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TITLE: The Identification and Characterization of Cytoplasmic Acetylated Lysines

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ABSTRACT

The Identification and Characterization of Cytoplasmic Acetylated Lysines

Lysine acetylation by histone acetyl transferases (HATs) is a reversible post-transcriptional modification that plays a key role in regulating several biological processes, such as the cell cycle, nuclear transport, and gene expression. Previous research has shown lysine acetylation contributes to regulation of almost all nuclear functions, but a recent study in Science determined that lysine acetylation also contributes to regulation of a large array of cytoplasmic functions. The goal of my research is to determine the function, recognition, regulation, and activity of the cytoplasmic acetylated lysines and the proposed isomerase that regulates them. A large number of enzymatic activity assays were completed using a synthesized acetylated peptide in the cis conformation. Isomerization, and therefore activity, was measured using absorbance on a spectrophotometer. Lysine acetylation has become an important target in cancer and neurodegenerative diseases and understanding the underlying mechanisms of acetylation could aid in the improvement of drug therapy.

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The Identification and Characterization of Cytoplasmic Acetylated Lysines

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A thesis performed by Kelsey Stein under the direction and mentorship of James Malter, Professor of Pathology, submitted in partial fulfillment of a Bachelor of Science degree in Genetics conferred with honors in research.

Abstract

Lysine acetylation by histone acetyl transferases (HATs) is a reversible post-transcriptional modification that plays a key role in regulating many nuclear events, such as the cell cycle, nuclear transport, and gene expression. Lysine acetylation has become an important target in cancer and neurodegenerative diseases and understanding the underlying mechanisms of acetylation could aid in the improvement of drug therapy. A recent study in Science suggested that lysine acetylation may also contribute to the regulation of a large array of cytoplasmic functions. The goal of my research was to determine if cytoplasmic proteins with acetylated lysines were recognized by isomerases akin to those that recognize and modulate Serine/Threonine-Proline peptide bonds. A number of enzymatic activity assays were completed using a synthesized acetylated peptide in the *cis* conformation. Isomerization to *trans* was measured using absorbance on a spectrophotometer. I found evidence that a protein was present in the cell that could accelerate the *cis* to *trans* isomerization of the peptide bond and that the acetylated lysine is required for isomerization. In addition, my data showed that this protein may be contained in organelles such as the Golgi apparatus or endoplasmic reticulum.

Introduction

Protein acetylation plays an important role in the regulation of many diverse cellular functions. More specifically, lysine acetylation is catalyzed by an acetyltransferase, which transfers the acetyl-group of acetyl-CoA to the epsilon-amino group of a lysine residue (11). Lysine acetylation is a reversible post-transcriptional modification that regulates the cell cycle, RNA transcription, splicing, nuclear transport, nuclear hormone signaling, and chromatin remodeling (1). As such, acetylation plays a key role in regulating epigenetics, or inherited gene expression (1). Because lysine acetylation results in the modification of core histone tails via histone acetyltransferases (HATs) or histone deacetylases (HDACs), it has become an

important drug target in cancer and neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, Huntington's disease, stroke and mood disorders (2- 3). The misregulation or hypoacetylation seen in cancer or neurodegenerative diseases can potentially be treated with HDAC or lysine deacetylase (KDAC) inhibitors (1).

Interestingly, multiple acetyltransferases interact with and acetylate one substrate. For example, p53 and Tat are acetylated by both p300 and PCAF (9). Some substrates, such as p53, MyoD and BCL6 associate with acetyltransferases as well as deacetylases, thus providing a mechanism for dynamic control of acetylation in vivo (10). However, the underlying mechanisms by which acetylation alters protein structure and function remains unknown. Therefore, understanding the underlying mechanisms of acetylation could aid in understanding fundamental biology as well as increase the number of targets available for drug therapy.

Previous research has identified an evolutionarily conserved motif known as the bromodomain that recognizes and has a specific affinity for acetylated lysine residues (8). Lysine acetylation generates specific docking sites for bromodomain proteins (8). For example, bromodomains of Gcn5, PCAF and TAF1 are able to recognize acetyllysine residues in histones, HIV Tat, p53, c-Myb or MyoD (8). The relationship between acetyllysine and bromodomains is reminiscent of the specific recognition of phosphorylated residues by phospho-specific binding modules such as SH2 domains and 14-3-3 proteins (8).

There are over 30 bromodomain proteins in humans and four different functions for these have been recognized (8). First, bromodomain proteins are important for chromatin acetylation by HATs (8). Second, bromodomains contribute to acetylation-dependant nucleosome assembly and remodeling (8). Third, bromodomains are involved in organizing chromosome or chromatin domains (8). Fourth, and pertinent to my research, bromodomains also recognize acetylated non-histone proteins (8). For example, acetylation of p53 promotes

its association with bromodomain-containing proteins (8).

Pin 1, or peptidyl-prolyl cis/trans isomerase, isomerizes only phosphorylated Serine/Threonine-Proline motifs (12). Thus the enzyme plays a role in post-phosphorylation control in regulating proline-directed kinases such as MAPK or CDK and consequently regulates cell proliferation and cell survival (13). Recent data also implicate Pin 1 as playing an important role in immune responses, at least in part by increasing the stability of cytokine mRNAs by stabilizing the protein complexes to which they bind (13).

Chouhary et al. showed the broad regulatory scope of lysine acetylation and identified 3,600 lysine acetylation sites on 1,750 proteins by mass spectroscopy (1). By quantifying acetylation changes in response to KDAC inhibitors on a global scale, they generated a lysine acetylome (1). The acetylome showed that the acetylation occurred predominately in large macromolecular complexes and regulated a multitude of dissimilar cellular functions. In addition to the regulation of almost all nuclear functions, Chouhary et al. showed that lysine acetylation also contributes to the regulation of a large array of cytoplasmic functions.

Acetylation has been widely studied for its role in transcriptional regulation in the nucleus, but no observation on translational regulation by acetylation has been described so far (11). However, in global proteomic studies a large number of translation initiation factors and ribosomal proteins have been found acetylated (14). Thus, a possible role of acetylation in translational control mechanisms remains to be discovered. Following translation, many newly synthesized proteins have to be assisted in folding correctly. This is ensured by chaperones. Chaperones are also implicated in the management of misfolded proteins, which arise following environmental stress (11). One chaperone, Hsp90, can be acetylated on several lysine residues and its activation state depends on its acetylation status (11). In its inactive ADP-bound state, Hsp90 associates with the cochaperone Hsp70, which prepares the misfolded protein for degradation by its association with a ubiquitin ligase.

Recent studies also identify acetylation on cytosolic metabolic enzymes in mammalian eukaryotes (14). In one study, five cytosolic metabolic enzymes were found to be lysine acetylated: aldolase, enolase, triosephosphate isomerase, phosphoglycerate mutase, and transketolase (14). Other studies describe acetylation and deacetylation events occurring in the lumen of the ER and Golgi apparatus, respectively (15). Bace1, the protease responsible for amyloid precursor protein cleavage, and the LDL receptor become acetylated in the Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) (15). Acetylation is essential for their stabilization and progression along the secretory pathway. Both proteins are deacetylated in the Golgi apparatus (15).

In total, these studies give evidence that acetylated lysines in the cytoplasm contribute to a wide array of cellular functions such as translational control mechanisms, chaperone activation, cytosolic metabolic enzyme activation, and progression along the secretory pathway. In addition, a variety of proteins could potentially recognize and interact with these acetylated lysines such as bromodomain proteins, other chaperones or isomerases.

The purpose of my research was to determine if cytoplasmic proteins with acetylated lysines were recognized by isomerases akin to those that recognize and modulate Serine/Threonine-Proline peptide bonds. Here I hypothesize that lysine acetylation isomerases (Ac-Lys) may play a role in regulating the function of acetylated lysine proteins in the cytoplasm. My goal was to identify and characterize these Ac-Lys isomerase(s) if they exist. To that end, I modified one of Chouhary's consensus sequences and generated a peptide substrate that could be used in an Ac-Lys isomerase activity assay and in pull down experiments to isolate and identify the isomerase(s) of interest.

Methods

Homogenate Preparation - Cleared homogenate (cHom) was made from mouse cortex as described in Westmark et al. (4). Briefly the mouse was culled and the brain cortex was harvested and mixed with a GM buffer (0.25M sucrose, 5 mM Tris-HCl pH=7.5, 0.1 mM EDTA). The solution was centrifuged at 1000Xg for 10 minutes at 4 degrees Celsius to remove the non-lysed organelles such as nuclei and red blood cells. The homogenate was snap frozen and stored at -80 degrees Celsius.

Ac-Lys Substrate - The Ac-Lys activity assay employs a colorimetric mechanism whereby chymotrypsin cleaves off a colorimetrically detectable p-nitroaniline (pNA) from the substrate only *after* the substrate has been isomerized from its *cis*-form into its *trans*-form by the isomerase of interest present in the cHom (**Figure 1D**). Chymotrypsin digestion of substrates is sterically hindered when the target amino acid (i.e. phenylalanine or tyrosine) is in the *cis* conformation. Once the substrate is isomerized to the *trans* configuration, the bulky-side group of the amino acid no longer sterically obstructs the substrate from binding to the well-defined substrate binding pocket of chymotrypsin, leading to detectible catalytic cleavage of the pNA from the substrate (**Figure 1D**). Biotin was added to the N-terminus in order to use the substrate in a pull down assay to isolate potential Ac-Lys isomerases for identification by subsequent mass spectrometry (**Figure 1B**). The consensus sequence of amino acids found in cytoplasmic proteins with Ac-Lys sites was KKKNFEKYIKKKK (**Figure 1A**) (1). This sequence was modified (Biotin-KKKKNFEKY-pNA) to form the substrate used in the new isomerase assay (**Figure 1B**). The substrate was made in two forms by BiomerTech: one with the C-terminal lysine acetylated and the other without any acetylation (“unacetylated substrate”) (**Figure 1B, 1C**).

Ac-Lys Assay - An assay was performed to detect isomerase activity on the Ac-Lys peptide. The substrate was dissolved in 50 uL 1 M DMSO. 5 uL of the substrate in DMSO was added to

500 uL of a 480 mM LiCl solution. Chymotrypsin was activated by dissolving it in 0.001% HCl and incubated on ice for 10 minutes. For the assay itself, 5 uL of the substrate was mixed with 72 uL reaction buffer (5 mL buffer [100 mM NaCl, 50 mM Hepes, pH 7], 10 uL 1 M DTT, 20 uL 10 mg/mL BSA), 3 uL DMSO/Inhibitor, 5 uL diluted cHom and 5 uL chymotrypsin.

Measurements were taken every two seconds for 1.5 minutes. The initial slopes (K_f) were calculated to determine K ($K = (K_f/k_o) - k_o$) where k_o is the average K_f of the substrate alone. Individual groups were compared to cHom and deemed statistically significant if $P < 0.05$.

Percoll Gradient - To separate cellular components, cells were lysed and separated on a percoll-sucrose gradient. First, the cortex was removed from 17 day old mice that were killed by carbon dioxide affixation. The cortex was homogenized in cold GM buffer and then spun at 1000Xg for 10 minutes at 4 degrees Celsius to remove nuclei and unlysed cells. After the nuclei and unlysed cells were removed, the remaining homogenate was added to a percoll gradient that was 2 mL each 23%, 15%, 10% and 3% percoll in GM buffer. This was spun at 32,500XG for 5 minutes at 4 degrees Celsius.

BCA - After the cellular components were separated on the percoll gradient, protein content was quantified by BCA using the Pierce BCA Protein Assay kit (7). Each layer of the percoll gradient was diluted 1:10 with depc-water. Protein extraction buffer (5 mL 10% Triton X-100, 5 mL 10X GTIP buffer, 35 mL depc-water, 125 uL NP-40, 5 mL 10x protease inhibitor cocktail) was diluted 1:10 with depc-water. 2 uL of the protein extraction buffer was mixed with 2 uL of each sample. Standards containing 0, 1, 2, 3, 4, 5, 6, 7 uL BSA (1.52 uL/ug) were added to 500 uL depc-water. Each sample was also added to 500 uL depc-water. 0.5 mL working reagent (5.33 mL MA, 5.24 mL MB, 0.213 mL MC [all found in kit]) was added to all standards and samples. All standards and samples were incubated at 60 degrees Celsius for one hour, cooled to room temperature and measured at 562 nM. The standards were plotted and linearized and protein content from the samples was then determined.

Pulldown Assay - A pulldown assay with Streptavidin beads was used to isolate potential isomerases from the supernatant. The beads were washed three times with 1X IP buffer (5 mL 1 M Hepes [pH 7.4], 100 mL 1 M NaCl, 30 mL 0.5 M EDTA [pH 8.0], 25 mL 10% Triton X-100). The cHom was either pre-cleared or not with either beads or beads bound with the unacetylated substrate. The homogenate samples were then incubated with acetylated lysine substrate bound beads, unacetylated lysine substrate bound beads, or unbound beads overnight at either 4 degrees Celsius or 37 degrees Celsius. The beads were then washed 4 times with IP Buffer to remove any unbound proteins. Any bound isomerase(s) were then separated from the beads with 4x SDS loading buffer. These samples were collected and run in a polyacrylamide gel using a 4% stacking gel and a 12% separating gel. The gel was stained with a Coomassie stain for one hour. The gel was then de-stained overnight so the proteins remained stained but the gel was unstained. Bands were cut out and processed using the ProteaseMAX Superfactant procedure (5). Trypsin was used to in-gel digest the proteins in the band into small peptides, which then diffused out of the gel and were collected. The fragments were analyzed by mass spectrometry to identify the proteins present.

Mass Spectrometry - Mass spectrometry was used to determine the mass of the proteins isolated from the pull down assay. Using a dilution specific to the mass spectrometer, a reading was taken in the form of a graph. From this graph the molecular mass of the charged particle was found, and from this the composition of the protein was deduced.

Western Blots – Total protein concentration was measured by BCA assay. Cellular extracts were diluted to the same protein concentration in depc-water and subjected to 10% SDS-PAGE. They were then transferred to 0.2 mM Nitrocellulose membranes. Blots were probed at 4 degrees Celsius overnight with primary antibodies in 5% milk/TBST.

Results

Several controls were used in every assay:

1. +cHom, -chymotrypsin

This control detects endogenous protease activity that may be present in the lysates. In addition, it ensures that the pNA is not spontaneously being released.

2. -cHom, +chymotrypsin ("S")

This control detects spontaneous isomerization (isomerization that occurs without the binding of the isomerase to the Ac-Lys residue).

As shown in **Figure 2**, there was significant activity present in the cHom when compared to spontaneous isomerization of the substrate (S). It is worth noting that there was pNA-release even without the addition of exogenous chymotrypsin to the assay, but pNA-release was significantly increased when chymotrypsin was added. The cleavage in the reaction without chymotrypsin may be due to auto-cleavage of the pNA in the *trans* form due to an unstable electronic configuration of the substrate or due to endogenous proteases. Therefore, we looked at the cleavage rate in the presence of a broad spectrum protease inhibitor cocktail that included serine protease inhibitors that were not specific for chymotrypsin (**Figure 3**). Presence of the broad spectrum protease inhibitor cocktail slightly reduced the isomerization activity of the substrate (**Figure 3**).

Next, cHom was untreated or treated with 100 nM or 500 nM of trichostatin A (TA) ($IC_{50} = 20$ nM) for five minutes followed by assay of isomerization. TA specifically inhibits mammalian histone deacetylases, which causes accumulation of highly acetylated histone molecules in mammalian cells. This induces cell differentiation, cell cycle arrest, apoptosis and is able to modulate transcription. Thus, we were testing to see if the release of pNA was the result of deacetylation due to endogenous deacetylases or if it was due to the isomerization of the substrate. **Figure 4** shows that pNA was released at the same level as cHom even when cHom was treated with 100 nM TA.

The unacetylated substrate was then tested for isomerization. Because no Ac-Lys was present, we expected reduced activity since the proposed isomerase would hypothetically not be able to bind the acetyl group and catalyze isomerization. **Figure 5** shows that isomerization was significantly reduced when the unacetylated substrate was used in the activity assay.

To determine if the unacetylated substrate competes with the acetylated substrate, a competition assay was completed (**Figure 6**). 0.75 ug cHom was assayed for isomerase activity in the presence of 1x acetylated lysine substrate and increasing amounts of unacetylated substrate (0x to 40x). Activity with 0x or 5x of the unacetylated substrate was the same as the acetylated substrate alone (**Figure 6**). Activity with 20x and 40x of the unacetylated was slightly lower but not statistically different from 0x (**Figure 6**).

To determine the cellular location with the most isomerization activity, cHom was applied to a percoll gradient and centrifuged to separate the cellular components (**Figure 7**). The bands were assayed by BCA to determine protein concentration. After protein concentration was normalized so that each band contained equal amounts of protein, the isomerization activity was determined. **Figure 7** shows the expected band composition. As shown in **Figure 8** and summarized in **Table 1**, the percoll gradient was a relatively crude purification so we see minor cellular, nuclear and organelle contamination in the fractions. The fractions contained expected protein markers, i.e. Bands 1-3 were mainly broken membranes, Band 4 was membranes and synaptosomes, Band 5 was synaptosomes, and Band 6 contained whole organelles. As seen in **Figure 9**, the fraction with the most isomerase activity was Band 6, which contained whole organelles.

To identify the isomerase of interest, a pull down assay was completed using 1 mg cHom and 126 ug Band 6 (**Figure 10**). This was completed using streptavidin magnetic beads and the biotinylated substrate. Lanes 1, 3, 5, and 7 were no substrate controls and show non-

specific binding (**Figure 10**). Unique bands in lanes 2, 4, 6, and 8 were submitted to the UW Biotechnology Center for in gel digestion and Mass Spec analysis, but the results received from the UW Biotechnology Center were inconclusive.

Discussion

In designing an Ac-Lys substrate that could be used in an isomerization assay, the following requirements had to be met:

1. The substrate had to be sequence specific for the isomerase(s) of interest.
2. The amino acids adjacent to the pNA had to be “locked” into *cis* or *trans* position (thus needed to have side groups that prevented free rotation). Therefore Chouhary’s full length substrate could not be used in an isomerase activity assay. Rather, the substrate sequence from Chouhary et al. was truncated and the pNA attached to the tyrosine, which was adjacent to the lysine of interest (**Figure 1B**).
3. The unisomerized substrate had to be “bulky” enough to cause steric hindrance to prevent binding and subsequent cleavage by chymotrypsin or other potential endogenous proteases.
4. The substrate had to be sequence specific for recognition by chymotrypsin in the *trans* form in order for enzymatic pNA cleavage and subsequent colorimetric detection to occur (**Figure 1D**).
5. The substrate needed a colorimetric indicator of protease cleavage of the substrate when in the *trans* form. To satisfy this requirement, pNA was attached to the C-terminal of the tyrosine so that when cleavage of the substrate occurred a free pNA would be released and could be measured at 390 nM (**Figure 1D**).

Figure 2 provides evidence that an isomerase was present in cHom and that it could accelerate the *cis* to *trans* isomerization of the peptide bond between the acetylated lysine and the tyrosine. Although **Figure 2** shows what appears to be cleavage of the pNA without chymotrypsin present, the reaction with chymotrypsin is significantly different than without. The cleavage in the reaction without chymotrypsin may be due to auto-cleavage of the pNA in the *trans* form due to an unstable electronic configuration of the substrate or due to endogenous proteases. Presence of the broad spectrum protease inhibitor cocktail slightly reduced the isomerization activity of the substrate (**Figure 3**). This suggests that some of the endogenous proteases may be inhibited, but not all. Because the results are not statistically significant, it is possible that none of the endogenous proteases are being inhibited and that this protease inhibitor cocktail is ineffective at blocking the proteases that cleave the lysine-tyrosine bond.

As seen in **Figure 4**, there was significant activity present in the cHom when compared to spontaneous isomerization of the substrate. TA had no effect on the activity rates of the cHom indicating that the production of pNA was not the result of deacetylation, due to the presence of endogenous deacetylases, but more likely, the isomerization of the substrate. Here, 100 nM TA was more ideal than 500 nM. To inhibit an enzyme completely, it is usually recommended to try a concentration 5-20 fold higher than the IC_{50} . Given that the IC_{50} for TA is 20 nM, 500 nM was drastically high. I did this on purpose to ensure that I inhibited all of the deacetylase completely. However, 500 nM was too high and non-specific inhibition of other proteins may have occurred. Since the 500 nM was not statistically different we may be able to surmise no non-specific inhibition occurred at 100 nM, but complete inhibition of the deacetylase. **Figure 6** showed that the unacetylated substrate is not a competitor of the acetylated substrate. This supports our hypothesis of an Ac-Lys-Tyr isomerase that is *specific* for the acetylated lysine residue.

Because the unacetylated peptide had reduced isomerization activity (**Figure 5**), we can conclude that the isomerization activity requires an acetylated lysine on the substrate. However, because our mass spectrometry results were inconclusive, we cannot definitively say what is potentially interacting with that acetyl group. Based on past research, I suggest that a bromodomain protein(s) recognizes the acetyl group and interacts to cause isomerization from *cis* to *trans*. This isomerization could potentially trigger a series of events via other protein interactions that leads to translational control, chaperone activation, cytosolic metabolic enzyme activation, and/or progression along the secretory pathway.

In the future, our lab will re-do the Biotin-Streptavidin pull down assay and run the mass spectrometry analysis in an attempt to identify the isomerase of interest. In addition, the lab will test with a broad spectrum protease inhibitor cocktail again. A different protease inhibitor cocktail would ideally inhibit all proteases except for chymotrypsin. If other proteases are present and can cleave when the sequence is in *cis*, they could be cleaving off the pNA without isomerization. Other future work includes doing *in vitro* expression of the isomerase. Genomic DNA from a mouse would be isolated and the gene of interest would be amplified using Real Time PCR. The gene would then be cloned into an expression vector, transformed into BL21 bacterial cells, and expressed in the BL21 cells. The protein would then be purified so it could be characterized.

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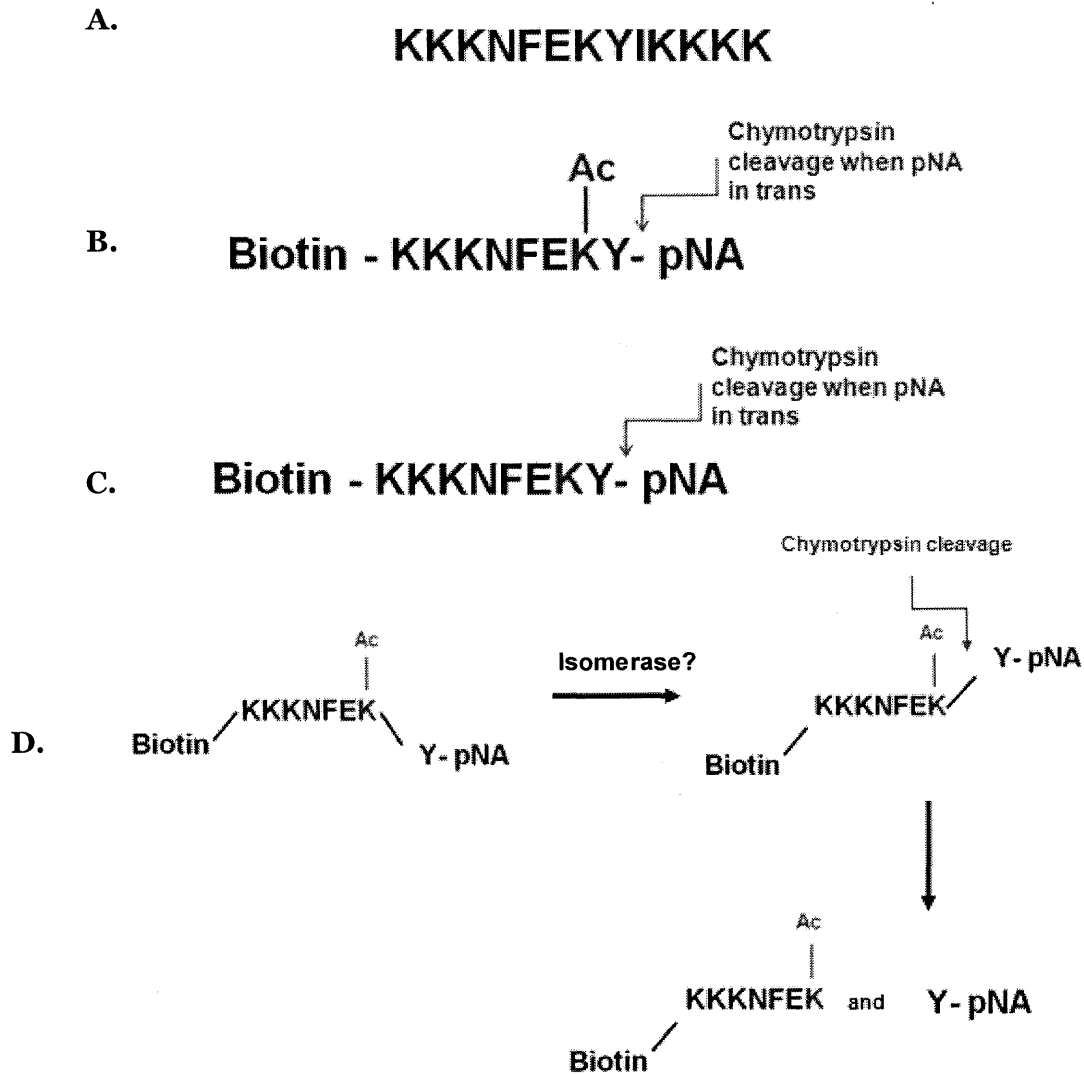


Figure 1: Substrate Sequence

A shows the consensus sequence identified by Chouhary et al. **B** is the modified acetylated substrate sequence used in the isomerization assays. Letters denote amino acids. Biotin was added to the N-terminus in order to use the substrate in a pull down assay to isolate potential Ac-Lys isomerases for identification by subsequent mass spectrometry. **C** is the modified unacetylated substrate sequence used in the isomerization assays. **D** shows the *cis* to *trans* isomerization between the lysine and tyrosine bond. Cleavage by chymotrypsin between the tyrosine and pNA only occurs when the substrate is in *trans*. Presumably, this isomerization is a result of an isomerase binding to the Ac-Lys.

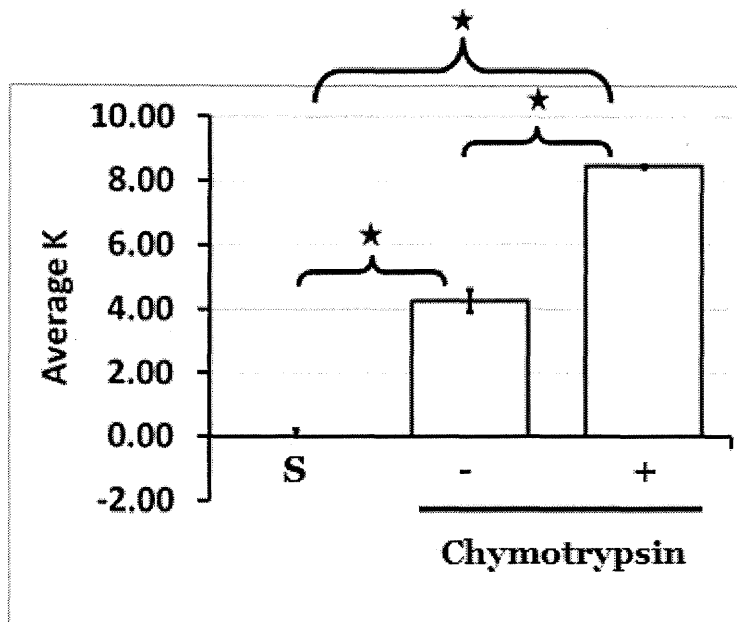


Figure 2: cHOM Activity

There was significant activity present in the cHOM when compared to spontaneous isomerization of the substrate (S). Activity occurred without the addition of exogenous chymotrypsin to the assay, but activity was significantly greater when chymotrypsin was present. ★ denotes $p < 0.05$, $n=3$, \pm SEM.

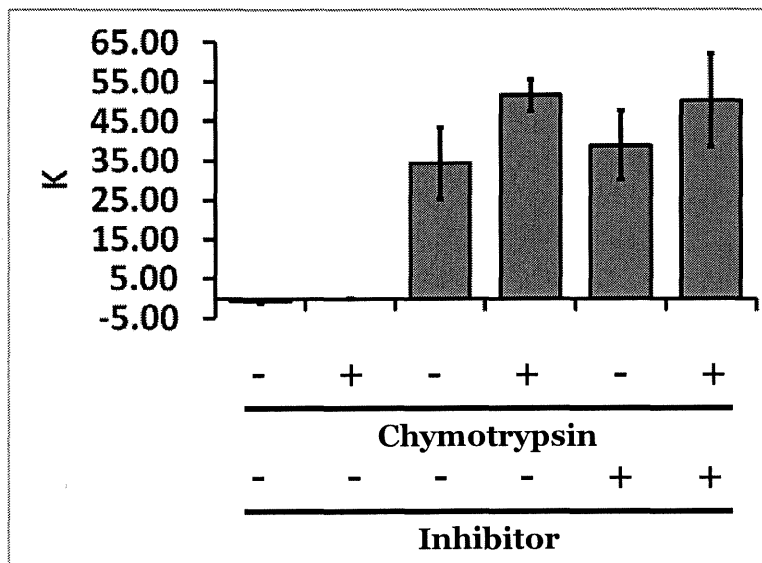


Figure 3: Effect on isomerization in the presence of a protease inhibitor cocktail cHom and/or chymotrypsin were incubated with the acetylated substrate in the presence or absence of a 1X broad spectrum protease inhibitor cocktail and assayed for the presence of isomerase activity. Presence of the inhibitor slightly reduced activity ($p > 0.05$ in all cases). $n = 3$, \pm SEM.

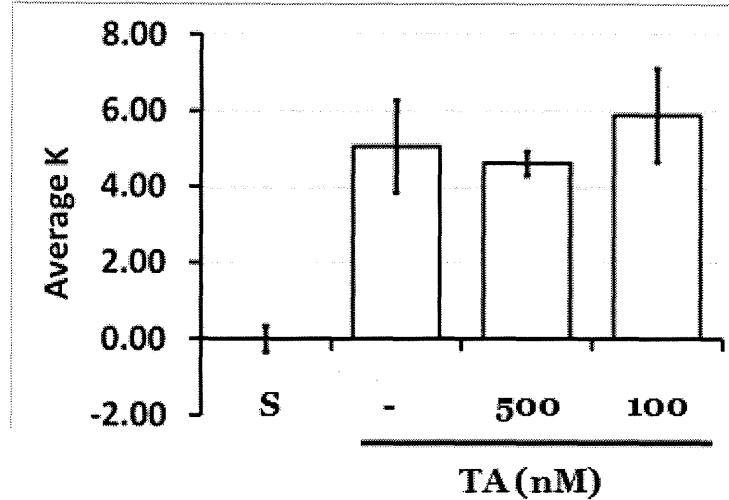


Figure 4: Treatment with TA

cHom was untreated or treated with the 100 nM or 500 nM TA ($IC_{50} = 20$ nM) for 5 minutes followed by assay of isomerase activity. There was increased activity when cHom was treated with 100 nM of TA. The spontaneous isomerization (S) of the substrate was determined, $n=3$, \pm SEM. Individual groups are compared to cHom (P value > 0.05) in all cases.

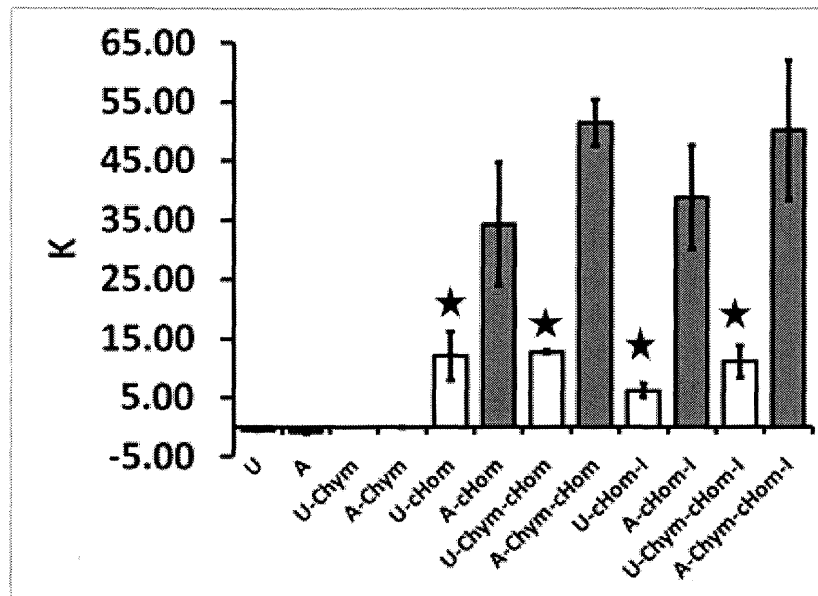


Figure 5: Unacetylated Substrate Activity

During the activity assay, cHom was either mixed with acetylated or unacetylated substrate. The unacetylated peptide results are shown in with the white bars. Less isomerization occurred with the unacetylated substrate. ★ denotes $p < 0.05$, $n=3$, \pm SEM.

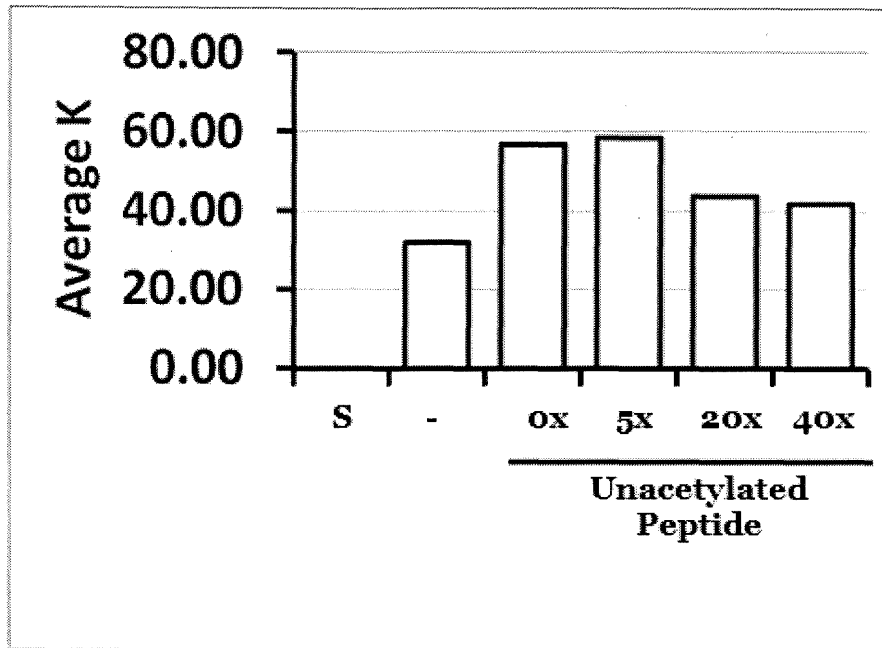
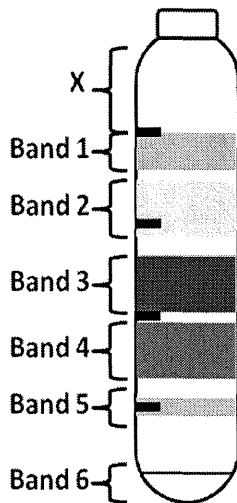


Figure 6: Competition experiment results

cHom was assayed for the presence of isomerase activity in the presence 1x acetylated lysine substrate and increasing amounts of unacetylated substrate (ox to 40x). ox or 5x of the unacetylated substrate was the same as the acetylated substrate alone. 20x and 40x were slightly lower but not statistically different from ox ($p > 0.05$ in all cases). $n = 4$, \pm SEM.



Band Number	Component
1	Membranes
2	Myelin & Membranes
3	Myelin & Membranes
4	Synaptosomes & Membranes
5	Synaptosomes
6	Whole organelles (golgi, ER, mitochondria)

Figure 7: Percoll Gradient Bands and Expected Band Composition

Mouse cortex was homogenized and separated on a percoll gradient giving rise to six bands, all with different cellular components.

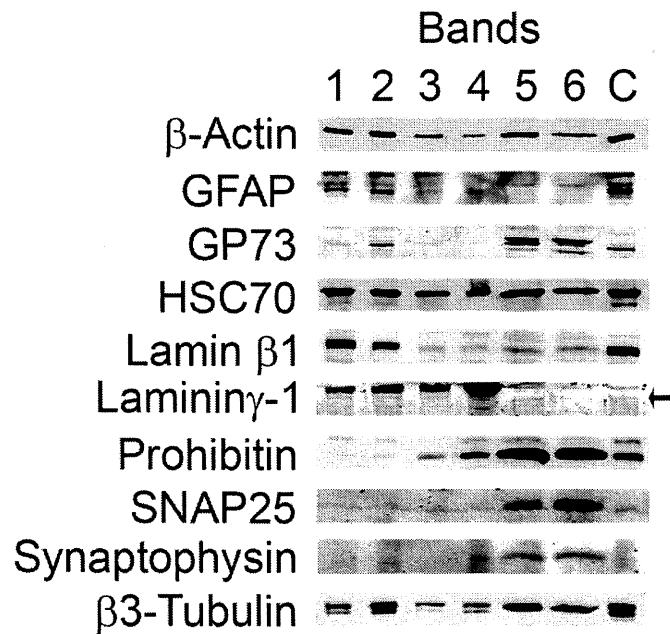


Figure 8: Western blot analysis of percoll-gradient fractions of centrifuged mouse cHom. Mouse cortex was homogenized and separated on a percoll-gradient into 6 bands or fractions. C is the control. Each band was run on a 12% acrylamide gel, transferred to membrane and incubated with their respective antibodies. Western blot performed by Pamela Westmark.

	<u>Band 1</u>	<u>Band 2</u>	<u>Band 3</u>	<u>Band 4</u>	<u>Band 5</u>	<u>Band 6</u>	<u>cHom</u>	<u>Marker</u>
b-Actin	+++	+++	+++	+++	+++	+++	+++	Structural
GFAP	+++	+++	+	+++			+++	Astroglia
GP73	+	+++			+++	+++	+++	Golgi
HSC70	+++	+++	+++	+++	+++	+++	+++	Cytosolic and nuclear
Lamin-B1	+++	+++	+	+	+	+	+++	Nuclear
Laminin γ-1	+++	+++	+++	+++	+++	+++	+++	Basement membranes
Prohibitin			+	+++	+++	+++	+++	Mitochondrial
SNAP25					+++	+++	+++	Synaptic
Synaptophysin				+++	+++	+++	+++	Synaptic
β3-Tubulin	+++	+++	+++	+++	+++	+++	+++	Neuron-specific cytoskeletal protein

Table 1: Summary of percoll gradient band composition

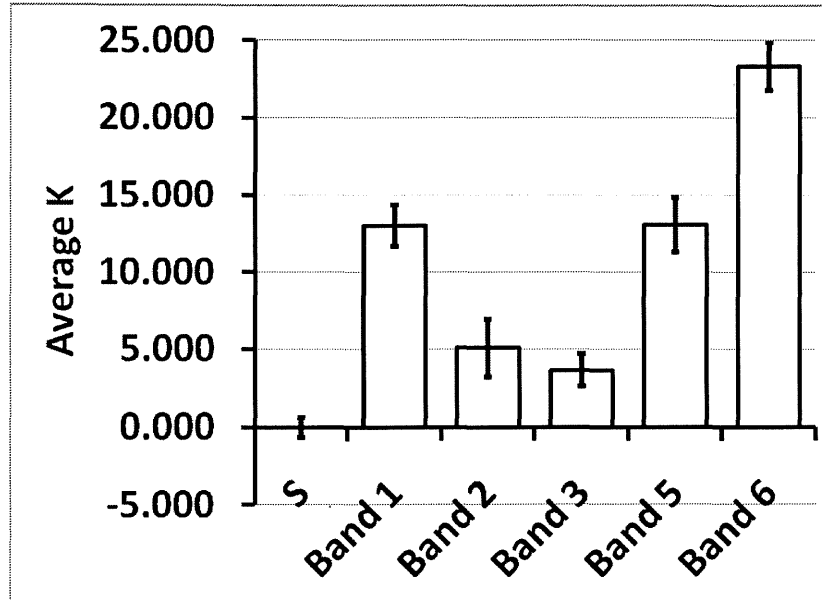


Figure 9: Activity of Percoll Gradient Bands

Isomerase activity of fractions from mouse cHom bands separated on a percoll gradient. Band 6 showed the highest activity. N=4, ±SEM.

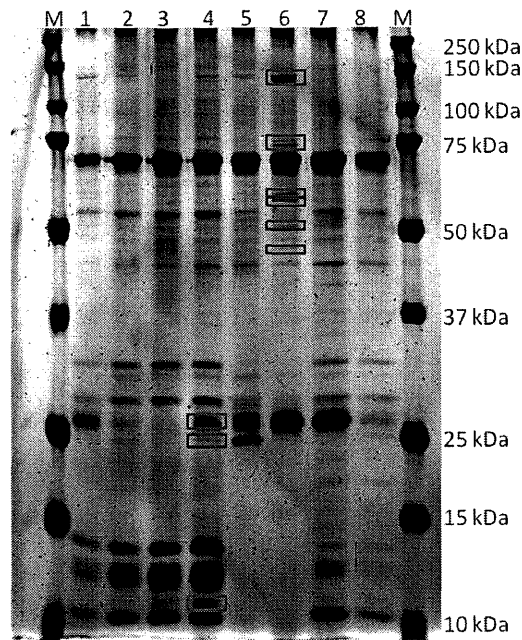


Figure 10: Pull Down Assay Results

Pull Down Assay of 1 mg cHom (lanes 1-4, 7-8) and 126 ug Band 6 (lanes 5-6) using streptavidin magnetic beads and the biotinylated substrate. Lanes 1, 3, 5, and 7 were no substrate controls and show non-specific binding. Unique bands in lanes 2, 4, 6, and 8 were submitted to the UW Biotechnology Center for in gel digestion and Mass Spec analysis.