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Anopheles gambiae Ag55 cell line as a model for *Lysinibacillus sphaericus* Bin toxin action

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ABSTRACT

Binary toxin (Bin) produced by *Lysinibacillus sphaericus* is toxic to *Culex* and *Anopheles* mosquito larvae. It has been used world-wide for control of mosquitoes that vector disease. The Bin toxin interacts with the glucosidase receptor, Cpm1, in *Culex* and its orthologue, Agm3, in *Anopheles* mosquitoes. However, the exact mechanism of its mode of action is not clearly understood. It is essential to understand mode of action of Bin toxin to circumvent the resistance that develops over generations of exposure. A suitable model cell line will facilitate investigations of the molecular action of Bin toxin. Here we report Bin toxin activity on Ag55 cell line that has been derived from an actual target, *Anopheles gambiae* larvae. The Bin toxin, both in pro and active forms, kills the Ag55 cells within 24 h. Bin toxin internalizes in Ag55 cells and also induces vacuolation as tracked by Lysotracker dye. The dose response studies showed that 1.5 nM of Bin toxin is sufficient to induce vacuolation and Ag55 cell death. Presence of α-glucosidase gene (Agm3) expression in the Ag55 cells was also confirmed. Thus, Ag55 cells constitute an appropriate model system to decipher the mode of Bin action in mosquito larvae.

1. Introduction

Mosquitocidal proteins produced by strains of *Bacillus thuringiensis* (*Bt*) subsp. *israelensis* and *Lysinibacillus sphaericus* (*Ls*) have played significant roles in combating mosquitoes that vector human diseases such as dengue, chikungunya, filariasis, malaria and West Nile fever (Berry, 2012). *Bt israelensis* is highly active against *Aedes*, *Anopheles* and *Culex* mosquito larvae while *Ls* is active against *Culex* and *Anopheles* larvae. An advantage of *Ls* is that, in contrast to *Bt israelensis*, it can survive and multiply in polluted aquatic environments (Nicolas et al., 1987). *Ls* produces different types of insecticidal toxins viz. binary (Bin), mosquitocidal (Mtx), Cry48/Cry49 and sphaericolysin toxins. Highly active strains produce Bin and Mtx toxins whereas mildly active strains mostly produce Mtx toxins. The *bin* toxin genes are present only in a subset of *Ls* strains and sequences of the encoded proteins are highly conserved between strains (Hire et al., 2009; Humphreys and Berry, 1998; Priest et al., 1997). The Bin toxin is composed of two polypeptides, BinA (41.9 kDa) and BinB (51.4 kDa) (Baumann et al., 1988; Hindley and Berry, 1987). Although BinA alone has some toxicity to *Culex* larvae, both Bin polypeptides are required for maximal toxicity to *Culex* and *Anopheles* larvae (Hire et al., 2009; Nicolas et al., 1993). The bacterium *Ls*, its use in insect control and the action of its insecticidal toxins are the subject of recent in-depth reviews (Berry, 2012; Silva-Filha et al., 2014).

After sporulation of *Ls* cells, the parasporal crystal remains associated with the spore encased in the exosporium. When ingested by mosquito larvae, the crystal dissolves releasing BinA (41.9 kDa) and BinB (51.4 kDa) protoxins which are then activated by proteases, to 39 kDa and 43 kDa core toxins, respectively. Activation of Bin protoxin occurs in susceptible and non-susceptible insects (Nicolas et al., 1990), suggesting that while processing of Bin protoxins to toxins is important, it may not be a major determinant of mosquito toxicity. The specificity of Bin toxin to various mosquito species is due to a single class of receptors on larval brush border membrane. An α-glucosidase called Cpm1 was identified as a Bin receptor in *Culex pipiens* (Silva-Filha et al., 1999). The 60 kDa Cpm1 protein is tethered to the brush border by a glycosylphosphatidyl inositol (GPI) anchor. Importantly, loss of GPI-anchorage of Cpm1 is a mechanism of *Culex* resistance to Bin (Darboux et al., 2002). Cqm1, the homologue of Cpm1 in *Culex*

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quinquefasciatus, is also considered a functional Bin receptor (Darboux et al., 2002; Romao et al., 2006). BinB of the Bin complex recognizes the receptor in susceptible larvae (Nielsen-Leroux and Charles, 1992), and the current model is that BinB binds to Cpm1 on the mosquito midgut membrane and then active BinA binds to the Cpm1 receptor-BinB complex. Binding interactions between Bin toxin and brush border membrane of *Anopheles gambiae* larvae are more complex than in *Culex* species as both BinA and BinB bind specifically to larval brush border membrane (Charles et al., 1997). With respect to a Bin receptor in *A. gambiae*, α-glucosidase Agm3 is the homologue of Cpm1 and is a putative Bin receptor in *A. gambiae* larvae (Opota et al., 2008). BinA/B work in concert for optimal toxicity to mosquito larvae, yet it is unclear whether an oligomeric form (BinA2.BinB2) is pre-formed prior to membrane binding (Pauchet et al., 2005; Smith et al., 2005), or whether this complex is unstable in solution (Hire et al., 2014; Kale et al., 2013) and then a complex forms at the membrane level.

Observed events in Bin toxin action include binding to a receptor, possibly pore formation, and internalization of Bin into the target cells, yet with the exception of receptor binding the importance and details of these events in Bin action are unclear. Evidence of pore formation was first reported by Cokmus et al. (1997) when patch-clamping of *C. quinquefasciatus* cells showed an increase in current caused by Bin toxin. BinA and BinB formed two sizes of voltage-dependent ion channels in planer lipid bilayers (Schwartz et al., 2001). Cpm1 was shown to be essential for Bin toxin-induced pore formation as demonstrated in cultured mammalian epithelial Madin-Darby Canine Kidney (MDCK) cells by expressing the Cpm1 (Pauchet et al., 2005). The Bin toxin also induces cytoplasmic vacuolation and autophagy in these cells expressing α-glucosidase Cpm1 (Opota et al., 2011). A recent report suggests that Bin toxin also induces apoptosis (Tangsongcharoen et al., 2015).

Studies in the 1980s introduced insect cultured cells as tools for investigating mechanisms of Bt Cry toxin action. For example, Haider and Ellar (1987) measured the effects of protease processing on lepidopteran-active CryA1 protein specificity using cultured cells. Trypsin-activated Cry1 protein was toxic to the lepidopteran *Choristoneura fumiferana* CF-1 cells, but not *Aedes albopictus* cells. In a recent study, the same CF-1 cell line was used to examine Cry1A toxin mediated events including receptor binding, oligomerization and pore formation (Portugal et al., 2014). Results from their study suggested that some steps in toxin action are conserved between susceptible larval midgut cells and CF-1 cells. Several recent studies demonstrated how cultured mosquito cells can be used 'as is' or as a host cell for expressing receptors for mosquito-active Cry toxins. In a study analyzing the effects of gut juice and trypsin activation on the processing and cytotoxicity of Cry toxins, Cry4Aa and Cry11Aa toxins were cytotoxic at high concentrations to lepidopteran and dipteran (*Aedes aegypti* C6/36) cultured cells, suggesting that some amount of Cry receptor is present on both cell types (Teixeira Correa et al., 2012). Interestingly, C6/36 cells constitutively expressing a heterologous *Aedes* cadherin showed increased susceptibility to Cry4Aa and Cry11Aa, but not Cry4Ba toxin (Lee et al., 2015).

Bin toxins are cytotoxic to tissue culture grown cells of *C. quinquefasciatus*, *A. gambiae* and *A. aegypti*, but not to cells of the lepidopteran *Spodoptera frugiperda* (Broadwell and Baumann, 1987). We investigated cultured Ag55 cells derived from *A. gambiae* as a model that may yield insights into mechanisms of Bin toxin action, especially the action of Bin in *Anopheles* relative to better studied *Culex* species. Ag55 cells were established from neonate first instar larvae of *A. gambiae* (Pudney et al., 1979). The cells have been investigated as a potential model for *Plasmodium* ookinete binding to adult mosquito midgut (Wilkins and Billingsley, 2010) and established as a cell suitable for RNA inhibition-based silencing of target mosquito genes (Konet et al., 2007; Smith and Linser, 2009). Bin protoxins and trypsin-activated toxins prepared from native *Ls* ISPC-8 inclusions are highly cytotoxic to Ag55 cells. Fluorescently labeled Bin toxin internalizes inside Ag55 cells and induces vacuolation, and toxin is localized to lysosomal vacuoles. The response of Ag55 cells to Bin toxin observed in this study supports the further development of Ag55 cells as a model for investigating Bin toxin action, especially with respect to Bin action in *Anopheles* relative to *Culex* species.

2. Materials and methods

2.1. Organisms and growth conditions

L. sphaericus (formerly, *B. sphaericus*) strain ISPC-8 was isolated from diseased *Culex fatigans* larvae. The sporulating culture was grown as described earlier (Hire et al., 2010). The culture was harvested when it showed >90% sporulation as observed under phase-contrast microscopy. *A. gambiae* (UGAL) strain was maintained at 27 °C with a light–dark photoperiod of 16 h: 8 h as previously described (Zhang et al., 2008).

2.2. Purification of Bin protein

The binary protein of *Ls* ISPC-8 was purified as described earlier (Hire et al., 2010). Purified Bin protoxin comprising BinA (41.9 kDa) and BinB (51.4 kDa) was activated using trypsin (Sigma) in the ratio of 20:1 (Bin: trypsin) and incubated at 37 °C for 5 h. The activated Bin was loaded onto a HighQ cartridge (BioRad). The bound protein was eluted using 0.1–1 M NaCl gradient in buffer B (50 mM Tris–HCl pH 9.5, 1 M NaCl, 2 mM dithiothreitol) over six column volumes. Activated Bin was separated by SDS-(12%) PAGE, stained with Coomassie blue and protein visualized using an imaging station (AlphaInnotech). Activated Bin protein was dialyzed against 20 mM sodium phosphate buffer pH 8.0 and used for cytotoxicity assays against Ag55 cultured cells. Protein concentrations were determined by BioRad protein assay using BSA as a standard.

2.3. Ag55 cell line and cytotoxicity assays

A. gambiae larval Ag55 cell line (Pudney et al., 1979) was a kind gift from Dr. Paul Linser, University of Florida, USA. Cells were cultured in 25 cm^2 flasks (Corning) at 28 °C in Leibovitz's L-15 media (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS, Atlanta Biologicals) and 1% (v/v) penicillin–streptomycin solution (10,000 U/ml and 10 mg/ml, respectively) (Sigma). Cell culture medium was changed on alternate days.

Ag55 cells showing >70% confluence in flasks were used in Bin cytotoxicity assays. Initial assays tested the cytotoxicity of Bin protoxin and activated toxin (50 nM each in 20 mM Na-phosphate buffer, pH 8.0) against Ag55 cells. The cytotoxicity of activated Bin against Ag55 cells was assessed after incubation periods of 0 h, 12 h, 24 h, 36 h and 48 h. Bin was serially diluted in 20 mM Na-phosphate buffer (pH 8.0) and 100 µl was added into 2 ml cell culture medium to achieve a final range of 1.5–50 nM toxin. Ag55 cells were cultured in wells of 12-well plates (Corning) and the medium was replaced with fresh medium alone or medium plus buffer or Bin. The number of viable cells was determined by a trypan blue exclusion assay (Altman et al., 1993). Cells were re-suspended in culture medium and 50 µl was transferred to a microfuge tube containing the same volume of 0.4% trypan blue solution in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO4, 1.4 mM KH_2PO_4, pH 7.6). After 3–4 min at room temperature, 10 µl ($\sim 1 \times 10^4$) of suspended cells were counted in a Hausser Bright-Line counting chamber (Fisher

Scientific) using an inverted light microscope. Viable (excluding trypan blue) and non-viable (trypan stained) cells in 4 large squares (1 mm × 1 mm) were counted and the average numbers of live cells were calculated. Cytotoxicity assays were independently repeated three times and data from replicate samples were pooled for analysis and plotted using SigmaPlot (v. 11.0).

2.4. RNA extraction, sequencing and data analysis

Total RNA was extracted from fourth instar *A. gambiae* (UGAL strain) larval guts and Ag55 cells using TRIzol (Ambion). Fifty larval guts and 1×10^7 cells (1 flask of 25 cm^2) were pooled separately and homogenized in 200 µl of TRIzol reagent using a cordless motor-driven pellet pestle (Grainger) and processed for total RNA according to the manufacturer's instructions (Ambion). The purity and concentrations of RNA samples were determined using a NanoDrop spectrophotometer (N-1000) and samples were submitted to the Georgia Genomic Facility (GGF), University of Georgia. GGF performed RNA integrity determination, Poly (A) enrichment of mRNA, cDNA synthesis, library preparations. The sequencing was performed using 100 bp paired-end Illumina HiSeq 2000 v3 platform. Extraction of RNA from larval guts and Ag55 cells was replicated three times.

RNA-seq raw datasets were analyzed using the protocol developed by Trapnell et al. (2012). The 6 samples (paired-end reads, including biological replicates) were independently mapped onto the *A. gambiae* genome (*Anopheles-gambiae*-PEST_CHROMOSOMES_AgamP4.fa.gz) downloaded from (VectorBase) by using Tophat v2.0.13 (Kim et al., 2013; Trapnell et al., 2009), which uses Bowtie2 (Langmead et al., 2009) as an aligner. Due to high sequencing base quality at both 3′ and 5′ ends according to the FastQC output (Mortazavi et al., 2008) and availability of a reference genome, preprocessing steps were not performed. After the alignment, Cufflinks v2.2.1 (Trapnell et al., 2010) was used to produce one file of assembled transfrags for each replicate (Trapnell et al., 2012) and cufflinks v2.2.1 (cuffmerge) was used to merge all the cufflinks for the assembled file of replicates. Cufflinks v2.2.1 was used to estimate the expression values of the transcripts in FPKM (Fragments Per Kilobase per Million mapped reads) with the Cuffdiff 2 default geometric normalization.

2.5. Labeling of Bin toxin with Alexa 488 dye

Activated and purified Bin toxin was labeled with Alexa 488 (Al^{488}) dye as per manufacturer's protocol (Molecular Probes Inc., Eugene, OR, USA). Briefly, 50 µl of 1 M bicarbonate was added to 0.5 ml of Bin protein (1.5 mg/ml in PBS). The Bin protein solution was transferred to the vial of Al^{488} reactive dye, stirred for 1 h and then Bin-Al^{488} separated from free Al^{488} using a BioGel P-30 gel-filtration column. The concentration of Bin-Al^{488} was calculated as per the manufacturer's protocol (Alexa Fluor 488 Protein Labeling Kit, Molecular Probes). Activated Bin and Bin-Al^{488} were separated by SDS-(12%) PAGE and protein visualized using an imaging station (AlphaInnotech). The labeled Bin protein was used for confocal imaging experiments.

2.6. Confocal microscopy

The Bin-Al^{488} toxin was used to assess internalization of toxin into Ag55 cells. Labeled Bin (3 µl) in PBS was added to growing Ag55 cells in chambered coverslips (ibidi, GmbH) to yield a 25 nM final Bin concentration and the cells were incubated for 18 h at 28 °C. After 18 h, treated Ag55 cells were observed with a LSM 710 confocal microscope (Carl Zeiss) at 495 nm excitation and 519 nm emission. For visualizing vacuolation, Bin-Al^{488} treated Ag55 cells were further treated with Lysotracker deep red dye (10 nM) for 2 h as described in the manufacturer's protocol (Molecular Probes). Treatment culture medium was discarded; the cells were washed three times with PBS and then observed with a Zeiss LSM 710 confocal microscope. Two to three images were recorded per treatment and all confocal experiments included independent biological replicates.

2.7. Toxicity of Bin to A. gambiae larvae

The toxicities of Bin protoxin, toxin, and Bin-Al^{488} toxin to third-instar larvae of *A. gambiae* (UGAL strain) were verified by bioassay. Soluble Bin at 60 nM, 120 nM and 180 nM final concentrations in 2 ml deionized water was compared against no treatment or buffer controls in 12-well Costar culture plates (Corning). Ten early fourth instar mosquito larvae were added to each well and the plates kept at 27 °C with a light–dark photoperiod of 16 h: 8 h. Each treatment was replicated three times, and the bioassays were conducted two times. Larval mortality was recorded after 48 h.

3. Results and discussion

3.1. Purification and toxicity of Bin toxins to A. gambiae larvae

The Bin component of *Ls* is critical to the efficacy of *Ls*-based biopesticide formulations for controlling *Culex* and *Anopheles* mosquitoes. Bin protoxin is composed of BinA (41.9 kDa) and BinB (51.4 kDa) (Broadwell et al., 1990; Nicolas et al., 1993; Oei et al., 1990) and soluble Bin is toxic to *A. gambiae* larvae with a LC_{50} of 360 ng/ml (4.3 nM), a value about 4-fold that to *C. pipiens* (Davidson, 1989). We purified Bin protoxin from *Ls* ISPC-8 using a combination of chromatographic columns (Hire et al., 2010) and then activated Bin protoxin using trypsin, and purified the BinA/B mixture on an anion exchange column. Activated Bin toxin was also labeled with Al^{488}. Concentrations of 60 nM, 120 nM and 180 nM were toxic to *A. gambiae* larvae resulting in mortality within 48 h (Supplement Fig. 1). Purified Bin and Bin-Al^{488}at 60 nM concentration caused about 50% *A. gambiae* larval mortality as compared to LC_{50} = 4.3 nM reported previously (Davidson, 1989). Fig. 1 shows purified Bin (stained with Coomassie Blue) and Bin-Al^{488} (visualized for fluorescence), after separation by SDS–PAGE. The Bin components migrated near where expected for proteins of 39 kDa (BinA) and 43 kDa (BinB).

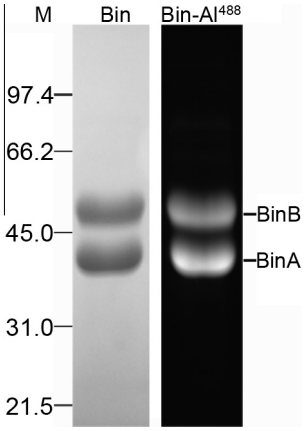

Fig. 1. SDS-(12%) PAGE of purified Bin and Bin-Al^{488}. The Bin lane was stained with Coomassie blue; the Bin-Al^{488} lane was imaged for fluorescence. BinA has an expected size of 39 kDa and BinB 43 kDa.

3.2. Toxicity of Bin protoxin and toxin to Ag55 cells

Bin protoxin and toxin solutions were tested at 50 nM each for toxicity against cultured Ag55 cells grown to about 75% confluency. By 24 h the untreated cells had retained their original shape and size, whereas Bin-treated cells became round, showed what appeared to be loss of cytoplasmic content, and cell lysis had reduced the cell numbers (Fig. 2). As indicated by the reduced number of intact cells, activated Bin toxin was qualitatively more toxic than protoxin to Ag55 cells; consequently, activated Bin toxin was used in subsequent experiments with Ag55 cells. Cytotoxicity was determined by counting live cells (excluding trypan blue) and dead cells (trypan blue stained) in a cell counting chamber at 12 h intervals for 48 h. As shown in Fig. 3A the percentage of live cells dramatically decreased after treatment with 50 nM activated Bin, whereas the cells in medium with buffer increased in number. Ag55 cells were also treated with Bin toxin at concentrations ranging from 1.5 nM to 50 nM and cell viability measured by trypan blue exclusion assay at 12 h intervals for 48 h. A concentration response effect was evident (Fig 3B) with cell mortality observed at the lowest 1.5 nM Bin concentration and increasing to the maximal 50 nM tested. A 6 nM concentration of soluble Bin caused a 50% reduction in cell viability at 48 h as measured by the trypan blue assay (Fig. 3B).

3.3. Low expression of Agm3 α-glucosidase in Ag55 cells

Agm3 α-glucosidase is a putative receptor of Bin toxin in *A. gambiae* larvae (Opota et al., 2008). The Agm3 protein is localized to the brush border membrane of posterior midgut in 4th instar *A. gambiae* larvae (Opota et al., 2008; Zhang et al., 2013). RNA-seq analysis of expressed genes in Ag55 cells showed that Agm3 transcripts were expressed with a FPKM value of 0.391, a level 750-fold lower than in gut of 4th instar larvae where the calculated FPKM value was 300. Additional experimentation is needed to determine if Agm3 functions as a Bin receptor in Ag55 cells and *in vivo* in *A. gambiae* larvae.

3.4. Bin internalization and Lysotracker-positive vesicles

The intoxication of *Culex* mosquito cells by Bin is associated with toxin uptake and vacuole formation [reviewed in (Silva-Filha et al., 2014)]. However, Bin toxin was not internalized in *A. gambiae* gut cells (Davidson, 1989). Cultured MDCK cells expressing Cpm1 on the cell surface bind to Bin and Bin induces pore and vacuole formation, but not cell lysis (Pauchet et al., 2005). The model in MDCK cells is that Bin is internalized to recycling endosomes, but not vacuolating lysosomes and thereby avoids degradation; overall this is thought to be part of an autophagic response induced by Bin (Opota et al., 2011).

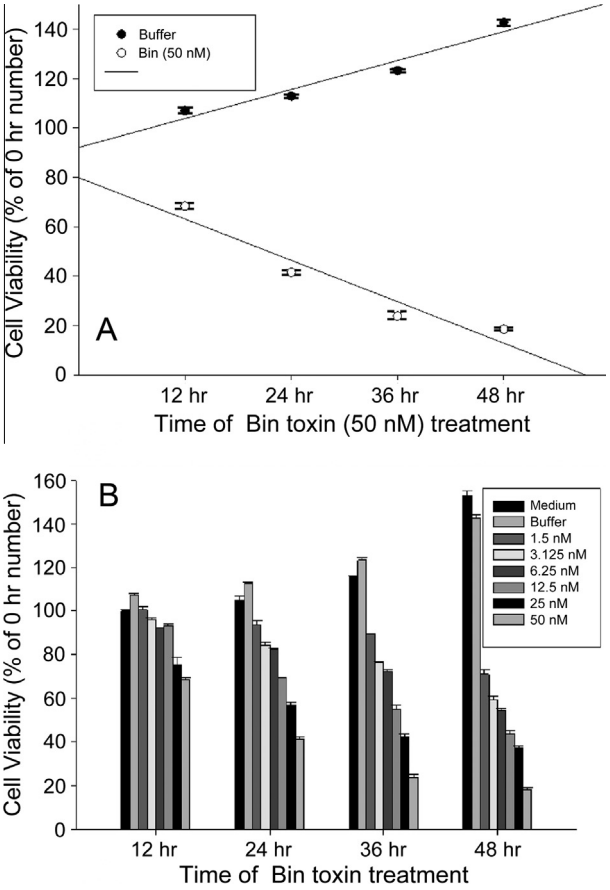

Fig. 3. Change in Ag55 cell viability after treatment with Bin toxin. Panel A. Ag55 cells (~1×10^6) were exposed to buffer or Bin toxin (50 nM final concentration) for 48 h and viability measured by trypan blue exclusion at 12 h intervals. Panel B shows the results from cytotoxicity assays of cells treated with 1.5–50 nM Bin for 48 h as measured by trypan blue exclusion. Viable cells in four squares of the cell counting chamber (total ~1×10^4 cells) were counted for each treatment. Cell viability is expressed as the % of live cells relative to the time 0 viable cell number. Error bars show standard error of the means.

We investigated whether some of these events occur with the Bin-Al^{488}-Ag55 cell combination. Bin-Al^{488} was cytotoxic to Ag55 cells inducing granulation and vacuole formation (Fig. 4A). Ag55 cells were also treated with the acidophilic dye Lysotracker, a dye that localizes to late endosomes and lysosomes (Via et al., 1998) and the dye localized to discrete organelles (Fig. 4A–C). When cells were exposed to Bin-Al^{488} for 18 h and then treated with Lysotracker, the Bin-induced vacuolization was more apparent (Fig. 4A) and Lysotracker was contained within many of the

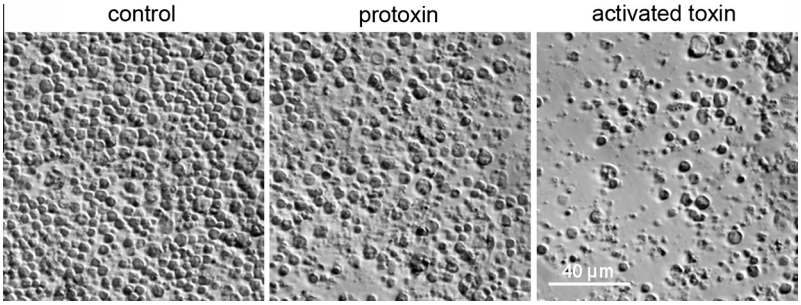

Fig. 2. Bin protoxin and activated toxin are cytotoxic to Ag55 cells. Ag55 cells in wells of a culture plate were incubated with 25 nM Bin for 24 h and then observed with the 40× brightfield objective of an inverted microscope (Leica DM IRE2). Cells incubated with Bin protoxin or Bin toxin were rounded and many appeared to have lost their cytoplasmic content. There were fewer intact Ag55 cells and more debris after treatment with Bin toxin relative to protoxin treatments. Bar indicates 40 µm.

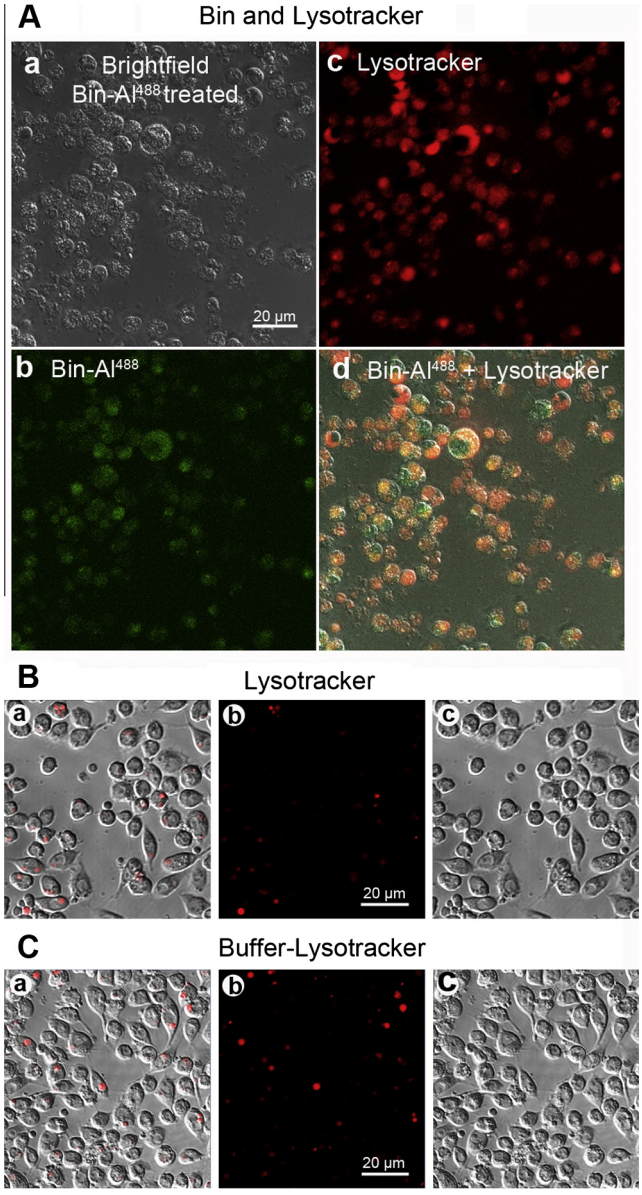

Fig. 4. Localization of Bin toxin in Ag55 cells. Ag55 cells were incubated with Bin-Al^{488} (25 nM) (Panel A), no treatment control (medium only) (Panel B), or buffer control (Panel C) for 18 h. After 18 h the cells were treated with 10 nM Lysotracker Red and observed under a Carl Zeiss confocal microscope with 495 nm excitation and 519 nm emission. Treated cells were observed under brightfield (Aa, Ba, Ca), green filter for Bin-Al^{488}(Ab), red filter for Lysotracker (Ac, Bb, Cb) and merged images (Ad, Bc, Cc). Bin toxin induced the vacuolation (Ac) and toxin localized into vacuoles inside the cells (Ac, Ad). The vacuoles were not induced in untreated (medium only control) and buffer treated (buffer control) cells clearly indicating that the vacuolation is because of Bin toxin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vacuoles (Fig 4Ac). While Bin and Lysotracker co-localized within some vacuoles there were also vacuoles that either contained Bin-Al^{488} or Lysotracker (Fig. 4Ad). While the internalization of Bin toxin into Ag55 cells and vacuole formation are similar to results obtained with cultured *Culex* cells and MDCK-Cpm1 cells, there is a substantial difference in the fate of the cells as Ag55 cells lyse upon Bin intoxication while MDCK-Cpm1 cells recover. Possibly this difference is accounted for by the epithelial nature of the MDCK cells.

4. Summary

In conclusion we demonstrated that Bin toxin kills Ag55 cells and the process is associated with internalization of toxin and the induction of vacuolation. Internalized Bin is present in vesicles and the process of cell death has features of autophagy. Thus Ag55 cells are an appropriate model cell line for studying the Bin toxin action. Further investigation is needed to determine how similar the mode of Bin action on Ag55 cells is to events which occur when *Anopheles* and *Culex* mosquito larvae in ingest Bin protein.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2015.09.009.

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