTEP1.1, ADE45332.1; BgTEP1.2, ADE45339.1; BgTEP1.3, ADE45340.1; BgTEP1.4, ADE45341.1 and BgTEP1.5, ADE45333.1.
Figure 1—figure supplement 3.
| Score          | Expect | Method                      | Identities | Positives | Gaps  |
|---------------|--------|-----------------------------|------------|-----------|-------|
| 1143 bits(2957) | 0.0    | Compositional matrix adjust. | 538/572(94%) | 555/572(97%) | 0/572(0%) |

CLUSTAL O (1.2.4) multiple sequence alignment

| A0A182YTN9 | MLVQFLFAATTLLQVSSQCTYSSWWYSFDTPGSKCNEINSYINALDRNDVWADDDLSN | 60 |
| A0A182YTZ4 | MFLQ1FVATTLQVSSQCTYSSWWYSFDTPGSKCNEINSYINALDRNDVWADDDLSN | 60 |
| A0A182YTN9 | LEGVQCCRPAPWNNVEQQVYVEDWATLDSLTVAFCRYGFQLGLYRSDTGWPRFKGY | 120 |
| A0A182YTZ4 | LEGVQCRRPPAPWNNVEQQVYVEDWATLDSLTVAFCRYGFQLGLYRSDTGWPRFKGY | 120 |
| A0A182YTN9 | LFNLESARCTKPANPLVNGCQIDVSSOMGRKQCSCPGGYFLGLYRADDLDYLFLK | 180 |
| A0A182YTZ4 | LFNLESARCTKPANPLVNGCQIDVSSOMGRKQCSCPGGYFLGLYRADDLDYLFLK | 180 |
| A0A182YTN9 | KIRCCTPAAKPLEMDEKSIQTRIMDTTLWNMATLHLYGWCYGRGVAYGVEDFTNG | 240 |
| A0A182YTZ4 | KIRCCTPAAKPLEMDEKSIQTRIMDTTLWNMATLHLYGWCYGRGVAYGVEDFTNG | 240 |
| A0A182YTN9 | FTWAADVTRTFGGCEGDKNGLNLVFDGMGFVKEI1YKSV1EDLQASEVSDGVLNLV | 300 |
| A0A182YTZ4 | FTWAADVTRTFGGCEGDKNGLNLVFDGMGFVKEI1YKSV1EDLQASEVSDGVLNLV | 300 |
| A0A182YTN9 | RASSPVTEDESRTKTIEETVTHSTTSTFTNSHELGIELNFEIASVGSAYTTKFEYHSA | 360 |
| A0A182YTZ4 | RASSPVTEDESRTKTIEETVTHSTTSTFTNSHELGIELNFEIASVGSAYTTKFEYHSA | 360 |
| A0A182YTN9 | TTNEKSIQTAGFTKKSSITLGPMEGAKYE1IMSKRSTTVYTAII1TTRFSTEKGLFRLW | 420 |
| A0A182YTZ4 | TTNEKSIQTAGFTKKSSITLGPMEGAKYE1IMSKRSTTVYTAII1TTRFSTEKGLFRLW | 420 |
| A0A182YTN9 | EDGNGERHQQYRTNSGRTYNYRTFGDSSVPPFYRLKALKKQSDNNEGVMWGMLFQKFPDARR | 480 |
| A0A182YTZ4 | EDGNGERHQQYRTNSGRTYNYRTFGDSSVPPFYRLKALKKQSDNNEGVMWGMLFQKFPDARR | 480 |
| A0A182YTN9 | VINLTDGQFQFIKGLKEGTSVNYVKEIKLNRDVSGNADPNSI1TT1Y1ASGP | 540 |
| A0A182YTZ4 | VINLTDGQFQFIKGLKEGTSVNYVKEIKLNRDVSGNADPNSI1TT1Y1ASGP | 540 |

Figure 1—figure supplement 4. The alignments of two Biomphalysin variants

(UniProtKB/TrEMBL: A0A182YTN9 and A0A182YTZ4) identified by LC-MS/MS.

Peptides identified from A0A182YTN9 and A0A182YTZ4 are highlighted in gray and underline, respectively. Two red oval boxes representing differences in peptide sequences that distinguish two Biomphalysin variants.
CLUSTAL O (1.2.4) multiple sequence alignment

BbFREP1 PARTIAL
BbFREP2 PRE
BbFREP3-2 PRE
BbFREP4 PRE
BbFREP5 PARTIAL
BbFREP6 PARTIAL
BbFREP7.1 PRE
BbFREP8 PARTIAL
BbFREP9 PARTIAL
BbFREP10 PARTIAL
BbFREP11 PARTIAL
BbFREP12 1 PRE
BbFREP12-FBG2
BbFREP13 1 PRE
BbFREP14
BbFREP1 PARTIAL
BbFREP2 PRE
BbFREP3-2 PRE
BbFREP4 PRE
BbFREP5 PARTIAL
BbFREP6 PARTIAL
BbFREP7.1 PRE
BbFREP8 PARTIAL
BbFREP9 PARTIAL
BbFREP10 PARTIAL
BbFREP11 PARTIAL
BbFREP12 1 PRE
BbFREP12-FBG2
BbFREP13 1 PRE
BbFREP14
BbFREP1 PARTIAL
BbFREP2 PRE
BbFREP3-2 PRE
BbFREP4 PRE
BbFREP5 PARTIAL
BbFREP6 PARTIAL
BbFREP7.1 PRE
BbFREP8 PARTIAL
BbFREP9 PARTIAL
BbFREP10 PARTIAL
BbFREP11 PARTIAL
BbFREP12 1 PRE
BbFREP12-FBG2
BbFREP13 1 PRE
BbFREP14

HFSGSSEIIKERIETVTLNCTYQALKHQRNE--------------

NFSGNSEIIKERVETLTLNCTFQVLNQDQNETSSLQSLYILHE

VKGDNSNERNISLFAKKAVEYETNSTALIEEIQRCKVSKCQCSLENNDSSSNYKRSRV

LSRYDETLKEFLDTLEAKTLNLSDLTVQVFNYSLISFGNVFISLTILHPTKSDAKVYRCK

LSRYNETIREFDELIALDLSLNLKQFVRFKYSQISFGNRYTTLLILHNPTFDARITYKCN

MATFLYVILVVALVTSLSTSELVIDVQPNVI

MATNLLLRLVFFQSLLPLLSSELVIDVQPDIISAEITAQLVINCSTVNNQVHLDOIRSLT

LSRYNETIRDFEVMXLLDLNLKQLKVLQFNYSLISFGNVFISLTILHPTKSDAKYRCS

VEGDKTNAASSSVAKKEVEYRTNMTALIEIERLLKVKEKNDQCSLXKXEELTSYHQKTKL

LSRYNQTLRDFEDITALDLLTLNLKDVLQVFNYSLISFGNVFISLTILHPTKSDAKYRCS

LSRYNETIREFDELIALLASLTLNLKQFNYSLISFGNVFISLTILHPTKSDAKYRCS

LSRYNETIREFDELIALLASLTLNLKQFNYSLISFGNVFISLTILHPTKSDAKYRCS

LSRYNETIREFDELIALLASLTLNLKQFNYSLISFGNVFISLTILHPTKSDAKYRCS

VBGNSETIREL1QPKKLCTTFQKSNDSDNSQVLFMSTYHETKVIASISKMQOTPS

YFSGSDIIKERIELPQKCTTFQKURLTDQNETSRLQLSLYILHESKVIAVYKNDQPVVT

SFNANVEKINEVIXPLMTCSFESVRNSDQVNTQVLMDHETFQVFIATTKDNQTVG

SBRNITANIKDRQVLT

NSRITANIKDRQVLT

NFSGSEIIKERETVLTNCQVLNQDQNETSSLQSLYILHETGVIANINKGQPVLT

HSVGSKVIKELIEPIITLCTSLQNLKDNSTVQFMYTLHESGNIATIMKDVQPVT

9

HSVGSKVIKELIEPIITLCTSLQNLKDNSTVQFMYTLHESGNIATIMKDVQPVT

KFSGNAELKELIEPIITLCSLKSFSNETNEHFVK1MFTQIETGVISTLSDKQAVV

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**Figure 1—figure supplement 5.** Alignment of multiple *BgFREP* amino acid sequences and distribution of identified peptides. Peptides identified by LC-MS/MS are highlighted in gray. The GenBank accession numbers of each entry are: *BgMFREP1* partial, AAK13549; *BgMFREP2* precursor, AAK13550; *BgFREP3-2* precursor, AAK28656; *BgMFREP4* precursor, AAK13551; *BgMFREP5* partial, AAK13546; *BgMFREP6* partial, AAK13552; *BgMFREP7.1* precursor, AAK28657; *BgMFREP8* partial, AAK13553; *BgMFREP9* partial, AAK13554; *BgMFREP10* partial, AAK13555; *BgMFREP11* partial, AAK13556; *BgFREP12.1* precursor, AAO59918; *BgFREP12-FBG2*, AAT58639; *BgFREP13.1* precursor, AAO59922 and *BgFREP14*, ABO61860.
CLUSTAL O (1.2.4) multiple sequence alignment

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sequences and distribution of identified peptides. Peptides identified by LC-MS/MS are highlighted in gray, the overlapping region of peptides are bold underline. GenBank annotations of *BgFREP3.2* (AAK28656.1) are used for locate the individual *BgFREP3* domains: orange, signal peptide; green, IgSF1 domain; blue, small connecting region; pink, IgSF2 domain; black, ICR; red, FBG domain.

Figure 1—figure supplement 6. Alignment of multiple *BgFREP3* amino acid
Figure 4—figure supplement 1.