MINIREVIEW

Structure and function of human α-lactalbumin made lethal to tumor cells (HAMLET)-type complexes

Ann-Kristin Mossberg[1], Kenneth Hun Mok[1,2], Ludmilla A. Morozova-Roche[3] and Catharina Svanborg[1,4]

1 Department of Microbiology, Immunology and Glycobiology (MIG), Institute of Laboratory Medicine, Lund University, Sweden
2 School of Biochemistry and Immunology, Trinity College Dublin, Ireland
3 Department of Medical Biochemistry and Biophysics, Umeå University, Sweden
4 Singapore Immunology Network (SIgN), A*STAR, Singapore

Keywords
α-lactalbumin; cancer; cell death; ELOA; HAMLET; lysozyme; oleate; oleic acid; protein folding

Correspondence
A.-K. Mossberg, Department of Microbiology, Immunology and Glycobiology (MIG), Institute of Laboratory Medicine, Lund University, Sölvegatan 23, S-223 62 Lund, Sweden
Fax: +46 46 13 74 68
Tel: +46 46 222 71 85
E-mail: Anki.Mossberg@med.lu.se

(Received 30 April 2010, revised 18 August 2010, accepted 2 September 2010)

doi:10.1111/j.1742-4658.2010.07890.x

Human α-lactalbumin made lethal to tumor cells (HAMLET) and equine lysozyme with oleic acid (ELOA) are complexes consisting of protein and fatty acid that exhibit cytotoxic activities, drastically differing from the activity of their respective proteinaceous compounds. Since the discovery of HAMLET in the 1990s, a wealth of information has been accumulated, illuminating the structural, functional and therapeutic properties of protein complexes with oleic acid, which is summarized in this review. *In vitro*, both HAMLET and ELOA are produced by using ion-exchange columns preconditioned with oleic acid. However, the complex of human α-lactalbumin with oleic acid with the antitumor activity of HAMLET was found to be naturally present in the acidic fraction of human milk, where it was discovered by serendipity. Structural studies have shown that α-lactalbumin in HAMLET and lysozyme in ELOA are partially unfolded, 'molten-globule'-like, thereby rendering the complexes dynamic and in conformational exchange. HAMLET exists in the monomeric form, whereas ELOA mostly exists as oligomers and the fatty acid stoichiometry varies, with HAMLET holding an average of approximately five oleic acid molecules, whereas ELOA contains a considerably larger number (11– 48). Potent tumoricidal activity is found in both HAMLET and ELOA, and HAMLET has also shown strong potential as an antitumor drug in different *in vivo* animal models and clinical studies. The gain of new, beneficial function upon partial protein unfolding and fatty acid binding is a remarkable phenomenon, and may reflect a significant generic route of functional diversification of proteins via varying their conformational states and associated ligands.

Introduction

The native fold of a protein is commonly regarded as its only relevant functional state [1]. However, over the past decade it has become increasingly clear that partial unfolding allows common proteins to adopt new, physiologically relevant functions. Several examples suggest that new functional properties may arise from partial unfolding of a previously native protein in response to new extracellular environments, and that local cofactors that stabilize or further define the fold may be involved [2]. These findings offer a new

Abbreviations
ANS, 8-anilinonaphthalene-1-sulfonic acid; BAMLET, bovine α-lactalbumin made lethal to tumor cells; ELOA, equine lysozyme with oleic acid; ER, endoplasmic reticulum; HAMLET, human α-lactalbumin made lethal to tumor cells; MAL, multimeric α-lactalbumin.

way of resolving the enigma arising from the 'one gene – one protein – one function' argument, and a new mechanism of diversifying protein function. Thus, in addition to alternative splicing of mRNA transcripts, post-translational modifications and changes in tertiary structure of specific domains, partial unfolding of a previously native protein is becoming recognized as a mechanism to generate functional diversity [3].

This review summarizes the information on two well-studied proteins that change function after partial unfolding and binding to fatty acid cofactors. The first example is human α-lactalbumin, which by unfolding can form a tumoricidal complex with oleic acid – human α-lactalbumin made lethal to tumor cells (HAMLET) – with tumoricidal activity and documented therapeutic use [2,4,5]. The second is equine lysozyme, a relative of α-lactalbumin, which partially unfolds while forming a fatty acid complex – equine lysozyme with oleic acid (ELOA) – with cytotoxic functions [6].

HAMLET – a complex of partially unfolded α-lactalbumin and oleic acid

The HAMLET-type complexes, with their strong potential to target undesirable cells, were discovered only two decades ago and since then the HAMLET field has widened in scope, acquiring new members and enriching our understanding of the basic principles underlying protein self-assembly and acquisition of new functionality. HAMLET key features are related to the intrinsic properties of proteins to possess varying functions depending on their conformational states and associated ligands.

HAMLET was discovered by serendipity [7]. During studies of antiadhesive molecules in human milk, tumor cells were shown to undergo substantial morphological changes when mixed with casein. The tumoricidal activity in the casein fraction was obtained after low pH precipitation of human milk [2,7] and the protein component of the casein fraction was identified as α-lactalbumin, a whey protein acting as a substrate specifier in the lactose synthase complex [8], which is needed for lactose production, but with no known tumoricidal activity.

To further characterize the active component, casein was fractionated by ion exchange chromatography, yielding five casein peaks eluting with increasing salt (0–0.3 M), but without tumoricidal activity. The active component remained on the column and was subsequently eluted after raising the salt concentration in the elution buffer to 1 M NaCl. The major component of the eluate was α-lactalbumin and the fraction was named multimeric α-lactalbumin (MAL) due to its oligomeric nature on SDS/PAGE [7,9]. Native α-lactalbumin was shown to lack tumoricidal activity, suggesting that α-lactalbumin in the MAL fraction was structurally modified. As no post-translational modifications were detected, the folding state of α-lactalbumin in MAL was examined with CD and binding of the hydrophobic dye 8-anilinonaphthalene-1-sulfonic acid (ANS). The results showed that MAL contained partially unfolded α-lactalbumin, possibly resulting from the low pH precipitation of the complex from milk.

MAL was tumoricidal under conditions where α-lactalbumin reverts to the native fold, suggesting that the partially unfolded state of α-lactalbumin in MAL was stabilized by a cofactor, which prevented it from reverting to the native state. We identified the cofactor as oleic acid and the conditions required for complex formation were defined by deliberate conversion of native α-lactalbumin to an active complex on an ion exchange column conditioned with oleic acid [2]. The complex was named HAMLET and was defined as a complex between partially unfolded α-lactalbumin and oleic acid.

Human α-lactalbumin is a globular 14.2 kDa milk protein (123 amino acids), expressed in secretory cells of the lactating mammary gland [8,10] during the whole lactating period [11]. After folding in the endoplasmic reticulum (ER), α-lactalbumin is transported to the Golgi apparatus, where it binds to the galactosyltransferase complex and acts as a substrate specifier in lactose production. The α-lactalbumin gene has been proposed to originate from an ancestral lysozyme gene, by gene duplication, 300–400 million years ago; α-lactalbumin shares $\sim 40\%$ sequence identity with human lysozyme [12,13].

The native fold of α-lactalbumin is stabilized by the high-affinity calcium-binding site, coordinated by the side chains of asparagines 82, 84, 87 and 88 and lysine 79 [14]. The α-helical domain contains three major α-helical (amino acids 5–11, 23–34 and 86–98) and two short 3_{10}-helical domains. The smaller β-sheet domain consists of a triple-stranded antiparallel β-sheet (amino acids 40–50). One disulfide bond connects α-helical and β-sheet domains (amino acids 73–91) and three additional disulfide bonds are located in the α-helical (amino acids 6–120, 28–111) and the β-sheet domains (amino acids 61–77) [14]. The protein forms relatively stable folding intermediates with a native-like secondary structure but lacking the specific tertiary side chain packing and with exposed hydrophobic surfaces. Partially unfolded states of α-lactalbumin revert to the native fold when the solvent conditions or temperature

are normalized (Ca^{2+}, temperature or pH) (reviewed in [15]).

ELOA – a complex of equine lysozyme and oleic acid

Recently, a new member was added to the HAMLET field – ELOA [6]. Its constituting component, equine lysozyme, belongs to an extended family of structurally homologous lysozymes and α-lactalbumins, occupying a special position in its family tree. Specifically, equine lysozyme contains the active site involved in the hydrolysis of peptidoglycan residues of bacterial cell walls and acts as a bacteriolytic enzyme similar to all lysozymes, ubiquitous proteins in many body fluids. However, equine lysozyme possesses the conserved, high-affinity calcium-binding site of α-lactalbumins, usually absent in noncalcium-binding c-type lysozymes, and is consequently viewed as an evolutionary bridge between lysozymes and α-lactalbumins. Similar to α-lactalbumins, equine lysozyme is less stable and cooperative than noncalcium-binding lysozymes and forms equilibrium partially folded states of a molten globule type [16–18]. However, like c-type lysozymes, it populates well-defined transient kinetic intermediates [19], possessing some characteristics of equilibrium molten globules. Partially folded states of equine lysozyme serve as precursors for spontaneous self-assembly into amyloid oligomers and fibrils with a very distinctive ring-shaped and linear morphology and the former display cytotoxic activity, causing an apoptotic type of cell death [20,21]. Similar to α-lactalbumins, equine lysozyme is highly abundant in milk. All these unique features make equine lysozyme a strong candidate to possess the properties of HAMLET-type forming proteins.

Methodologies for producing protein–fatty acid complexes

A schematic of the HAMLET production process is shown in Fig. 1A and a schematic structure is shown in Fig. 1B. The method to reproducibly generate HAMLET in the laboratory from its pure constituents has been described [2,7]. Briefly, it involves (a) preconditioning of a DEAE Trisacryl-M matrix with oleic acid; (b) Partial unfolding of α-lactalbumin by removing the Ca^{2+} ion with EDTA; (c) ion exchange

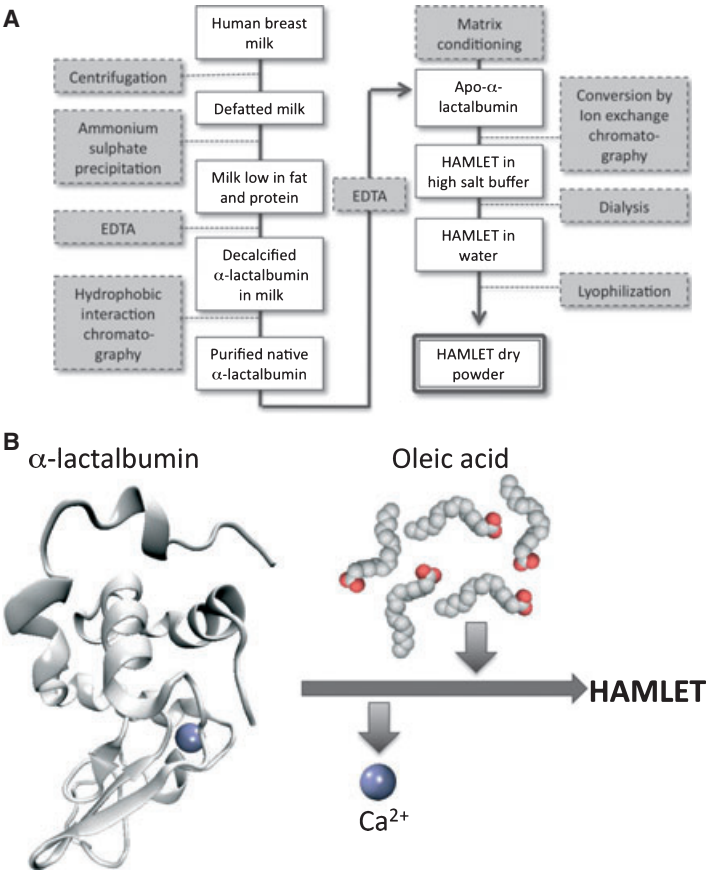

Fig. 1. (A) Flow chart of the purification of human α-lactalbumin and conversion to HAMLET. To form HAMLET, α-lactalbumin must be partially unfolded prior to the addition to the oleic acid-conditioned matrix. Native α-lactalbumin is not retained on the column matrix, elutes in the void volume and does not form active complexes. (B) Schematic of HAMLET complex formation. Native α-lactalbumin is partially unfolded by EDTA, removing the calcium ion. The EDTA-treated protein is subjected to ion exchange chromatography on an oleic acid (C18:1)-conditioned ion exchange matrix and the complex eluted by high salt has incorporated the fatty acid.

chromatography; (d) elution of the protein–fatty acid complex with high salt (from 0.3 to 1.0 M NaCl). HAMLET is structurally stable and maintains tumoricidal activity after storage, especially when lyophilized (A. Mossberg, manuscript in preparation). The method has successfully been developed to meet industrial scale, good manufacturing practice (GMP) requirements.

The molecular interactions between oleic acid and the ion exchange matrix are not fully understood. The active group of the matrix, the DEAE group, is positively charged and might therefore bind negative molecules. At pH 8.5 (the pH of the conversion step) the fatty acid is deprotonated, resulting in a negative net charge [22], potentially allowing the carboxyl-group of the fatty acid to bind to the matrix and leaving the hydrophobic tails facing the water phase [23]. Consistent with this mechanism, the anion exchange matrix, DEAE Trisacryl M, has so far been superior to other matrices in supporting HAMLET conversion. Removal of the DEAE head group from the matrix (Trisacryl-M G50) prevents HAMLET conversion and a cation exchange matrix (CM-Trisacryl-M) is not suitable for HAMLET conversion (A. Mossberg, manuscript in preparation).

Equine lysozyme readily forms ELOA on ion exchange chromatography matrices preconditioned with oleic acid. In contrast to HAMLET, the protein does not require unfolding prior to the chromatographic step to form complexes [6]. The Sepharose matrix is positively charged under the experimental conditions and oleic acid is bound to the matrix before ELOA formation. It is speculated that during interaction with the solid–liquid interface in the column, the hydrophobic residues of equine lysozyme become exposed, facilitating its partial unfolding to the molten globule state and oleic acid binding and, as a result, ELOA formation.

Several groups have attempted to form HAMLET or ELOA by simple mixing and co-incubation of apo α-lactalbumin or equine lysozyme in solution with oleic acid either under native, mildly denaturing acidic (pH 2 and 4.5) or basic (pH 9) conditions. In our early work [24], titration of oleic acid to apo or native α-lactalbumin did not yield an active complex at a protein/lipid ratio of 1 : 1, as monitored by ^{1}H-NMR. However, heat treatment of human or bovine α-lactalbumin at temperatures of 50, 60 or even 80 °C have resulted in the generation of cytotoxic HAMLET or bovine α-lactalbumin made lethal to tumor cells (BAMLET) complexes [25,26]. Titration of apo human α-lactalbumin with oleic acid accompanied by determination of the critical micelle concentration of oleic acid has also resulted in the formation of complexes with different stoichiometries at different temperatures (2.9 at 17 °C and 9 at 45 °C) [27]. In contrast to Kamijima *et al.* [25], Tolin *et al.* [28] observed that complexes were formed after 1 h by mixing protein at pH 7.4 with 10–15 molar equivalents of oleic acid, with activity similar to complexes obtained by the chromatographic method. Zhang *et al.* [29] pointed out parallels between their method to prevent amyloid formation at low pH and the casein precipitation method used to purify MAL [30].

Structural aspects of HAMLET-type complexes

The hallmark spectroscopic signatures of the molten globule state are present in HAMLET: far- and near-UV CD spectra suggesting a retention of secondary structure but near-complete loss of tertiary interactions, respectively, together with the enhancement of fluorescence upon binding of ANS, indicating increased exposure of hydrophobic segments [2]. The ^{1}H-NMR spectrum of HAMLET exhibited broad peaks with poor chemical shift dispersion, indicating a protein in conformational exchange on the millisecond timescale. The NMR signals corresponding to oleic acid were detected in the spectrum and the signal was broader than oleic acid alone, suggesting that the fatty acid was integrated into the protein [2,31]. Recombinant wild-type α-lactalbumin, expressed in *Escherichia coli*, showed identical CD and ANS spectra as the native protein and was readily converted to HAMLET on an oleic acid-conditioned column [2]. Pulsed-field gradient NMR techniques [32] have provided an estimation of the hydrodynamic radii of HAMLET (R_h = 26.9 Å), which is intermediate of the hydrodynamic radii of the acidic molten globule state of α-lactalbumin (R_h = 20.9 Å) and the theoretically extreme expansion state of this protein (= α-lactalbumin with all four disulfide bridges eliminated through a Cys to Ala substitution in 8.0 M urea at pH 2.0; R_h = 33.3 Å) [31]. As the hydrodynamic radius of native human α-lactalbumin is 17.1 Å [32], the protein moiety of HAMLET appears to be largely monomeric and, interestingly, a further radius expansion of the protein is observed from the classical molten globule forms.

A combination of hydrogen/deuterium exchange and limited proteolysis coupled with MS was used to study the conformation of HAMLET in solution [33]. Proteolysis experiments were performed using trypsin, chymotrypsin, V8 and AspN endoproteases, subtilisin and endoprotease K as proteolytic probes. Proteolytic conditions were carefully selected in order to ensure maximum stability of the protein conformation, and

cleavage sites were assigned based on the fragments identified by MS (ES- or MALDI-MS). The proteolysis experiments revealed that HAMLET and apo α-lactalbumin are both accessible to proteases in the α-domain, but showed substantial differences in the kinetics of enzymatic digestion. The hydrogen/deuterium exchange clearly showed that HAMLET and apo α-lactalbumin might correspond to two distinct conformational states. On the basis of these data, a putative binding site of the C18:1 fatty acid was proposed to involve the β-sheet domain of α-lactalbumin.

Similar to human α-lactalbumins in HAMLET, equine lysozyme in ELOA is also present in a molten globule state, as evident from a range of its conformational properties reflected in (a) near- and far-UV CD spectra, resembling closely those of equine lysozyme molten globule, (b) uniform broadening of the NMR spectrum, indicative of conformational mobility typical for a molten globule state and (c) binding of ANS, probing the exposure of hydrophobic surfaces in partially unfolded states [6].

Important insights into the nature of interactions of equine lysozyme and oleic acid within ELOA complexes were obtained by NMR spectroscopy. Direct evidence that oleic acid molecules constitute an integral part of ELOA was derived from the one-dimensional ^{1}H NMR spectrum of ELOA, showing up-field shifts of the resonance of bound oleic acid compared with those of free molecules. The ^{1}H NOESY spectrum of ELOA demonstrated the presence of cross-peaks between the protons of lysozyme aromatic residues and oleic acid, indicative of the direct interactions between oleic acid and the aromatic residues [6]. In addition, ELOA is characterized by similar thermal stability to equine lysozyme, its thermal unfolding occurred within the same broad temperature range from 30 to 80 °C. However, two consecutive transitions with the population of partially folded state at $\sim$ 57 °C, characteristic for equine lysozyme, were not observed, indicating that the conformational changes in ELOA and equine lysozyme alone may have different origins. It is interesting to note, that HAMLET is less stable towards thermal denaturation than human α-lactalbumin in the presence of calcium [24]. These observations suggest that association within the HAMLET-type complexes significantly perturbs the structure of its constituting proteinaceous compounds.

Partial unfolding alone does not make α-lactalbumin tumoricidal

Partially unfolded apo α-lactalbumin reverts to the native state at Ca^{2+} concentrations present in cell culture media and for this reason it has been difficult to assess if α-lactalbumin unfolded by EDTA, pH or temperature becomes cytotoxic in the absence of bound fatty acid. To address this question, we used the D87A Ca^{2+} site mutant [34], which fails to bind Ca^{2+} and remains partially unfolded at physiological solvent conditions. The mutant formed a tumoricidal HAMLET-like complex with oleic acid, but the partially unfolded protein alone did not kill the tumor cells, suggesting that oleic acid is needed for tumoricidal activity. To further examine if a return to the native state may occur upon interaction with certain tumor cell compartments, a variant α-lactalbumin with all four of its disulfide bridges 'crippled' through a Cys to Ala site substitution was employed. The resulting 'α-lactalbumin$^{\text{all-Ala}}$' mutant possesses the properties of a molten globule at physiological solvent conditions. Despite such drastic non-native character, the derivatized protein–fatty acid complex analogue (termed rHLA$^{\text{all-Ala}}$-OA) displayed similar cytotoxic properties to HAMLET, unequivocally showing that a new biological function was present upon the partial unfolding of α-lactalbumin [31]. Notably, NMR spectroscopic experiments showed that despite the equivalence in biological activity, HAMLET possessed greater native-like structural features than rHLA$^{\text{all-Ala}}$-OA, suggesting that the partially unfolded nature of the protein moiety could span a continuum of conformational ensembles that share the cytotoxic activity [31].

Fatty acid binding to α-lactalbumin and equine lysozyme

The conformational change obtained by removing Ca^{2+} enables the protein to interact with fatty acids [2]. The fatty acid specificity in HAMLET was studied using fatty acids differing in chain length, saturation and orientation of the double bond. Only C16–C20 and *cis*-unsaturated fatty acids formed complexes with partially unfolded α-lactalbumin, suggesting that stereospecificity might be involved. The HAMLET complex with oleic acid or vaccenic acid complexes killed tumor cells efficiently, whereas the C16 or C20 *cis*-fatty acid complexes with α-lactalbumin showed low or intermediate activity [35].

Bovine α-lactalbumin has also been shown to interact with lipids, including saturated C18:0 (stearic acid) and its spin-labeled (doxyl) analog [36]. By intrinsic protein fluorescence and electron spin resonance methods, the apo protein was shown to have a stronger affinity for the fatty acid than the native protein and it was suggested that apo α-lactalbumin possesses two fatty acid binding sites. In contrast, the Ca^{2+}-free

protein was shown to have the same binding site for oleic and palmitic acids, with a higher affinity for oleic acid [37]. Yang *et al.* [38] studied the interaction between bovine apo α-lactalbumin and oleic acid at different pHs and found that oleic acid induces a dimeric protein intermediate at pH 4.0 and 7.0. In addition, the molten globule content increased remarkably at pH 3.0 [38]. Tolin *et al.* [28] recently showed that oleic acid is incorporated by several α-lactalbumin peptides, as shown after limited proteolysis and separation by reversed-phase HPLC, suggesting that there is no single fatty acid binding site in HAMLET.

The protein/lipid stoichiometry in HAMLET has been estimated by amino acid analysis/GC-MS and independently by peak integration of the ^{1}H NMR spectra. The mean molar ratio was 1 : 5.4 (protein/fatty acid; SD 1.5) from chemical analysis and 1 : 5.1 (protein/fatty acid) in NMR experiments, resulting in good agreement [31]. It should be noted that in preparing HAMLET, extensive dialysis and/or gel filtration is performed subsequent to the chromatographic preparation step to ensure that unbound fatty acid is removed. Studies from other laboratories have shown that the number of fatty acids in other HAMLET-like complexes depends on the method of production [27]. The stoichiometry of oleic acid in the complexes probably significantly modifies the mechanism of cytotoxicity and the tumor selectivity of the complexes.

In the case of ELOA, the one-dimensional ^{1}H NMR spectrum resulted in a value varying from 11 to 48 oleic acids per protein molecule, depending on the specific chromatographic conditions during the complex formation [6]. In general, increasing the saturation of the column with oleic acid resulted in the formation of ELOA with a higher oleic acid content. The number of equine lysozyme molecules in ELOA was determined by pulsed-field gradient NMR diffusion measurements and varied from four to 30 protein molecules in different preparations, with four to nine in most cases [6]. Thus, the number of oleic acid and protein molecules can vary significantly within the ELOA complexes and the largest ELOA lies at the upper scale among the HAMLET-type complexes.

Based on these diverse methods and results, a question remains how narrow or broad the definition of 'HAMLET', 'ELOA' and related complexes should be. HAMLET has been most extensively defined, has been shown to be highly reproducible even under conditions of large-scale production and has been shown to successfully target and kill tumor cells in humans and animals. In view of this extensive documentation, we propose that it would be useful if HAMLET were used as a standard positive control when studying α-lactalbumin/oleic acid complexes. Collaborations between various laboratories will then help to reveal if different production methods result in the formation of the same molecule, or if the cell death mechanisms differ. It will be especially important to distinguish the unspecific effects of high lipid concentrations (1 : 120 molar equivalents) on membranes and the resulting cell lysis, from the mechanisms of cell death in response to protein–lipid complexes such as HAMLET. High amounts of free oleic acid should ideally be removed by a further purification step to separate protein-associated lipid from the total lipid in the sample.

Interaction of HAMLET and ELOA with phospholipid membrane vesicles

HAMLET and ELOA interact with tumor cell membranes and the nature of this interaction probably determines the subsequent death response [39,40]. HAMLET interacts with membranes prepared from egg yolk or soybean phospholipids and perturbs their structure, as shown by leakage of fluorescent, small molecules from membrane vesicles. Although HAMLET showed a uniform binding to artificial membranes, we observed a punctate binding pattern in tumor cell plasma membrane vesicles, indicating that HAMLET may bind with higher affinity to distinct membrane areas of the tumor plasma membrane. We did not detect uptake of HAMLET into the vesicles, however, suggesting that critical cellular components were not present in the artificial vesicle preparations. Similarly, by using a range of biophysical techniques, such as quartz crystal microbalance with dissipation and confocal laser scanning microscopy, we observed nondisruptive binding and accumulation of ELOA, but not equine lysozyme, on the surface of giant unilamellar vesicles [40]. Structural characterization of ELOA on interaction with lipid membranes by fluorescence spectroscopy and CD suggested a conversion of ELOA towards a more native-like state, although complete refolding was not observed.

Mechanisms of tumor cell death in response to HAMLET and ELOA

HAMLET is internalized by tumor cells, targets distinct cellular organelles and activates several cell death pathways (Fig. 2A). However, healthy differentiated cells tested so far have been resistant to HAMLET's lethal effects. In tumor cells, HAMLET enters the cytoplasm of tumor cells and accumulates in the nuclei [2,30,41,42]. Healthy cells, in contrast, only take up

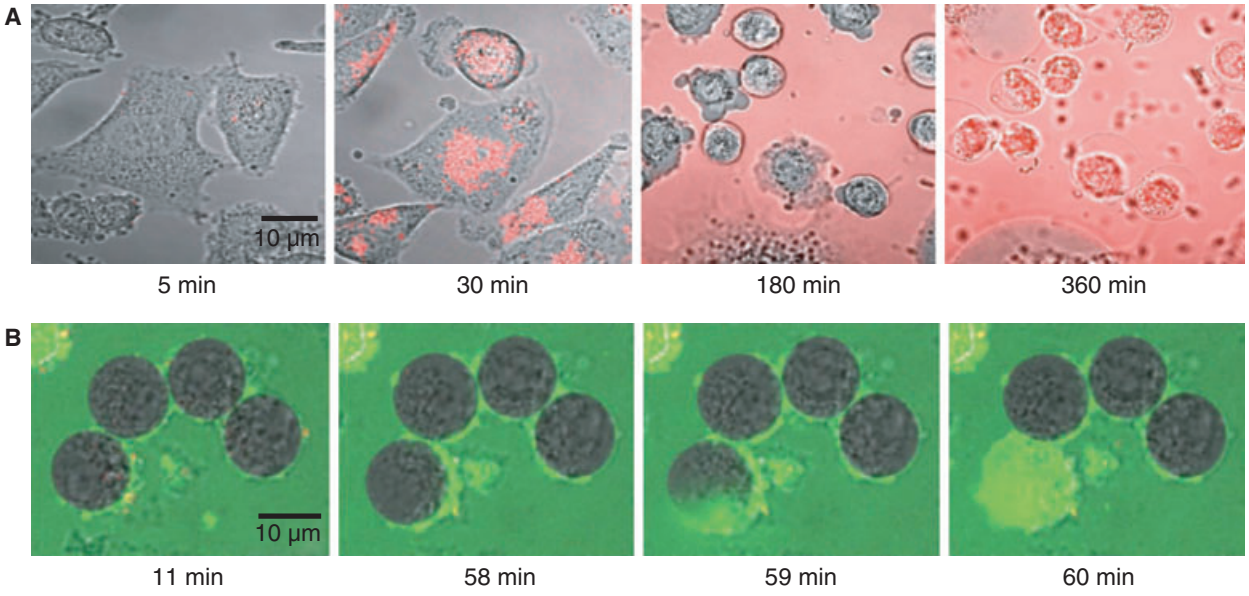

Fig. 2. (A) Progressive Alexa 568-HAMLET (red stain) internalization by tumor cells from 30 min to 6 h. HAMLET is initially bound to the membrane of the cells and subsequently transported into the cells. The cells maintain cellular integrity for a long period of time (180 min), but are eventually filled with HAMLET. (B) Imaging ELOA interaction with live cells. Time-dependent accumulation of Alexa 488 (bright green) in the vicinity of live PC12 cells during 58 min of co-incubation. At 59 min, the cell wall was ruptured, allowing ELOA to stream in and fill the cell interior (60 min).

small amounts of HAMLET and there is no evidence of nuclear translocation [41,42]. Native α-lactalbumin differs from HAMLET in that only small amounts are internalized [2,9,42], suggesting that unfolding of α-lactalbumin and oleic acid binding are both required for uptake into tumor cells. Metaphorically, we have proposed that HAMLET resembles a Lernean Hydra, attacking its prey with many, functionally distinct heads, thus ensuring that HAMLET targets cell death pathways, which are more active in tumor cells than in normal, differentiated cells [43,44].

Proteasome inhibition in response to unfolded α-lactalbumin in HAMLET

The massive invasion of a partially unfolded protein into tumor cells is expected to trigger ER stress and a disruptive, 20S proteasomes response, based on the roles of the ER and proteasomes in unfolded protein homeostasis [45]. HAMLET was shown to bind directly to isolated 20S proteasome subunits *in vitro* and to cause a rapid structural change in intact proteasomes, leading to inhibition of proteasome activity. In addition, *in vitro* proteolysis experiments showed that unfolded α-lactalbumin in HAMLET is resistant to proteolysis by proteasomal enzymes compared with the partially unfolded, fatty acid free protein. In this way, HAMLET acts as a proteasome inhibitor.

Nuclear receptors and chromatin interactions of HAMLET

HAMLET accumulates in the nuclei of tumor cells and histones have been identified as nuclear receptors for HAMLET [41]. High-affinity interactions with histone H3 and weaker interactions with H4, H2A and H2B have been documented with isolated histones in nuclear extracts and by confocal microscopy. Furthermore, histones and HAMLET have been shown to colocalize in the nuclei of tumor cells. HAMLET, histones and DNA form virtually insoluble complexes and this interaction disrupts transcription. The accessibility of the chromatin for HAMLET is controlled by acetylation and deacetylation of the histone tail. Histone deacetylases, which close the chromatin, are often over-expressed in tumor cells and histone deacetylase inhibitors are therefore used to treat malignancies. HAMLET acts in synergy with histone deacetylase inhibitors by enhancing the hyperacetylation response to the histone deacetylase inhibitors and by promoting cell death [46]. Interestingly, it has been suggested that α-lactalbumin does not have to be converted to HAMLET to bind to histones *in vitro* and that the

interaction is based on electrostatic interactions [47]. In this study, several α-lactalbumin molecules bound to each histone protein, indicating nonsite-specific binding. It should be noted that the authors acknowledged that native α-lactalbumin would not reach the nuclei of intact tumor cells, and that there is clear evidence that HAMLET – not the native protein independently – is translocated to the nuclei in living tumor cells.

Apoptosis and macroautophagy in response to HAMLET

HAMLET-treated cells show characteristics of apoptosis with typical changes in morphology and DNA fragmentation [7]. A tentative mechanism was provided when HAMLET was shown to interact with mitochondria, causing mitochondrial swelling and loss of mitochondrial membrane potential [48,49], accompanied by cytochrome *c* release, proapoptotic caspase activation and exposure of phosphatidylserine on the cell surface [49]. Apoptosis was not the cause of cell death, however, as caspase inhibitors did not rescue HAMLET-treated cells from dying [48–50]. This conclusion was further supported by studies focusing on the Bcl-2 family of proteins and the p53 tumor suppressor. Both gene families are involved in apoptosis and the altered death response of tumor cells has been explained by mutations or other changes in the expression levels of those genes. Using stably transfected or mutant cell lines, HAMLET was shown to kill tumor cells regardless of their Bcl-2 and p53 status [50]. This is consistent with apoptosis being a cellular response, but not the cause of death.

HAMLET-treated tumor cells also show signs of macroautophagy; a mechanism used to degrade and reutilize long-lived proteins and organelles, especially in response to starvation [51]. Extensive macroautophagy may also cause programmed cell death [52,53]. Double-membrane vesicles, LC3 translocation and accumulation typical of macroautophagy were observed in tumor cells after HAMLET treatment and inhibition of macroautophagy by Beclin 1 and Atg5 siRNAs significantly reduced HAMLET-induced cell death, suggesting that macroautophagy is one component of cell death in response to HAMLET.

Cytotoxicity of ELOA complexes

Similar to HAMLET, the assembly of equine lysozyme and oleic acid into ELOA complexes led to cytotoxic activity. ELOA effectively reduced the viability of mouse embryonic fibroblast and liver cell cultures, neuroblastoma cell line SH-SY5Y and a rat pheochromocytoma cell line PC12 [6]. This effect was dose and time dependent and ELOA added within a 1.0–10 μM range decreased the cell survival by ~ 70–80% after 5–24 h. Similar to the α-lactalbumin component in HAMLET, equine lysozyme alone did not kill mouse embryonic liver cells, and the reduction in cell viability induced by the oleic acid equivalent of ELOA did not exceed ~ 10%. The same marginal effect was observed when a mixture of oleic acid and equine lysozyme at their equivalent concentration in the ELOA complex was added to cells [6]. These observations emphasize the importance of the complex formation and the protein conformational change in producing the cytotoxic effects.

Combined staining of mouse embryonic liver cells with acridine orange and ethidium bromide indicated that ELOA induces apoptotic-type cell death as previously observed with HAMLET. In order to reveal the cellular targets of ELOA, the interactions of ELOA with live cells were monitored by confocal laser scanning and fluorescence correlation spectroscopy, providing nondestructive observation of molecular interactions in live cells with single-molecule sensitivity [54]. The Alexa Fluor 488-labeled ELOA complex initially accumulated in the vicinity of the cell membrane of rat pheochromocytoma PC12 cells, reaching a 10-fold higher local concentration than in solution. During this accumulation, cells 'resisted' ELOA and significant uptake of the complex into cells did not take place. The internalization of ELOA occurred only when the cell membranes were completely disrupted. It is important to note that ELOA is an oligomeric complex compared with monomeric HAMLET and, therefore, they may act via differing mechanisms (Fig. 2B).

HAMLET as a therapeutic agent

HAMLET is an interesting candidate drug, with selectivity for tumor cells *in vitro*. The tumoricidal effect of HAMLET and the selectivity for tumor tissue has also been documented *in vivo* in animal models and in clinical studies.

Human glioblastoma xenografts

In a rat glioblastoma xenograft model that reproduces the invasive growth of human tumors with glioblastoma cells obtained from surgical specimens, HAMLET or α-lactalbumin were infused into the tumor graft area for 24 h [42]. By magnetic resonance imaging, HAMLET was shown to reduce the tumor size and to delay the development of pressure-related symptoms without toxic side-effects. HAMLET caused

apoptosis in the tumor, as determined by terminal deoxynuclotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining, but there was no apoptotic response in surrounding healthy tissues.

Placebo-controlled study of human skin papillomas

The effect of HAMLET was further studied in a placebo-controlled and double-blind study of skin papillomas [5]. Patients with severe, therapy-resistant papillomas on hands and feet received HAMLET or saline solution daily for 3 weeks and the effect on lesion volume was recorded. At the end of the placebo-controlled study, the HAMLET-treated patients showed a decrease in lesion volume by at least 75% and after 2 years most of the lesions had resolved (83% of the patients). We conclude that HAMLET has beneficial effects on skin papillomas without detected side-effects.

Human bladder cancer

We selected to study the response of bladder cancers to HAMLET as a variety of topical treatments are used for intravesical instillation to prevent or delay cystectomy. Nine patients received five daily HAMLET instillations prior to scheduled surgery [55]. HAMLET caused a rapid shedding of dead tumor cells, as determined by Trypan blue exclusion and the cells showed signs of apoptosis (Fig. 3). At surgery, a reduction in tumor size was observed in six patients and four of the patients had positive TUNEL staining in biopsies from the remaining tumor. The results thus show that HAMLET has a direct effect on bladder cancer tissue *in vivo* [55]).

To examine the therapeutic effects of HAMLET, we subsequently used an orthotopic mouse bladder cancer model [4]. Tumor cells were installed via catheter into the bladder of anesthetized mice, followed by five intravesical instillations of HAMLET. We found that the tumor area was significantly reduced in HAMLET-treated animals compared with controls. By whole body imaging, uptake and retention of HAMLET was specific for tumor tissue as visualized using Alexa-labelled HAMLET. We concluded that HAMLET shows therapeutic potential and delays bladder cancer progression in the mouse model.

Conclusions

Although protein misfolding and aggregation have been associated with tissue toxicity and disease, partial protein unfolding is becoming recognized as a mechanism to generate beneficial functional diversity [2]. It is well accepted that a nascent polypeptide chain released from the ribosome folds to its global free energy minimum where the native three-dimensional structure is defined and where its native – and almost always beneficial – biological function is displayed [1]. In contrast, partially folded intermediates and/or their misfolded species are usually considered to lack 'biological purpose' [56]. For those examples where biological activity can be attributed to misfolded species, for example

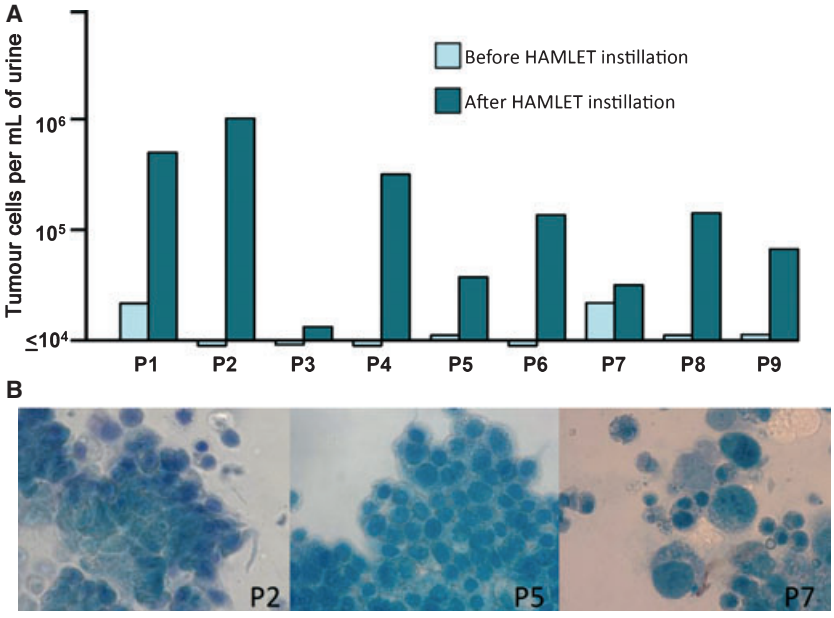

Fig. 3. HAMLET triggers cell shedding into the urine of patients with bladder cancer. (A) The mean number of shed cells in urine before (light blue) and after (dark blue) the HAMLET instillations. (B) Examples of dead (Trypan blue) cell aggregates found in the urine after HAMLET instillations. Figure reproduced from [55].

upon formation of oligomeric amyloid prefibrils, the result has almost always been detrimental to the host cell [57], apart from a few, recent exceptions, such as the Pmel17 protein in melanosomes [58] or the *Saccharomyces cerevisiae* Sup35 prions [59]. By describing the form and function of novel complexes such as HAMLET and ELOA, we have provided new evidence that a loss of native structure can endow proteins and their complexes with distinct and beneficial functions substantially different from the native protein.

Acknowledgements

Ludmilla Morozova-Roche acknowledges the support of VR-M and Insamlingsstiftelsen, Umeå. The HAMLET group in Lund acknowledges the support of the Swedish Cancer Society, the Lund Family Grant from the American Cancer Society, Swedish Medical Research Council, Swedish Natural Science Research Council, Swedish Pediatric Cancer Society, the Österlund Foundation, the Lund Hospital Foundation, Royal Physiographic Society, Anna-Lisa, Sven-Erik Lundgren Foundation, Knut and Alice Wallenberg Foundation, Inga-Britt and Arne Lundbergs Foundation and the John and Augusta Person Foundation for Medical Research.

References

1 Anfinsen CB (1973) Principles that govern the folding of protein chains. *Science* **181**, 223–230.
2 Svensson M, Håkansson A, Mossberg A-K, Linse S & Svanborg C (2000) Conversion of α-lactalbumin to a protein inducing apoptosis. *Proc Natl Acad Sci USA* **97**, 4221–4226.
3 Pettersson-Kastberg J *et al*. (2009) Can misfolded proteins be beneficial? The HAMLET case *Ann Med* **41**, 162–176.
4 Mossberg A-K, Hou Y, Svensson M, Holmqvist B & Svanborg C (2010) HAMLET treatment delays bladder cancer development. *J Urol* **183**, 1590–1597.
5 Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK & Svanborg C (2004) Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid. *N Engl J Med* **350**, 2663–2672.
6 Wilhelm K, Darinskas A, Noppe W, Duchardt E, Mok KH, Vukojevic V, Schleucher J & Morozova-Roche LA (2009) Protein oligomerization induced by oleic acid at the solid–liquid interface: equine lysozyme cytotoxic complexes. *FEBS J* **276**, 3975–3989.
7 Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H & Svanborg C (1995) Apoptosis induced by a human milk protein. *Proc Natl Acad Sci USA* **92**, 8064–8068.
8 Hill RL & Brew K (1975) Lactose synthetase. *Adv Enzymol Relat Areas Mol Biol* **43**, 411–490.
9 Svensson M, Sabharwal H, Hakansson A, Mossberg AK, Lipniunas P, Leffler H, Svanborg C & Linse S (1999) Molecular characterization of α-lactalbumin folding variants that induce apoptosis in tumor cells. *J Biol Chem* **274**, 6388–6396.
10 Stinnakre MG, Vilotte JL, Soulier S & Mercier JC (1994) Creation and phenotypic analysis of alpha-lactalbumin-deficient mice. *Proc Natl Acad Sci USA* **91**, 6544–6548.
11 Qasba PK, Dandekar AM, Horn TM, Losonczy I, Siegel M, Sobiech KA, Nakhasi HL & Devinoy E (1982) Milk protein gene expression in the rat mammary gland. *Crit Rev Food Sci Nutr* **16**, 165–186.
12 Nitta K & Sugai S (1989) The evolution of lysozyme and alpha-lactalbumin. *Eur J Biochem* **182**, 111–118.
13 Qasba PK & Kumar S (1997) Molecular divergence of lysozymes and alpha-lactalbumin. *Crit Rev Biochem Mol Biol* **32**, 255–306.
14 Acharya KR, Ren JS, Stuart DI, Phillips DC & Fenna RE (1991) Crystal structure of human alpha-lactalbumin at 1.7 A resolution. *J Mol Biol* **221**, 571–581.
15 Kuwajima K (1996) The molten globule state of alpha-lactalbumin. *FASEB J* **10**, 102–109.
16 Morozova-Roche LA (2007) Equine lysozyme: the molecular basis of folding, self-assembly and innate amyloid toxicity. *FEBS Lett* **581**, 2587–2592.
17 Morozova LA, Haynie DT, Arico-Muendel C, Van Dael H & Dobson CM (1995) Structural basis of the stability of a lysozyme molten globule. *Nat Struct Biol* **2**, 871–875.
18 Morozova-Roche LA, Arico-Muendel CC, Haynie DT, Emelyanenko VI, Van Dael H & Dobson CM (1997) Structural characterisation and comparison of the native and A-states of equine lysozyme. *J Mol Biol* **268**, 903–921.
19 Morozova-Roche LA, Jones JA, Noppe W & Dobson CM (1999) Independent nucleation and heterogeneous assembly of structure during folding of equine lysozyme. *J Mol Biol* **289**, 1055–1073.
20 Mališauskas M, Zamotin V, Jass J, Noppe W, Dobson CM & Morozova-Roche LA (2003) Amyloid protofilaments from the calcium-binding protein equine lysozyme: formation of ring and linear structures depends on pH and metal ion concentration. *J Mol Biol* **330**, 879–890.
21 Mališauskas M, Ostman J, Darinskas A, Zamotin V, Liutkevicius E, Lundgren E & Morozova-Roche LA (2005) Does the cytotoxic effect of transient amyloid oligomers from common equine lysozyme *in vitro* imply innate amyloid toxicity? *J Biol Chem* **280**, 6269–6275.
22 Raymond KW (2008) *General, Organic, and Biological Chemistry: an Integrated Approach*. Wiley, Hoboken, NJ.

23 Mossberg A-K (2010) *Human Milk as a Source of Tumor Killing Molecules*. Lund University, Lund.
24 Fast J, Mossberg AK, Nilsson H, Svanborg C, Akke M & Linse S (2005) Compact oleic acid in HAMLET. *FEBS Lett* **579**, 6095–6100.
25 Kamijima T, Ohmura A, Sato T, Akimoto K, Itabashi M, Mizuguchi M, Kamiya M, Kikukawa T, Aizawa T, Takahashi M *et al*. (2008) Heat-treatment method for producing fatty acid-bound alpha-lactalbumin that induces tumor cell death. *Biochem Biophys Res Commun* **376**, 211–214.
26 Lišková K, Kelly AL, Nora OB & Brodkorb A (2010). Effect of denaturation of α-lactalbumin on the formation of BAMLET (bovine alpha-lactalbumin made lethal to tumor cells). *J Agric Food Chem* **58**, 4421–4427.
27 Knyazeva EL, Grishchenko VM, Fadeev RS, Akatov VS, Permyakov SE & Permyakov EA (2008) Who is Mr. HAMLET? Interaction of human α-lactalbumin with monomeric oleic acid. *Biochemistry* **47**, 13127–13137.
28 Tolin S, De Franceschi G, Spolaore B, Frare E, Canton M, Polverino de Laureto P & Fontana A (2010) The oleic acid complexes of proteolytic fragments of α-lactalbumin display apoptotic activity. *FEBS J* **277**, 163–173.
29 Zhang M, Yang F Jr, Yang F, Chen J, Zheng C-Y & Liang Y (2009) Cytotoxic aggregates of α-lactalbumin induced by unsaturated fatty acid induce apoptosis in tumor cells. *Chem Biol Interact* **180**, 131–142.
30 Hakansson A, Andreasson J, Zhivotovsky B, Karpman D, Orrenius S & Svanborg C (1999) Multimeric α-lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp Cell Res* **246**, 451–460.
31 Pettersson-Kastberg J, Mossberg AK, Trulsson M, Yong YJ, Min S, Lim Y, O'Brien JE, Svanborg C & Mok KH (2009) α-Lactalbumin, engineered to be non-native and inactive, kills tumor cells when in complex with oleic acid: a new biological function resulting from partial unfolding. *J Mol Biol* **394**, 994–1010.
32 Redfield C, Schulman BA, Milhollen MA, Kim PS & Dobson CM (1999) α-Lactalbumin forms a compact molten globule in the absence of disulfide bonds. *Nat Struct Biol* **6**, 948–952.
33 Casbarra A, Birolo L, Infusini G, Dal Piaz F, Svensson M, Pucci P, Svanborg C & Marino G (2004) Conformational analysis of HAMLET, the folding variant of human α-lactalbumin associated with apoptosis. *Protein Sci* **13**, 1322–1330.
34 Svensson M, Fast J, Mossberg AK, Düringer C, Gustafsson L, Hallgren O, Brooks CL, Berliner L, Linse S & Svanborg C (2003) α-Lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human α-lactalbumin made lethal to tumor cells). *Protein Sci* **12**, 2794–2804.
35 Svensson M, Mossberg AK, Pettersson J, Linse S & Svanborg C (2003) Lipids as cofactors in protein folding: stereo-specific lipid–protein interactions are required to form HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Protein Sci* **12**, 2805–2814.
36 Cawthern KM, Narayan M, Chaudhuri D, Permyakov EA & Berliner LJ (1997) Interactions of α-lactalbumin with fatty acids and spin label analogs. *J Biol Chem* **272**, 30812–30816.
37 Barbana C, Pérez MD, Sánchez L, Dalgalarrondo M, Chobert JM, Haertle T & Calvo M (2006) Interaction of bovine α-lactalbumin with fatty acids as determined by partition equilibrium and fluorescence spectroscopy. *Int Dairy J* **16**, 18–25.
38 Yang F Jr, Zhang M, Chen J & Liang Y (2006) Structural changes of α-lactalbumin induced by low pH and oleic acid. *Biochim Biophys Acta* **1764**, 1389–1396.
39 Mossberg A-K, Puchades M, Halskau O, Baumann A, Lanekoff I, Chao Y, Martinez A, Svanborg C & Karlsson R (2010) HAMLET interacts with lipid membranes and perturbs their structure and integrity. *PLoS ONE* **5**, e9384.
40 Nielsen SB, Wilhelm K, Vad B, Schleucher J, Morozova-Roche LA & Otzen D (2010). The interaction of equine lysozyme:oleic acic complexes with lipid membranes suggests a cargo off-loading mechanism. *J Mol Biol* **398**, 351–361.
41 Duringer C, Hamiche A, Gustafsson L, Kimura H & Svanborg C (2003) HAMLET interacts with histones and chromatin in tumor cell nuclei. *J Biol Chem* **278**, 42131–42135.
42 Fischer W, Gustafsson L, Mossberg AK, Gronli J, Mork S, Bjerkvig R & Svanborg C (2004) Human α-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res* **64**, 2105–2112.
43 Mok KH, Pettersson J, Orrenius S & Svanborg C (2007) HAMLET, protein folding, and tumor cell death. *Biochem Biophys Res Commun* **354**, 1–7.
44 Hallgren O, Aits S, Brest P, Gustafsson L, Mossberg AK, Wullt B & Svanborg C (2008) Apoptosis and tumor cell death in response to HAMLET (human alpha-lactalbumin made lethal to tumor cells). *Adv Exp Med Biol* **606**, 217–240.
45 Gustafsson L, Aits S, Onnerfjord P, Trulsson M, Storm P & Svanborg C (2009) Changes in proteasome structure and function caused by HAMLET in tumor cells. *PLoS ONE* **4**, e5229.
46 Brest P, Gustafsson M, Mossberg A-K, Gustafsson L, Düringer C, Hamiche A & Svanborg C (2007) Histone

deacetylase inhibitors promote the tumoricidal effect of HAMLET. *Cancer Res* **67**, 11327–11334.
47 Permyakov SE, Pershikova IV, Khokhlova TI, Uversky VN & Permyakov EA (2004) No need to be HAMLET or BAMLET to interact with histones: binding of monomeric α-lactalbumin to histones and basic poly-amino acids. *Biochemistry* **43**, 5575–5582.
48 Kohler C, Gogvadze V, Hakansson A, Svanborg C, Orrenius S & Zhivotovsky B (2001) A folding variant of human α-lactalbumin induces mitochondrial permeability transition in isolated mitochondria. *Eur J Biochem* **268**, 186–191.
49 Kohler C, Hakansson A, Svanborg C, Orrenius S & Zhivotovsky B (1999) Protease activation in apoptosis induced by MAL. *Exp Cell Res* **249**, 260–268.
50 Hallgren O, Gustafsson L, Irjala H, Selivanova G, Orrenius S & Svanborg C (2006) HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53. *Apoptosis* **11**, 221–233.
51 Yorimitsu T & Klionsky DJ (2005) Autophagy: molecular machinery for self-eating. *Cell Death Differ* **12**(Suppl. 2), 1542–1552.
52 Baehrecke EH (2005) Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol* **6**, 505–510.
53 Gozuacik D & Kimchi A (2007) Autophagy and cell death. *Curr Top Dev Biol* **78**, 217–245.
54 Renvoize C, Biola A, Pallardy M & Breard J (1998) Apoptosis: identification of dying cells. *Cell Biol Toxicol* **14**, 111–120.
55 Mossberg A-K, Wullt B, Gustafsson L, Månsson W, Ljunggren E & Svanborg C (2007) Bladder cancers respond to intravesical instillation of HAMLET (human α-lactalbumin made lethal to tumor cells). *Int J Cancer* **121**, 1352–1359.
56 Monod J (1970) *Le Hasard et la Necessité*. Le Seuil, Paris.
57 Chiti F & Dobson CM (2006) Protein misfolding, functional amyloid, and human disease. *Ann Rev Biochem* **75**, 333–366.
58 Fowler DM, Koulov AV, Alory-Jost C, Marks MS, Balch WE & Kelly JW (2006) Functional amyloid formation within mammalian tissue. *PLoS Biol* **4**, e6.
59 Shorter J & Lindquist S (2005) Prions as adaptive conduits of memory and inheritance. *Nat Rev Genet* **6**, 435–450.