COVER SHEET

TITLE: identification of acetylated lysine residues on the ER chaperone BiP.

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ABSTRACT

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Alzheimer's disease (AD) pathogenesis involves the abnormal production of a small peptide called Aβ. The rate-limiting enzyme for the generation of Aβ is BACE1 and, as such, its down-regulation decreases Aβ levels. Dr. Puglielli's laboratory has recently identified new aspects of BACE1 metabolism that involve transient acetylation in the lumen of the ER and deacetylation in the Golgi apparatus. While dissecting the biochemical machinery responsible for the transient acetylation of nascent BACE1, Dr. Puglielli and co-workers have discovered that the ER-based chaperone BiP –vitally important for cellular and ER homeostasis– also undergoes lysine acetylation. The purpose of this project was to identify the lysine residues that undergo acetylation on BiP, and future experiments using mutagenesis strategies will investigate how the acetylation status of BiP affects its cellular functions. Here, we show that six acetylated lysine residues were identified: Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}, Lys^{585} and Lys^{621}.

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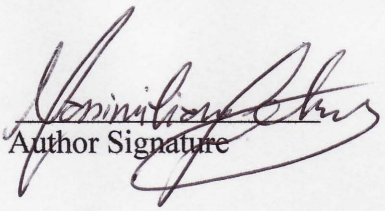

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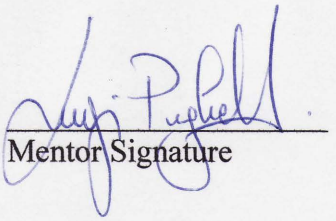

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Date

IDENTIFICATION OF ACETYLATED LYSINE RESIDUES ON THE ER CHAPERONE BIP

by

Massimiliano Lehnus

A thesis submitted in partial fulfillment of the Honors requirements for the degree of

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Dedicated to my parents, ***Marco Lehnus*** *and* ***Rossana Lo Monaco****, for being such attentive listeners and caring counselors. You have indulgently given me so much, and I need you both to know that I am so deeply grateful for all your sacrifices.*

~

Dedicato ai miei genitori, ***Marco Lehnus*** *e* ***Rossana Lo Monaco****, per essere ed essere stati degli attenti ascoltatori e premurosi consiglieri che mi hanno accompagnato con cura ed amore durante tutto questo percorso. Mi avete dato cosi tanto, e voglio che sappiate che sono profondamente grato per tutti i vostri sacrifici.*

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I would also like to thank Dr. **Mariana Pehar** for all your assistance. Despite your busy schedule, you have always managed to set aside some time to teach me new techniques and to help me interpret new results. You have been an excellent guide. I have learned so much from our discussions, and I consider you a great friend.

You have been superb instructors, and I look up to you both.

THESIS ABSTRACT

Alzheimer's disease (AD) is the most prevalent form of dementia in the world. The pathogenesis of the disease involves the abnormal production of a small peptide called Aβ. The rate-limiting enzyme for the generation of Aβ is BACE1 and, as such, its down-regulation decreases Aβ levels. Dr. Puglielli's laboratory has recently identified new aspects of BACE1 metabolism that involve transient acetylation in the lumen of the ER and deacetylation in the Golgi apparatus. While dissecting the biochemical machinery responsible for the transient acetylation of nascent BACE1, Dr. Puglielli and co-workers have discovered that the ER-based chaperone BiP – vitally important for cellular and ER homeostasis– also undergoes lysine acetylation. The purpose of this project was to identify the lysine residues that undergo acetylation on BiP, and future experiments will investigate how the acetylation status of BiP affects its cellular functions. Six acetylated lysine residues were identified: Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}, Lys^{585} and Lys^{621}. A mutagenesis strategy of these residues has already been devised, and future transfection of different cell lines containing the mutated BiP gene will allow for the investigation of the function of the acetylation of BiP in relation to the physiology of the cell. Overall, this project will further investigate the role of lysine acetylation, and will analyze how BiP acetylation affects cell function and its possible involvement in Alzheimer's disease pathology.

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LIST OF ABBREVIATIONS

α: alpha

Aβ: amyloid-β peptide

AD: Alzheimer's disease

ApoE: apolipoprotein E

AT-1: acetyl-CoA transporter 1

ATase: acetyltransferase

β: beta

BACE1: β-site APP cleaving enzyme 1

bp: base pair

BSA: Bovine Serum Albumin

°C: degrees Celsius

cDNA: complementary DNA

CHO: Chinese Hamster Ovary

CMV: cytomegalovirus

CoA: coenzyme A

DAC: deacetylation

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide triphosphate

EDTA: ethylenediaminetetraacetic acid

EOAD: early onset Alzheimer's disease

ER: endoplasmic reticulum

ERAD: ER-associated degradation

ERGIC: endoplasmic reticulum/Golgi intermediate compartment

FBS: fetal bovine serum

γ: gamma

G418: geneticin

Gin: glutamine

HAT: histone acetyltransferase

hr: hour(s)

K: lysine

kb, kbp: Kilobase pairs

kD: Kilodalton

kDa: Kilodalton

MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight

mg: milligram

min: minute(s)

ml: milliliter

mM: millimolar

mRNA: messenger RNA

LDL: low density lipoprotein

LDLR: low density lipoprotein receptor

LOAD: late onset Alzheimer's disease

Lys: lysine

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PCSK9: proprotein convertase subtilisin/kexin type 9:

P/S: penicillin/streptomycin

Q: glutamine

R: arginine

RNA: ribonucleic acid

rpm: revolutions per minute

RTPCR: real-time PCR

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

µg: microgram

µl: microliter

µm: micrometer

µM: micromolar

WT: wild type

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INTRODUCTION

Overview of Alzheimer's Disease

Alzheimer's disease (AD), the most common form of dementia, is a brain disease causing a person to have increasing difficulty in thinking and speaking, remembering people and things, and learning new concepts (Reisberg, 1983). AD currently affects approximately 35 million individuals worldwide. In fact, the prevalence of AD increases sharply after the age of 60 and doubles with every decade of life reaching ~50% in individuals that are 85 years of age or older (Puglielli, 2008). AD exists in two forms, "familial" (also called early-onset AD) and "sporadic" (also called late-onset AD). Familial AD (FAD), accounts for ~3% of all AD cases, and has so far been linked to mutations in the genes for the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) (Bertram and Tanzi, 2008). Sporadic/late-onset AD accounts for ~97% of all AD cases, and aging is the single most important risk factor associated with its development (Puglielli, 2008). From a genetics perspective, sporadic/late-onset AD does not show a clear pattern of inheritance. Nonetheless, the inheritance of the ε4 allele of the apolipoprotein E gene (APOE) is currently the most important genetic risk factor for the sporadic form of the disease (Bertram and Tanzi, 2008). In terms of AD development, the most significant known consequence of all these mutations is the unbalanced production of the amyloid-β (Aβ) peptide, which is a 39–43 amino acid long peptide (Puglielli *et al.*, 2003). Aβ is generated from APP after it has been cleaved by the β-secretase and

γ-secretase enzymes (Bertram and Tanzi, 2008), and it is key in AD neuropathology as it aggregates in the form of amyloid plaques (also called "senile plaques"), an essential hallmark of AD dementia. In fact, as reported by Tomski (1991), among the most considerable findings in the brain of AD patients are plaques, where, as noted earlier, Aβ can be found, and tangles. Plaques are located between nerve cells, while tangles build up inside dying cells, and are formed by a protein called tau (Tomski, 1991). When the human tau protein functions properly in a healthy individual, its primary role is that of assembling and stabilizing microtubules in the brain. However, hyperphopshorylation of this protein in Alzheimer's disease patients causes it to be incapable of carrying out its duties (Puglielli, 2008). As a direct consequence, microtubules tend to get disrupted and neurofibrillary tangles start to form, which in the long run causes loss of neuronal connectivity and, therefore, the typical symptoms caused by the disease will begin to appear and worsen as more neurons die (Iqbal *et al.*, 2009).

Lysine Acetylation Overview

The process of acetylation is a type of cellular regulatory mechanism that involves the addition of an acetyl group into a compound. N^{α}, N^{ε}, and O are three different types of acetylation that have been discovered so far. N^{α}-acetylation takes place on the N-terminal of nascent proteins in the cytoplasm, and has been observed on alanine, serine, methionine, threonine and glycine (Polevoda, 2003). Most eukaryotic proteins undergo N^{α}-acetylation, but its effects are still unknown (Polevoda *et al*., 2000). O-acetylation has not been described in humans (Clarke *et*

al., 1992) and it affects serine and threonine (Mittal *et al.*, 2006 and Mukherjee *et al.*, 2006). N^{ε} –acetylation, or more commonly, "lysine acetylation", refers to the acetylation of the ε amino group of a lysine residue. Lysine acetylation was first observed on histone proteins. When these are acetylated by histone acetyltransferases enzymes (HAT's), the level of DNA transcription increases as chromatin decondenses, and vice versa when these proteins are deacetylated by histone deacetylases enzymes (HDAC) (Kimura *et al.*, 2005 and Henikoff *et al.*, 2005). More recently, this type of reversible lysine acetylation has also been discovered in non-histone proteins in the cytoplasm, nucleus, mitochondria (Yang and Gregoire, 2007, Schwer *et al.*, 2006 and Polevoda and Sherman, 2002) and ER (Costantini *et al.*, 2007), where it seems to affect both activity and stability of the modified polypeptides (Polevoda and Sherman, 2002 and Kouzarides, 2000). These non-histone proteins comprise chaperons, enzymes, cytoskeletal proteins, transcription factors, DNA recombination and repair proteins, and signaling proteins (Yang *et al.*, 2007). Although acetylation of lysine residues does not appear to be a random process, a consensus motif has not yet been found (Costantini *et al.*, 2007 and Spange *et al.*, 2009). In addition to lysine acetylation, the ε-NH_2 group of lysine residues can also undergo biotinylation, phosphorylation, methylation, sumoylation, ubiquitination, propioylation and butyrylation (Yang *et al.*, 2007), most of which still have undetermined effects. However, once any of these modifications is present, no other one can occur simultaneously, thereby underlining the importance of when and where lysine acetylation takes place.

Transient Lysine Acetylation of Nascent BACE1

The membrane protein beta-site APP cleaving enzyme 1 (BACE1) is the rate-limiting enzyme in Aβ formation (Costantini *et al*., 2007). Dr. Luigi Puglielli's laboratory has recently discovered a new type of post-translational regulation of BACE1, involving its transient acetylation in the endoplasmic reticulum's (ER) lumen (Costantini *et al*., 2007). Acetylation of nascent BACE1 requires ATase1 and ATase 2, two ER-based acetyl-CoA:lysine acetyltransferases (Ko and Puglielli, 2009), as well as AT-1, an ER membrane acetyl-CoA transporter (Jonas *et al*., 2010). The latter transports the charged and membrane impermeable acetyl-CoA, donor of the acetyl-group during the lysine acetylation reaction, from the cytosol to the ER's lumen (Costantini *et al*., 2007). AT-1 regulates the acetylation status of ER transiting proteins (BACE1, low-density lipoprotein receptor (LDLR), and APP), is essential for cell viability, and is present at high levels in the brain of late-onset/sporadic AD patients (Jonas *et al*., 2010). The acetylated intermediates of BACE1 are able reach the Golgi apparatus, where a Golgi-resident deacetylase removes the acetyl group (Costantini *et al*., 2007). This is in contrast with the non-acetylated intermediates, which are degraded in a post-ER compartment, with a high chance of the latter being the ER Golgi intermediate compartment (ERGIC), following a pathway involving the serine protease proprotein convertase substilin kexin type 9/neural-apoptosis-regulated convertase 1 (PCSK9/NARC-1) (Jonas *et al*., 2008). Alterations in the levels of ATase1/ATase2 (Ko and Puglielli, 2009) and of PCSK9/NARC-1 (Jonas *et al*., 2008) affect those of BACE1, thereby influencing those of Aβ generation as well, and consequently AD development.

Transient Lysine Acetylation of the ER Chaperone BiP

Dr. Puglielli's laboratory initially discovered this novel form of post-translational regulation of nascent membrane proteins in 2007 while studying BACE1 metabolism (Costantini *et al*., 2007). However, it is now evident that additional membrane and secreted proteins are affected as well. Therefore, it became logical to ask whether the ER-based transient acetylation of the ε-amino group of a lysine residue is limited to membrane/secreted proteins (which only transit through the ER) or if ER-resident proteins undergo this modification as well. And if that were the case, it would be imperative to investigate lysine acetylation further because of its potentially critical biological meaning. The identification of novel substrates of the ER-based acetylation machinery is -at this stage- essential to understand this form of post-translational regulation. In fact, it can help assess the broad spectrum of proteins that are modified and acquire more information on the exact function of the lysine acetylation. With this in mind, Dr. Puglielli's laboratory has recently used a small-scale proteomic approach to identify new substrates of the ER acetylation machinery. This strategy was coupled to both Matrix Assisted Laser Desorption/Ionization (MALDI) and collision-induced dissociation analysis and resulted in the identification of several ER-resident proteins that appear to be acetylated in one or more lysine residues. Surprisingly, the ER-resident protein BiP (also known as glucose-regulated protein 78kD, "GRP78", or heat shock 70kD protein 5, "HSPA5") was found as a possible target of the acetylation machinery. BiP is located on chromosome 9q33.3, and it is a chaperone in the ER lumen, where it is required for proper protein folding and maturation. BiP is upregulated when misfolded proteins

collect in the ER as a result of environmental and/or metabolic stress; it is released from transmembrane ER signaling proteins and creates a positive feedback loop (Zhang and Kaufman, 2006). Downregulation of BiP has been shown to have interesting effects in the reduction of the formation of certain types of cancer, underscoring a possible role as a negative regulator of apoptosis (Tanimoto, R., *et al.,* 2010). Importantly, significant down-regulation of BiP in the cell results in cell death, further stressing the significance of this protein for the biology of the cell. In the present project I identified the lysine residues that undergo acetylation on BiP, and planned a mutagenesis strategy of these residues to examine how their inability to undergo acetylation affects the chaperone's cellular functions.

MATERIALS AND METHODS

Plasmid construction

Human BiP (NM_005347) cDNA was obtained from OriGene (SC108086) and cloned into pcDNA^{TM}3.1/myc-His C (Invitrogen), a vector for mammalian expression under the control of the CytoMegalovirus (CMV) promoter and with a his-myc tag added to the C-terminal domain of the protein. The his-myc tag allowed us to perform the purification of the transgenic protein. Restriction enzymes BamHI (R0136) and XhoI (R0146) (New England BioLabs Inc.) were used to digest the original BiP plasmid and the pcDNA3.1/myc-His vector. The DNA fragments –BiP cDNA and digested vector– were purified by agarose gel electrophoresis followed by gel extraction using the QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's protocol. Dephosphorylation of the linearized vector was carried out using Calf Intestinal Alkaline Phosphatase (CIAP) (18009-019, Invitrogen), and the gel purification/extraction procedure was repeated. Dephosphorylated pcDNA^{TM}3.1/myc-His C vector and BiP cDNA fragment were then joined using T4 DNA Ligase (15224, Invitrogen).

Plasmid DNA Isolation and Purification

The transformation procedure was carried out using 2µl DNA from ligation reactions, in the case of unmutated BiP DNA, or 2µl DNA obtained from PCR

reactions to mutate BiP acetylated lysine residues. These were added directly to 100µl of MAX Efficiency® DH5α™ Competent Cells (18258-012, Invitrogen) on ice for 30 min. The cells were then heat-shocked for 45 sec at 42 °C, and S.O.C. Medium (15544-034, Invitrogen) was used to culture the cells at 37 °C for 1 hr at 225 rpm. LB agar (L3027, Sigma Aldrich) supplemented with 100µg/ml ampicillin (used as a selection marker for the presence of the plasmid), was utilized to grow the bacteria at 37 °C overnight. Isolated colonies were then grown at 37 °C for 12 hr at 225 rpm in LB Broth (L3152, Sigma Aldrich) supplemented with 100µg/ml ampicillin. The plasmid was purified using PureYield™ Plasmid Miniprep System (A1223, Promega), following manufacturer's protocol.

Cell cultures

Transgenic BiP was overexpressed in Human Neuroglioma (H4) cells or Chinese Hamster Ovary (CHO) cells by stable transfection using Lipofectamine2000™ (Invitrogen). Stable transfected cells were grown in DMEM media (Mediatech, Inc) supplemented with 10% fetal bovine serum (FBS), 1% Glutamine/Penicillin/Streptomycin solution (Mediatech, Inc.) and 0.7% G418 Sulphate (Mediatech, Inc; to select for stably transfected cells). Cells were grown in a humidified atmosphere with 6% CO_2 at 37 °C.

Cell extraction and protein purification

Cell monolayers were washed twice with Phosphate-Buffered Saline (PBS) and then scraped and centrifuged in 50ml conical tubes at 1,000 rpm for 5 min. Cell lysis was performed on ice in GTIP buffer (100 mM Tris-pH 7.6, 20 mM EDTA, 1.5 M NaCl) with 1% Triton X-100 (Roche), 0.25% NP40 (Roche), and a protease inhibitor cocktail (Roche). Lysed cells were then centrifuged at 14,500 rpm and the supernatant was collected. Protein concentration was determined using the BCA Protein Assay (Pierce). Prior to being separated and analyzed by reducing SDS-PAGE, protein samples were incubated at 70ºC for 10 min in SDS sample buffer (Invitrogen) with β-mercaptoethanol. BiP was purified from total cell extracts by affinity chromatography using the ProFound c-Myc-Tag IP/Co-IP kit (Pierce).

Antibodies and Western blot analysis

Electrophoresis was conducted on 4-12% Bis-Tris SDS-PAGE systems (NuPAGE; Invitrogen). The gels were transferred to nitrocellulose membranes (Invitrogen), which were then blocked for 1 hr in Tris Buffered Saline-0.1% Tween®20 (TBST) with 5% nonfat dry milk. The membranes were incubated with primary antibody diluted in 5% BSA in TBST overnight , then washed with TBST, incubated with peroxidase-conjugated secondary antibody (Amersham Biosciences) for 1 hr, and developed using the LumiGLO chemiluminescent detection system (KPL). The antibodies used for the Western Blots in this

investigation are the following: anti-BiP (1:1000; Cell Signaling); anti-c-Myc (1:1000; Sigma); anti-c-His (1:1000; Cell Signaling); anti-actin (1:1000; Cell Signaling). Secondary antibodies (Amersham) were diluted at 1:6000.

Mass Spectrometry

Purified BiP was trypsin-digested and analyzed by HPLC using a C18 reverse phase column. The separated tryptic peptides were analyzed by μLC-MS/MS using a Micromass Q-TOF2 spectrometer (University of Wisconsin Biotechnology Center, Mass Spectrometry Facility). As peptides elute from the HPLC-column/electrospray source, MS/MS spectra were collected, and acetylated or non-acetylated peptide signals on BiP were differentiated, allowing us to identify the modified amino acids.

Mutagenesis

BiP mutagenesis was performed with QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's protocol. The primers used are listed in Table 2. The presence of the mutations was confirmed by sequencing (University of Wisconsin Biotechnology Center, DNA Sequencing Facility). Transfection of H4 cells was carried out by using Lipofectamine2000^{TM} (Invitrogen) as described above.

RESULTS

BiP overexpression in H4, CHO and AT-1 cell lines

In order to identify the acetylated lysine residues on BiP, we first overexpressed the protein in three different cell lines, Human Neuroglioma (H4) cells, Chinese Hamster Ovary (CHO) cells and CHO cells overexpressing the acetyl-CoA transporter AT-1 (AT-1 cells). To make sure that the protein would be properly expressed in the cells before proceeding with a stable transfection, we conducted a transient transfection with the pcDNA^{TM}3.1/myc-His C plasmid containing the human BiP cDNA with a C-terminal his-myc tag. Figures 3, 4 and 5 illustrate the Western Blot analyses of total cell lysates from transient transfected H4, CHO and AT-1 cells, respectively. We chose these cell lines to investigate the difference in expression of BiP in cells that are part of the nervous system (H4), mammalian somatic cells (CHO) and in AT-1 cells, which are CHO cells that overexpress AT-1. AT-1 has an important role in the acetylation of ER transiting proteins, like for instance BACE1 (Jonas *et al*., 2010). Hence, we are interested in studying the effects that its simultaneous expression would have on BiP acetylation levels. The cells were transiently transfected with 4μg of BiP-pcDNA plasmid or empty-pcDNA plasmid, as a negative control. Antibodies against BiP, as well as to its added his-myc tags, were used to detect BiP expression levels in the cells. Anti-actin antibodies were used as a loading control. The upper bands, visible in the cells transfected with BiP, represent the levels of the overexpressed fusion protein with the his-myc tag; while the lower band correspond to the endogenous BiP levels.

Similar results were obtained for the subsequent stable transfection, which was carried out on H4 and CHO cells only. AT-1 cells transfection was postponed for later studies. Figures 6 and 7 show the Western Blot analyses of total cell lysates from transgenic H4 and CHO cells, respectively. Similar procedures described above for transiently transfected cells (figures 3 through 5) were followed; except this time 2µg, as well as 4µg, of BiP DNA were used. The results obtained show that the difference in the levels of expression in the cells transfected with either amount is negligible. Overall, figures 3-8 show strong BiP overexpression in all three types of transgenic cell lines, indicating that all three of them can be used for comparison in future experiments analyzing BiP acetylation.

Next, we used total cell extracts from the stably transfected cells (figures 6 and 7) to purify the BiP protein for further analysis. Figure 8 and 9 show the Western Blot analysis of the first three elutions of the BiP protein, in H4 and CHO cells, respectively, after it had been purified by affinity chromatography using the ProFound c-Myc-Tag IP/Co-IP kit (Pierce). As a negative control, we performed the same purification procedure on cell lysates obtained from cells stably transfected with the empty pcDNA plasmid. The c-myc epitope allowed us to purify the protein using anti-c-Myc antibodies to detect the levels of expression. Fast blue staining of the gels (data not shown) containing the protein samples showed no additional bands other than that of Bip (around 70kDa), suggesting that the purification procedure was successful. Figure 8 shows that the first elution of the H4 cells containing the BiP plasmid has a significantly larger band than the other two, indicating that it contains greater levels of the purified protein. Figure 9 shows that only the first elution of the

CHO cells containing the BiP plasmid appears to have the purified BiP protein. This might suggest that CHO cells actually have a lower level of expression of the BiP plasmid as compared to H4 cells, and therefore there may have not been enough protein leftover to attach to the beads during the column purification procedure in the second and third elutions.

Identification of acetylated lysine residues on the ER chaperone BiP

The first elution of purified BiP (figure 8), obtained from total cell extracts of stably transfected H4 cells (figure 6), was sent for mass spectrometry analysis to the Mass Spectrometry Facility at the University of Wisconsin Biotechnology Center. Acetylated or non-acetylated peptide signals on BiP were differentiated, and this allowed us to identify the modified amino acids. Figure ? shows the disorder prediction of the BiP protein sequence that was conducted with DRIP-PRED analysis (http://www.sbc.su.se/~maccallr/disorder/) of the Stockholm Bioinformatics Center, Stocholm University, Sweden. Amino acids in dark blue represent highly ordered regions, while underlined ones indicate problable disorder. The circled lysines (K) are the residues that were found to be acetylated: Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}, Lys^{585} and Lys^{621}.

Figure 10 shows a three-dimensional (3-D) view of the ATPase-binding domain of BiP. Figure 10 A illustrates with red dots where the first four acetylated lysine residues are present on the protein (K81, K154, K164, and K213), while figure 10 B is meant to emphasize the position of the acetylated residues (in yellow) within

the 3-D structure of the protein. The letter "C", in white, indicates the C-terminal domain of BiP. The structural and graphical illustrations were prepared with the Entrez Molecular Modeling Database (MMDB) available at http://www.ncbi.nlm.nih.gov/Structure/.

Table 1 illustrates the mutagenesis strategy that we adopted after this point. The first four amino acids were found on the ATPase-binding domain of the protein (figure 10), while the last two are in the nucleotide-binding domain. Thus, we decided to create two groups of cell lines, each containing three different combinations of mutated acetylated lysine residues. The first group would contain mutations causing lysine residues to change into arginine (R) ones, which prevent acetylation without modifying the structure of the protein (loss of acetylation mutants). The second group would contain lysine residues mutating into glutamines (Q), which simulate a gain of acetylation, since glutamine mimics the structure of acetylated lysine residues. Both of these groups (lysine to arginine mutations, or lysine to glutamine mutations), are composed of mutants for the first four acetylated lysine residues found (Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}), the last two (Lys^{585} and Lys^{621}), and all six residues mutated together. This mutagenesis scheme will allow us to investigate the function of the acetylation of BiP. Loss- and gain- of acetylation mutants will be stably transfected into H4 cells in future experiments.

The lysine residues were mutated using QuickChange® Lightning Site-Directed Mutagenesis Kit, and the presence of mutations was confirmed by the DNA Sequencing Facility at the University of Wisconsin Biotechnology Center. Table 2 lists the primer sequences used to carry out seven rounds of mutagenesis to obtain the

combinations of mutations described above. The codons that underwent mutagenesis are shown in red, while the nucleotides that are underlined and in bold represent the actual substituted bases after the mutagenesis. Figure 2 offers a view of the complete nucleotide sequence of the BiP ORF, as well as the position of the primers (underlined and in bold) used for the mutagenesis, and the codons corresponding to the acetylated lysine residues (in red), with respect to the ORF.

Figure 1

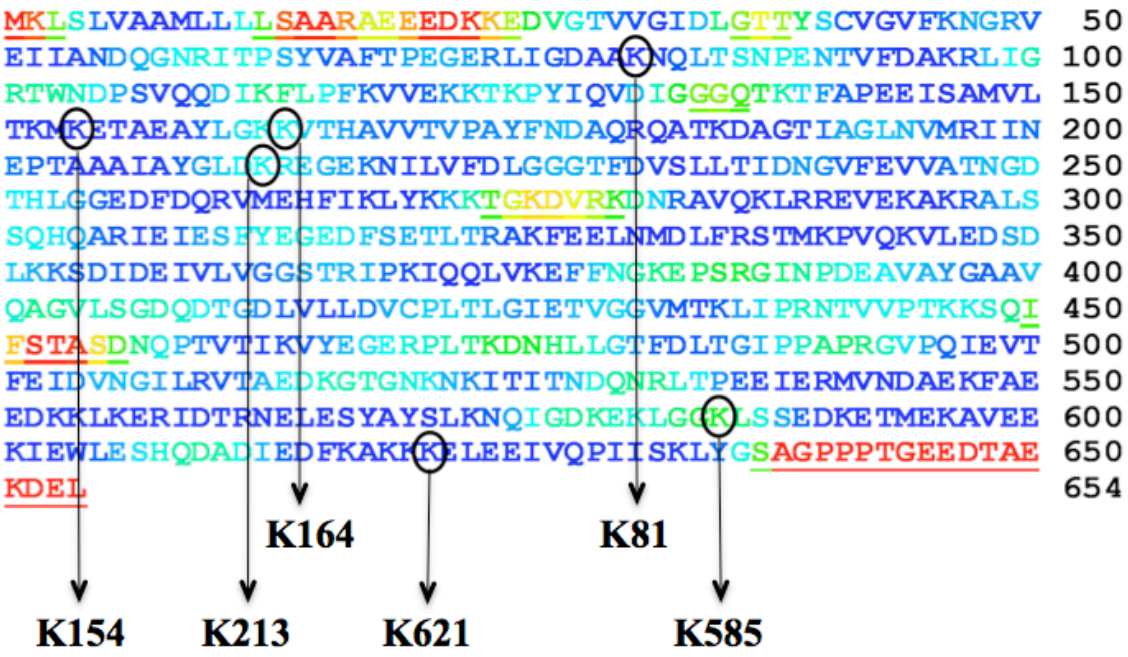
MKLSLVAAMLLLLSAARAEEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRV 50
EIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRLIG 100
RTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVL 150
TKMKETAEAYLGKKVTHAVVTVPAYFNDAQRQATKDAGTIAGLNVMRIIN 200
EPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGD 250
THLGGEDFDQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKRALS 300
SQHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRSTMKPVQKVLEDSD 350
LKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAV 400
QAGVLSGDQDTGDLVLLDVCPLTLGIETVGGVMTKLIPRNTVVPTKKSQI 450
FSTASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIPPAPRGVPQIEVT 500
FEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAE 550
EDKKLKERIDTRNELESYAYSLKNQIGDKEKLGGKLSSEDKETMEKAVEE 600
KIEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAGPPPTGEEDTAE 650
KDEL 654
K154
K213
K164
K621
K81
K585

Figure 1. Acetylated Lysine Residues on BiP.

Figure 1 shows the amino acid sequence of the protein BiP and the disorder prediction of the BiP protein sequence (DRIP-PRED analysis). Ordered and disordered regions are represented as dark blue or underlined, respectively. The circled lysines (K) are the residues that were found to be acetylated following BiP purification from transgenic H4 cell extraction and mass spectrometry analysis: Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}, Lys^{585} and Lys^{621}.

Figure 2

ATGAAGCTCTCCCTGGTGGCCGCGATGCTGCTGCTGCTCAGCGCGGCGCGGGCCGAGGAG
GAGGACAAGAAGGAGGACGTGGGCACGGTGGTCGGCATCGACCTGGGGACCACCTACTC
CTGCGTCGGCGTGTTCAAGAACGGCCGCGTGGAGATCATCGCCAACGATCAGGGCAACCG
CATCACGCCGTCCTATGTCGCCTTCACTCCTGAAGGGGAACGTCTGATTGG**CGATGCCGC**
CAAG**AACCAGCTCACC**TCCAACCCCGAGAACACGGTCTTTGACGCCAAGCGGCTCATCG
GCCGCACGTGGAATGACCCGTCTGTGCAGCAGGACATCAAGTTCTTGCCGTTCAAGGTGG
TTGAAAAGAAAACTAAACCATACATTCAAGTTGATATTGGAGGTGGGCAAACAAAGACA
TTTGCTCCTGAAGAAATTTCTGCCATGGTTCTCA**CTAAAATG**AAA**GAAACCGCTGAGGC**
TTATTTGGGAAAGAAG**GTTACCC**ATGCAGTTGTTACTGTACCAGCCTATTTTAATGATGC
CCAACGCCAAGCAACCAAAGACGCTGGAACTATTGCTGGCCTAAATGTTATGAGGATCAT
CAACGAGCCTACGGCAGCTGCTATT**GCTTATGGCCTGGAT**AAG**AGGGAGGGGG**AGAAG
AACATCCTGGTGTTTGACCTGGGTGGCGGAACCTTCGATGTGTCTCTTCTCACCATTGACA
ATGGTGTCTTCGAAGTTGTGGCCACTAATGGAGATACTCATCTGGGTGGAGAAGACTTTG
ACCAGCGTGTCATGGAACACTTCATCAAACTGTACAAAAAGAAGACGGGCAAAGATGTC
AGGAAAGACAATAGAGCTGTGCAGAAACTCCGGCGCGAGGTAGAAAAGGCCAAACGGGC
CCTGTCTTCTCAGCATCAAGCAAGAATTGAAATTGAGTCCTTCTATGAAGGAGAAGACTTT
TCTGAGACCCTGACTCGGGCCAAATTTGAAGAGCTCAACATGGATCTGTTCCGGTCTACTA
TGAAGCCCGTCCAGAAAGTGTTGGAAGATTCTGATTTGAAGAAGTCTGATATTGATGAAA
TTGTTCTTGTTGGTGGCTCGACTCGAATTCCAAAGATTCAGCAACTGGTTAAAGAGTTCTT
CAATGGCAAGGAACCATCCCGTGGCATAAACCCAGATGAAGCTGTAGCGTATGGTGCTGC
TGTCCAGGCTGGTGTGCTCTCTGGTGATCAAGATACAGGTGACCTGGTACTGCTTGATGTA
TGTCCCCTTACACTTGGTATTGAAACTGTGGGAGGTGTCATGACCAAACTGATTCCAAGG
AACACAGTGGTGCCTACCAAGAAGTCTCAGATCTTTTCTACAGCTTCTGATAATCAACCAA
CTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAAGACAATCATCTTCTGG
GTACATTTGATCTGACTGGAATTCCTCCTGCTCCTCGTGGGGTCCCACAGATTGAAGTCAC
CTTTGAGATAGATGTGAATGGTATTCTTCGAGTGACAGCTGAAGACAAGGGTACAGGGAA
CAAAAATAAGATCACAATCACCAATGACCAGAATCGCCTGACACCTGAAGAAATCGAAA
GGATGGTTAATGATGCTGAGAAGTTTGCTGAGGAAGACAAAAAGCTCAAGGAGCGCATT
GATACTAGAAATGAGTTGGAAAGCTATGCCTATTCTCTAAAGAATCAGATT**GGAGATAAA**
GAAAAGCTGGGAGGTAAA**CTTTCCTCTGAAGATAAGG**AGACCATGGAAAAAGCTGTA
GAAGAAAAGATTGAATGGCTGGAAAGCCACCAAGATGCTGACATTGAA**GACTTCAAAGC**
TAAGAAGAAG**GAACTGGAAGAAATTGTTCAACC**AATTATCAGCAAACTCTATGGAAGT
GCAGGCCCTCCCCCAACTGGTGAAGAGGATACAGCAGAAAAAGATGAGTTGTAG

Figure 2. Acetylated Lysine Codons and Mutagenesis Primers on BiP's Open Reading Frame.

Figure 2 represents the Open Reading Frame (ORF) of the protein BiP. The codons shown in red correspond to the acetylated lysine residues that were identified in this study. The sequences that are underlined and in bold illustrate the primers used for BiP mutagenesis.

Table 1

Domain	*Acetylated* Lysine (K) residues	*Loss*-of-function mutation (Arginine, R)	*Gain*-of-function mutation (Glutamine, Q)
1. ATPase domain	**K81**	AAG → AGG	AAG → CAG
	K154	AAA → AGA	AAA → CAA
	K164	AAG → AGG	AAG → CAG
	K213	AAG → AGG	AAG → CAG
2. Nucleotide-binding domain	**K585**	AAA → AGA	AAA → CAA
	K621	AAG → AGG	AAG → CAG
3. Entire BiP protein	**All six residues**	See above	See above

Table 1. Mutagenesis Strategy.

In order to investigate the function of the acetylation of BiP, we formulated a mutagenesis strategy, as shown in table 1. The lysine residues were mutated using QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene). The presence of mutations was confirmed by sequencing (University of Wisconsin Biotechnology Center, DNA Sequencing Facility). Mutations causing lysine residues to change into arginine (R) will prevent acetylation without modifying the structure of the protein (loss of acetylation mutants). In contrast, lysine residues mutating into glutamine (Q) will simulate a gain of acetylation, since glutamine mimics the structure of acetylated lysine residues. Loss- and gain- of acetylation mutants will then be stably transfected into H4 cells.

Table 2

Position	Rounds	Mutation	Primer sequences
81	1st	K → R	CGATGCCGCCAGGAACCAGCTCACC
		K → Q	CGATGCCGCCCAGAACCAGCTCACC
154	2nd	K → R	CTAAAATGAGAGAAACCGCTGAGGCTTATTTGGGAAAG AGGGTTACCC
164		K → Q	CTAAAATGCAAGAAACCGCTGAGGCTTATTTGGGAAAG CAGGTTACCC
213	3rd	K → R	GCTTATGGCCTGGATAGGAGGGAGGGGG
		K → Q	GCTTATGGCCTGGATCAGAGGGAGGGGG
585	4th, 6th	K → R	GGAGATAAAGAAAAGCTGGGAGGTAGACTTTCCTCTGA AGATAAGG
		K → Q	GGAGATAAAGAAAAGCTGGGAGGTCAACTTTCCTCTGA AGATAAGG
621	5th, 7th	K → R	GACTTCAAAGCTAAGAAGAGGGAACTGGAAGAAATTGT TCAACC
		K → Q	GACTTCAAAGCTAAGAAGCAGGAACTGGAAGAAATTGT TCAACC

Table 2. List of primers used for BiP mutagenesis.

BiP mutagenesis was carried out on seven different rounds with the QuickChange® Lightning Site-Directed Mutagenesis Kit (Stratagene). K81 was mutated on the first round, K154 and K164 on the second, and K213 on the third. These first four mutated residues correspond to one type of the transgenic H4 cell lines with mutated BiP that were subsequently transfected. Next, K585 and K621 were mutated on the fourth and fifth round, respectively. Altogether, the six mutated residues represent a second type of transgenic H4 cell line with mutated BiP. Finally, a sixth and seventh round of mutagenesis were conducted to mutate K585 and K621 only, to eventually obtain the last type of the desired transgenic H4 cell lines with mutated BiP. After each mutagenesis round, the presence of each mutation was verified by DNA sequencing. The codons that underwent mutagenesis are shown in red, while the nucleotides that are underlined and in bold represent the actual substituted bases following mutagenesis.

Table 3

Peptide	Modified Residues	Sequence with modification	Mass Calculated
75-96	K81	LIGDAAKNQLTSNPENTVFDAK	2387.1968
153-164	K154	MKETAEAYLGKK	1409.7224
164-181	K164	KVTHAVVTVPAYFNDAQR	2057.0694
198-214	K213	IINEPTAAAIAYGLDKR	1856.9996
582-596	K585	LGGKLSSEDKETMEK	1692.8240
620-633	K621	KKELEEIVQPIISK	1694.9818

Table 3. List of identified tryptic peptides with acetylated lysine residues.

Figure 3

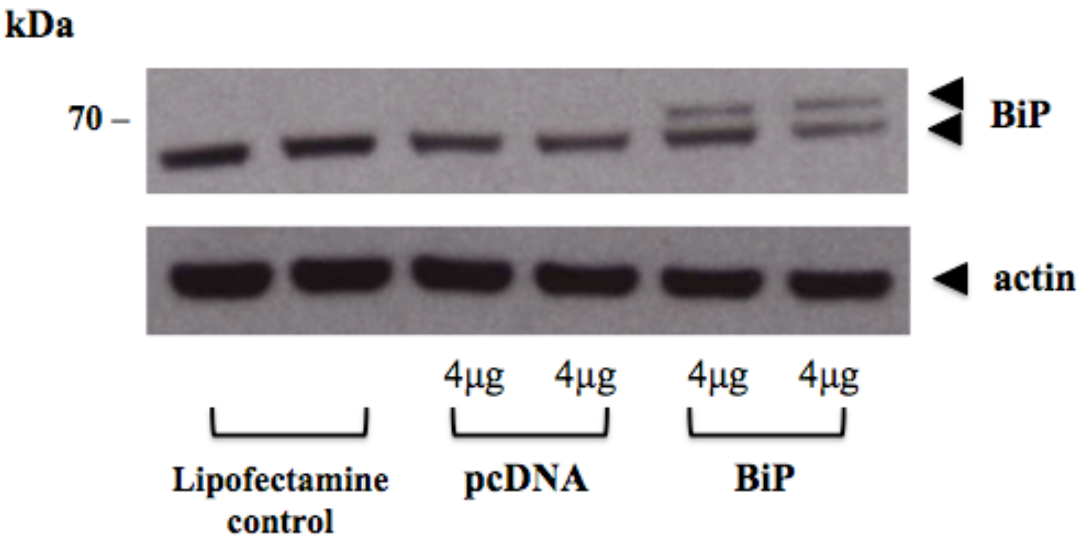
kDa
70 –
BiP
actin
4µg
4µg
4µg
4µg
Lipofectamine control
pcDNA
BiP

Figure 3. Western Blot analysis of total cell lysates - Transient H4 transfection.

Figure 3 shows the Western Blot analysis of total cell lysates from transgenic Human Neuroglioma (H4) cells that were transiently transfected with Lipofectamine2000^{TM} (Invitrogen), as a negative control, and with 4µg of BiP DNA. As an additional negative control, we performed the same purification procedure on cell lysates obtained from cells transiently transfected with 4µg of the empty pcDNA plasmid. Antibodies to the BiP protein were used to detect its level of expression in the cells. Anti-actin antibodies were used as a positive loading control. The upper band, visible in the cells transfected with BiP, represents transgenic BiP, while the lower band corresponds to endogenous BiP.

Figure 4

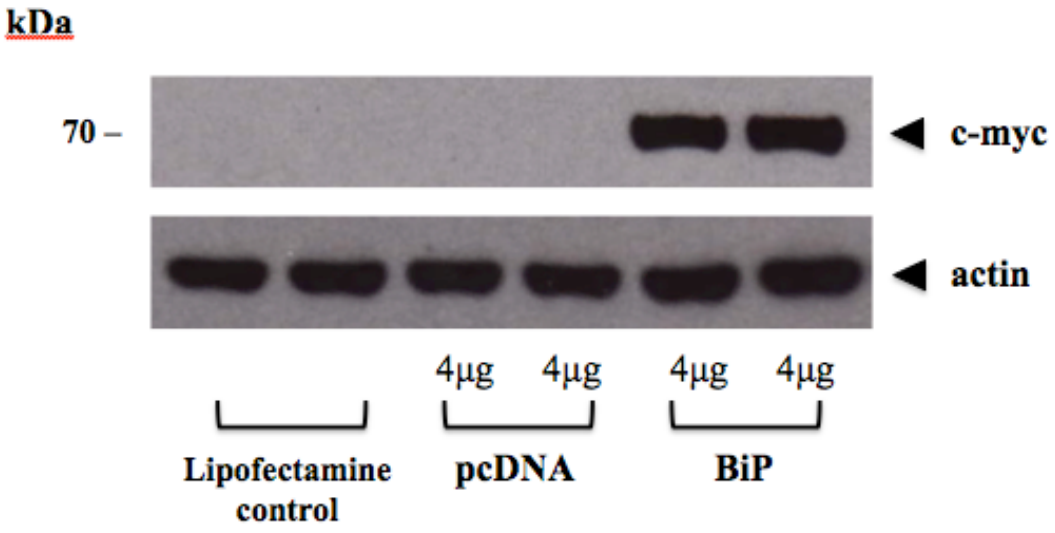
kDa
70 –
c-myc
actin
4µg
4µg
4µg
4µg
Lipofectamine control
pcDNA
BiP

Figure 4. Western Blot analysis of total cell lysates - Transient CHO transfection.

Figure 4 shows the Western Blot analysis of total cell lysates from transgenic Chinese Hamster Ovary (CHO) cells that were transiently transfected with Lipofectamine2000^{TM} (Invitrogen), as a negative control, and with 4µg of BiP DNA. As an additional negative control, we performed the same purification procedure on cell lysates obtained from cells transiently transfected with 4µg of the empty pcDNA plasmid. Antibodies to its added c-myc tag were used to detect transgenic BiP. Anti-actin antibodies were used as a positive loading control.

Figure 5

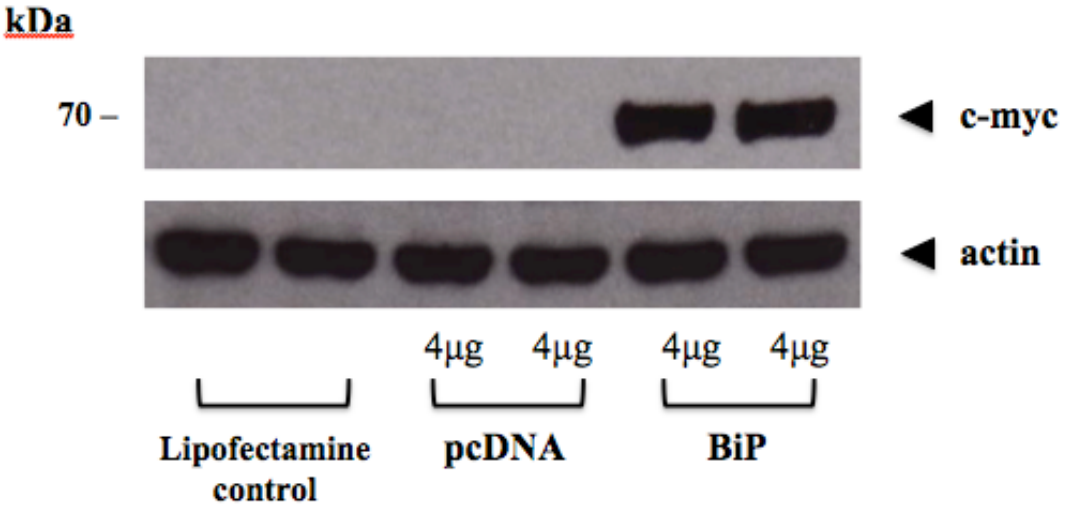
kDa
70 –
c-myc
actin
4µg
4µg
4µg
4µg
Lipofectamine control
pcDNA
BiP

Figure 5. Western Blot analysis of total cell lysates - Transient AT-1 transfection.

Figure 5 shows the Western Blot analysis of total cell lysates from transgenic AT-1 cells that were transiently transfected with Lipofectamine2000^{TM} (Invitrogen), as a negative control, and with 4μg of BiP DNA. As an additional negative control, we performed the same purification procedure on cell lysates obtained from cells transiently transfected with 4μg of the empty pcDNA plasmid. Antibodies to the myc tag were used to detect transgenic BiP levels of expression in the cells. Anti-actin antibodies were used as a positive loading control.

Figure 6

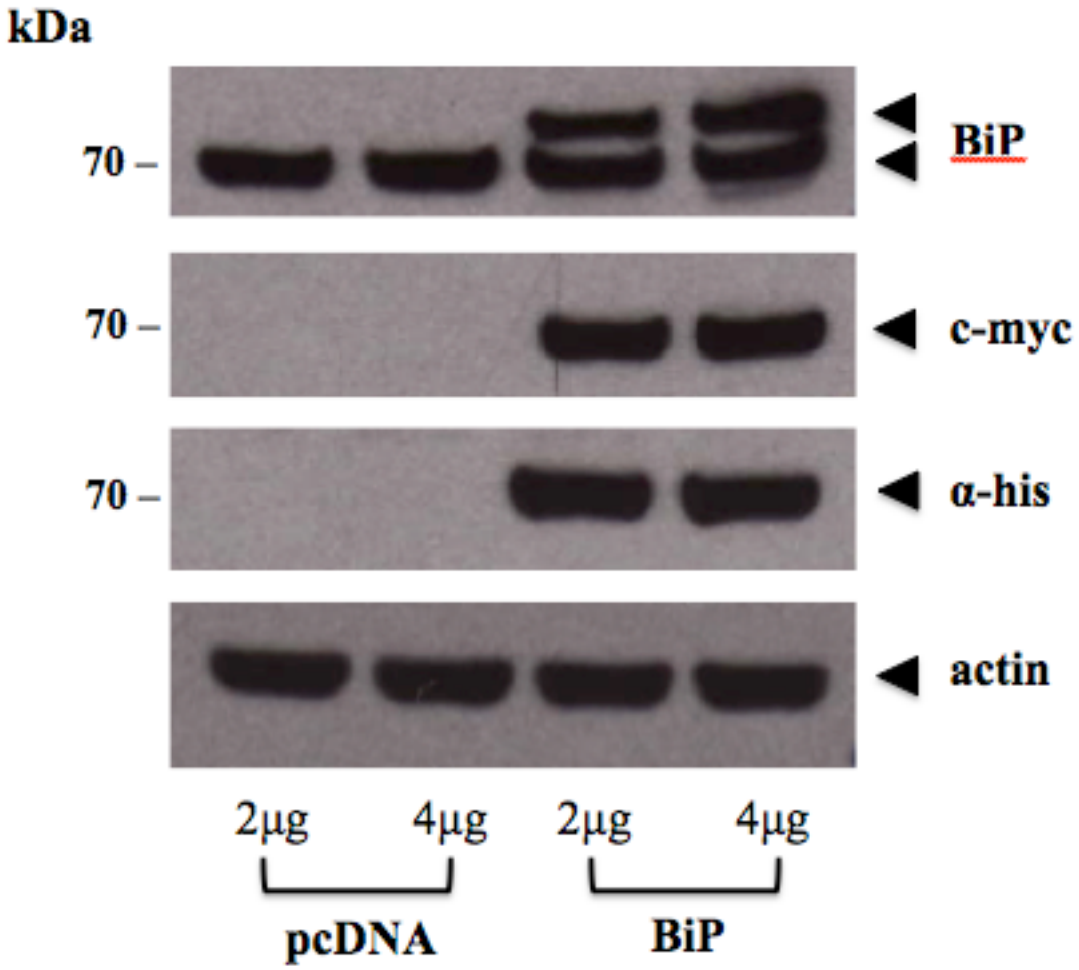

Figure 6. Western Blot analysis of total cell lysates - Stable H4 transfection.

Figure 6 shows the Western Blot analysis of total cell lysates from transgenic Human Neuroglioma (H4) cells that were stably transfected with Lipofectamine2000^{TM} (Invitrogen), and with 2µg and 4µg of BiP DNA. As a negative control, we performed the same purification procedure on cell lysates obtained from cells stably transfected with 2µg and 4µg of the empty pcDNA plasmid. Antibodies to the BiP protein, as well as to its added his-myc tags, were used to detect its level of expression in the cells. Anti-actin antibodies were used as a positive loading control. The upper band, visible in the cells transfected with BiP, represents transgenic BiP, while the lower band corresponds to endogenous BiP.

Figure 7

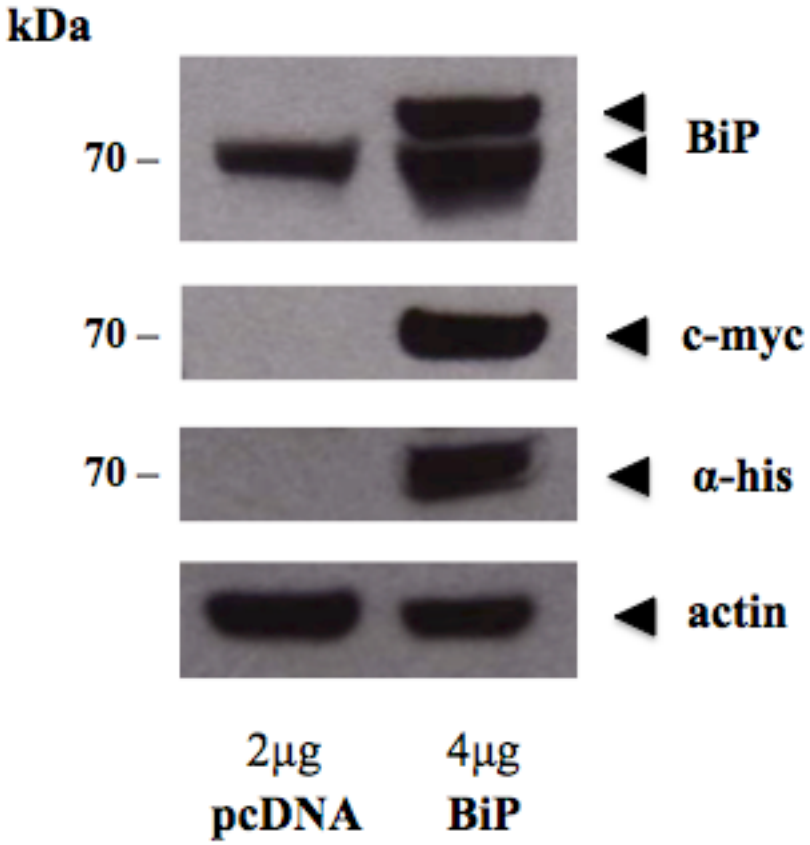

Figure 7. Western Blot analysis of total cell lysates - Stable CHO transfection.

Figure 7 shows the Western Blot analysis of total cell lysates from transgenic Chinese Hamster Ovary (CHO) cells that were stably transfected with Lipofectamine2000^{TM} (Invitrogen), and with 2µg and 4µg of BiP DNA. As a negative control, we performed the same purification procedure on cell lysates obtained from cells transiently transfected with 2µg and 4µg of the empty pcDNA plasmid. Antibodies to the BiP protein, as well as to its added his-myc tags, were used to detect its level of expression in the cells. Anti-actin antibodies were used as a positive loading control. The upper band, visible in the cells transfected with BiP, represents transgenic BiP, while the lower band corresponds to endogenous BiP.

Figure 8

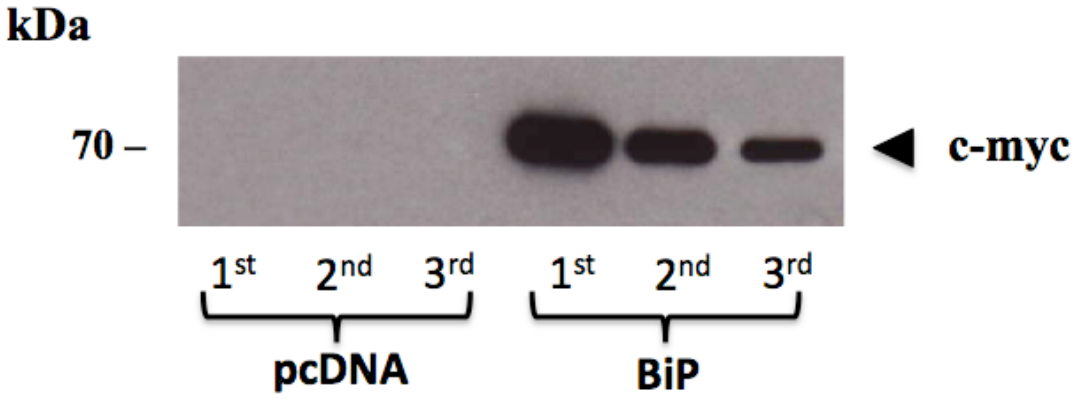

Figure 8. Western Blot analysis of total cell lysates – BiP purification from stable H4 transfection.

Figure 8 shows the Western Blot analysis of the first three elutions of the BiP protein –obtained from total cell extracts of Human Neuroglioma (H4) cells stably transfected with 2µg of BiP DNA– after it had been purified by affinity chromatography, using the ProFound c-Myc-Tag IP/Co-IP kit (Pierce). The first elution of the cells containing the BiP plasmid shows a significantly larger band than the other two, indicating that it contains greater levels of the purified protein. As a negative control, we performed the same purification procedure on cell lysates obtained from cells stably transfected with 4µg of the empty pcDNA plasmid. Anti-c-Myc antibodies were used to detect the levels of protein expression.

Figure 9

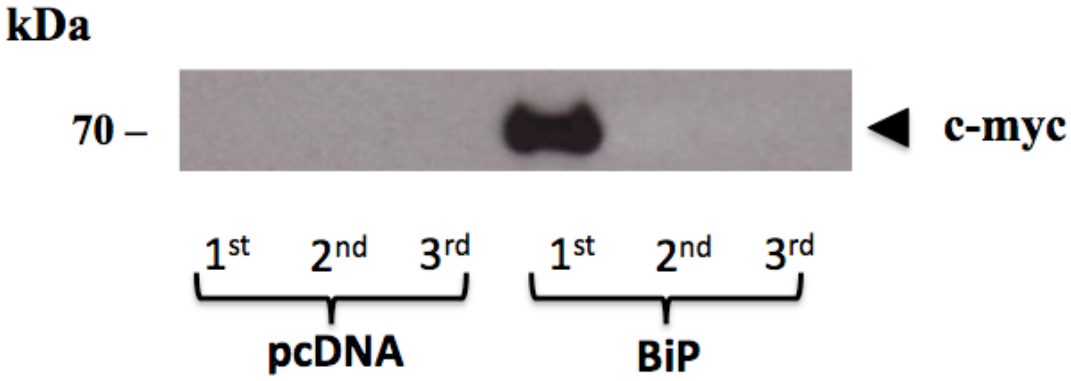

Figure 9. Western Blot analysis of total cell lysates – BiP purification from stable CHO transfection.

Figure 9 shows the Western Blot analysis of the first three elutions of the BiP protein –obtained from total cell extracts of Chinese Hamster Ovary (CHO) cells stably transfected with 4μg of BiP DNA– after it had been purified by affinity chromatography, using the ProFound c-Myc-Tag IP/Co-IP kit (Pierce). The first elution of the cells containing the BiP plasmid appears to be the only elution containing the purified BiP protein. As a negative control, we performed the same purification procedure on cell lysates obtained from cells stably transfected with 2μg of the empty pcDNA plasmid. Anti-c-Myc antibodies were used to detect the levels of protein expression.

Figure 10

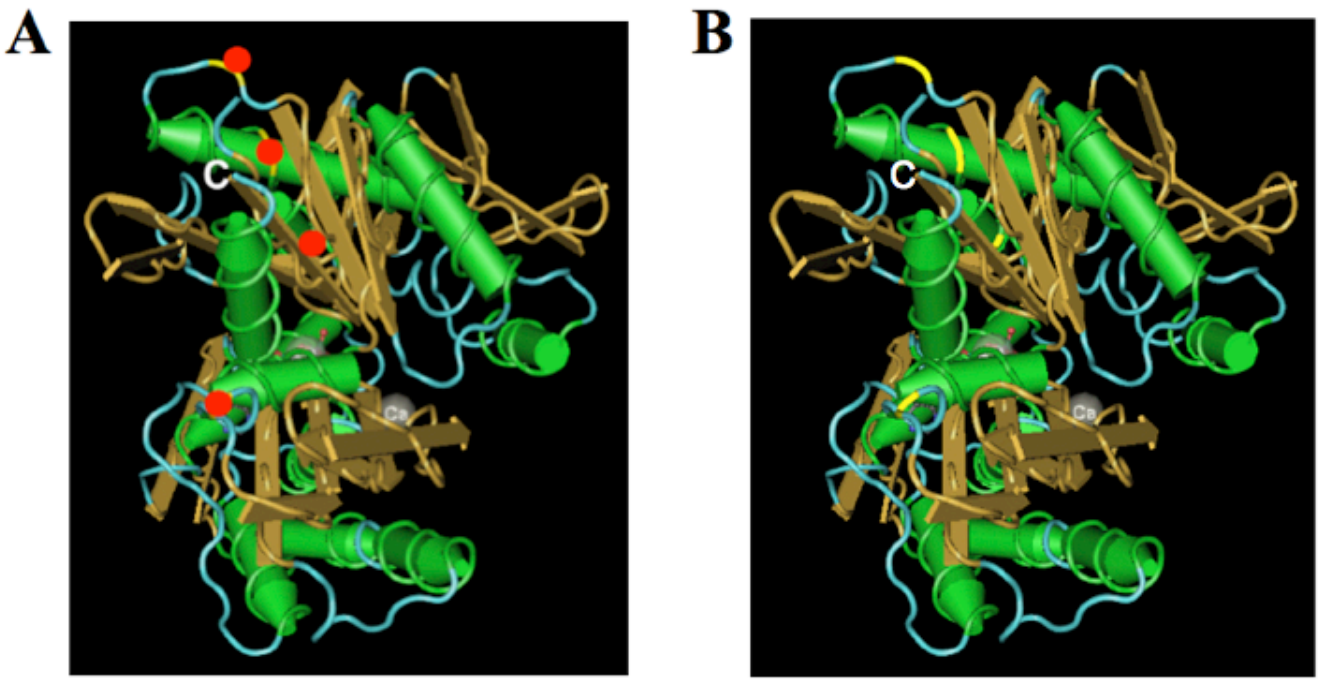

Figure 10. Acetylated Lysine Residues on BiP's ATPase domain.

Figure 10 illustrates a three-dimensional view of BiP with the acetylated lysine resdies. (A) shows the ATPase binding domain of BiP and, highlighted in red, the first four acetylated lysine residues present on the protein: K81, K154, K164, and K213. (B) offers a different visual perspective of the residues (in yellow) to call attention to their location within the three-dimensional structure of the BiP protein. The letter "C", in white, indicates the C-terminal domain of the protein.

CONCLUSION AND DISCUSSION

Human BiP was successfully cloned into a vector for mammalian expression and overexpressed in H4, CHO and AT-1 cell lines. Following a purification procedure and mass spectrometry analysis, six acetylated lysine residues were identified on BiP: Lys^{81}, Lys^{154}, Lys^{164}, Lys^{213}, Lys^{585} and Lys^{621}. The first four acetylated residues are located on the ATPase-binding domain, as illustrated in figure ? A and B. The last two residues are within the nucletotide-binding domain (data not shown). These results match our expectations that an ER-resident protein, BiP, can undergo acetylation. Additionally, we were not surprised to notice that the location of the residues that underwent the modification do not seem to follow a particular acetylation pattern, as similarly observed during the study of the acetylated residues of BACE1 (Costantini *et al.*, 2007 and Spange *et al.*, 2009). Previously, it was shown that N^{ε} –acetylation is not limited to histone proteins, but that several other substrates in various cellular compartments are subjected to this type of modification (Yang and Gregoire, 2007, Schwer *et al.*, 2006 and Polevoda and Sherman, 2002). Importantly, lysine acetylation has significant effects on the stability and activity of its targets; e.g. preventing BACE1 from undergoing N^{ε} –acetylation has been shown to cause it to be degraded (Jonas *et al*., 2008). BACE1 is the rate-limiting enzyme in Aβ formation (Costantini *et al*., 2007), thus, if BACE1 levels can be regulated using an acetylation-deacetylation strategy, the onset of AD can perhaps be controlled as well. The data obtained in this study confirms our initial hypothesis that the process of lysine acetylation can be expanded to ER-resident proteins as well. Thus, in view of the

facts that 1) this type of modification serves a crucial function in other systems (regulation of DNA transcription in histone proteins, Yang and Gregoire, 2007, and degradation of BACE1, Jonas *et al*., 2008), and that 2) once a lysine residue is acetylated it can no longer be subjected to other types of post-translational modifications (e.g. methylation, sumoylation, ubiquitination, propioylation and butyrylation, Yang *et al.*, 2007), the discovery that such a vitally important chaperone like BiP is also able of becoming acetylated opens up a very promising area of study.

Figure 8 and 9 show the Western Blot analyses of the three elutions of purified BiP. Unlike H4 cells, CHO cell lines did not appear to contain the purified protein in the second and third elutions following the ProFound c-Myc-Tag IP/Co-IP kit (Pierce) purification (the experiment was repeated twice but only representative results are shown in figure 9). These results might perhaps indicate that H4 cell lines, as nervous system cells, allow for a greater expression of the BiP plasmid. This may be due to certain post-translational modifications ensuring the proper expression of BiP that are more favorable in these cells as opposed to the somatic CHO cells. Nonetheless, it is difficult to speculate further at this point, especially considering that the levels of BiP expression appeared to be almost identical in Western Blot analyses that were conducted earlier (figures 3-7).

In order to investigate the function of the acetylation of BiP, we formulated a mutagenesis strategy (Table 1) causing select lysine residues to act as if they were always (Lys to Gln) or never (Lys to Arg) acetylated. We chose to mutate together 1) the residues located on the ATPase-binding domain of the protein, 2) those in the nucleotide domain, and finally 3) all of the six acetylated residues identified. The

residues located on the ATPase-binding domain are perhaps the critical ones conferring the stability of BiP after it undergoes acetylation, but at this moment in time it is still not possible to draw too many conclusions. Loss- and gain- of acetylation mutants will be stably transfected into H4 cells, and several studies examining the role of acetylation will be led. These will include exploring the effect of the acetylation status on BiP activity and ER functions. BiP is an important chaperone in the ER lumen and it is required for proper folding and maturation of nascent proteins. Therefore, changes in BiP activity could have important consequences for the physiology of the cell. Once we have identified the critical lysine residues present on BiP that regulate its activity, we will perform another transfection of this mutant form of BiP into AT-1 cells. This will allow us to find out if overexpressed AT-1 levels can regulate the acetylation status of BiP, as it does on BACE1 (Jonas *et al*., 2010). In addition to AT-1 levels, we are going to analyze the effect of increased expression of the Atases on BiP acetylation, but all along being mindful of the possibility that its acetylation may be controlled by different acetyltransferases.

Overall, this project continues to investigate the role of lysine acetylation, and will analyze how acetylated BiP –which has recently been identified as an important substrate of the ER-based acetylation machinery- affects cell function and Alzheimer's disease progression.

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