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Dimeric but not monomeric α-lactalbumin potentiates apoptosis by up regulation of ATF3 and reduction of histone deacetylase activity in primary and immortalised cells

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ABSTRACT

α-lactalbumin is a protein of dual function found in milk of most mammals. α-lactalbumin binds β-1,4-galactosyltransferase to form the regulatory subunit for lactose synthesis and has also been shown to cause cell death. This study shows, for the first time, that α-lactalbumin isolated in a rare 28 kDa dimeric form induces cell death, while 14 kDa monomeric α-lactalbumin is inactive. In contrast to the casein derived and chemically induced α-lactalbumin variants, MAL and HAMLET/BAMLET, the effects of 28 kDa α-lactalbumin are calcium independent and, unlike MAL and HAMLET, 28 kDa α-lactalbumin dimer causes cell death of primary mammary cells and a variety of immortalised cell lines, which are committed to cell death pathways within 1–4 h of exposure. Microarray analysis confirmed that cell death was the result of an apoptotic response. Functional assays determined that the mechanism by which 28 kDa α-lactalbumin kills cells involved inhibition of histone deacetylase activity mediated by NF-kB. We also show that 28 kDa α-lactalbumin occurs naturally in the milk of cows, goats and sheep, is low in concentration during mid-lactation, but accumulates during milk stasis, consistent with a role in involution.

1. Introduction

α-lactalbumin (LALBA) is a protein with a molecular mass of 14 kDa present in the milk of most mammals [1]. The protein is a metallo-protein with a high affinity for calcium, which is essential for folding and structural stability [2]. One biological function of LALBA is to act as a specifier protein for lactose synthetase in the formation of lactose from UDP-galactose and glucose [3]. More recently LALBA has also been identified as a potent inducer of cell death and has been implicated in the process of mammary gland involution during milk stasis [4–6].

Chemically induced forms of LALBA (MAL; multimeric alpha lactalbumin, HAMLET/BAMLET; human/bovine alpha lactalbumin made lethal to tumours) have also been reported to kill transformed cells, while sparing healthy epithelial cells [7,8]. The formation of these complexes are due to extraction procedures and do not naturally occur in milk [9]. MAL was isolated from human casein by ion-exchange chromatographic fractionation at low pH, and is comprised of both monomeric and oligomeric components [7,10]. The death-inducing component of MAL is a partially unfolded LALBA variant that undergoes a conformational change toward a molten globule-like state, which is stabilised by the incorporation of oleic acid and requires calcium for activity [11]. The technique to convert native LALBA to an active form was later optimised to produce HAMLET/BAMLET through removal of bound calcium with EDTA, followed by chromatography through an oleic acid conditioned ion-exchange matrix and elution with a high concentration of salt [9,12]. The unfolded state of both MAL and HAMLET is unstable in the absence of fatty acids, as the proteins revert back to their native state in the presence of serum and under physiological solvent conditions

Abbreviations: MAL, multimeric alpha lactalbumin; HAMLET/BAMLET, human/bovine alpha lactalbumin made lethal to tumours; LALBA, α-lactalbumin; HDAC, histone deacetylation; HDACi, Histone deacetylase inhibitor; TSA, Trichostain A; FIL, feed back inhibitor of lactation.

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[13]. The unfolding of LALBA alone is not the cause of cell death, however, as mutation of the LALBA calcium binding site to stabilize the structure into the apo-state did not induce cell death unless complexed with oleic acid [13].

Investigation of bovine whey proteins has also shown that some commercial preparations of bovine LALBA (bLALBA) cause cell death of IEC-6 cells (a non-transformed crypt cell line derived from the rat small intestine) [14], RAW cells (Mouse leukemic monocyte macrophage cell line) [5], HT-29, Caco-2 (human colon adenocarcinoma cell lines) [6] and primary seal mammary epithelial cells [4].

The potentiation of cell death can be conveyed within the cell in a variety of ways. The specific process of cell death initiated by HAMLET/BAMLET has been somewhat elusive. HAMLET/BAMLET has been reported to induce cell death via a variety of methods including apoptosis [15,16] macroautophagy [17] and activation of a lysosomal cell death program [18]. More recently it has been suggested that HAMLET/BAMLET exerts toxic effects on the cell [19,20]. It has also been proposed that the active component of HAMLET/BAMLET is the oleic acid co-factor and not the protein which is acting as a carrier [21]. Both HAMLET/BAMLET and native LALBA are capable of binding histones which suggests that these proteins interfere with chromatin organization [22,23].

In the current study, we present data of the structural requirements and functional activity of 28 kDa LALBA, a novel and naturally occurring dimeric protein which accumulates in milk at weaning. We show that 28 kDa LALBA kills a variety of cell types and describe the molecular basis of apoptosis pathways induction and other biofunctions mediated by 28 kDa LALBA. We confirm that apoptosis occurs via inhibition of histone deacetylase activity, and explore the possible implications on mammary gland involution and potential therapeutic benefits for cancer treatment.

2. Materials and methods

2.1. Isolation of 28 kDa bLALBA

bLALBA used in this study was derived from the whey fraction of milk, and purchased from Sigma (Product no# L5385). The 28 kDa and 14 kDa forms of bLALBA were separated using size exclusion chromatography. 10 mg of bLALBA (Sigma) were dissolved in 20 mM Sodium Phosphate buffer pH = 7.4 and 150 mM NaCl and then applied on Superdex 75 HiLoad 26/20 (GE Healthcare) equilibrated in the same buffer. The dimer and monomer of bLALBA eluted as two distinct and well-separated peaks at the expected elution volume. To sequester fatty acids 10 mg/ml bLALBA was incubated with 10 mg/ml bovine serum albumin (BSA) at 37 °C for 1 h. Denaturation of bLALBA protein was achieved by heat treatment of 95 °C for 5 min. Apo bLALBA was generated from 10 mg/ml of native bLALBA and dissolved in 3.5 mM EDTA to remove bound calcium. All bLALBA samples were sterilized by tissue culture grade, SpinX centrifuge tube filters (COSTAR) before application in cell culture. Ovine LALBA was isolated from the whey fraction of milk using methods described by [24]. Goat LALBA was also isolated from the whey fraction of milk and was a generous gift from Dr. JP Vilotte, France.

2.2. Mammary epithelial cells and cell lines

Mammary tissue obtained from a pregnant wallaby and a pregnant cow was immediately transferred to Medium 199 with Earle's salts (Gibco BRL Life Technologies) with 10 μg/ml penicillin/streptomycin (Gibco BRL Life Technologies) and 2.5 μg/ml Fungizone (Gibco BRL Life Technologies) on ice and transported back to the laboratory for enzymatic digestion to harvest mammary epithelial cells as described elsewhere [25]. Cancer cell SkBr3, MDA-MD-453, T47D, HS578T, MDA-MD-231 and BT578T lines were generously supplied by Dr. Erik Thompson, University of Melbourne, Department of Surgery, St. Vincent's Hospital. Primary mammary epithelial cells, mouse mammary HC11, human embryonic kidney HEK293 and human mammary cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% FCS, 5% penicillin streptomycin and 5% glutaraldehyde. Human intestinal AGS cells were cultured in Roswell Park Memorial Institute medium (RPMI; Gibco) supplemented with 10% FCS, 5% penicillin streptomycin and 5% gluteraldehyde. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37 °C. The cells used were within the 50th passage.

2.3. Cell proliferation assay

Cells (1×10^4 cells/ml) suspended in culture medium (DMEM or RPMI + 10% FCS) were seeded into 96-well plates (100 ul/well), incubated overnight then replaced with medium containing different concentrations of bLALBA ranging from 0.025 mg/ml – 1.6 mg/ml, and cultured at 37 °C for 7 days. Following incubation, adherent cells were fixed in situ at day 1, 2, 3, 4 and 7 day intervals and stained using the sulphorhodamibe B assay [26]. Optical densities were read on an automated Multiscan Ex spectrophotometric plate reader (Thermo Electron Corporation) at a wavelength of 540 nm.

2.4. Light microscopy

Cells (1×10^4 cells/ml) suspended in culture medium (DMEM or RPMI + 10% FCS) were seeded into a 10 cm petri dish (NUNC) that had a glass slide placed inside and grown at 37 °C overnight. The following day culture medium was replaced with medium containing 1 mg/ml bLALBA and incubated for 7 h. The adherent cells on glass slides were fixed in 4% paraformaldehyde for 20 min and stained with hematoxylin and eosin according to [27]). Sections were coverslipped and examined for cyto-evalution using a Coolscope digital (Nikon) microscope.

2.5. SDS-Page

SDS-PAGE was performed using 15% and 4–12% polyacrylamide gels [28]. Proteins (20 μg) were dissolved in the sample buffer (0.01 M Tris-HCl buffer pH 6.8 containing 20% glycine, 1% SDS and 5 mM EDTA plus 5% β-mercaptoethanol) by heating at 100 °C for 15 min and run in a Tris glycine buffer pH 8.3 with 0.1% SDS. The SeeBlue2 (Sigma) molecule weight standard marker was used. After electrophoresis the gel was stained with either Coomassie Brilliant Blue (CBB) or silver stain according to standard procedures [29].

2.6. Immunoblotting

Proteins separated by SDS-PAGE were transferred on to a nitrocellulose membrane (Amersham Biosciences) by electrophoresis using semi-dry transfer (BioRad). For immunoassay, the membrane was rinsed two times then incubated at room temperature with tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl) for 1 h. After rinsing two times with TBS plus 0.05% Tween 20 (TBST) the membrane was incubated at 4 °C overnight with 1:4000 diluted goat anti-bovine LALBA antibody. The membrane was then rinsed twice with TBST, and incubated for 1 h with 1:2000 diluted polyclonal rabbit anti goat immunoglobulin antibody DakoCytomation), then rinsed again twice with TBST. The antibody complex was visualized with ECL Western blotting detection reagents (Amersham Biosciences) and exposed to film for 5 min.

2.7. Mass spectrometry

Following SDS-PAGE, the protein bands were visualized by Coomassie Blue stain and the 14 kDa and 28 kDa bLALBA bands were cut out for mass spectrometry following a DTT reduction and iodoacetamide alkylation. Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) was used for peptide mass fingerprinting. The dimer fraction of bLALBA isolated by size exclusion chromatography

was analyzed by mass spectrometry without and with DTT using an Agilent QTOF LC/MS with a C8 column and a gradient of ACN 5–75% in 0.1% formic acid.

2.8. Statistical analysis

Statistical analysis was performed by one- and two-way ANOVAs followed by post-hoc Tukey's multiple comparison tests, or student *t*-test (unpaired, one tailed). In all analyses, *p*-values <0.01 were considered statistically significant.

2.9. Transcriptomics

The Affymetrix mouse microarray was used to examine global gene expression in cells following treatment with bLALBA. HC11 cells (1×10^4 cells/ml) suspended in culture medium (DMEM + 10% FCS) were seeded into T25 flasks (NUNC), and incubated overnight at 37 °C. The media was replaced with medium containing bLALBA (1 mg/ml) and cultured at 37 °C for 5 h. Total RNA from control HC11 cells (no bLALBA treatment), and treated HC11 cells were isolated using a Lipid RNA Extraction kit (Qiagen). The experiment was repeated with another biological replicate, and Affymetrix mouse microarrays were used to analyse RNA from both experiments (Australian Genome Research Facility, Melbourne, Australia). Data was normalized using the Affymetrix package of Bioconductor [30], using standard RMA techniques [31] and the Lima package was used for differential expression analysis [32]. Genes were considered differentially expressed if there was at least a 1.5-fold increase or decrease in intensity between slides with a *p*-value <0.01. Datasets were analyzed for function using the Ingenuity Pathways Analysis software (Ingenuity® Systems, http://www.ingenuity.com).

2.10. qRT-PCR validation

HC11 cells (1×10^4 cells/ml) suspended in culture medium (DMEM + 10% FCS) were seeded into 24 well plates (NUNC) in triplicate, and incubated overnight at 37 °C. The media was replaced with medium containing bLALBA (1 mg/ml) and cultured at 37 °C for 6.5 h. RNA was isolated as described above and ~100 ng of total RNA per sample was used to synthesis cDNA using iScript cDNA Synthesis Kit (Biorad) following manufacturer instructions. Real time qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) and CFX Connect Real-Time PCR Detection System (Bio-Rad). The reaction containing diluted cDNA, 1× master mix and Forward and Reverse primers (Supplementary Table 1). Expression levels were determined by normalizing expression against GAPDH.

2.11. HDAC activity assay

MCF7 cells suspended in culture medium (DMEM + 10% FCS) were seeded into 96-well plates (2.5×10^4/well), incubated overnight then replaced with medium containing different concentrations of bLALBA ranging from 0.25 mg/ml – 2 mg/ml, and cultured at 37 °C for 24 h. Specificity of the reaction was tested by performing each treatment in the presence and absence of TSA (2 h/37 °C) as recommend by the manufacturer. Histone deacetylase activity of each sample was determined using HDAC Cell-based Activity Assay kit (Caymen Chemical Company, MI, USA) according to manufacturer's instructions. Assays were performed in duplicate and repeated on two separate occasions.

2.12. Immunohistochemistry

MCF7 cells suspended in culture medium (DMEM + 10% FCS) were seeded into 96-well plates (2.5×10^4/well), incubated overnight then replaced with medium containing ± bLALBA (1 mg/ml) for 2 h. Cells were washed in PBS, fixed in 4% paraformaldehyde/PBS (10 min), washed in PBS, permeabilized with 0.1% Triton X/PBS (5 min), washed with PBS, blocked with 1%BSA/PBS (1 h) and incubated with rabbit anti-human primary antibody (NF-κB p65 Cell Signaling 1:100) in blocking buffer overnight at 4 °C. Cells were washed in PBS and incubated with anti-rabbit FITC labelled secondary antibody (Bethyl, 1:1000) for 1 h. Cells were then washed and counterstained with Hoechst stain (1 μg/ml). Images were captured using Zeiss fluorescent laser microscope (Carl Zeiss, Yena, Germany).

3. Results

3.1. Identification and quantification of LALBA monomeric and dimeric forms

Previous studies have identified bLALBA as an apoptotic factor found in milk [4,5,33]. Our previous studies confirmed that apoptosis occurs via DNA fragmentation [4]. In order to further define this activity, bLALBA was extracted and separated from milk by SDS-PAGE and showed that the sample comprised both 14 kDa monomeric and 28 kDa dimeric forms (Fig. 1a). Observed bands were confirmed to be LALBA by MALDI-TOF mass spectrometry. The monomeric and dimeric forms shared 70% and 58% sequence coverage with the amino acid sequence of the native 14 kDa LALBA, respectively, indicating that the 28 kDa bLALBA form shares position identity with the monomeric form.

3.2. Identification of 28 kDa LALBA in other species

To determine if 28 kDa LALBA was also found in the milk of other species, milk from goat and sheep was examined. SDS-PAGE and Western analysis identified 14 kDa, 21 kDa and 28 kDa forms in goat and sheep preparations of LALBA (Fig. 1b). These corresponded to monomeric, glycosylated and dimeric LALBA forms respectively. Apoptotic LALBA has previously been implicated in the involution process [4] so in order to determine the abundance of the naturally occurring 14 kDa and 28 kDa bLALBA form during the entire bovine lactation cycle, we analyzed casein and whey fractions of milk from dairy cows during peak lactation and the weaning period (drying off). Immunoblot analysis of milk samples showed that although the amount of 14 kDa LALBA

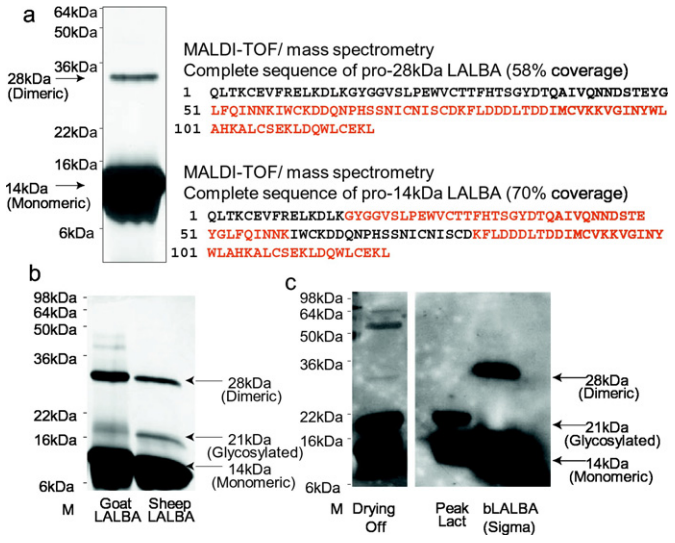

Fig. 1. Identification and structure of apoptotic 28 kDa LALBA. a Silver stain SDS-PAGE gel and MALDI-TOF/mass spectrometry identifying 14 kDa and 28 kDa forms of bLALBA in a commercial preparation of LALBA isolated from bovine milk. The complete amino acid sequence of bLALBA is shown overlaid with peptides (red). b Silver stain of an SDS-PAGE gel of LALBA isolated from goat and sheep milk. c Immunoblot of bovine milk samples showed that 28 kDa LALBA is recognised by anti-LALBA antibodies during the drying off period (weaning) and commercial preparation of bLALBA but not during peak lactation in the dairy cow.

remained relatively constant between peak lactation and weaning, the 28 kDa LALBA form was more abundant during weaning (Fig. 1c), suggesting that the 28 kDa LALBA is specific to the weaning period.

3.3. Native 28 kDa LALBA, but not 14 kDa LALBA induces cellular apoptosis

To investigate the individual apoptotic activity of both the 14 kDa and 28 kDa LALBA forms, the proteins were separated by size exclusion chromatography and quantified. This analysis showed that 28 kDa dimeric LALBA comprised only 3% of the total protein (Fig. 2a) consistent with the results for analysis of total whey. Following separation by HPLC each fraction was tested for apoptotic potential. Statistical analysis of the different treatments showed that only fractions containing 28 kDa bLALBA induced a significant amount of apoptosis in HC11 cells (Fig. 2b), while fractions containing 14 kDa LALBA did not induce apoptosis. Based on this observation we tested the hypothesis that dimerization causes the change in LALBA apoptotic potential. In order to test this, 14 kDa LALBA was separated from 28 kDa LALBA by size exclusion chromatography and dimerization of 14 kDa LALBA was induced by heating 80 °C/2 h. This method causes approximately 50% of the protein to convert from the monomeric form into the dimeric form (Fig. 2c). Both monomeric and heat induced dimeric forms of LALBA were tested for apoptotic activity but both preparations failed to induce apoptosis (data not shown) suggesting that dimerization alone is not responsible for the apoptotic properties of 28 kDa LALBA.

We analyzed the 28 kDa bLALBA separated from the monomeric bLALBA without and with DTT by QTOF LC/MS. The identified molecular weight of purified bLALBA dimer without DTT is 28,338.84 (Fig. 2d), and in the presence of DTT it is 28,353.83 (Fig. 2E) accounting for a reduction of 8 disulfide bonds (4 in each monomer), thus a shift of approximately +16. Interestingly, the identified molecular weight of the bLALBA dimer differs from the theoretical expected mass 28,356 of two monomeric bLALBA (taking into account 4 disulfide bonds present in each monomer) by −17. According to a Data Base of Protein Post Translational Modifications (www.abrf.org) [34,35], it suggests a possible covalent cross link between asparagine or glutamine of one monomer and lysine of another monomer. Interestingly, this type of cross-linking is known to occur after secretion and spontaneously without involvement of specific catalyst [34]. A covalent asparagine/glutamine to lysine cross link would account for remarkable structural integrity of bLALBA dimer. The reported crystal structure of bLALBA (Sigma) contains six crystallographically independent molecules suggesting possible modes of dimer

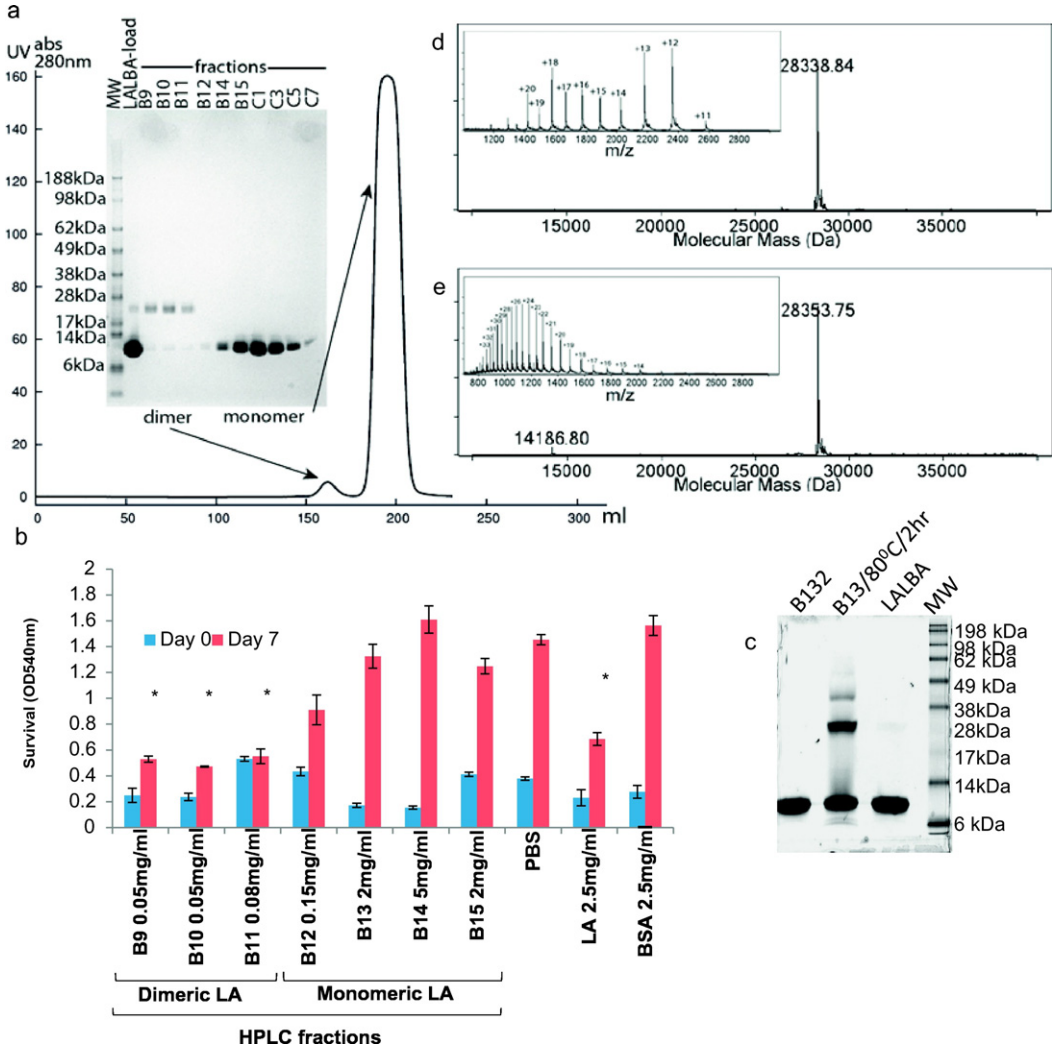

Fig. 2. Separation of 14 kDa and 28 kDa bLALBA by size exclusion chromatography and apoptotic potential. a The Superdex 75 HiLoad 26/60 elution profile of bLALBA (Sigma). Inset: 4–12% SDS PAGE gel of the fractions (B9, B10, B11, B12, B14, B15, C1, C3, C5, C7) showing successful separation of 14 kDa and 28 kDa bLALBA. b HC11 cells were incubated with bLALBA HPLC separated fractions (B9 0.05 mg/ml; B10 0.5 mg/ml; B11 0.8 mg/ml; B12 0.15 mg/ml; B13 2 mg/ml; B14 5 mg/ml; B15; 2 mg/ml) for 7 days at 37 °C in DMEM supplemented with 10% FCS. Cell growth was quantified by SRB assay. Each value represents mean ± SEM. *Indicates a significant difference of $p < 0.01$ at day 7 compared to PBS treatment. Only fractions containing 28 kDa LALBA showed apoptotic activity. c Fraction B13 was incubated at 80 °C/h to promote dimerization, but did not exhibit apoptotic activity. d Deconvoluted ESI-TOF mass spectrum of 28 kDa bLALBA isolated using Superdex 75 without DTT and e with DTT and raw MS data plotted as intensity versus mass-to-charge ratio is shown as insets.

formation at high concentrations [36]. By inspecting the structure we identified that Gln 39 from one monomer can form a covalent link with Lys 98 from a different monomer without the need for any structural rearrangement except for a change in side chain orientations.

In our previous study we treated HC11 with bLALBA (28 kDa and 14 kDa combined) and used in situ detection of DNA fragmentation which was accompanied by changes in cell morphology and cell death to demonstrate apoptosis [4]. Here the confirmation of effects of 28 kDa LALBA in the absence of 14 kDa LALBA was shown to be apoptotic by examination of cell morphology and subsequent death of cells. H&E staining of HC11 cells showed normal morphology with large nuclei and cytoplasm prior to the addition of 1 mg/ml bLALBA (Fig. 3a). Following bLALBA exposure for 24 h the few cells that remained displayed apoptotic morphology where cells were small and shrivelled with no detectable cytoplasm (Fig. 3b).

During our studies, consistent with Xu et al., 2005 [14] we also found that commercial preparation of bLALBA differed in activity and were batch-dependent. Some batches showed apoptotic activity while other batches showed no activity (data not shown). SDS-PAGE analysis suggested that the presence of 28 kDa LALBA within the sample of inactive LALBA did not appear to be responsible for this activity (data not shown) but may be related to issues with commercial production which interfere with preservation of protein activity.

3.4. 28 kDa LALBA structural requirements

Previous studies have shown that both MAL and HAMLET are active in the apo-conformation and are stabilised with a fatty acid co-factor [11]. In addition, calcium is involved in the apoptotic signal response to MAL [7]. In order to determine if calcium was required in the media for 28 kDa bLALBA apoptotic activity, cells were incubated in the presence and absence of calcium. Furthermore, to determine if calcium was required for 28 kDa bLALBA activity the molecule was incubated in the presence of EDTA in order to remove the bound ion. A two-way ANOVA showed that regardless of whether HC11 cells were incubated in the presence or absence of calcium, a highly significant apoptotic effect was observed when cells were exposed to 28 kDa bLALBA incubated with or without EDTA (Fig. 3c). Therefore, the apoptotic activity of the protein does not require the presence of either bound or free calcium, which suggests 28 kDa bLALBA functions differently to MAL.

The function of HAMLET is dependent on its fatty acid co-factor [37]. To determine if 28 kDa bLALBA had a similar requirement the protein was pre-incubated with BSA to sequester any fatty acids [12]. It should also be noted that the media for all experiments included 10% FCS, which was effective in sequestering fatty acid co-factors from HAMLET and bovine LALBA made lethal to tumour cells (BAMLET) [37]. A one-way ANOVA of the different treatments showed that regardless of treatment with BSA or FCS, samples that contained bLALBA still induced a highly significant level of apoptosis in HC11 cells ($p < 0.001$; Fig. 3d). However, there was no significant difference in the level of apoptosis in cells cultured in media that contained heat-treated bLALBA, and control media (Fig. 3d). Therefore, although a conformational state is required, the presence of BSA or FCS has no effect on the activity of the protein, which suggests 28 kDa bLALBA is dissimilar to MAL and BAMLET/HAMLET.

The length of exposure of cells to bLALBA required to induce apoptosis ranged from 1 to 4 h and was found to differ between cell types (Fig. 3e-g). Cells were incubated with bLALBA for 1, 2, 3, 4 and 5 h periods, after which medium that contained bLALBA was removed, the wells washed with PBS, and fresh medium without bLALBA was added for a further 4 days before cell survival was measured. Both HC11 cells and wallaby mammary epithelial primary cells required < 1 h of bLALBA

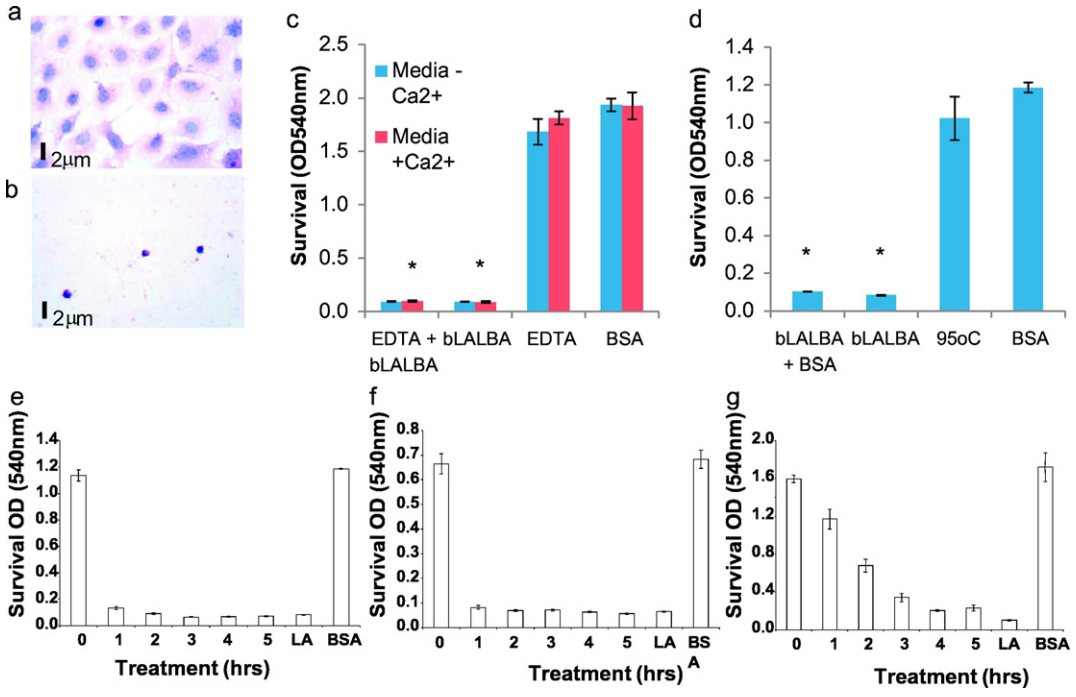

Fig. 3. Characterization of 28 kDa bLALBA apoptotic activity. a Morphology of hematoxylin and eosin stained HC11 cells cultured in the absence and b presence of bLALBA (1 mg/ml) in DMEM/10% FCS medium. c Incubation of bLALBA in the absence of calcium does not prevent apoptotic activity. bLALBA (1 mg/ml) was incubated with 10 mM EDTA for 1 h at room temperature to remove bound Ca^{2+} before addition to culture. Cell survival was quantified by SRB assay after 4 days. Post-hoc analysis revealed that there was a significant amount of apoptosis in HC11 cells exposed to any treatment that contained bLALBA. Each value represents mean ± SEM ($n = 4$). ** $p < 0.001$. d Incubation of bLALBA with BSA does not prevent apoptotic activity, however heat treatment does. bLALBA (1 mg/ml) was incubated with BSA for 1 h at 37 °C to sequester co-factors before addition to culture media. Cell survival was quantified by SRB assay after 4 days. Post-hoc analysis showed that there were significant differences in the level of apoptosis between treatments. Each value represents mean ± SEM ($n = 4$). ** $p < 0.001$. e Examination of the temporal effect of bLALBA on apoptosis on HC11 cells, f wallaby primary cells (WalP) and g bovine primary cells (BMEC). Cells were incubated with bLALBA (1 mg/ml) and removed hourly (1–5 h). In control cultures the bLALBA (1 mg/ml) and BSA (1 mg/ml) were not removed from media. Cell growth was quantified by SRB assay after 4 days. Each value represents mean ± SEM ($n = 4$).

exposure to induce irreversible apoptosis with no recovery (Fig. 3e and f). Although bovine mammary epithelial primary cells showed some apoptosis following 1 h of bLALBA exposure (measured 4 days later), the cells required a longer exposure time of 4 h to induce apoptosis of the entire cell population with no recovery (Fig. 3g).

3.5. Characterization of 28 kDa apoptotic effects

Native bLALBA has been previously found to exert an apoptotic effect on IEC-6 intestinal cells [14], RAW cells [5] and primary mammary epithelial cells from a fur seals [4]. Our current study now suggests that this apoptotic activity is due to the presence of a naturally occurring dimeric 28 kDa LALBA form, while the most abundant 14 kDa LALBA form is inactive. In order to extend these studies, 28 kDa LALBA apoptotic activity was measured using primary bovine cells and primary wallaby cells, immortal HC11, AGS and Hek293 cell lines and a variety of human breast cancer cell lines. The observed apoptotic effects of 28 kDa bLALBA were found to be concentration dependant for all cell types examined. Cells were continuously exposed to titrations of bLALBA that ranged from 0.25–1.6 mg/ml for a total of 7 days, and measurements were taken at 1, 2, 3, 4 and 7 days of culture. The sensitivity of primary mammary epithelial cells to bLALBA depended on the species of origin (Fig. 4a and b). Apoptosis of the entire population of wallaby primary mammary epithelial cells occurred with exposure to bLALBA at a concentration of 0.8 mg/ml, and the cells did not exhibit any recovery. bLALBA induced apoptosis in only a proportion of these cells at a lower concentration of 0.4 mg/ml. The 'lag' effect in cell growth observed by day 7 of incubation was not due to cell recovery, but due to the proliferation of a small population of cells that survived (Fig. 4a). In contrast, bovine primary mammary epithelial cells (BMEC) required a bLALBA concentration of 1.6 mg/ml to induce apoptosis of the entire cell population, and a lower concentration of 0.8 mg/ml was only potent

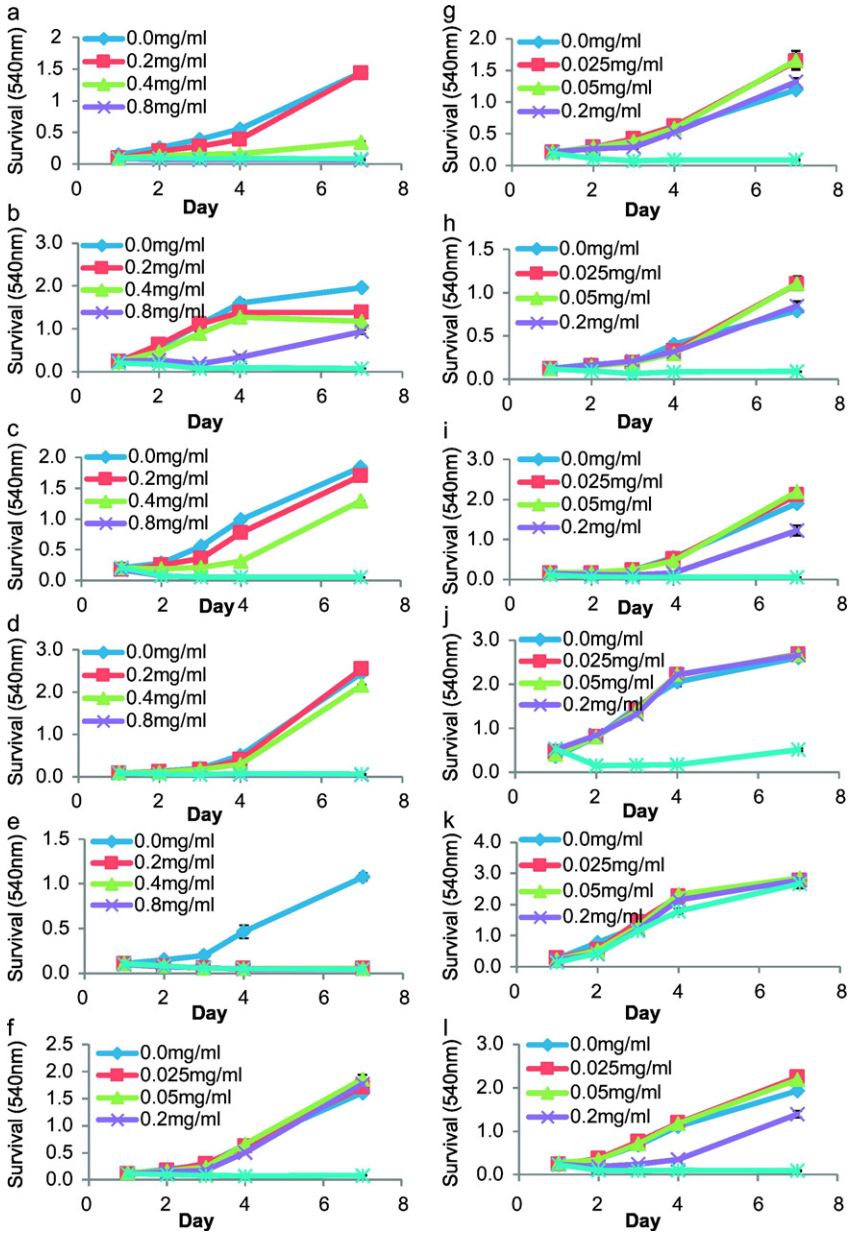

Fig. 4. Effect of bLALBA concentrations on apoptosis of primary epithelial cells and immortal and cell lines. a wallaby primary cells, b bovine primary cells, c AGS cells, d Hek293 cells, e HC11 cells, f MCF7 cells, g SkBr3 cells, h MDA-MD-453 cells, i T47D, j HS578T cells, k MDA-MD-231 cells and l BT578T cells. Cell growth was quantified by SRB assay. Each measurement of cell survival was performed in quadruplicate ($n = 4$).

enough to induce apoptosis in a proportion of cells (Fig. 4b). Again the cells that had not died as a result of bLALBA treatment were able to proliferate so that the cell population had increased by day 7 of incubation.

Similarly, the apoptotic effect of bLALBA differed between immortalised cell lines and was dose-dependent (Fig. 4c–l). Apoptosis of the entire population of intestinal AGS cells and Hek293 kidney cells occurred at a bLALBA concentration of 0.8 mg/ml (Fig. 4c and d), and the cells did not exhibit any recovery. A concentration of 0.4 mg/ml was sufficient to induce apoptosis in a proportion of AGS cells, however the remaining population of cells had increased by day 7. The mammary epithelial HC11 cell line was the most sensitive to the effects of bLALBA, and apoptosis of the entire cell population occurred at a concentration of 0.2 mg/ml (Fig. 4e). Apoptosis of the breast cancer cell lines MCF7, SkBr3, MDA-MB-453, T47D, HS578T and BT578T occurred at a bLALBA concentration of 0.8 mg/ml (Fig. 4f–l), with the exception of MDA-MB-231 cells which required a higher concentration of 2 mg/ml bLALBA in order to elicit apoptotic effects (data not shown). A bLALBA concentration of 0.2 mg/ml was potent enough to induce apoptosis in a proportion of SkBr3 cells and BT578T cells, of which the surviving cells proliferated to increase the population density by day 7 of incubation (Fig. 4g and l). All cells types displayed similar apoptotic morphology after exposure to bLALBA for 24 h (data not shown). Exposure of bLALBA for longer periods caused cells to break apart with only cell debris remaining.

3.6. Apoptosis pathways and biofunctions initiated by 28 kDa bLALBA

In order to characterize the apoptotic pathways stimulated by 28 kDa bLALBA, mouse Affymetrix microarrays were used to examine transcript expression profiles of HC11 cells exposed to 28 kDa bLALBA. Genes were considered differentially expressed if there was a significant differential expression (p-value < 0.001) with 1.5-fold or more increase or decrease of intensity between samples with a. HC11 cells incubated in DMEM and 10% FCS (control) were compared with HC11 cells incubated in the same media with the addition of bLALBA (1 mg/ml) for 5 h (treatment). Cells exposed to bLALBA showed 451 genes were up-regulated and 1531 genes were down-regulated. Gene expression profile datasets were integrated and analyzed to investigate functional pathways affected in HC11 cells exposed to bLALBA using the Ingenuity Pathways Analysis software (Ingenuity® Systems, http://www.ingenuity.com). An analysis of biofunctions based on IPA downstream effects showing a prediction of function based on a comparison of the direction of gene change in the data with expectations derived from the literature was performed (Table 1). This analysis predicts that functions in the categories of DNA Replication, embryonic development, organismal development, organismal survival, cellular assembly and organization, and gene expression all had increased activation states associated with cell death after the addition of bLALA. Decreased repair of DNA and decreased DNA damage response with an increase in damage of chromosomes was also observed in addition to decreased initiation of transcription and increased translation of RNA.

Canonical pathways were also explored. The top 5 pathways were: Nuerotrophin/TRK signaling, Prolactin signaling, IGF-1 signaling, Growth hormone signaling and ERK5 signaling. Many of the genes in these pathways were down regulated suggesting a reduction in cellular activity at this time point. In order to further investigate functional changes within the cells exposed to bLALBA a list of the predicted activation status of transcription factors was generated (Table 2). Notably, NFκB/p65 was predicted to be activated while histone deacetylation (HDAC) was predicted to be inhibited. Analysis of the top molecules showing the largest fold change (Table 3) showed that many of these genes are associated with a response to inhibition of HDAC, which was shown to be inhibited in Table 3.

3.7. Validation of transcriptional responses

LALBA-induced transcriptional responses determined by microarray were verified by qRT-PCR. Validation was performed on RNA isolated from an independent experiment using HC11 cells exposed to 1 mg/ml LALBA for 6.5 h (Fig.5). Expression of selected genes by qRT-PCR supported the microarray-based observations.

3.8. Effect of LALBA on HDAC activity of MCF7 cells

To investigate the inhibitory effects of bLALBA on HDAC activity in cells we treated MCF7 cells with increasing concentrations of bLALBA for a 24 h period. After 24 h treatment cells exposed to 2.0 mg/ml LALBA showed typical rounding of cells, while lower concentrations (0.25–1.0 mg/ml) displayed no obvious signs of morphological changes (data not shown). The HDAC activity of treated cells was shown to be inhibited in a dose dependent manner (Fig. 6a), implying that the apoptotic effects of bLALBA was closely related to the inhibition of HDAC activity. Specificity of this reaction was confirmed by a supplementary treatment with Trichostain A (TSA), a known HDACi (Histone deacetylase inhibitor) which further reduced HDAC activity (Fig. 6b). bLALBA HDAC inhibitory activity was also shown to be effective (but somewhat reduced) over a two hour period (Fig. 6c). The concentration of effective dose of 28 kDa LALBA was calculated to be 2.14 mM (28 kDa LALBA is 3% of total LALBA, effective dose of total LALBA at 2 mg/ml). The inhibitory effects of 14 kDa LALBA alone, and an inactive batch of bLALBA (batch # 035 K044) on HDAC activity were also measure and showed no effect (Supplementary Fig. 1b).

Previous studies have shown that HDACIs activate NF-κB in diverse cell types [38–40], which was predicted to be activated in our studies. In order to validate this phenomenon MCF7 cells were treated ± bLALBA (1 mg/ml) for 2 h and NF-κB localization was analyzed using immunocytochemistry (Fig. 5d–g). These studies showed that bLALBA exposure caused NF-κB translocation to the nuclear fraction.

Table 1
Biofunctions of HC11 cells following exposure to bLALBA.

Category	Functions annotation	Predicted activation state	Regulation z-score	p Value	# Molecules
DNA replication	Repair of DNA	Decreased	−2.106	1.71E-09	124
	DNA damage response	Increased	2.191	3.19E-04	69
	Damage of chromosomes	Increased	2.143	3.43E-03	23
Embryonic development	Size of embryo	Decreased	−3.835	4.41E-12	173
	Death of embryo	Increased	2.192	1.07E-06	61
Organismal development	Delay in growth of organism	Increased	2.121	8.74E-03	11
Organismal survival	Death of embryo	Increased	2.192	1.07E-06	61
	Organismal death	Increased	2.223	2.18E-06	924
	Viability	Decreased	−2.774	6.10E-04	39
Cellular assembly and organization	Association of chromosome compartments	Decreased	−2.599	6.30E-04	8
	Association of chromatin	Decreased	−2.200	5.67E-03	5
Gene expression	Initiation of transcription	Decreased	−2.191	2.37E-07	69
	Translation of mRNA	Increased	2.345	7.94E-4	27

Table 2
Predicted activation status of transcription factors of HCll cells exposed to bLALBA.

Transcription regulator	Predicted activation	Regulation z-score	Molecular type
CTNNB1	Activated	3.385	Transcription regulator
RELA (p65)	Activated	3.159	Transcription regulator
NR5A2	Activated	2.963	Ligand-dependent nuclear receptor
Ap1	Activated	2.817	Complex
CREBBP	Activated	2.788	Transcription regulator[a]
CREB1	Activated	2.762	Transcription regulator[b]
HNF4A	Activated	2.711	Transcription regulator
FOXL2	Activated	2.682	Transcription regulator
NFKB1	Activated	2.634	Transcription regulator
ELK1	Activated	2.596	Transcription regulator
NFATC3	Activated	2.506	Transcription regulator
ID1	Activated	2.423	Transcription regulator
HMGA1	Activated	2.407	Transcription regulator
PDX1	Activated	2.384	Transcription regulator
MTPN	Activated	2.387	Transcription regulator
GLI1	Activated	2.354	Transcription regulator
NFkB (complex)	Activated	2.212	Complex
SP1	Activated	2.167	Transcription regulator
GATA4	Activated	2.165	Transcription regulator
KDM5B	Activated	2.074	Transcription regulator
NANOG	Activated	2.042	Transcription regulator
ATF2	Activated	2.02	Transcription regulator
ZFP36	Inhibited	−2.15	Transcription regulator
ZNF217	Inhibited	−2.36	Transcription regulator
TAF4	Inhibited	−2.408	Transcription regulator
DACH1	Inhibited	−2.473	Transcription regulator
FOSL1	Inhibited	−2.545	Transcription regulator
Hdac	Inhibited	−2.706	Group
TBX2	Inhibited	−2.886	Transcription regulator

[a] Basal transcription machinery.
[b] ATM signaling.

4. Discussion

Death of cells by chemically induced forms of LALBA isolated from the casein fraction of human milk, MAL and HAMLET, has been reported previously [12,33]. Both these forms of LALBA induce cell death in a variety of tumour cells and immature cells, but have no effect on differentiated, non-transformed cells [9]. The naturally occurring 28 kDa bLALBA complex, however, had indiscriminate apoptotic activity and possessed markedly different properties. We also discovered that although 28 kDa LALBA was a functionally stable complex, it was more than just a dimeric structure. Formation of 28 kDa bLALBA simply by stimulating dimerization of 14 kDa bLALBA did not activate its apoptotic properties, suggesting a co-factor may also be required for 28 kDa LALBA activity. We suggest that the proposed co-factor is bound to the dimeric 28 kDa LALBA structure and thus is protected from sequestration by FCS or BSA. This property is of particular significance if LALBA is to be used in future therapies. In contrast, the inactivation of MAL/HAMLET/BAMLET by serum components might be a limiting factor in their potential use as a therapy.

Examination of HC11 cell morphology and biofunction, in addition to our previous results showing in situ detection of DNA fragmentation as assayed by using the classical apoptosis Tunnel stain [4], suggests cell death induced by 28 kDa bLALBA is a result of apoptosis. A number of individual genes implicated in apoptosis were also upregulated; ID2, has been implicated in promoting apoptosis [41] while antisense inhibition of *Id2* expression has been shown to protect cells from cell death, supporting the likelihood of a physiological role for Id2 in mediating apoptosis; EGR2, found to induce apoptosis in various cancer lines by direct transactivation of BNIP3L and BAK [42,43]; and FOS (a member of the AP-1 complex, also shown to be activated here) [44]. Down regulated genes involved in apoptosis include FANCF which is involved in maintaining a DNA-damage response pathway [45].

Analysis of predicted activation status of transcription factors following exposure to bLALBA suggested that histone deactylation (HDAC) was inhibited. Subsequent analysis of HDAC activity of MCF7 exposed to bLALBA for 24 h supported this conclusion showing that bLALBA inhibited HDAC activity in a dose dependent maner. Histone deacetylases are essential components of co-repressor complexes as they provide the enzymatic activity for active repression [46]. HDAC are a class of enzymes that remove acetyl groups from an ε-*N*-acetyl lysine amino acid on a histone. Removal of acetyl groups gives chromatin a tightly-bound structure that prohibits transcription. Inclusion of acetyl groups decreases the affinity of histones for DNA and allows transcription to occur. Inhibition of HDACs regulate chromatin structure and act to increase histone acetylation by preventing HDAC activity leading to relaxation of the DNA conformation and an increase in transcription activity and subsequent upregulation of certain genes [47]. This effect can cause cell growth arrest and induction of apoptosis [48,49]. Gene transcript analysis revealed that in the top 10 differentially expressed genes (up or down regulated) a number of genes were also associated with a response to inhibition of histone deacetylase proteasome inhibition, namely, ID2 [50], HSP70 [51], ATF3 [52], FOSB [53], AREG/AREGB [54]. Notably, elevated FOSB expression has been identified by microarray analysis as a potential marker of valproic acid (an established HDAC inhibitor) sensitivity [53].

HDAC inhibitors (HDACi) are capable of inhibiting HDACs with varying efficiency (at nanomolar to millimolar concentrations). It appears that 28 kDa bLALBA effectively causes HDAC inhibition (either directly or indirectly) at 2.1 mM which is within this range. Our assays also showed that bLALBA reduced HDAC activity of HCll cells after only 2 h in culture. When compared to the inhibitory effects of the prototype HDAC inhibitor TSA, bLALBA effects were reduced, however we predict that this inhibitory effect may be improved in future studies by increasing the dose of bLALBA.

Our data suggest that Rel/NFkB activation is involved in the 28 kDa bLALBA stimulated apoptosis. The transcription factor NFkB is a critical immediate early response gene involved in modulating cellular responses to apoptosis following diverse environmental injuries. The activation of NFkB is widely accepted to play an anti-apoptotic role in cellular response to injury, but it is also involved in histone deacetylase

Table 3
Top molecules with greatest gene expression changes of HCll cells exposed to bLALBA.

Up-regulated	Fold	Function	Down-regulated	Fold	Function
Id2	31.6	Regulator of cell proliferation and Id2 mediated apoptosis	Cstf2	−19.3	3′ end cleavage and polyadenylation of pre-mRNAs
Fos	20.9	Proto-oncogene and mediator of cMyc induced apoptosis	Kbtbd7	−12.9	Activators mitogen-activated protein kinase (MAPK) signaling
Hspa1a	14.6	Inhibits apoptosis via Apaf-1	Socs6	−12.9	Cell proliferation
Atf3	14.6	Induced after DNA damage to aid survival	Fancf	−11.1	DNA repair protein
Nfkbia	10.3	Inhibits NFKB/REL formation	Zfp871	−11.0	Unknown
Sprr1a	8.8	Regeneration associated gene	Fosl2	−10.7	Cellular proliferation and apoptosis
Egr2	8.3	Increases apoptosis	Has2	−10.6	Extracellular matrix protein, tissue repair
Malat1	8.3	Non-coding RNA	Emp2aiP1	−10.5	Dual Specificity Phosphatase involved in Lafora disease
Fosb	7.7	Transcription factor and activation-induced cell death	Fem1c	−10.3	Cell death and associated with histone deacetylase activity
Gadd45g	7.6	Stress responsive gene and DNA damage response	Tspyl1	−10.3	Nuclear import and the formation of nuclear bodies

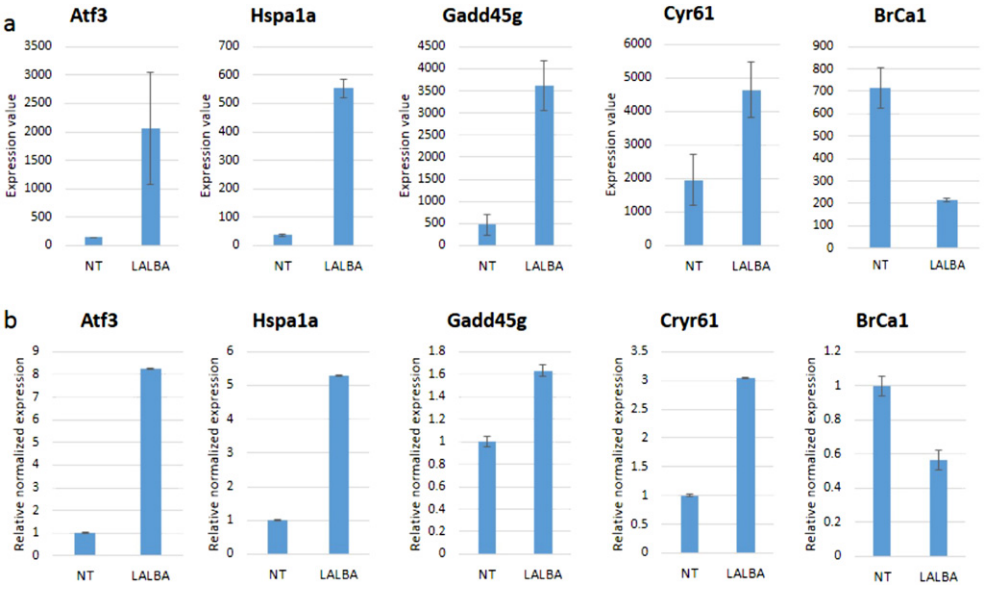

Fig. 5. Validation of LALBA-induced transcriptional responses by qRT-PCR. Transcriptional responses of HC11 cells following treatment with 1 mg/ml LALBA as observed by microarray were validated by repeating the experiment and performing qRT-PCR. Expression intensities of Atf3, Hspa1a, Gadd45g, Cyr61 and BrCa1 from (a) Affymetrix mouse microarray probe sets and (b) qRT-PCR analysis. For qRT-PCR each value represents mean ± SEM ($n = 3$) and normalized to GAPDH. Expression changes were $p < 0.001$.

inhibitor (HDACI)-mediated NFkB activation [55]. Our data also suggest that Ataxia-telangiectasia mutated (ATM) signaling via CREBBP is activated. HDAC inhibitors regulate chromatin structure and activate the DNA damage checkpoint pathway involving ATM [56]. This result suggests that ATM signaling is also used as part of the DNA damage response to bLALBA treatment.

Similar to 28 kDa bLALBA, cells treated with HAMLET display apoptotic morphology [7]. The exact nature of cell death induced by MAL/HAMLET/BAMLET complexes has been somewhat mixed, with reports stating that cell death occurs via an apoptosis-like mechanism [15,16,57] macroautophagy [17], which is upregulated during cytoxic response, activation of a lysosomal cell death program [18] and toxicity [19]. HAMLET induced apoptosis is therefore reported to be a cellular response and not the cause of death (for review see [20]). This observation is consistent with HDACi effects observed here. Although cell death in HAMLET treated cells results in caspase activation and cytochrome *c* release [58], caspase inhibitors do not prevent cell death [57]. Furthermore, experiments using Bcl-2 and p53 tumour suppressor transgenic cell lines do not rescue HAMLET treated cells [16]. HAMLET has been shown to bind to the cell surface [19,59] and enter the cell cytoplasm where it interacts with histones and chromatin in cell nuclei, locking the cells into the death pathway through irreversible disruption of chromatin organization [22]. Although HAMLET/BAMLET has not been measured for direct HDACi activity it has been reported that HAMLET cell death effects are enhanced by HDACi's such as TSA [60]. Cells pre-treated with TSA and then exposed to HAMLET showed greater H4 acetylation than cells treated with TSA alone, and HAMLET pre-treatment prevented the hyperacetylation response to TSA. In contrast, our results showed that hyperacetylation by TSA was not inhibited following 24 h bLALBA exposure to cells. The further inhibition of HDAC by TSA following bLALBA exposure indicates that the assay measuring HDAC activity was specific.

Studies have shown that 14 kDa LALBA does not have to be converted to HAMLET to bind to histones in vitro, however, it cannot enter cells to translocate to the nuclei [8,61]. Whether 28 kDa LALBA can translocate the plasma membrane and be transported to the nucleus is unknown. However, the reduced HDAC activity of LALBA treated cells may suggest that it also enters the nucleus and binds histones. The difference in efficacy of 28 kDa LALBA and HAMLET/BAMLET toward non-transformed cells suggests that these complexes have different mechanisms of action. The variation in sensitivity to different cell lines such as MDA-MD-231 suggests a mechanism that relies on specific receptors that may be expressed at varying levels regulating 28 kDa LALBA uptake or movement to the nucleus.

Apoptosis is critical for the removal of the milk secreting alveolar epithelial cells at weaning, so that the architecture of the tissue can be remodelled prior to a successive lactation. Mammary derived signaling axes are induced during the first phase of involution and a number of mechanisms have been postulated, including accumulation of apoptotic factors in milk at weaning [62–65].This first phase typically comprises down regulation of milk protein gene expression while the second phase of involution is marked by high rates of apoptosis. The 28 kDa LALBA complex is an attractive candidate for initiation of involution. The current study showed that anti-LALBA antibodies only recognised 28 kDa bLALBA in the whey from cows during the drying-off period. Furthermore, we found that although 28 kDa LALBA is potent and fast acting, it is also dose dependant. Thus, it is plausible that low concentrations of 28 kDa LALBA present in milk during mid-lactation would not induce involution in the mammary gland during the lactation period or digestive tract of the recipient young. Similar to the mode of regulation employed by FIL (feed back inhibitor of lactation) [66,67], 28 kDa LALBA may be sensitive to the frequency and completeness of milk removal. As lactation ceased and milk accumulated in the gland, 28 kDa LALBA could increase to a concentration which becomes effective to cells. Microarray analysis suggests that in HC11 cells treated with bLALBA the prolactin pathway is down regulated and a number of apoptotic pathways are activated, implicating LALBA in both the first and second phases of the involution process. The observed NFkB activation of LALBA treated cells in our studies correlates with a growing body of evidence implicating NFkB in the involution process (for review see [68]). It has been reported that NFkB is activated in response to milk stasis [63] and by the signal transducer and activator of transcription (STAT3) [69]. STAT3 is the earliest transcription factor to be activated in involution within a few hours following forced weaning [63]. More recently NFkB activation has been shown to reduce milk production and increase the cleavage of caspase-3, an indicator of apoptosis [70].

Several other lines of evidence also support a role for LALBA in mammary gland involution. The fur seal lactation phenotype shows mothers

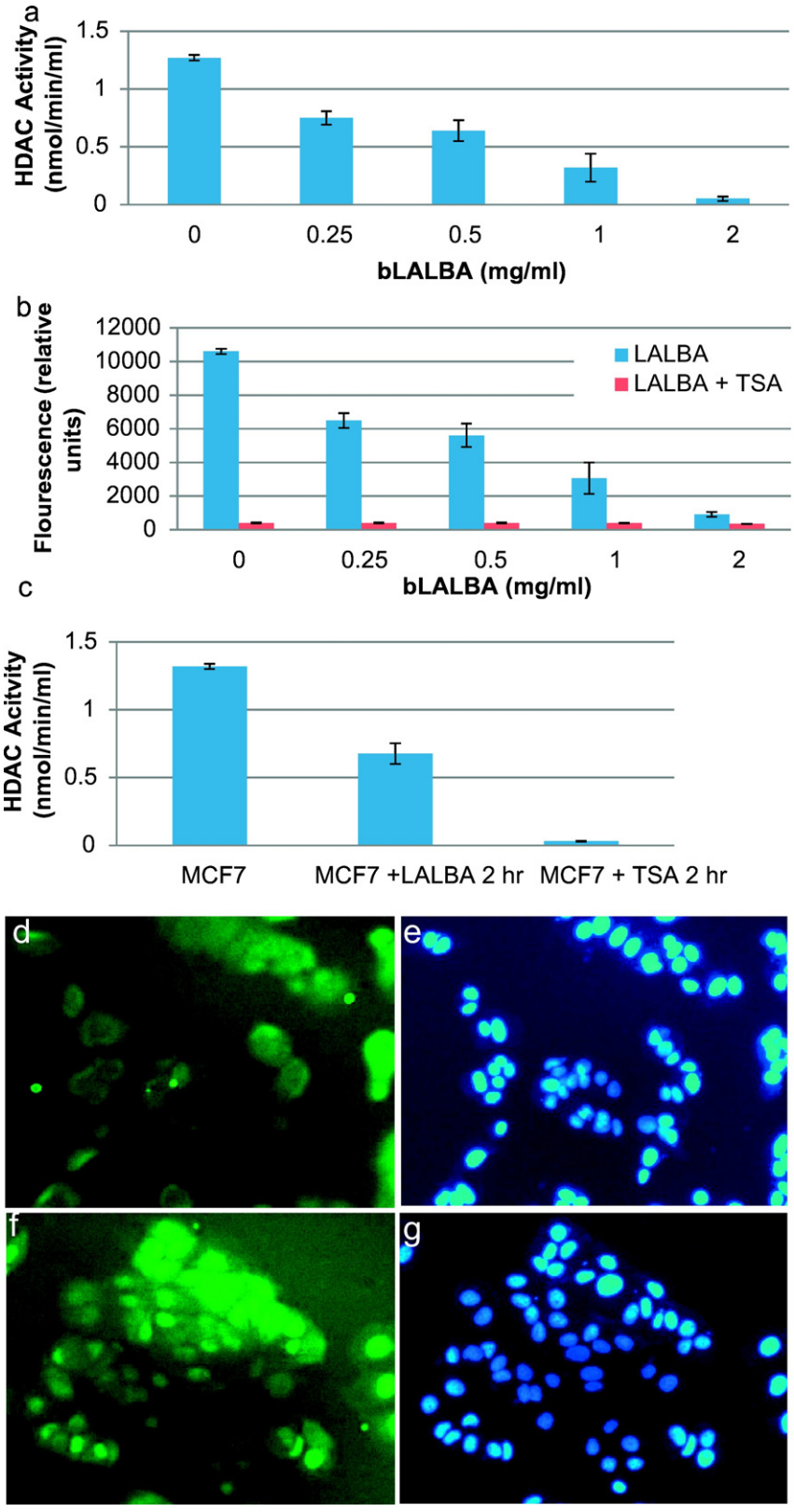

Fig. 6. bLALBA histone deacetylase activity and NF-κB nuclear localization. a bLALBA (0–2.0 mg/ml) was applied to MCF7 cells for 24 h and HDAC activity was determined. b Specificity of HDAC reaction was tested by adding bLALBA (2 mg/ml) to MCF7 cells for 24 h followed by a 2 h treatment with TSA (red) or without TSA (blue). Data is shown as fluorescence at 460 nm. c HDAC inhibition of MCF7 cells exposed to bLALBA was compared to TSA over a 2 h time period. Data are expressed as mean ± SEM ($n = 2$). $p \leq 0.01$ for all treatments compared to untreated cells. Representative assay performed twice. e Fluorescent microscopic analysis of untreated MCF7 cells showing p65 localization (*green* florescence) to the cytoplasm and e Hoechst co-stain (*blue* fluorescence) of the same cells showing location of nuclei. f Nuclear localization of p65 in MCF7 cells exposed to bLALBA (1 mg/ml, 2 h). g and Hoechst co-stain (*blue* fluorescence) of the same cells showing location of nuclei.

can endure long foraging trips to sea (up to 23 days) in the absence of involution, returning to shore to deliver copious quantities of milk to their young [71]. Examination of LALBA gene function shows the gene is spliced incorrectly, is poorly expressed and fails to be translated [4], and it was suggested that the lack of LALBA in fur seal milk enables uncoupling of milk stasis and mammary gland involution [4]. The fur seal protein product, if translated, is predicted to comprise a truncated protein which is terminated at amino acid 104. Interestingly, the suggested covalent cross linking between Gln 39 from one monomer with Lys 98 from a different monomer to form the highly stable apoptotic dimer would still occur in the fur seal LALBA product if it was correctly expressed and translated. This suggests that the combination of down regulation and lack of translation of LALBA protein has led to the loss of involution in fur seals, rather than incorrect splicing which may have occurred earlier in the fur seals evolutionary history to limit lactose synthesis.

Similarly it has been observed that LALBA-deficient mice maintain mammary tissue in an active state with normal milk protein composition and mammary structure after 5 days of lactation, even though pups are unable to remove milk from the gland [72]. Thus, the absence of LALBA may explain the delay in mammary gland involution of LALBA deficient mice.

It has also been reported that although a high concentration of LALBA (3 mg/ml, compared with 0.8 mg/ml in mouse milk) is observed in the tammar wallaby (*Macropus eugenii*) and a didelphid marsupial (*Monodelphis domestica*), the concentration of total lactose decreases to zero during late lactation [73]. As the major function of LALBA is to act as a specifier protein for lactose synthetase in the formation of lactose, the continued presence of LALBA in milk during late lactation when no lactose is secreted is puzzling, and could be consistent with a role in mammary gland involution. Certainly, a local autocrine factor such as LALBA present in accumulated milk would explain how the tammar wallaby can have both an actively lactating and involuting gland existing in the same animal [65,74]. Consequently, immunisation of dairy cows against the 28 kDa LALBA form may improve lactational persistency, as the milk production decline after peak lactation is the result of cell loss by apoptosis [75].

5. Conclusion

The MAL/HAMLET and 28 kDa LALBA are clearly different variants of the same LALBA molecule. Therefore, similar to the chemically induced form of HAMLET, the naturally occurring 28 kDa LALBA has potential use as a therapeutic agent against tumours and as a topical cream to remove papillomas [37]. The 28 kDa LALBA complex could also be used for the topical treatment of skin melanomas and therapeutic/cosmetic treatment of either any skin lesion/disease or treatment that enhances the health of skin and that requires rapid removal of cells. Furthermore, 28 kDa LALBA could be used to treat tumours through various drug delivery systems and/or by the stimulation of endogenous 28 kDa LALBA production by tumour cells. The high potency of 28 kDa LALBA and its natural occurrence in milk without further chemical modification may also make it a more attractive alternative to HAMLET.

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