UPTEC X07 009
Examensarbete 20 p
Mars 2007

Characterization of the interaction between HAMLET and alpha-actinin-4

Maria Trulsson

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 07 009	**Date of issue 2007-03**

Author

Maria Trulsson

Title (English)

Characterization of the interaction between HAMLET and alpha-actinin-4

Title (Swedish)

Abstract

HAMLET (human α-lactalbumin made lethal to tumour cells) is a protein-lipid complex that kills tumour cells but leaves healthy, differentiated cells unaffected. In this study we show that HAMLET bound to α-actinin-4 (αA4) in tumour cell extracts and co-localized with αA4 in the cell periphery. The interaction was characterized using synthetic peptides covering most of the αA4 molecule. HAMLET showed specificity for the actin domain and the spectrin repeats, suggesting that HAMLET might interfere with the binding of αA4 ligands. We discuss if this mechanism might be important for tumour cell detachment in response to HAMLET.

Keywords

HAMLET, alpha-actinin-4, tumour cells, cell death, peptide binding assay

Supervisors

Catharina Svanborg

Institute of Laboratory Medicine, Department of MIG, Lund University

Scientific reviewer

Helena Jernberg Wiklund

Department of Genetics and Pathology, Uppsala University

Project name	Sponsors
Language **English**	Security Secret until 090301
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **26**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

Characterization of the interaction between HAMLET and alpha-actinin-4

Maria Trulsson

Sammanfattning

HAMLET (human α-lactalbumin made lethal to tumour cells) är ett protein-lipid-komplex, som består av α-lactalbumin och oleinsyra. HAMLET inducerar celldöd i tumörceller och odifferentierade celler medan de enskilda komponenterna inte påverkar cellerna. HAMLET upptäcktes av en slump och det är ännu inte helt klart hur HAMLET tas upp av tumörcellerna och varför inte friska celler påverkas. Mekanismen för celldöd är också ännu oklar. HAMLET-behandlade celler visar tecken på apoptos men tidigare studier har visat att HAMLET inte inducerar celldöd via de klassiska apoptosvägarna. Man har däremot sett att komplexet interagerar med histonerna i kärnan samt att proteasomerna påverkas.

I ett försök att identifiera proteiner som interagerar med HAMLET, fann vi att alpha-actinin-4 band till HAMLET. I det här projektet karakteriserades interaktionen mellan HAMLET och α-actinin-4. Specifika bindningsdomäner för HAMLET på α-actinin-4 identifierades med hjälp av ett bibliotek med syntetiska peptider från α-actinin-4, domänerna var fördelade över hela proteinet. Inbindningen var oberoende av α-lactalbuminets veckning och/eller närvaro av fettsyran. Genom att reducera uttrycket av α-actinin-4 visade vi också att α-actinin-4 inte är viktigt för upptaget av HAMLET eller för cellernas känslighet men att interaktionen kan påverka om cellerna ska lossna från sin omgivning, vilket kan vara ett första steg i celldödsprocessen.

Examensarbete 20 p
Civilingenjörsprogrammet i Molekylär bioteknik

Uppsala universitet februari 2007

Table of contents

Table of contents…….. 2
1. Introduction……....3
1.1 General background……... 3
1.2 HAMLET……... 3
1.2.1 α- Lactalbumin……3
1.2.2 Oleic acid……4
1.2.3 Modes of action……4
1.3 Alpha-actinin-4……... 4
1.3.1 Interaction of HAMLET with alpha-actinin-4……5

2. Aims of the project……5

3. Materials and methods……...6
3.1 HAMLET production……..6
3.2 Cell lines……..6
3.3 Apoptosis assay……...6
3.4 ATPlite……6
3.5 Peptide binding assay……..7
3.6 Molecular modelling……... 8
3.7 Immunocytochemistry…… 8
3.8 Overexpression of α-actinin-4 by transient transfection…….……8
3.9 Western blot……9
3.10 siRNA transfection and RT-PCR……...10

4. Results……... 11
4.1 HAMLET interacts with peptides from α-actinin-4…… 11
4.2 HAMLET binding domains in α-actinin-4……11
4.3 Alpha-actinin-4 binding to α-lactalbumin variants……...13
4.4 Cellular localization of α-actinin-4……... 15
4.5 Overexpression of α-actinin-4……...17
4.6 Alpha-actinin-4 knockdown by siRNA did not affect the sensitivity to HAMLET……...18

5. Discussion……..20

6. Conclusions.....23

7. Acknowledgements.....24

8. Abreviations.....24

9. References.....25

1. Introduction

1.1 General background

Human breast milk is very important for newborns and infants. Mainly because it provides them with nutrients, but milk also contains molecules that are important for both short-term and long-term health of the babies. A foetus or a newborn has not yet a fully developed immunologic defence. The foetus receives IgG antibodies from the mother to compensate for this lack of protection and human breast milk provides infants with several immunologic factors. Secretory IgA from the milk gives protection by binding to potential pathogens and preventing them from attaching to the infant's cells. Human breast milk also contains leukocytes, such as macrophages and neutrophils, which are a good protection against microbial pathogens. A small amount of lymphocytes such as T cells, natural killer cells and antibody-producing B cells are also present. In addition, breastfeeding may decrease the risk of allergies and autoimmunity [1].

Several studies have shown that breastfeeding decreases the risk of childhood cancers such as childhood acute lymphoblastic and non-lymphoblastic leukaemia, Hodgkin´s disease and non-Hodgkin´s lymphoma. Furthermore, some studies indicate that the duration of breastfeeding is related to cancer risk [2], suggesting that breast milk might contain molecules that participate in the defence against cancer. A group at the University of Lund was studying the antibacterial properties of human milk when they observed cell death and morphological changes in some cancer cell lines [3]. The morphological changes resembled those in cells undergoing apoptosis, with chromatin condensation, DNA fragmentation, phosphatidylserine exposure and formation of apoptotic bodies [4]. The group first identified the active component as multimeric α-lactalbumin (MAL) and showed that MAL induced apoptosis in different tumour cells and immature mammalian cells but no other cells [3]. The active component of MAL was later identified as a protein lipid complex consisting of α-lactalbumin and oleic acid and was named HAMLET (human α-lactalbumin made lethal to tumour cells).

1.2 HAMLET

1.2.1 α-Lactalbumin

α-Lactalbumin is a 14-kDa protein that is present in the whey of human milk. It is secreted by the mammary epithelium and functions as a coenzyme in the synthesis of lactose. α-Lactalbumin has a binding site with high affinity for Ca^{2+} ions and the ion is essential for the protein to maintain its native folding and structure [5]. The protein has four α-helices and a triple stranded anti-parallel β-sheet. α-Lactalbumin in its native form with the Ca^{2+} ion bound does not induce apoptosis and cannot convert into HAMLET. Unfolding is necessary for the antitumour activity and is triggered by the removal of the Ca^{2+} ion after addition of EDTA or a reduction in pH. The partially unfolded state is also called the molten globule state of α-lactalbumin. The fatty acid is a necessary component of the complex, as the unfolded protein alone does not induce apoptosis (Figure 1) [6].

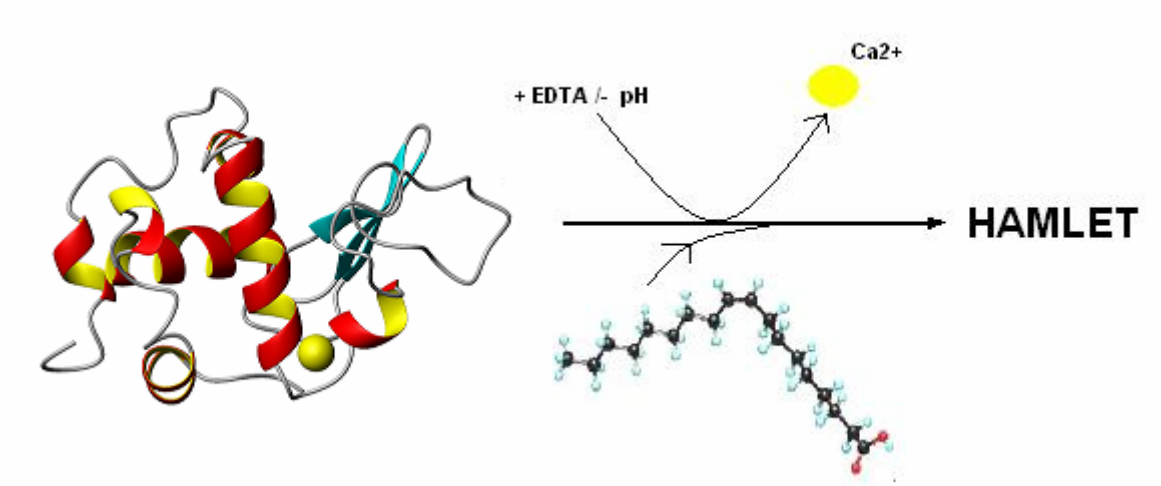

Figure 1: A schematic picture of the conversion of α-lactalbumin to HAMLET. *In vitro*, a folding change is induced by removal of the Ca^{2+} ion with EDTA. The partially unfolded protein is bound to oleic acid on a conditioned ion exchange matrix and the complex is eluted with high salt.

1.2.2 Oleic acid

Oleic acid is an essential cofactor for HAMLET, and prevents apo α-lactalbumin (the molten globule state) from reverting to the native, inactive state at neutral pH and in the presence of Ca^{2+} [7]. In a study made by Svensson *et al*. (2003), it was concluded that only unsaturated C18 fatty acids in the cis conformation can form a HAMLET-like complex and that C18:1:9cis (oleic acid) is the most efficient one. They also proposed a binding pocket for the C18:1:9cis fatty acid between the α-helical and β-sheet domains of the apo α-lactalbumin [8].

1.2.3 Modes of action

HAMLET binds to the cell surface, enters the cell, travels through the cytoplasm to the nucleus and accumulates there [6]. It is still to be revealed exactly how this happens and how HAMLET induces cell death. Tumour cells treated with HAMLET show some characteristic signs of apoptosis. p53 is a tumour suppressor that controls apoptosis in cells with irreparable DNA damage and is often mutated in cancer cells. Hallgren *et al*. showed that HAMLET induces apoptosis independently of p53, that is, the cells died even if p53 was mutated. The Bcl-2 family is another important group of proteins in the apoptosis-like cell death pathway. Bcl-2 and Bcl-xl protect the cell from apoptosis and are over-expressed in many tumours. HAMLET does not decrease the expression of Bcl-2 and over-expression of Bcl-2 or Bcl-xl does not rescue the cells [4]. Caspases are enzymes that normally play an important role in the induction of apoptosis. HAMLET-treated cells show an increase in caspase activity but inhibition of caspases does not rescue the cells [9]. It has also been shown that HAMLET interacts with histones in the nucleus. This prevents DNA from binding to the histones and thereby disturbs the chromatin structure and prevents transcription, replication and recombination [10].

1.3 Alpha-actinin-4

Alpha-actinin is the major F-actin binding and cross-linking protein in human cells. It has an N-terminal actin binding domain consisting of two calponin homology domains, a central rod domain consisting of four spectrin repeats and a C-terminal calmodulin-like domain with two

EF-hand motifs. The central rod domain interacts with the rod domain of a second α-actinin molecule to form a functional anti-parallel homodimer (Figure 2) [11].

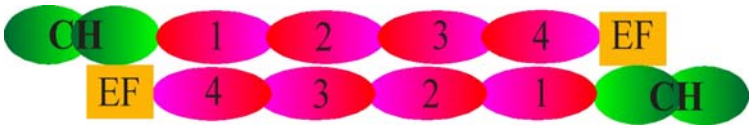

Figure 2: *α*-Actinin exists as a homodimer of anti-parallel subunits. α-Actinin has an N-terminal actin-binding domain containing two calponin homology domains (CH), a central rod domain consisting of four spectrin repeats (1-4) and a C-terminal calmodulin-like domain containing two EF-hand motifs.

There are four known isoforms of α-actinin with a molecular weight of 94-103 kDa [11]. Isoform 2 and 3 can be found in muscle cells while isoforms 1 and 4 are non-muscle isoforms. The isoforms differ in Ca^{2+}-sensitivity. Binding of Ca^{2+} to the calmodulin-like domain of the non-muscle isoforms regulates the actin binding activity. The calmodulin-like domain of the muscle isoforms can not bind Ca^{2+} and is instead believed to be regulated by the binding of phosphatidylinositol 4,5-biphosphate (PIP_2) [12]. The cellular localization of α-actinin-4 differs depending on cell type and culture conditions. Unlike α-actinin-1, α-actinin-4 does not localize to focal adhesion plaques or adherence junctions but has been detected at points of cell-cell contact where it co-localizes with BP180 and other adherence junction proteins [13]. α-Actinin-4 has also been shown to localize in the cytoplasm at sharp extensions and in migrating cells. Increased expression of α-actinin-4 leads to increased cellular motility and cancer metastasis. α-Actinin-4 is also translocated to the nucleus in some cancer cell lines, naturally or upon inhibition of PI_3 kinase or actin depolymerization [14, 15].

1.3.1 Interaction of HAMLET with alpha-actinin-4

HAMLET was discovered by serendipity and one of the most important questions now is how HAMLET enters the tumour cells and why it does not affect healthy differentiated cells. A proteomic screen of binding partners for HAMLET was performed in an attempt to solve these questions. Total cell extracts were obtained from tumour cells, and separated into membrane, cytoplasmic and nuclear fractions using Qproteome Cell Compartment Kit (QIAGEN, Hilden, Germany). The fractions were run on a gel and blotted onto a membrane that was later incubated with a HAMLET-solution. Bound HAMLET was then detected by antibodies and the interacting proteins were identified by mass-spectroscopy. One of the proteins in the membrane fraction of tumour cells was identified as alpha-actinin-4 (unpublished).

2. Aims of this project

The primary aim of this project was to screen a library with synthetic peptides from α-actinin-4 to identify which domains in α-actinin-4 that interact with HAMLET. In addition, we also wanted to study the functional consequences of these interactions. The third aim was to examine if variations in protein folding influenced the affinity or specificity of HAMLET for α-actinin-4.

3. Materials and methods

3.1 HAMLET production

HAMLET was produced as described earlier [7]. Briefly, native α-lactalbumin was purified from human milk whey. It was done by ammonium sulphate precipitation followed by phenyl-Sepharose chromatography. Adding EDTA to remove the Ca^{2+} ion then generated the molten globule state of α-lactalbumin. The partially unfolded protein was finally converted to HAMLET on an oleic acid-conditioned ion-exchange matrix (Figure 1).

3.2 Cell lines

The A549 cell line is a human lung carcinoma cell line from the American Type Culture Collection (ATCC). The cells were cultured in cell culture medium RPMI 1640 supplemented with 5% foetal calf serum (FCS), 1:100 of nonessential amino acids, 1 mM sodium pyruvate and 50 µg/ml gentamycin (GibcoBRL, Life Technology Ltd. Paisley, Scotland, U.K).

3.3 Apoptosis assay

The apoptosis assay was the most important tool to examine the effect of HAMLET on tumour cells. The cells were grown to about 80% confluency and detached from the culture flasks by adding 10 ml versene (0.5 mM EDTA in 200 ml H_20 and 800 ml PBS). PBS (phosphate-buffered saline, 1/15 M, pH 7.2) was added up to 45 ml to wash the cells (centrifugation at 1200 rpm for 7 minutes). The cells were resuspended in cell culture medium (without FCS since HAMLET is inactivated by serum albumin) at a concentration of 1 x 10^6 cells/ml and seeded into a 24-well plate at a density of 1 x 10^6 cells per well. HAMLET dissolved in PBS was then added to the wells and the plates were incubated at 37˚C in 5% CO_2. After 1 hour, 50µl of FCS (5%) was added to each well. Cell viability was determined after another 4 or 2 hours of incubation by Trypan blue exclusion. That is, 10µl cell suspension was mixed with 5µl of a 0.2% Trypan blue solution and the number of stained cells (dead cells) per 100 cells was determined.

3.4 ATPLite

Metabolically active cells contain ATP, which may be used as a marker for cell viability. The concentration of ATP decreases rapidly when cells undergo apoptosis or necrosis. The amount of ATP in the cells can be measured using luminescence with an ATPLite assay kit (PerkinElmer, Wellesley, MA, USA). The cells are lysed to release the ATP. The substrate solution contains D-Luciferin and luciferase and light is produced when ATP reacts with these two components according to the reaction scheme:

$$ATP + D - Luciferin + O^2 \xrightarrow{Luciferase, Mg^{2+}} Oxyluciferin + AMP + PP_i + CO_2 + Light$$

The cells were diluted to 1x10^6 cells/ml and 35 µl was added to each well in a white 96-well plate with clear bottom (Corning Inc., Corning, NY, USA). HAMLET and serum was added in the same way as when cell viability was determined by Trypan blue exclusion. After the incubation, medium was added to a total volume of 100 µl. The ATP level was measured with the ATPLite assay kit according to the manufacturer's instructions. The luminescence was detected using a luminometer (LUMIstar, BMG Labtech, Offenburg, Germany).

3.5 Peptide binding assay

Earlier experiments had shown that HAMLET interacts with α-actinin-4. A library of overlapping peptides from α-actinin-4 (Innovagen, Lund, Sweden) was used to further study this interaction. Each peptide was 20 amino acids in length and overlapping by 5 amino acids on both sides. The peptides were labelled with biotin and the peptides that bound to HAMLET were detected using a streptavidin- alkaline phosphatase (AP) conjugate. Alkaline phosphatase is an enzyme that produces a yellow colour when the phosphatase substrate (4-nitrophenyl phosphate) is added. The colour intensity corresponds to the amount of bound streptavidin and can be detected by measuring the absorbance. The basic principle is illustrated in figure 3.

Figure 3: This figure illustrates the difference between a positive and a negative peptide in the peptide binding assay. The well is coated with HAMLET (blue) and the biotinylated (red) peptides are allowed to bind to HAMLET. Bound peptides are detected with streptavidin that has high affinity for biotin. Streptavidin is conjugated to alkaline phosphatase (green) and can thus be detected by addition of phosphatase substrate.

It was desired to avoid any blocking steps with substances containing serum albumin since HAMLET interacts with and is inactivated by serum albumin. The first step was therefore to perform a regular ELISA to determine how much HAMLET had to be added to cover the entire surface of the well. A plate was coated with four different concentrations of HAMLET, 2 µg/ml, 5 µg/ml, 10 µg/ml and 20 µg/ml over night at 4°C. The plate was washed twice with PBS before the primary antibody, anti-α-lactalbumin (Bethyl Laboratories, Montgomery, TX, USA), was added (diluted 1:5000 and 1:15000 in 0.05% PBS-Tween). The plate was incubated for 2 hours at room temperature and then washed three times with 0.05% PBS-Tween before the secondary antibody was added. The secondary anti-goat antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was conjugated with AP and after 1 hour incubation, the plate was washed three times with 0.05% PBS-Tween before 150 µl substrate solution (one tablet Phosphatase substrate (Sigma-Aldrich, St. Louis, MO, USA) in 5 ml 1M diethanolamine pH 9.8) was added. The plate was read with an ELISA reader at 405 nm after 30 min incubation in the dark. There was no difference in absorbance between the different concentrations of HAMLET, suggesting that the wells are fully coated already at a HAMLET-concentration of 2µg/ml and therefore this concentration was used in the peptide-binding assay.

The peptides were dissolved in a DMSO solution (80% DMSO (Sigma Aldrich, St. Louis, MO, USA) and 20% H_2O) to a concentration of 10 mg/ml and further diluted in water. The wells of a Maxi Sorp 96-well plate with flat bottom (Nunc, Roskilde, Denmark) were coated with 150 µl HAMLET solution (2µg/ml in PBS) over night in 4˚C. The next day, all wells were washed 2x with PBS before 50 µl peptide solution (200ng/ml in 0.05% PBS-Tween) was added. The plate was incubated for 2 hours at room temperature, washed three times with 0.05% PBS-Tween and then incubated with 100µl streptavidin-alkaline phosphatase conjugate (Mabtech, Stockholm, Sweden) diluted 1:1000 in 0.05% PBS-Tween. The plate was incubated for 1 hour in room temperature and then washed three times with 0.05% PBS-Tween. Thereafter, 150 µl substrate solution prepared as described above was added. The plate was incubated for 30 min in the dark (room temperature) and read with an ELISA reader

at 405 nm. Native α-lactalbumin and the mutant all-Ala α-lactalbumin were also used for coating. In all-Ala α-lactalbumin, all cysteine residues in α-lactalbumin have been replaced by alanine residues, and therefore the molecule lacks disulphide bridges and is partially unfolded. The mutant was also tested after conversion to all-Ala HAMLET with the fatty acid.

3.6 Molecular modelling

No structural information covering the entire human α-actinin-4 protein is available. α-Actinin from chicken gizzard shows 85% sequence identity with α-actinin-4 and was used for molecular modelling instead. Structural information for α-actinin from chicken gizzard (1SJJ) was obtained from the Protein Data Bank (PDB, Protein Data Bank: www.pdb.org, 3 Oct, 2006). The molecular visualization program MolMol [16] was used to create a three-dimensional model of α-actinin. The 3D-model was used to visualize the localization of the HAMLET-binding α-actinin peptides in the protein. Distances between residues in α-actinin were also measured with MolMol.

3.7 Immunocytochemistry

Confocal microscopy is an excellent tool to study the morphology of cells and the distribution of proteins within the cells. The distribution of HAMLET within cells can be studied if HAMLET is labelled. In this assay, HAMLET was labelled with ALEXA Flour 568 (Molecular Probes Inc. Eugene, OR, USA) according to the manufacturer's instructions. A549 cells were grown on Lab-Tek 8-well chamber slides (Nunc, Roskilde, Denmark) overnight in 37˚C and 5% CO_2. On day 2, all chambers were washed with PBS and new medium without FCS was added. Some cells were treated with a mixture of unlabelled HAMLET and HAMLET labelled with ALEXA (20% of the total amount of HAMLET) and some cells were treated only with ALEXA-HAMLET. The cells were treated with HAMLET for different lengths of time in order to see what happens to the cells before they are completely filled with HAMLET. The medium was collected after HAMLET exposure to make sure that the detached cells were saved. Attached cells and detached cells were fixed with 3.7% formaldehyde/ethanol. Cytospin, 1500 rpm for 5 minutes, was used to transfer detached cells onto poly-lysine slides. The cells were circled with a blocking pen before washing with PBS for 5 minutes was performed. The cells were permeabilized with 0.25% Triton/5% FCS/PBS and then incubated with the primary anti-actinin-4 antibody (Alexis Corporation, Lausen, Switzerland) overnight in fridge. The slides were then washed in 5% FCS/PBS for 2 x 5 minutes. They were further incubated with the secondary Alexa Fluor® 488 goat anti-rabbit antibody (Molecular Probes, Eugene, Oregon, US) for 1 h. The slides were then washed again with 5% FCS/PBS for 2 x 5 minutes and then finally washed with PBS for 5 minutes. The slides were analyzed by confocal microscopy (Carl Zeiss, Germany).

3.8 Overexpression of alpha-actinin-4 by transient transfection

Overexpression of α-actinin-4 is one way to examine how the molecule is involved in the biological effects of HAMLET. A plasmid containing the ACTN4 gene was kindly provided by Fathia Mami-Chouaib [11]. The plasmid was amplified using One Shot® TOP10 Competent cells (Invitrogen, Carlsbad, CA, USA) according to the manual with minor changes. One vial of cells was divided into two and 0.5 µl plasmid was added to one of them while the other half was used as a negative control. The cells were transformed using chemical transformation. That is, the vials were incubated on ice for 30 minutes and then for exactly 30 seconds in a 42˚C water bath and then placed on ice again. Pre-warmed S.O.C medium was added to each

vial. The vials were then incubated in a shaking incubator at 37˚C for 1 hour at 225 rpm. 100 µl from each transformation was spread on separate LB agar plates. The plasmid also contained a resistance gene for ampicillin and the plates were supplemented with ampicillin to select for transfected cells. The plates were incubated overnight at 37˚C. The cells were plated twice to get pure colonies. When single colonies were obtained, one colony was transferred to LB broth supplemented with 100 µg/ml ampicillin and incubated in a shaking incubator at 37˚C overnight at 180 rpm. The plasmid was then purified with the QIAGEN EndoFree Plasmid Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A549 cells were transiently transfected using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A plasmid containing GFP was used as a transfection control and a pcDNA3.1-V5HisA plasmid was used as a negative control. The expression of α-actinin-4 was examined by Western blot and the transfected cells were examined for HAMLET sensitivity in an apoptosis assay. Some cells were also cytospinned onto slides to look at the distribution of α-actinin-4 by confocal microscopy as described earlier.

3.9 Western blot

Western blot is a method for detecting proteins in a given sample. Denatured proteins are separated by mass using gel electrophoresis and then transferred to a membrane where they may be detected by specific antibodies. This method makes it possible to compare the amount of protein in different samples. The cells were removed from the wells and cell density was determined. An equal amount of cells were harvested by centrifugation (400x g for 10 minutes at 4˚C). The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.8, 1% NP40, 2 mM EDTA, 150 mM NaCl) supplemented with protease inhibitors (cocktail). The tubes were mixed vigorously and incubated on ice for 1 hour (mixed a few times during the incubation). The tubes were then centrifuged at 16100x g for 20 min at 4˚C. The suspension was transferred to a new 1.5 ml-tube and the protein concentration was determined using the DC Protein Assay (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The fractions were separated on a NuPage® Novex Bis-Tris Gel 4-12% (Invitrogen, Carlsbad, CA, USA) using the XCell *SureLock*™ Mini-Cell (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The experiment was performed in MES buffer and the MultiMark® Multi-Colored Standard (Invitrogen, Carlsbad, CA, USA) was used as size marker. The gel was then blotted onto a PVDF membrane (Osmonics Inc., Minnetonka, MN, USA) using the XCell *SureLock*™ Mini-Cell according to Invitrogen's Western Transfer Protocol. The membrane was stained with amido black (0.1% Naphtol Blue Black (Sigma Aldrich, St. Louis, MO, USA) in 10% methanol and 2% acetic acid) for 20 minutes and destained with destaining buffer (50% methanol and 7% acetic acid) for 10 minutes. Thereafter, the membrane was blocked with 3% BSA/PBS for 1 hour and then washed 3x 10 minutes with 0.05% PBS-Tween. The membrane was incubated with the primary α-actinin-4 antibody (1:2000 in PBS-Tween) over night at 4°C and then washed 3x 10 minutes with 0.05% PBS-Tween. The bound primary antibodies were detected with the secondary HRP-conjugated swine-anti-rabbit antibody (DakoCytomation, Glostrup, Denmark) diluted 1:2000 in PBS-Tween (1 hour incubation). After the incubation, the membrane was washed 3x 10 minutes with 0.05% PBS-Tween and then placed in 100 mM Tris solution. The blot was then developed using an Enhanced Chemiluminescence detection system (ECL, PerkinElmer, Wellesley, MA, USA) and a GelDoc device (BioRad Laboratories, Hercules, CA, USA).

3.10 siRNA transfection and RT-PCR

Gene silencing by RNA interference (siRNA) occurs naturally in many organisms. Double stranded RNA rarely occurs in cells but when it does, it is cut into fragments by a ribonuclease enzyme. These fragments are called short interfering RNA (siRNA) and are about 21-25 bp long. The strands in the fragment separate and the antisense strand binds to a complementary region of another RNA molecule and thus triggers cleavage of this double stranded RNA (Figure 4). If the other RNA molecule is an mRNA molecule, the gene expression of the corresponding gene will be blocked. Addition of siRNA has become an important method to suppress gene expression in knockdown experiments [17].

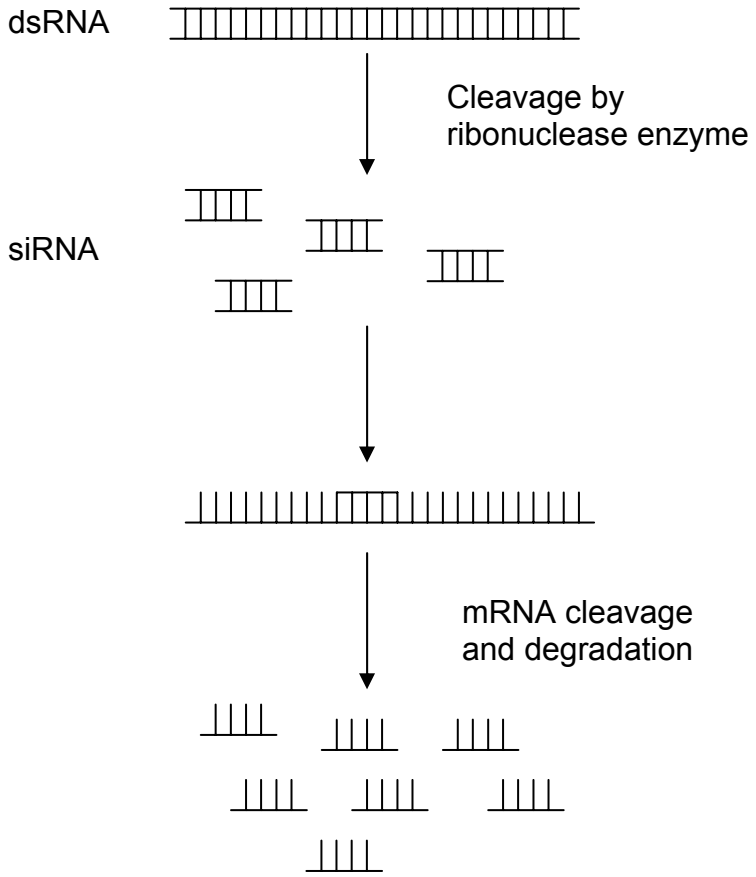

Figure 4: The mechanism of knockdown by siRNA is illustrated in this picture.

A549 cells were plated on 24-well plates (40000 cells per well) in medium without gentamycin and allowed to attach overnight. The cells were transfected with siRNAs using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The siRNA against α-actinin-4 was ordered from Qiagen (Qiagen, Hilden, Germany). Cells were also transfected with AllStar Negative Control siRNA (Qiagen, Hilden, Germany). After 48 hours, some cells were detached from the 24-well plates and transferred to Lab-Tek 8-well chamber slides where they were allowed to grow over night. These slides were used for immunocytochemistry (approximately 70 hours after transfection) as described above to examine knockdown of α-actinin-4. New medium was added to the cells that were not used for immunocytochemistry. After approximately 70 hours, knockdown was examined by RT-PCR. cDNA was made with SuperscriptFree (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The amount of GAPDH was measured

using a GAPDH assay (Applied Biosystems, Foster City, CA, USA) and the amount of α-actinin-4 was measured with QuantiTect (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Knockdown was also examined by Western blot as described earlier. Transfected cells were tested for HAMLET sensitivity in an apoptosis assay as described earlier; both Trypan blue exclusion and ATP levels were used as endpoints.

4. Results

4.1 HAMLET interacts with peptides from α-actinin-4

To study the interaction between HAMLET and α-actinin-4, a synthetic peptide library was used. The peptides were 20 amino acids long with a 5 amino acid overlap, covering most of the α-actinin-4 sequence. HAMLET was used to coat 96-well plates, the biotinylated peptides were added and bound peptides were detected with streptavidin-AP. Using 0.2 absorbance units as the cut off, ten peptides with affinity for HAMLET were detected (See table 1). Peptide 4 and 12 bound most strongly to HAMLET. Peptides 19, 24 and 33 showed intermediate binding and peptides 6, 34 and 59 bound more weakly. The weakest binding was shown by peptides 10 and 53 but they could clearly be distinguished from the negative peptides (about 0.12 absorbance units).

Table 1: Ten peptides out of 58 were found to interact with HAMLET in the peptide binding assay. These peptides are listed below as well as the average absorbance, corresponding residues, domains and amino acid sequences.

Peptide	Absorbance units	Residues	Amino acid sequence	Domain in α-actinin-4
4	1.27	46-65	PAWEKQQRKTFTAWCNSHLR	CH 1
6	0.34	76-95	EDFRDGLKLMLLLEVISGER	CH 1
10	0.22	136-155	DGNAKMTLGMIWTIILRFAI	
12	0.79	166-185	KEGLLLWCQRKTAPYKNVNV	CH 2
19	0.55	271-290	AQKAETAANRICKVLAVNQE	
24	0.54	346-365	KVQEKCQLEINFNTLQTKLR	R1
33	0.58	481-500	NELDYYDSHNVNTRCQKICD	R2
34	0.27	496-515	QKICDQWDALGSLTHSRREA	R2
53	0.21	781-800	HGGALGPEEFKACLISLGYD	EF
59	0.31	871-890	LPPDQAEYCIARMAPYQGPD	

4.2 HAMLET binding domains in actinin-4

The domains in actinin-4 recognised by HAMLET were identified using the Swiss-Prot/TrEMBLE database (O43707, www.expasy.org, 31 Oct. 2006) where the features for the protein are listed. The actin-binding domain stretches from amino acid 1-269 and contains two calponin homology domains. Peptides four and six corresponded to the first calponin homology domain, peptide 10 corresponded to the short link between the calponin homology domains and peptide 12 corresponds to the second calponin homolgy domain. Peptide 19 did not correspond to any specific domain. The central rod domain consists of four spectrin repeats and peptide 24 corresponded to the 1st spectrin repeat and peptide 33 and 34 corresponded to the 2nd spectrin repeat. Peptide 53 corresponded to the 1st EF-hand while peptide 59 did not correspond to any specific domain (Table 1).

The interacting domains were further examined by molecular modelling using the molecular visualization program MolMol. The localization of the residues corresponding to each peptide on a single α-actinin monomer is seen in Figure 5A. Peptides 4 and 6 are localized close to

each other on the actin-binding domain. Peptides 10 and 12 are also localized close to each other but on the other side of the actin-binding domain. Peptides 19 and 24 are localized next to each other on the central rod domain. There is a cavity between the actin-binding domain and the central rod domain. Peptides 4, 10, 19 and 24 are all facing this cavity and may therefore form a combined binding site for HAMLET. Peptide 33 and 34 are following each other (Figure 5B) and the absorbance is less for peptide 34 than for peptide 33. This might indicate that the last 5 amino acids on peptide 33 are involved in binding to HAMLET. Peptide 53 is not localized close to any other peptide on the monomer but in the dimer, it is localized close to peptide 19 in one end and peptide 12 in the other (the anti-parallel dimer looks slightly different at the ends). Peptide 59 is not localized close to another peptide either and is to some extent covered by the other subunit. It does, however, seem as if some residues in this peptide are available for binding close to peptide 4 on the other subunit. It is important to notice that none of the other peptides are covered by the other subunit when they are combined to form an anti-parallel dimer (Figure 6). The distances between HAMLET-binding peptides were measured to be able to propose possible interaction sites. HAMLET is about 21Å in diameter and the pocket between peptides 6, 19 and 24 is about 30Å which means that HAMLET perfectly fits in this pocket. HAMLET could also interact with peptide 10 in the same pocket (Figure 5C and D).

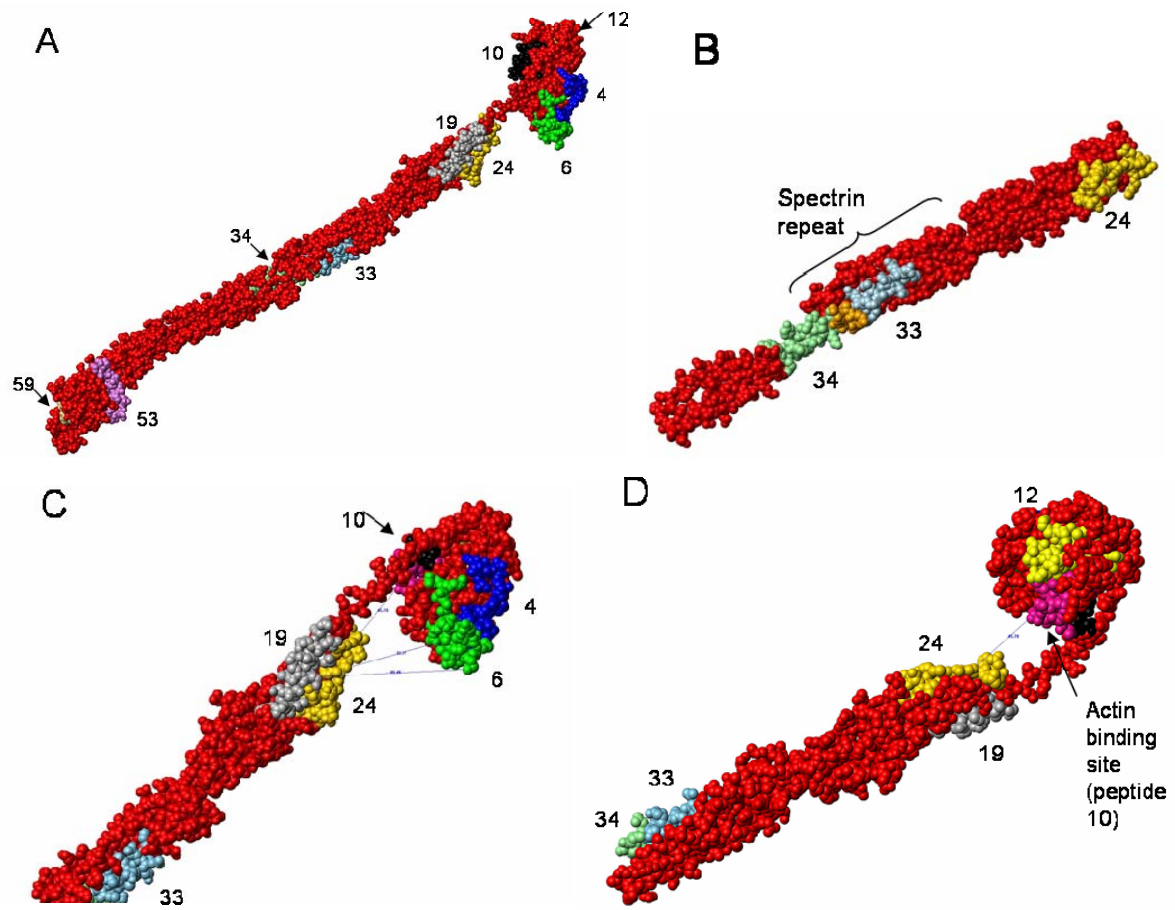

Figure 5: The peptides with affinity for HAMLET were localized on α-actinin-4 using MolMol. (A) A monomer of α-actinin-4 with the HAMLET binding peptides in colour. (B) The central rod domain with spectrin repeats. (C and D) A close-up view of the actin-binding domains and possible interaction sites for HAMLET.

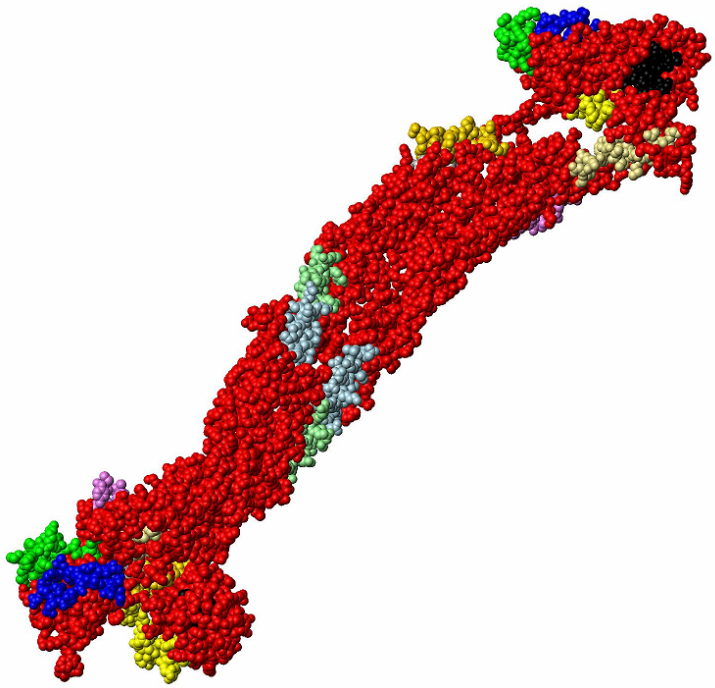

Figure 6: α-Actinin-4 was also examined as a dimer to show that the positive peptides were not covered by the other monomer. In the dimer, it was also seen that positive peptides in the N-terminal and C-terminal domains may be combined to form HAMLET binding sites.

4.3 Alpha-actinin-4 binding of α-lactalbumin variants

HAMLET consists of partially unfolded α-lactalbumin and oleic acid. To further understand the specificity of interaction with α-actinin-4, different structural variants of the proteins were examined. The interaction of native α-lactalbumin with α-actinin-4 was compared to the interaction with partially unfolded α-lactalbumins. Apo- α-lactalbumin was obtained by EDTA treatment, which removes the Ca ion that coordinates the native state, thus allowing a change in the tertiary structure of α-lactalbumin. Furthermore, we used pALAALA, which is an α-lactalbumin mutant lacking disulphide bonds, due to the substitution of cysteins for alanines.

To study the peptide interaction with α-lactalbumin folding variants, 96-well plates were coated with native or unfolded α-lactalbumins and the biotinylated peptides were added as described. The results are shown in figure 7. Peptide 4 corresponding to the actin-binding domain showed weaker binding to apo α-lactalbumin and to all-Ala α-lactalbumin. Peptide 6, also corresponding to the actin-binding domain, showed a small decrease in binding to α-lactalbumin when EDTA was added but showed a much stronger binding to all-Ala α-lactalbumin than to the native protein. Peptide 10, also corresponding to the actin-binding domain, did not change in affinity to any of the α-lactalbumin folding variants. Peptide 12 corresponding to the actin-binding domain showed a slightly decreased binding to α-lactalbumin when EDTA as added and the binding was much weaker to all-Ala α-lactalbumin. Peptide 19 does not correspond to any specific domain and showed only a small decrease in binding to α-lactalbumin when EDTA was added. Peptide 24 corresponding to the 1st spectrin repeat and peptide 33 corresponding to the 2nd spectrin repeat both showed a strong increase in binding to α-lactalbumin when EDTA was added but there were only small differences in binding to all-Ala α-lactalbumin compared to wild type α-lactalbumin. Peptide 34 corresponding to the end of the 2nd spectrin repeat and peptide 53 corresponding to the EF-hand also showed the same pattern but the increase in binding was not as high as for peptide 24 and 33. Finally peptide 59 does not correspond to any specific domain and did not show

any difference in binding between any α-lactalbumin folding variants. These three α-lactalbumins all have different tertiary structures (Figure 8A) and thus, the folding of α-lactalbumin influences the affinity of α-lactalbumin for α-actinin-4.

	Peptide	α-lactalbumin	α-lactalbumin + EDTA	all-Ala α-lactalbumin	HAMLET	all-Ala HAMLET
Actin-binding domain	4	2,76	2,01	2,2	2,57	2,21
	6	0,27	0,17	0,66	0,24	0,81
	10	0,11	0,11	0,11	0,1	0,1
	12	1,72	1,61	1,39	1,55	1,35
	19	1,39	1,23	1,39	1,35	1,16
Spectrin repeats	24	0,68	1,35	0,73	0,63	0,73
	33	0,82	1,55	0,71	0,77	0,67
	34	0,38	0,58	0,37	0,33	0,31
CaM domain	53	0,19	0,43	0,18	0,16	0,16
	59	0,81	0,83	0,76	0,76	0,55

Figure 7: The data from the peptide binding assay with α-lactalbumin and its folding variants is presented in this figure. The differences between the folding variants are highlighted. Green and pink is compared to α-lactalbumin while blue is compared to HAMLET. HAMLET was used on both plates and is therefore used for correlation.

The fatty acid bound forms of α-lactalbumins, HAMLET and all-Ala HAMLET, were subsequently compared. Peptide 4 corresponding to the 1st actin-binding domain shows less binding to all-Ala HAMLET than to HAMLET. Peptide 6, also corresponding to the 1st actin-binding domain, showed a big increase in binding to all-Ala HAMLET compared to HAMLET. Peptide 12 corresponding to the 2nd actin-binding domain showed a minor decrease in binding to all-Ala HAMLET. The rest of the peptides showed no differences in binding to HAMLET with or without cysteins. Even the fatty acid bound forms of α-lactalbumin show a difference in tertiary structure (Figure 8B) and a difference in affinity for α-actinin-4. This indicates again that the folding is important.

The α-lactalbumins were compared to the fatty acid complexes, to examine if the fatty acids might affect the binding of α-lactalbumins to α-actinin-4. The fatty acid appeared to decrease the affinity of native α-lactalbumin for α-actinin-4. Peptides 4 and 12 bound more strongly to native α-lactalbumin than to HAMLET but other peptides showed no change in binding. There are, however, more changes when HAMLET and apo α-lactalbumin are compared. Peptide 4 bound stronger to HAMLET than it did to apo α-lactalbumin. There is no difference in binding of peptide 6, 10, 12 and 59. Peptide 19 show a slightly increased binding when the fatty acid is present. Peptides 24, 33, 34 and 53 showed decreased binding when the fatty acid is bound to the apo-conformation of α-lactalbumin. It seems as if the fatty acid affects the affinity for α-actinin-4 since HAMLET and apo α-lactalbumin are quite similar in tertiary structure (Figure 8C) but there are quite big changes in affinity when the fatty acid is present. The only difference in peptide binding between all-Ala α-lactalbumin and all-Ala HAMLET is that peptide 6 binds stronger to the fatty acid bound form. This indicates that the fatty acid did not affect the binding to α-actinin-4 that much. all-Ala α-lactalbumin and all-Ala HAMLET are very similar regarding the tertiary structure (Figure 8D) and it is therefore not that surprising that they gave similar results.

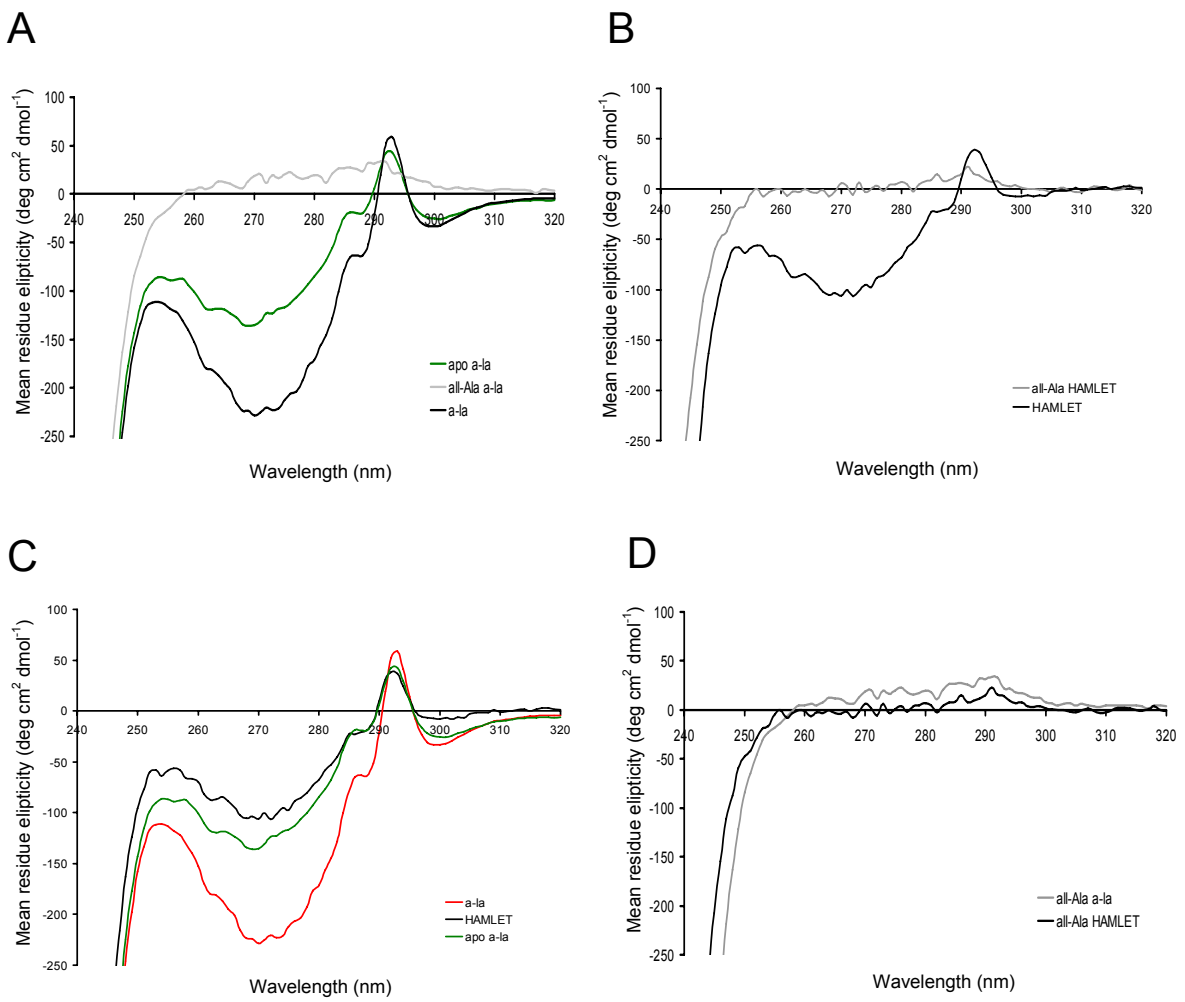

Figure 8: The difference in tertiary structure between α-lactalbumin folding variants may be investigated by circular dichroism spectroscopy. A: comparison between all-Ala α-lactalbumin, α-lactalbumin and apo α-lactalbumin. B: comparison between all-Ala HAMLET and HAMLET. C: comparison between α-lactalbumin, HAMLET and apo α-lactalbumin. D: comparison between all-Ala α-lactalbumin, all-Ala HAMLET. The data was kindly provided by Jenny Pettersson.

4.4 Cellular localization of α-actinin-4

Confluent layers of A549 cells were examined by confocal microscopy, after staining with antibodies specific for α-actinin-4. The localisation of α-actinin-4 was compared between control cells and cells exposed to HAMLET (0.5 mg/ml, 1 hour, 30 min and 10 min). To compare the cellular distribution of HAMLET and Actinin-4, Alexa flour labelled HAMLET was used. The results are shown in Figure 3. In control cells, α-actinin-4 staining was strong in the membrane of the A549 cells and weaker in the cytoplasm. Nuclear staining was not observed. After HAMLET treatment, α-actinin-4 appeared to be transferred from the cytoplasm to the cell periphery and especially into the membrane blebs (Figure 9). Co-localisation with HAMLET was observed in the cell periphery (Figure 10). The results suggest that α-actinin-4 is localised close to the cell membrane and interacts with HAMLET there.

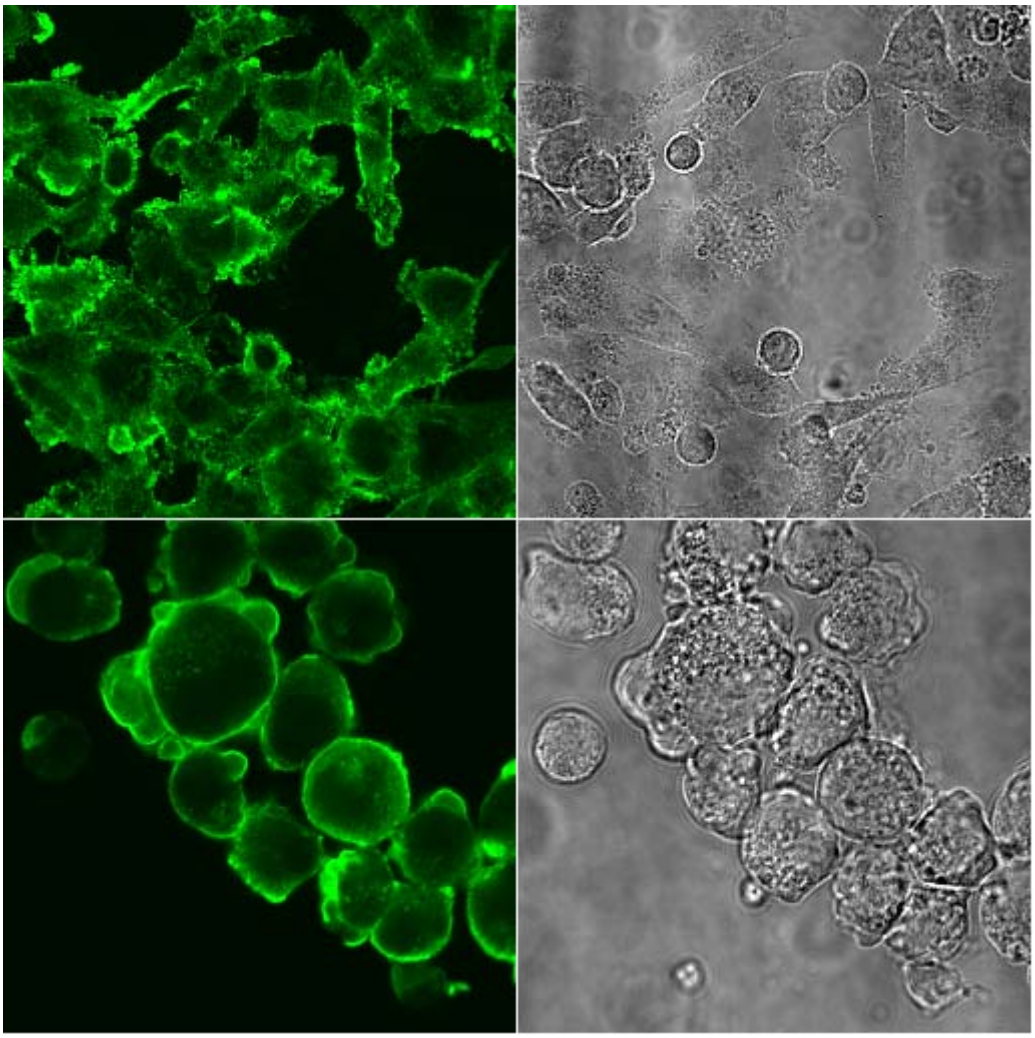

Figure 9: The cellular distribution of α-actinin-4 may be examined by confocal microscopy. Top pictures: cells before HAMLET treatment. α-actinin-4 staining was strongest in the cell periphery and weaker in the cytoplasm. Bottom pictures: Cells treated with 0.5 mg/ml HAMLET for 30 minutes. The staining in the cytoplasm is even weaker after HAMLET-treatment and α-actinin-4 is now localized to the membrane blebs.

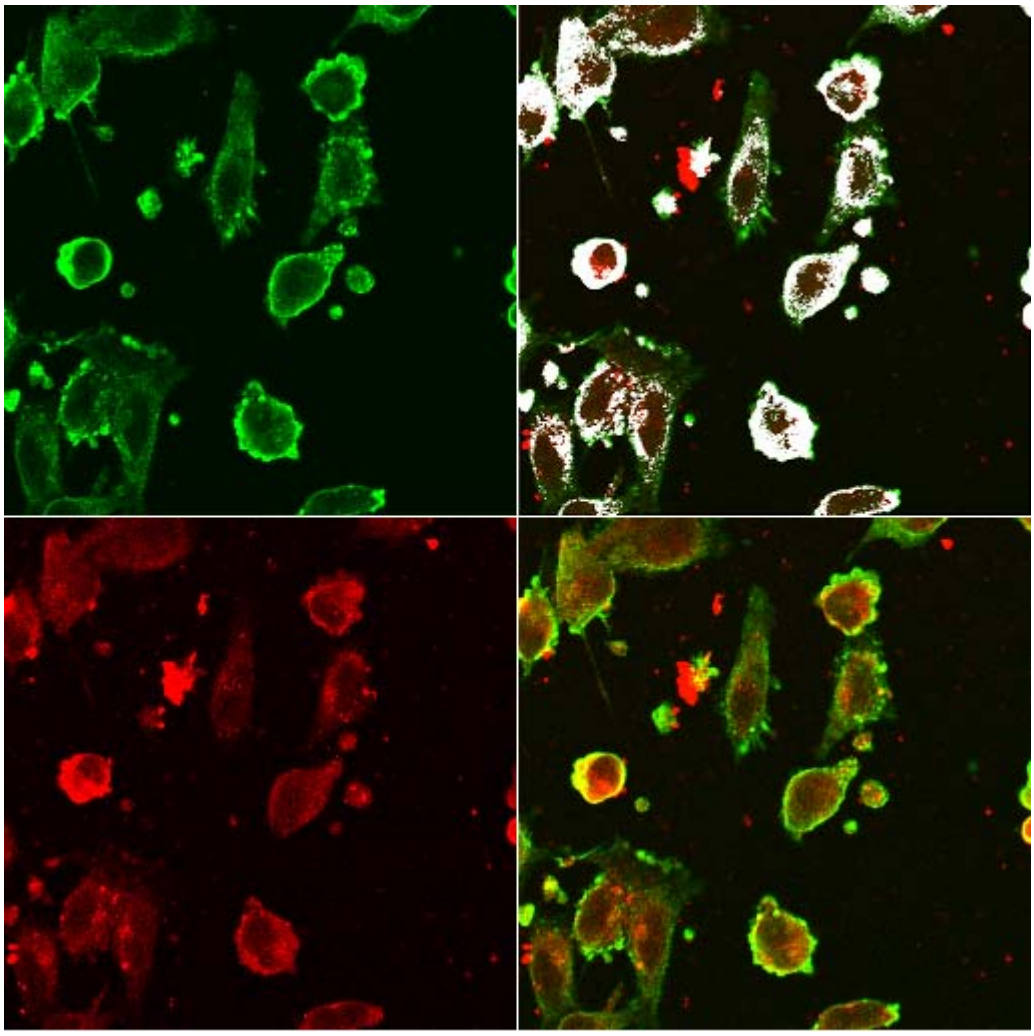

Figure 10: Confocal microscopy was used to study co-localization between proteins. The pictures to the left show the distribution of α-actinin-4 (top) and HAMLET (bottom). Co-localization of α-actinin-4 and HAMLET is shown to the right. In the top picture, white indicates co-localization according to the LSM Image program.

4.5 Over expression of alpha-actinin-4

The effect of α-actinin-4 on cell death in response to HAMLET was examined in A549 cells by transient transfection with a plasmid encoding α-actinin-4. A preliminary experiment is shown below. The transfection efficiency was about 60% according to the cells transfected with the plasmid encoding GFP. Apoptosis assays were done to determine whether increased expression of α-actinin-4 led to increased or decreased sensitivity to HAMLET. The first assay indicated that overexpression of α-actinin-4 leads to increased sensitivity to HAMLET (figure 11). The expression of α-actinin-4 was quantified by Western blot. α-Actinin-4 was constitutively expressed in A549 cells and it was therefore hard to say if actinin-4 was overexpressed (Figure 11). The second transfection showed only minor changes in viability and it was again hard to tell whether there actually was overexpression of α-actinin-4. Some cells were fixed with 4% paraformaldehyde and cytospinned onto slides, stained with antibodies against α-actinin-4 and examined by confocal microscopy. Comparisons between cells transfected with the ACTN4 plasmid and the V5HisA plasmid did not indicate any overexpression; in fact the staining seemed to be less intense in cells transfected with the ACTN4 plasmid. It should, however, be noted that these cells have been detached and cytospinned, not grown on slides and this might change the appearance of α-actinin-4 since it interacts with the cytoskeleton and adherence molecules.

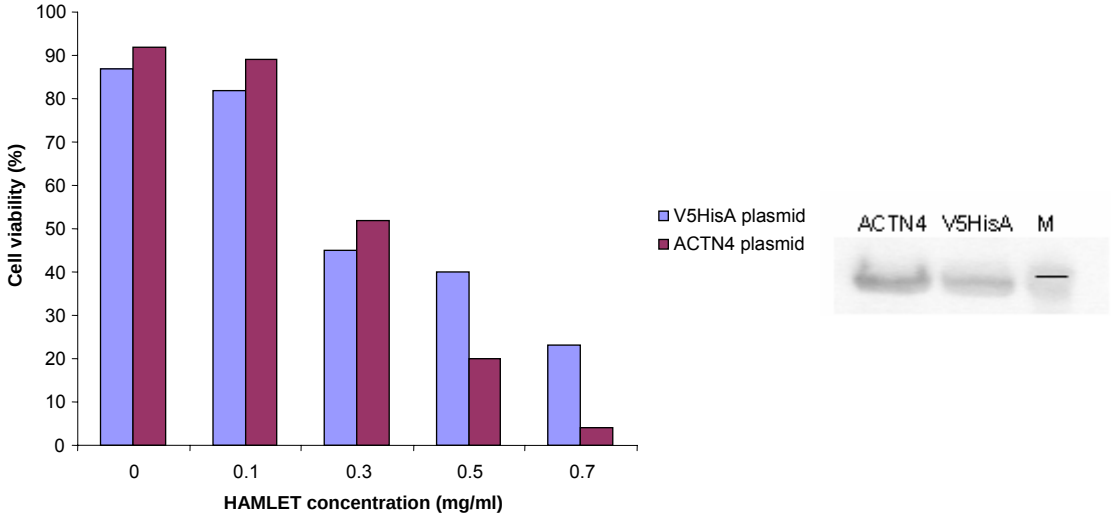

Figure 11: Effect of α-actinin-4 overexpression on HAMLET sensitivity was studied in an apoptosis assay. Transfected cells were more sensitive to HAMLET than cells transfected with the V5HisA plasmid. The difference in α-actinin-4 expression is shown by Western blot.

4.6 Alpha-actinin-4 knockdown by siRNA did not affect the sensitivity to HAMLET

Knockdown of protein expression by siRNA was detected in many ways. RT-PCR showed a decrease in mRNA levels and western blot also showed a decrease in expression of α-actinin-4 (figure 12). Immunocytochemistry showed decreased staining of α-actinin-4 in cells transfected with actinin-4 siRNA compared to cells transfected with control siRNA or cells that were not transfected (figure 13). An apoptosis assay was performed to examine whether the amount of α-actinin-4 affects the sensitivity to HAMLET. Trypan blue exclusion and ATP levels were used as endpoints and there were no differences in sensitivity to HAMLET (Figure 14).

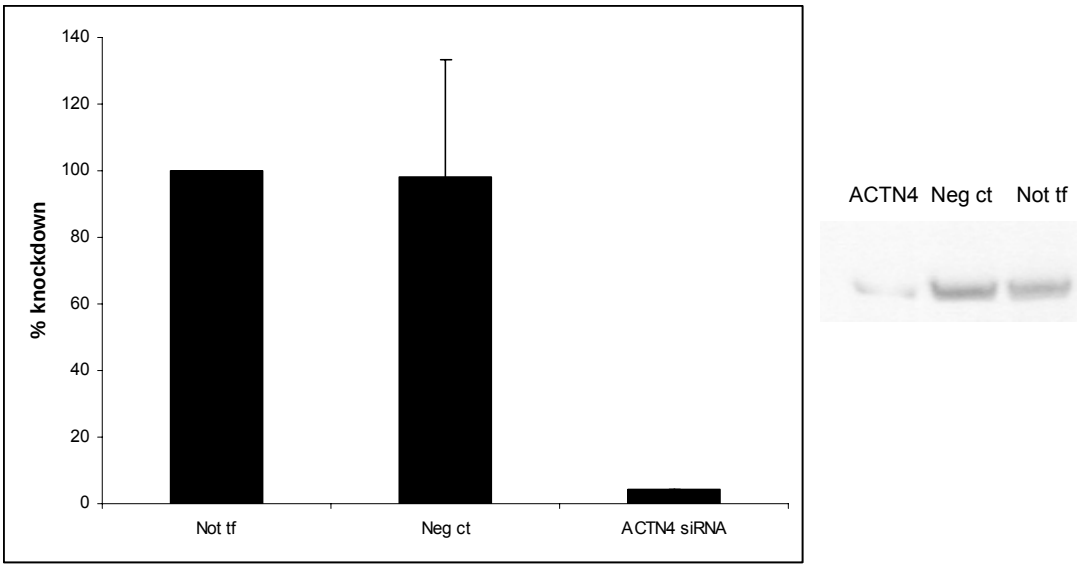

Figure 12: RT-PCR showed a decrease in mRNA levels while western blot showed a decrease in protein level.

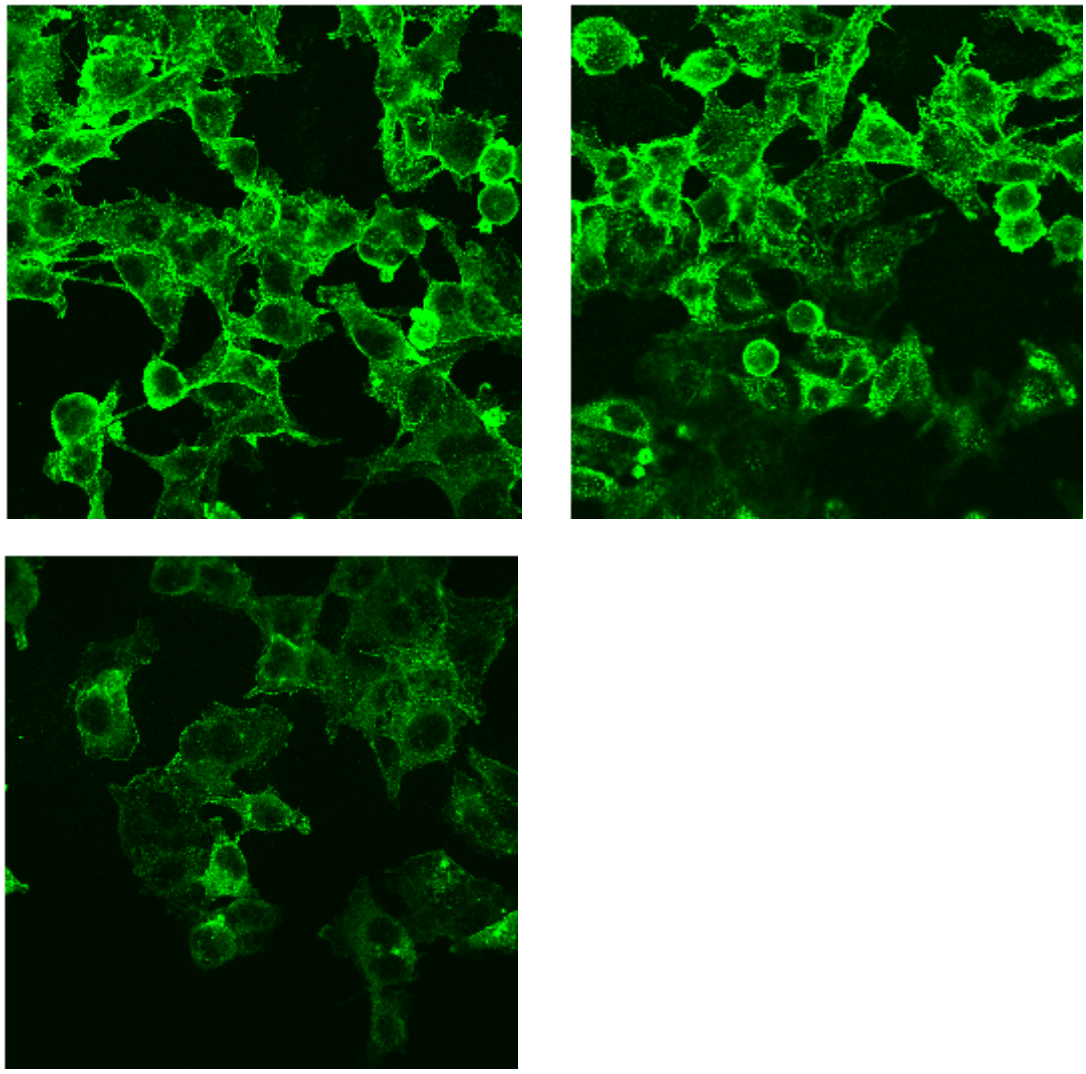

Figure 13: Top left: Nontransfected control cells. Top right: Cells transfected with control siRNA. Bottom left: Cells transfected with α-actinin-4 siRNA. The pictures are taken with the same gain and the cells are not treated with HAMLET. The staining is much less intense in cells transfected with siRNA against α-actinin-4.

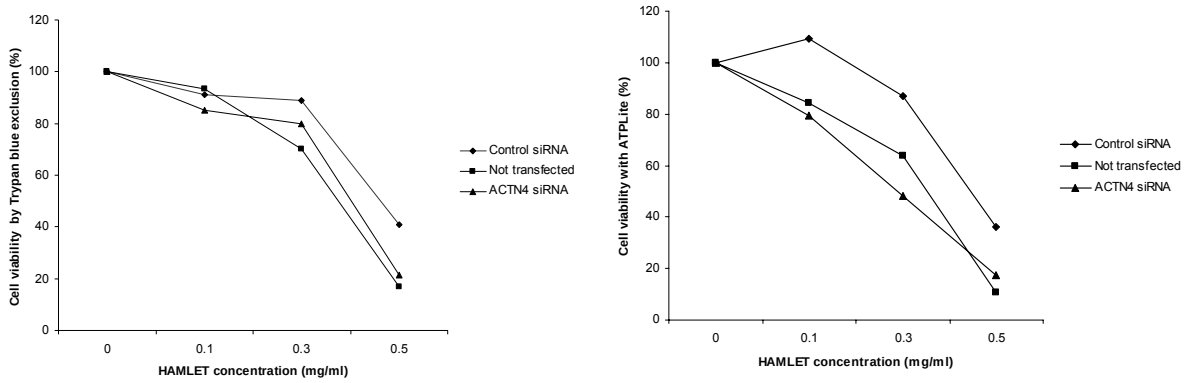

Figure 14: Effect of α-actinin-4 knockdown on HAMLET sensitivity was studied in an apoptosis assay. There was no difference in sensitivity to HAMLET when trypan blue exclusion (left) or ATP levels (right) were used as endpoints.

5. Discussion

HAMLET is a protein-lipid complex that kills tumour cells but leaves healthy, undifferentiated cells unaffected. It is still not fully understood how HAMLET enters cells and induces cell death and why healthy cells are unaffected. This study started with a HAMLET-overlay to identify interacting partners in tumour cells. Alpha-actinin-4 was identified in the membrane fraction. Since α-actinin-4 is involved in cytoskeletal architecture, cell adhesion and some cell death related responses, we have examined how HAMLET interacts with α-actinin-4 and if this interaction participates in cell death. The interaction between HAMLET and α-actinin-4 was characterized in a peptide-binding assay where some specific HAMLET-binding domains were identified. HAMLET was shown to interact with the actin-binding domain of α-actinin, with two areas on the central rod domain and with the calmodulin-like domain. HAMLET is the only protein known to interact with both the actin-binding domain and the spectrin repeats in this way.

We wanted to examine whether the interaction with α-actinin-4 was specific for HAMLET. Different forms of native α-lactalbumin were tested for binding to the positive peptides. Binding to the peptides was not entirely folding dependent, as both the native and the partially unfolded forms of the protein bound to the actin-binding and spectrin repeat domains, respectively. Minor differences were also observed. The partially unfolded forms of α-lactalbumin bound less well than native α-lactalbumin to the actin-binding domains, except for peptide 6, where all-Ala α-lactalbumin bound more strongly. all-Ala HAMLET also bound less well to the actin-binding domains than HAMLET, except for peptide 6 where it bound more strongly. Addition of the fatty acid increased the binding of the partially unfolded protein to the actin-binding domain but slightly decreased the binding compared to native α-lactalbumin. In contrast, the partially unfolded state bound more strongly to the central rod domain than α-lactalbumin did but the other variant with an even more open conformation did not show this increased binding. There were only small differences between HAMLET and all-Ala HAMLET in binding to this domain. Addition of the fatty acid decreased the binding of the partially unfolded protein while no differences were seen between native α-lactalbumin and HAMLET or between all-Ala α-lactalbumin and all-Ala HAMLET. The same pattern was seen with the CaM domain. This indicated that complete unfolding or addition of the fatty acid is not important for binding to α-actinin-4. Thus, the lethal effect of the unfolded form of α-lactalbumin for tumour cell death could not be explained by these interactions.

We also discussed if α-actinin-4 might be involved in the uptake of HAMLET. This is not likely as α-actinin-4 is not a transmembrane protein but α-actinin-4 may have an indirect effect on the uptake mechanism. Lanzetti *et al*. (2004) showed that α-actinin-4 interacts with RNtre and is involved in macropinocytosis. They also showed that siRNA against α-actinin-4 led to a decrease in circular ruffles [18]. There was no apparent decrease in HAMLET uptake after knockdown of α-actinin-4 by siRNA, thus indicating that HAMLET is not taken up by macropinocytosis and α-actinin-4 is not involved in HAMLET uptake. It remains possible, however, that HAMLET affects macropinocytosis through its interaction with α-actinin-4.

The two non-muscle isoforms of α-actinin show 87% sequence identity and most of the positive peptides in the peptide-binding assay are conserved in α-actinin-1, indicating that HAMLET quite likely also interacts with α-actinin-1. α-actinin-1 and -4 both cross-link actin filaments but the cellular localization of the two is somewhat different. α-Actinin-4 has been shown to localize to points of cell-cell contact, hemidesmosomes [13], sharp extensions and membrane ruffles [15] whereas α-actinin-1 has been shown to localize to focal adhesion plaques

and adherence junctions [13]. There were some indications of minor changes in the cellular localization of α-actinin-4 before and after HAMLET-treatment. Before HAMLET treatment, the staining of α-actinin-4 was strong in the cell periphery but there was also weak staining in the cytoplasm, consistent with the cellular functions of α-actinin-4. The α-actinin-4 staining in the cytoplasm was even weaker after HAMLET-treatment and more intense in the cell periphery. This might indicate two things. First, α-actinin-4 does not cross-link actin filaments to the same extent after HAMLET treatment. Second, the cells are sending its α-actinin-4 molecules to the cell periphery either to repair cell adhesion complexes or to try and increase cell adhesion.

The domains to which HAMLET binds indicate which of the biological functions of α-actinin-4 may be disrupted by HAMLET. HAMLET-treated cells detach from the underlying matrix and the interaction between HAMLET and α-actinin-4 might disrupt the actin cytoskeleton and facilitate this detachment. Several different actin-binding sites have been published. Honda *et al*. (1998) proposed amino acids 111-125 as an actin-binding site [14]. Franzot *et al*. (2005) proposed three actin-binding sites in α-actinin-3, i.e. residues 48-57 corresponding to peptide 4, residues 123-147 corresponding to peptide 10 and residues 153-172 corresponding to peptide 12 [12]. Binding of HAMLET to the actin-binding sites hinders α-actinin-4 from performing its major task, which is cross-linking of actin filaments. If HAMLET binds in the cavity between the actin-binding domain and the central rod domain, it may either prevent actinin-4 from binding to actin due to decreased flexibility in the neck region or it may prevent the release of α-actinin-4 from actin filaments. The CaM domain binds to the neck of the other subunit and it is possible that the CaM domain regulates the rearrangement of the calponin-homology domains when α-actinin binds to actin [12]. HAMLET might thus affect the behaviour of the CaM domain when binding to peptide 53 since it corresponds to a potential Ca-binding site. Disruption of the cytoskeletal architecture has been shown to induce a form of apoptosis called amorphosis and detachment from the extracellular matrix may induce an apoptosis-like cell death called anoikis [19]. Both anoikis and amorphism are possible cell death mechanisms following HAMLET treatment but both ways acts through Bcl-2. Hallgren *et al.* (2006) showed that HAMLET does not act through the Bcl-2 system. This suggests that HAMLET does not act via this mechanism and that α-actinin-4 is not directly involved in cell death.

The central rod domain of the monomers interacts to form a dimer. HAMLET does not interfere with this interaction since the peptides are facing out from the site. α-Actinin-4 is suggested to be an important scaffold protein because of the many proteins that interacts with the central rod domain and the spectrin repeats. α-Actinin-4 does for example interact with the cytoplasmic domain of integrins [20], ICAMs [21], L-selectin [22] and EpCAM [23] through the spectrin repeats. Integrins are important in cell-matrix adhesions. They interact with the cytoskeleton so that the cells bind properly to the extracellular matrix. One of the proteins that bind integrins to the cytoskeleton is α-actinin and the linkage leads to clustering of integrins and formation of focal adhesions [24]. It is not known where on the rod domain of α-actinin that integrin binds but it is possible that it is the same binding site as HAMLET occupies. Binding of HAMLET may then block the coupling of integrins to the cytoskeleton and thereby hinder the formation of functional focal adhesions (Figure 15). This may explain the detachment that is seen among HAMLET-treated cells.

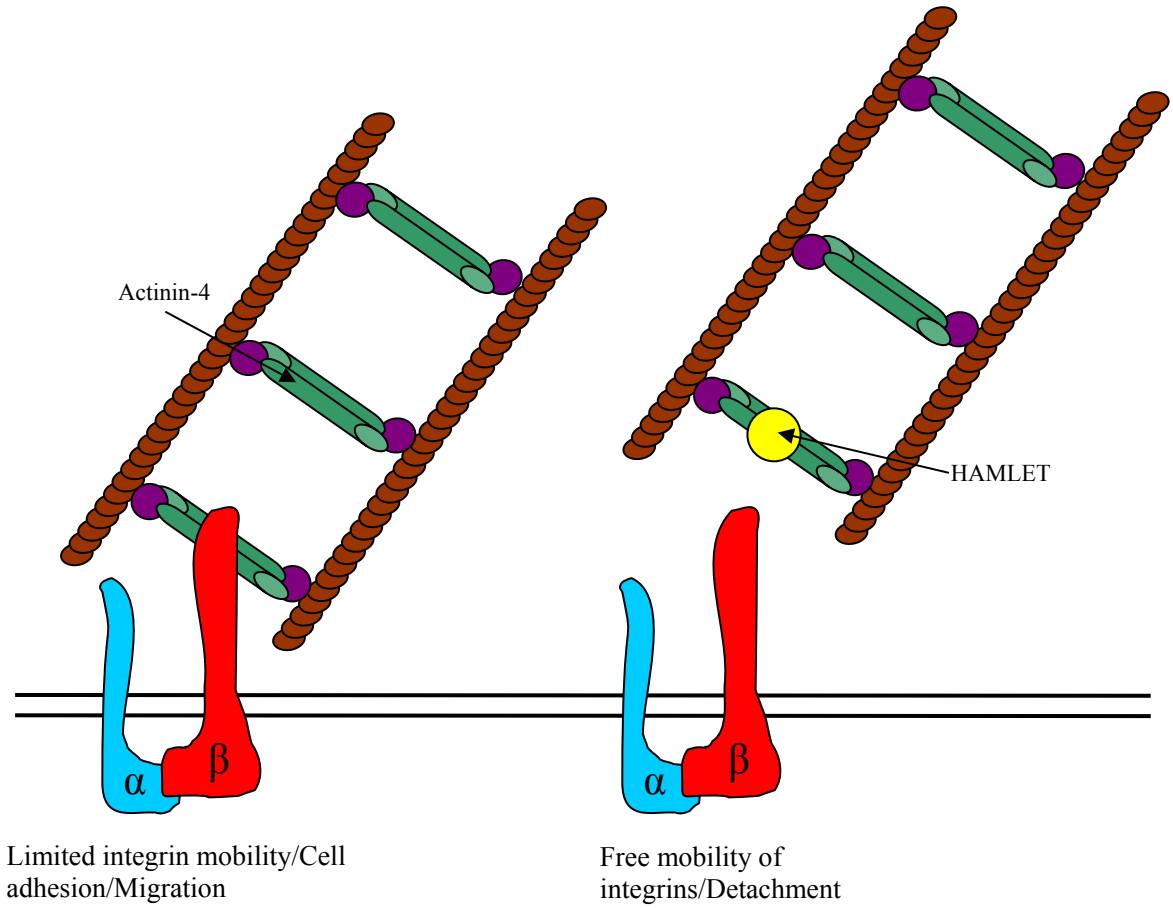

Figure 15: Hypothesis of HAMLET-mediated cell detachment. Cell detachment is one of the characteristics seen in HAMLET-treated cells. This might be explained by the interaction between HAMLET and α-actinin-4. The integrin β domain normally interacts with the central rod domain of α-actinin-4 and this promotes cell adhesion and migration. HAMLET also interacts with the central rod domain and possibly blocks the binding of integrin. When the integrins are not coupled to the cytoskeleton, the cells will detach.

There are other ways for HAMLET to interfere with cell adhesion. L-selectin is responsible for cell-cell interactions between white blood cells and endothelial cells in the bloodstream. Pavalko *et al*. (1995) showed that L-selectin is anchored to the actin cytoskeleton via binding to α-actinin-4 [22]. Additionally, integrins are also involved in the binding of blood cells to endothelial cells through the interaction with intercellular adhesion molecules (ICAMs) on the endothelial cells. Celli *et al*. (2006) additionally showed that ICAMs binds to the cytoskeleton via α-actinin [21]. Cadherins also mediate cell-cell adhesion and must, like integrins, be connected to the cytoskeleton to function properly and the anchor proteins are called catenins [24]. It has been shown that α-actinin-4 interacts with β-catenin. Hayashida *et al*. (2005) showed that α-actinin-4 interacts with β-catenin in the absence of E-cadherin [25], which potentially contradicts the hypothesis of cell detachment as HAMLET would increase the binding of β-catenin to E-cadherin and increase cell-cell adhesion. Furthermore, α-actinin co-localizes with BP180 in hemidesmosomes [13], which are important for the attachment of epithelial cells to the underlying matrix. The hemidesmosome consists of a number of components such as an integrin dimer, plectin, BP230 and BP180. Gonzales *et al*. (2001) suggests that α-actinin-4 function as a scaffolding protein in the formation of hemidesmosomes. Gonzales *et al*. (2001) also showed that BP180 interacts with α-actinin-4 somewhere between amino acid 711 and 822 containing the two EF-hands. One of the positive peptides, peptide 53, partly covers this fragment and binding of HAMLET to peptide 53 may block the binding of α-actinin-4 to BP180 and thus prevents the proper formation of hemidesmosomes [13]. This again may explain the detachment seen in HAMLET-treated cells.

α-Actinin-4 also interacts with the histone acetylation machinery through HDAC7 and HDAC5 [26]. The binding site on α-actinin-4 was localized to amino acids 832-911. These HDACs all associate with MEF2 transcription factors and thus suppress transcription. α-Actinin-4 competes for the same binding site on HDAC7 and thus enhances the transcription activity of MEF2. They also showed that α-actinin-4 interacts directly with MEF2 and enhance transcription. The MEF2 binding site was mapped to amino acids 1-449 [26]. Peptide 59 in the peptide binding assay corresponds to amino acids 871-890 suggesting that binding of HAMLET to this site may prevent α-actinin-4 from binding to HDAC7 which indicates that HAMLET indirectly suppress the transcription activity of MEF2. Peptides 4, 6, 10, 12, 19 and 24 are all localized to the proposed binding site for MEF2 so the likelihood of HAMLET blocking this interaction is quite big thus suppressing the transcription activity further.

Alpha-actinin-4 is also part of the CART complex that is required for efficient recycling of the transferrin receptor to the plasma membrane. The CART complex consists of Hrs, α-actinin-4, BERP and myosin V. Yan *et al*. (2005) showed that α-actinin-4 interacts with a protein called Hrs and the minimal fragment of α-actinin-4 required for binding corresponded to amino acids 357-469 [27]. Peptide 24 in our peptide binding assay covers amino acids 346-365. It is therefore possible that HAMLET may block the interaction between Hrs and α-actinin-4 and thereby disrupt the CART complex. α-actinin-4 also interact with BERP but it is not known what part of α-actinin-4 that is required for this interaction [27, 28]. Yan *et al*. (2005) suggests that a functional CART complex is important for many cellular functions and it is then possible that HAMLET impairs these cellular functions and thereby makes cells less tolerant to stress.

6. Conclusion

α-Actinin-4 was identified as a binding partner for HAMLET in cancer cells. Since α-actinin-4 is involved in cytoskeletal control, cell adhesion and some cell death related responses, we examined how HAMLET interacted with α-actinin-4 and if this interaction participates in cell death. Several conclusions could be drawn from the results presented in this study.

First, we identified interacting domains on α-actinin-4. HAMLET interacts with the actin-binding domain, the central rod domain and the calmodulin-like domain of α-actinin-4.

Second, different folding variants of α-lactalbumin were compared and the tertiary structure or the fatty acid was shown not to be important for binding to α-actinin-4. Thus, the binding to α-actinin-4 cannot explain why HAMLET and not α-lactalbumin kills tumour cells or why HAMLET is recognised by tumour cells.

Third, we investigated biological consequences of this interaction apart from recognition and uptake. Through the interaction with α-actinin-4, HAMLET might affect a diverse range of cellular functions, such as the cytoskeletal architecture due to the interaction with the actin-binding domain. The interaction with the central rod domain prevents integrin, L-selectin and β-catenin from being connected to the cytoskeleton and thus impairs cell adhesion to the underlying matrix and to other cells. HAMLET also prevents the interaction between α-actinin-4 and BP180, thus affecting cell adhesion through impairment of hemidesmosomes.

7. Acknowledgements

First of all, I would like to thank Catharina Svanborg for giving me the opportunity to perform my degree project in the HAMLET-group and for all her encouragement during the project. I would also like to thank Lotta Gustafsson, Anki Mossberg, Jenny Petterson and Sonja Aits for their help in the lab, what should I have done without you! I would also like to thank Mattias Gustafsson for performing my transfections and RT-PCR for the siRNA experiments. Finally I would like to thank my friends and my family to whom I can always turn.

8. Abreviations

all-Ala α-lactalbumin	α-lactalbumin mutant
all-Ala HAMLET	HAMLET mutant
AP	Alkaline phosphatase
ATP	Adenosine 5´-triphosphate
BSA	Bovine serum albumin
CaM domain	Calmodulin-like domain
CART complex	Cytoskeleton-associated recycling or transport complex
CH domain	Calponin homology domain
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelium cell adhesion molecule
FCS	Foetal calf serum
GFP	Green fluorescent protein
HAMLET	Human α-lactalbumin made lethal to tumour cells
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
ICAMs	Intercellular cell adhesion molecule
Ig	Immunoglobulin
LB	Luria broth
MAL	Monomeric α-lactalbumin
MALDI spectroscopy	Matrix-assisted laser desorption ionization spectroscopy
RNA	Ribonucleic acid
mRNA	messenger RNA
PBS	Phosphate buffered saline
PI_3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
RT-PCR	Reverse transcriptase- Polymerase chain reaction
siRNA	small interference RNA

9. References

1. Jackson, K. M. & Nazar, A. M. Breastfeeding, the immune response, and long-term health. J Am Osteopath Assoc 106, 203-7 (2006).
2. Martin, R. M., Gunnell, D., Owen, C. G. & Smith, G. D. Breast-feeding and childhood cancer: A systematic review with metaanalysis. Int J Cancer 117, 1020-31 (2005).
3. Hakansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H. & Svanborg, C. Apoptosis induced by a human milk protein. Proc Natl Acad Sci U S A 92, 8064-8 (1995).
4. Hallgren, O., Gustafsson, L., Irjala, H., Selivanova, G., Orrenius, S. & Svanborg, C. HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53. Apoptosis 11, 221-33 (2006).
5. Svensson, M., Sabharwal, H., Hakansson, A., Mossberg, A. K., Lipniunas, P., Leffler, H., Svanborg, C. & Linse, S. Molecular characterization of alpha-lactalbumin folding variants that induce apoptosis in tumor cells. J Biol Chem 274, 6388-96 (1999).
6. Svensson, M., Fast, J., Mossberg, A. K., Duringer, C., Gustafsson, L., Hallgren, O., Brooks, C. L., Berliner, L., Linse, S. & Svanborg, C. Alpha-lactalbumin unfolding is not sufficient to cause apoptosis, but is required for the conversion to HAMLET (human alpha-lactalbumin made lethal to tumor cells). Protein Sci 12, 2794-804 (2003).
7. Svensson, M., Hakansson, A., Mossberg, A. K., Linse, S. & Svanborg, C. Conversion of alpha-lactalbumin to a protein inducing apoptosis. Proc Natl Acad Sci U S A 97, 4221-6 (2000).
8. Svensson, M., Mossberg, A. K., Pettersson, J., Linse, S. & Svanborg, C. 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, 2805-14 (2003).
9. Kohler, C., Hakansson, A., Svanborg, C., Orrenius, S. & Zhivotovsky, B. Protease activation in apoptosis induced by MAL. Exp Cell Res 249, 260-8 (1999).
10. Duringer, C., Hamiche, A., Gustafsson, L., Kimura, H. & Svanborg, C. HAMLET interacts with histones and chromatin in tumor cell nuclei. J Biol Chem 278, 42131-5 (2003).
11. Menez, J., Le Maux Chansac, B., Dorothée, G., Vergnon, I., Jalil, A., Carlier, M. F., Chouaib, S. & Mami-Chouaib, F. Mutant alpha-actinin-4 promotes tumorigenicity and regulates cell motility of a human lung carcinoma. Oncogene 23, 2630-9 (2004).
12. Franzot, G., Sjoblom, B., Gautel, M. & Djinovic Carugo, K. The crystal structure of the actin binding domain from alpha-actinin in its closed conformation: structural insight into phospholipid regulation of alpha-actinin. J Mol Biol 348, 151-65 (2005).
13. Gonzalez, A. M., Otey, C., Edlund, M. & Jones, J. C. Interactions of a hemidesmosome component and actinin family members. J Cell Sci 114, 4197-206 (2001).
14. Honda, K., Yamada, T., Endo, R., Ino, Y., Gotoh, M., Tsuda, H.,Yamada, Y., Chiba, H. & Hirohashi, S. Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J Cell Biol 140, 1383-93 (1998).
15. Honda, K., Yamada, T., Seike, M., Hayashida, Y., Idogawa, M., Kondo, T., Ino, Y. & Hirohashi, S. Alternative splice variant of actinin-4 in small cell lung cancer. Oncogene 23, 5257-62 (2004).
16. Koradi, R., Billeter, M. & Wuthrich, K. MOLMOL: a program for display and analysis of macromolecular structures. J Mol Graph 14, 51-5, 29-32 (1996).

17. Aigner, A. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. J Biotechnol 124, 12-25 (2006).
18. Lanzetti, L., Palamidessi, A., Areces, L., Scita, G. & Di Fiore, P. P. Rab5 is a signalling GTPase involved in actin remodelling by receptor tyrosine kinases. Nature 429, 309-14 (2004).
19. Martin, S. S. & Vuori, K. Regulation of Bcl-2 proteins during anoikis and amorphosis. Biochim Biophys Acta 1692, 145-57 (2004).
20. Sampath, R., Gallagher, P. J. & Pavalko, F. M. Cytoskeletal interactions with the leukocyte integrin beta2 cytoplasmic tail. Activation-dependent regulation of associations with talin and alpha-actinin. J Biol Chem 273, 33588-94 (1998).
21. Celli, L., Ryckewaert, J. J., Delachanal, E. & Duperray, A. Evidence of a functional role for interaction between ICAM-1 and nonmuscle alpha-actinins in leukocyte diapedesis. J Immunol 177, 4113-21 (2006).
22. Pavalko, F. M., Walker, D. M., Graham, L., Gobeen, M., Doerschuk, C. M. & Kansas, G. S. The cytoplasmic domain of L-selectin interacts with cytoskeletal proteins via alpha-actinin: receptor positioning in microvilli does not require interaction with alpha-actinin. J Cell Biol 129, 1155-64 (1995).
23. Balzar, M. Bakker, H. A., Briaire-de.Bruijn, I. H., Fleuren, G. J., Warnaar, S. O. & Litvinov, S. V. Cytoplasmic tail regulates the intercellular adhesion function of the epithelial cell adhesion molecule. Mol Cell Biol 18, 4833-43 (1998).
24. Alberts, B. Molecular biology of the cell (Garland Science, New York, 2002).
25. Hayashida, Y., Honda, K., Idogawa, M., Ino, Y., Ono, M., Tsuchida, A., Aoki, T., Hirohashi, S. & Yamada, T. E-cadherin regulates the association between beta-catenin and actinin-4. Cancer Res 65, 8836-45 (2005).
26. Chakraborty, S., Reineke, E. L., Lam, M., Li, X., Liu, Y., Gao, C., Khurana, S., & Kao, H. Y. alpha actinin 4 potentiates MEF2 transcription activity by antagonizing histone deacetylase 7. J Biol Chem (2006).
27. Yan, Q., Sun, W., Kujala, P., Lotfi, Y., Vida, T. A. & Bean, A. J. CART: an Hrs/actinin-4/BERP/myosin V protein complex required for efficient receptor recycling. Mol Biol Cell 16, 2470-82 (2005).
28. El-Husseini, A. E., Kwasnicka, D., Yamada, T., Hirohashi, S. & Vincent, S. R. BERP, a novel ring finger protein, binds to alpha-actinin-4. Biochem Biophys Res Commun 267, 906-11 (2000).