

Specific Inhibition of Rac1 Activation

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biochemistry

by

Scott Beaurivage

April 27, 2006

APPROVED:

Stephen Miller, Ph.D.
Biochemistry and Mol Pharmacology
Umass Medical Center
Major Advisor

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

Rac1 is a member of a family of small GTPases that are primarily responsible for cellular motility and morphology. Activation of Rac1 by Rac-specific guanine nucleotide exchange factors (GEFs) is thought to be important for the metastasis of cancer cells. The scope of this project was to synthesize a minimal extended peptide sequence that would block interaction between Rac1 and the Rac1-specific exchange factor GEF-H1. Inhibition of Rac1 activation by Rac-specific GEFs would be a useful tool for the study of Rac-mediated cellular processes and may ultimately result in cancer therapeutics.

TABLE OF CONTENTS

ABSTRACT	2
TABLE OF FIGURES	4
BACKGROUND.....	6
GTPASES	6
RHO GTPASES	8
RAC SPECIFIC GEF INHIBITORY PEPTIDE.....	11
N-METHYLATED PEPTIDES	14
PROJECT PURPOSE.....	18
METHODS.....	19
CLONING OF RAC1	19
RAC1 EXPRESSION	20
PROTEIN PURIFICATION	21
PEPTIDE SYNTHESIS AND PURIFICATION	21
N-METHYLATION OF ASP ON SOLID SUPPORT	23
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)	23
CELL PERMEABILITY TESTS	24
BINDING ASSAYS	24
RESULTS.....	26
RAC1 EXPRESSION AND PURIFICATION	26
PEPTIDE SYNTHESIS.....	27
<i>β-Amyloid Peptide</i>.....	27
<i>Impurity Analysis</i>.....	28
<i>Inhibitory peptides</i>.....	30
<i>Purification</i>	31
CELL PERMEABILITY TEST	31
BINDING ASSAY.....	31
DISCUSSION.....	33
PROTEINS	33
PEPTIDES	34
CELL PERMEABILITY.....	35
FUTURE RESEARCH.....	35
BIBLIOGRAPHY	37

TABLE OF FIGURES

FIGURE 1: CDC42 IN COMPLEX WITH THE GTPASE BINDING DOMAIN OF ACK	7
FIGURE 2: SWITCHES AND β -SHEETS OF RAC1	8
FIGURE 3: BIOLOGICAL PATHWAYS INDUCED BY VARIOUS RHO FAMILY MEMBERS	9
FIGURE 4: CRYSTAL STRUCTURE MODEL FOR THE INTERACTION OF RAC1 WITH GEF TIAM1	10
FIGURE 5: RESULTS OF GEF BINDING SPECIFICITY. FROM GAO <i>ET AL.</i>	11
FIGURE 6: SCHEMATIC REPRESENTATION OF TESTED RAC1/CDC42 CHIMERAS. FROM GAO <i>ET AL.</i>	12
FIGURE 7: RAC IN COMPLEX WITH THE GTPASE BINDING REGION OF TIAM1	13
FIGURE 8: RESIDUES 45-60 OF RAC1 IN COMPLEX WITH THE GTPASE BINDING DOMAIN OF TIAM1	14
FIGURE 9: <i>N</i> -METHYLATED AND NON-METHYLATED PEPTIDE KLVFF	15
FIGURE 10: AGAROSE GEL OF PCR PRODUCT	26
FIGURE 11: PROTEIN PURIFICATION GELS	27
FIGURE 12: HPLC CHROMATOGRAM OF MANT-K(MEL)V(MEF)F BATCH 2	28
FIGURE 13: IMPURITY ANALYSIS	28
FIGURE 14: HPLC CHROMATOGRAM OF MANT-K(MEL)V(MEF)F BATCH 4	29
FIGURE 15: MALDI OF MANT-K(MEL)V(MEF)F	30
FIGURE 16: MALDI OF UNACETYLATED WDTAG	30
FIGURE 17: CELL PERMABILITY TEST USING MANT-K(MEL)V(MEF)F	31
FIGURE 18: PRELIMINARY INHIBITION ASSAY USING SYNTHESIZED PEPTIDES	32

ACKNOWLEDGEMENTS

First I would like to thank Professor Stephen Miller for letting me into his lab and assisting with report writing. I would also like to thank Anjan Bhunia, Henry Gillin, Katryn Harwood, and Karen Mruk for showing me procedures I was unfamiliar with and getting me accustomed to the lab. Thanks also goes out to Katryn Harwood for providing me with GEF-H1 and assisting with assay development. Thank you also to the Nucleic Acid and Peptide Core facilities at the University of Massachusetts Medical School for providing DNA sequences and a manufactured peptide respectfully. Finally, I would like to thank Professor David Adams for his assistance in setting this project up, and his help writing the report.

BACKGROUND

GTPases

GTPases are a large family of proteins that hydrolyze guanosine triphosphate (GTP) into guanosine diphosphate (GDP). The function of a GTPase at any given time is determined by the nucleotide to which it is bound. When a GTPase is bound to GDP it is in its “off” form and is unable to bind effectors. Binding GTP turns it “on” and allows effectors to bind. Most effector proteins are autoinhibited; the binding of these proteins to GTP bound GTPases activates the effector and enables it to perform biological effect such as rearrangement of the actin cytoskeleton. The switch between active and inactive forms can be seen by certain conformational changes that take place when the protein binds GTP. The process of switching GDP for GTP is mediated by a guanosine nucleotide exchange factor (GEF). The GEF allows the transfer of a GTP to replace GDP so that the GTPase is effectively reloaded. At this point the GTPase is free to hydrolyze the GTP, a process helped along by GTPase activating proteins, or GAPs. GAPs increase a GTPases’ intrinsic hydrolysis rate by forming a more stable transition state.

GTPases of the Ras superfamily contain two distinct regions that change conformation upon binding GTP. These regions are known as switch I and switch II. Switch I is an extended structure that participates in binding Mg^{2+} . The conformation of switch II is a loop attached to a helix (Fig. 1, left panel, white and red) [11]. These both undergo conformational changes that facilitate binding of effectors. When bound to GDP,

the switches are flexible and loose. After binding GTP, however, the structures become rigid and well defined (Fig. 1, right panel).

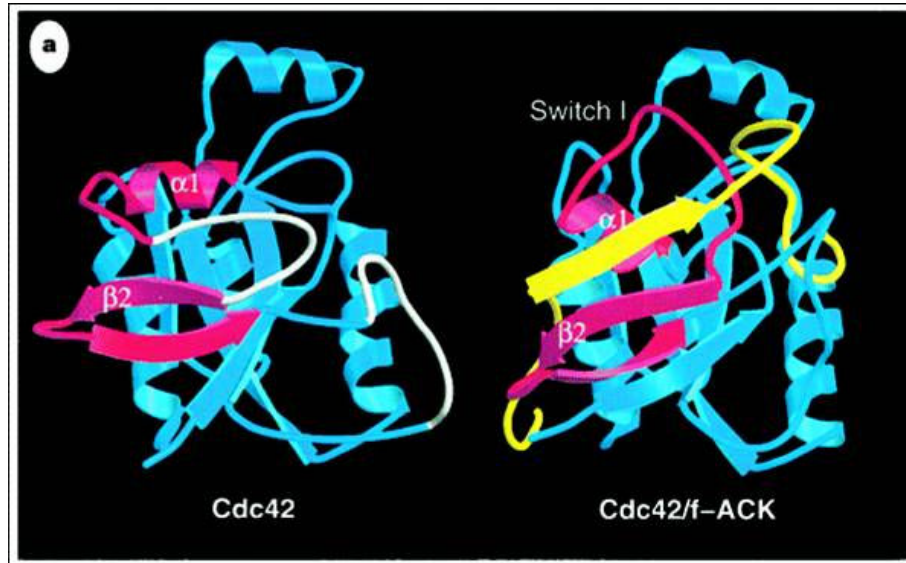


Figure 1: Cdc42 (left panel) and Cdc42 in complex with the GTPase binding domain of ACK, an effector protein (right panel). Switches I & II are both shown in white (left) and red (right). White represents random coil structure, red represents fixed structure. From Owen *et al* [8].

Between switch I and II in Rac are two β strands, $\beta 2$ and $\beta 3$ (See Fig. 2). These β strands have an extended conformation that resembles a flat sheet. Since the GTPase/effector interface has this specific conformation a mimicking molecule that displays this type of structure would be a good candidate for competitive inhibition.

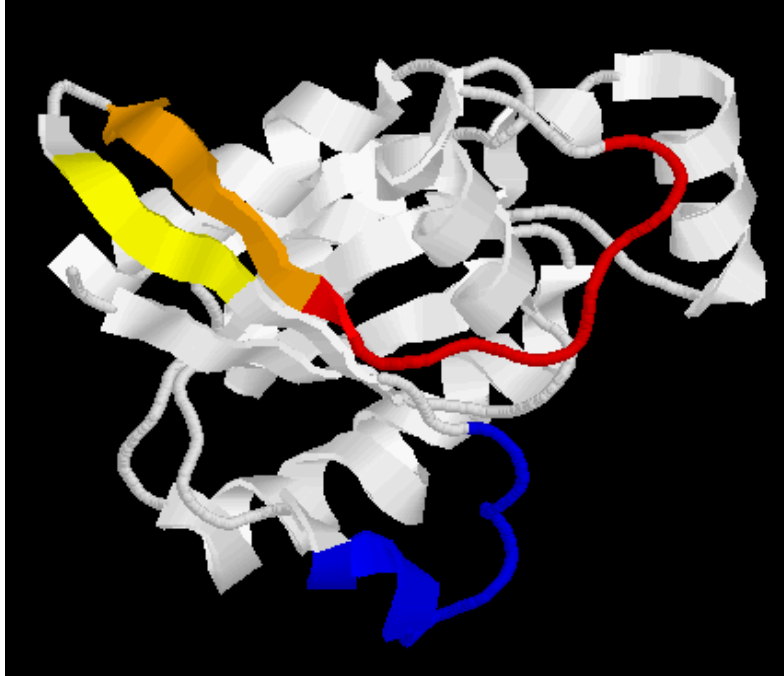


Figure 2: Switches and β -sheets of Rac1. Switch I shown in red; β 2 shown in orange; β 3 shown in yellow; switch II shown in blue; rest of Rac1 shown in gray.

Rho GTPases

The Rho family is comprised of 20-30 kDa GTPases and includes isoforms of Rho, Rac and Cdc42 among others. Rho GTPases have a large variety of functions in the cell including organization of actin filaments, signal transduction pathways, and making up components of larger complexes such as NADPH oxidase. This variety of functioning is due to the fact that there are approximately 22 Rho family GTPases, 80 GAPs and 80 GEFs. The three GTPases mentioned in Figure 3 (Rho, Cdc42, and Rac) are among the most thoroughly studied of the Rho family. The number of biological effects that any one GTPase may have shows the importance of specificity relating to these molecules. For example, Cdc42 and Rac have approximately 70% sequence homology [3]. It is not surprising that they share several effectors, exchange factors and GAPs. Despite their

similarities, there are also regulatory molecules that react specifically with either Cdc42 or Rac. These are the molecules of the most interest in this study.

	Actin	SRF	JNK/p38	NF- κ B	NADPH oxidase	G ₁ cell-cycle progression	Cell-cell contacts	Secretion	Cell polarity	Transformation
Rho	+	+	-	+	-	+	+	+	-	+
Rac	+	+	+	+	+	+	+	+	-	+
Cdc42	+	+	+	+	-	+	+	?	+	+

Figure 3: Biological Pathways Induced by Various Rho Family Members. The table shows various pathways that are regulated by Rho, Rac and Cdc42 [1].

As can be seen from Figure 3 Rac and Cdc42 are very similar in function yet still display some specificity, for example only Rac is present in NADPH oxidase and only Cdc42 shows an effect on cell polarity. One important role of Rac and Cdc42 is their effects on the actin cytoskeleton. Rearrangements of the actin cytoskeleton allow for cellular motility [12]. Lamellipodia and filopodia are cellular protrusions of the cytoskeleton which allows for cell motility; these are controlled by Rac1 and Cdc42 respectively. Cellular motility is important to biological process such as wound healing and angiogenesis. Metastasis, the movement of cancerous cells through tissues, relies heavily on lamellipodia/filopodia formation. This identifies Rac and Cdc42 as possible targets for prevention of cancer metastasis [14].

Guanosine Nucleotide Exchange Factors (GEFs)

There are at least three GEFs that interact specifically with Rac and not Cdc42. These are of particular interest considering there are such similarities between Rac and Cdc42's binding interactions. The GEFs are TiamI, Trio and GEF-H1 (which also binds Rho A, B and C). Each of these GEFs contains a certain sequence homology that has been shown to influence binding specificity and catalytic function. This similarity in

sequence is composed of two domains, the Dbl homology (DH) and the pleckstrin homology (PH). The PH domain almost always follows the Dbl domain, and the two together have been found to be the minimal sequence required for full GEF functionality [3,15]. While the DH domain seems to be the necessary domain for nucleotide exchange, the PH domain seems to both enhance the exchange process and specificity as well as influence cellular localization [10]. The crystal structure of the DH/PH domain of TiamI in complex with Rac1 has recently been published [13]. From this crystal structure one can identify the points of interaction between the two proteins.

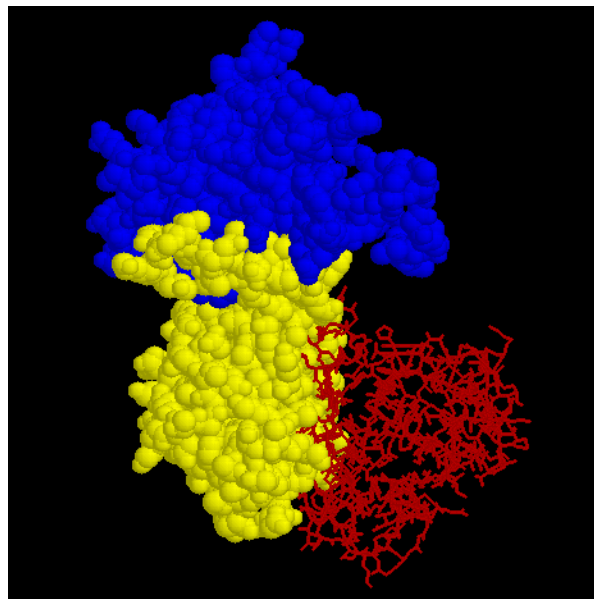


Figure 4: Crystal Structure Model for the Interaction of Rac1 with GEF TiamI. The DH domain of TiamI (yellow) shows several contacts with Rac1 (red). The PH domain (blue) shows no visible contacts.

Figure 4 shows that there is no interaction between Rac1 and the PH domain (blue) of TiamI and there is also only a small area that interacts with the DH domain (~residues 52-60 of Rac1) .

Rac Specific GEF Inhibitory Peptide

Gao *et al.* set out to determine which sections of Rac1 are necessary for specificity and binding to Tiam1, Trio and GEF-H1. This study was done before the crystal structure of Rac1/Tiam1 was published so it was not clear what sections of the proteins were interacting. They began their study by running simple assays to determine GEF nucleotide exchange activity on Rac1 and Cdc42. Figure 4 shows their results which clearly indicate that all their GEFs bind specifically to Rac1 only.

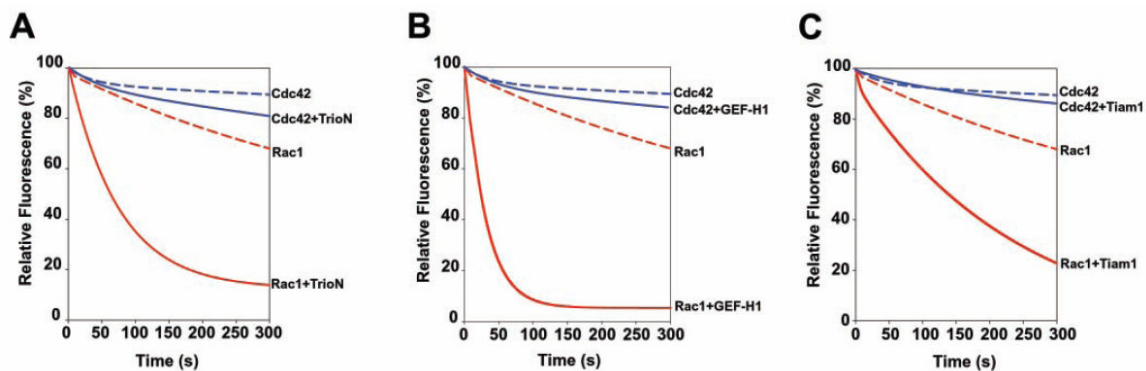


Figure 5: Results of GEF binding specificity. Decrease in fluorescence shows exchange of MANT-GDP for GTP and therefore indicates GEF activity. From Gao *et al* [3].

Due to the amount of evidence that shows switch I & II to be of high importance to binding, the researchers next made point mutations to both regions. They found that mutations of certain residues in both sections resulted in the inability to bind GEFs and the loss of ability to exchange nucleotide once bound.

Although these residues are important to GEF binding and activity, it is not likely that they allow for specificity between Rac and Cdc42. The reason for this is that switch I & II are highly conserved across most GTPases, especially between Rac and Cdc42. The only difference between switch I & II of Rac and Cdc42 is at residue 33; in Rac it is an isoleucine, in Cdc42 a valine, a highly conservative substitution [7]. To test for residues

that determine specificity a series of Rac1/Cdc42 chimeras (Fig. 6) were tested for their ability to bind GEF's. The assumption was that any chimera that contained the necessary sequence from Rac1 would bind GEFs.

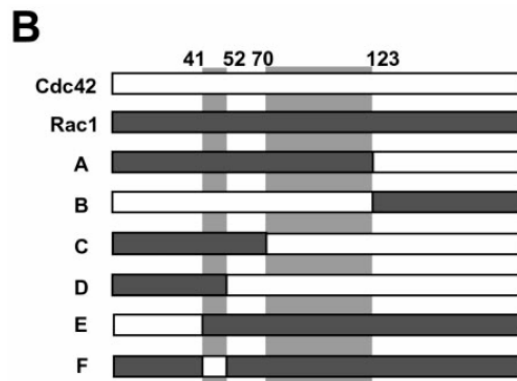


Figure 6: Schematic representation of tested Rac1/Cdc42 chimeras. The dark areas indicate the amino acid residues from Rac1; white designates residues from Cdc42. From Gao *et al* [3].

They found that chimeras containing residues 53-70 of Rac1 were able to bind and function, whereas chimeras lacking this sequence could not.

Next they determined which residues of this sequence were important to specificity between Rac and Cdc42. This was a relatively easy problem to solve because the only residue that is different between the two GTPases in this range is 56 which is a tryptophan in Rac1 and a phenylalanine in Cdc42. The crystal structure of Rac1 associated with TiamI shows that the W56F mutation results in the loss of a hydrogen bond to His1178 of TiamI. There is also a loss of a considerable amount of Van der Waals interactions due to the loss of the indole side chain of the tryptophan.

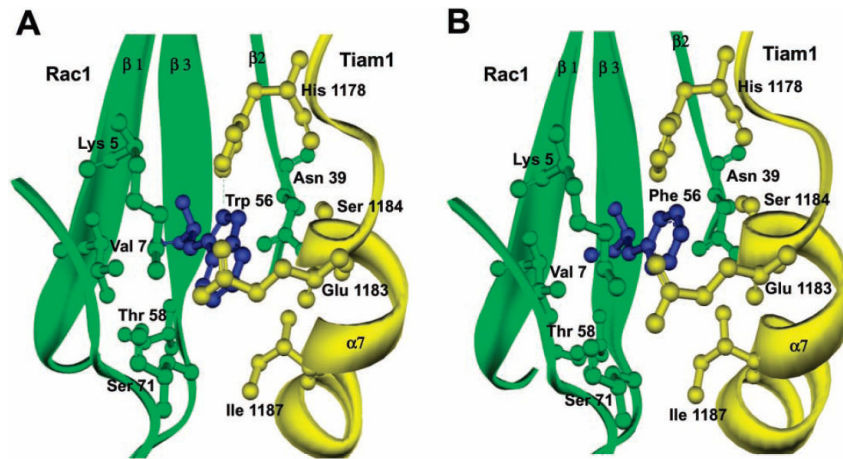


Figure 7: Left image shows wild type Rac1 in complex with GTPase binding domain of TiamI. Right image shows Rac1 W56F mutant in complex with the GTPase binding region of TiamI [13].

Since it appeared that Trp56 was the only residue that provided specificity between Rac1 and Cdc42, the researchers tested Rac1 W56F vs. Cdc42 F56W. The results showed no binding of GEFs to Rac1 W56F, but binding similar to that of wild type Rac1 by Cdc42 F56W.

Finally, they constructed two Rac1 based peptides to use for testing in an inhibition assay. The peptides were identical to residues 45-60 of Rac1 except in one the W56F mutation was made. Results showed a dose-dependent inhibition of Rac1 binding to Trio, TiamI and GEF-H1 when treated with the peptide containing Trp56. There was no inhibition by the Phe56 peptide [3].

The crystal structure of residues 45-60 of Rac1 in complex with TiamI is shown in Figure 8. The conformation is such that residues 45-51 are coiled and make no apparent contact with TiamI. Residues 52-60, however, adopt an extended conformation and have three points of contact with TiamI; Asn52, Trp56 and Gly60. Since these appear to be the main contact points of Rac1 to TiamI it should be possible to truncate this peptide to residues 52-60 and still retain the ability to inhibit interaction.

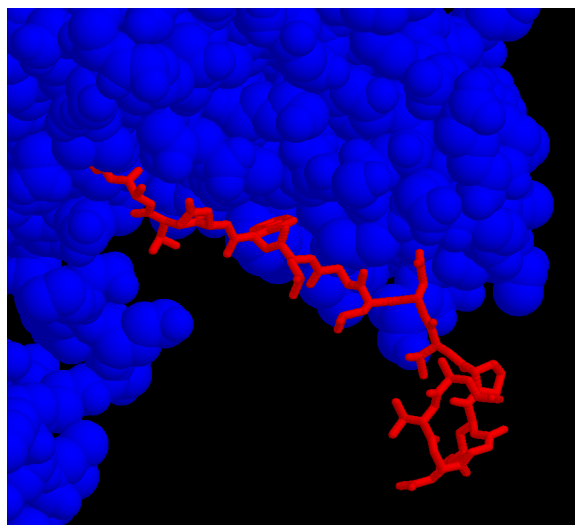


Figure 8: Residues 45-60 of Rac1 (red) in complex with the GTPase binding domain of Tiam1 (blue).

Furthermore, since Trp56 has been determined as the only residue to determine GTPase specificity, [3] it may also be possible to truncate even further depending on the roles of Asn52 and Gly60. There is also the potential to increase inhibition efficiency by modifying the peptide so that it always exhibits an extended conformation similar to that seen at the binding interface.

N-Methylated Peptides

The conformation of a peptide is very important to how active it is biologically [9]. While short peptides are generally unstructured, the conformation can be specifically hindered such that it gives a desired structure. These hindrances come primarily from amino acid side chains and alterations of groups that prevent rotation around peptide bonds. N-methylated amino acid substitutions have been shown to restrict the altered amino acid and the one preceding it to an extended conformation. N-methylation is done by replacing the amide hydrogen on a residue with a methyl group. Doing this locks the

conformation of the modified amino acid as well as the residue that precedes it [9]. Alternating sites of N-methylation, then, should lock the entire peptide in an extended conformation. Gordon *et al.* used this alternating N-methylation strategy when they constructed a peptide to inhibit A β 1-40 fibrillogenesis.

Gordon *et al.* synthesized a five amino acid sequence comprised of A β 16-20. They also synthesized the same sequence containing alternating N-methylations. The structure of these sequences was determined by circular dichroism, a method that measures the ellipticity of light. The spectra obtained showed that the methylated version of the peptide was a β -strand; the un-methylated version showed characteristics of a random coil [4].

Some other important characteristics arise because of N-methylation. These include increased cell permeability and reduced susceptibility to proteolysis both of which increase potential as inhibitory molecules in live cells. The reason these characteristics show up is because N-methylation reduces the number of available hydrogen bond donors (Fig. 9) [4].

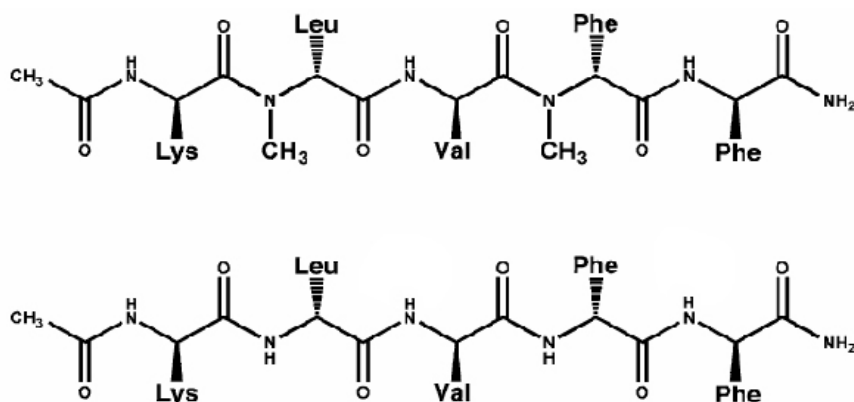


Figure 9: The top image is of an N-methylated peptide (K(Me)LV(Me)FF). The bottom image is of the same peptide sequence (KLVFF) with no methylation. From Gordon *et al.*

A peptide has to be desolvated of the water that is hydrogen bound to it before it can pass through the membrane. Reducing the amount of hydrogen bond donors reduces the energy required to desolvate the peptide and therefore increases its chances of crossing the membrane. Alternating N-methylation also produces a chain with two faces. On one face of the peptide there is normal bonding capabilities contributed from side chains and amide hydrogens; this face should function like a normal peptide chain. The second face has lost the hydrogen bonding capabilities of the amide hydrogens because they have been replaced by methyl groups which make no such hydrogen bonds. Removal of these hydrogen bond donors greatly changes the way this face interacts. The loss of hydrogen bonding capabilities also makes the molecule more hydrophobic. Gordon *et al.* also studied this aspect and found that N-methylated derivatives of their peptide were able to make it into cells while un-methylated versions could not [4]. These N-methylated peptides should also make for good inhibitors of intracellular β -strand interactions because of their extended conformation and increased cell permeability.

The design of a minimal peptide domain that will specifically inhibit GEF-H1's binding capabilities to Rac1 should incorporate all of these features. Since residues 45-51 of Rac1 make no contacts with the DH/PH domain of TiamI they should be able to be removed with little to no effect on inhibition. It is also likely that other truncation of residues 52-60 could be made and still be effective as long as they contain Trp56. Since residues 52-60 of Rac1 adopt a β -strand conformation when in complex with TiamI, it is likely that an N-methylated version of the inhibitory peptide would be more effective. If the peptide were already in an extended conformation the entropic cost of changing to an extended conformation would be reduced, so binding to the peptide would be favored.

N-methylation should also make the inhibitory peptide cell permeable and useful for *in vivo* assays. Given the information collected, construction of a truncated, Rac1 derived, N-methylated peptide sequence should provide better inhibition of GEF-H1/Rac1 interaction than the previously made inhibitory peptide. There is also potential for blocking other GTPase/GEF and GTPase/effector interactions that have similar binding interfaces.

PROJECT PURPOSE

The aim of this project was to synthesize potential inhibitory peptides that could block the interaction between Rac1 and GEF-H1. To accomplish this, Rac1 was expressed and purified, and GEF-H1 was obtained from a fellow member of the lab. Analysis and purification of peptides also required the use of analytical methods such as HPLC and MALDI. These proteins and peptides are now available to use in assays to test the inhibition ability of the peptides on the binding ability and function of GEF-H1.

METHODS

The primary preparative methods were solid phase peptide synthesis (SPPS) and protein expression & purification. Peptide purity and identity was assessed by high performance liquid chromatography (HPLC) and matrix assisted laser desorption/ionization was used to back up HPLC data with mass data. The two main routes of testing were pull down assays and fluorescence microscopy.

After research into the subject had been done and there was a plan of how to accomplish the goal, the first step was to produce the required materials. Since the aim of this project was to block interaction between Rac1 and GEF-H1 it was necessary to express Rac1 and GEF-H1 and also to synthesize candidates for inhibitory peptides. The lab in which this project took place was currently working on the expression of GEF-H1. The GEF-H1 used in assays was provided by Katryn Harwood.

Cloning of Rac1

The wild type Rac1 I.M.A.G.E clone was obtained from Open Biosystems. Polymerase chain reaction (PCR) was used to amplify the full-length Rac1 cDNA, incorporating 5' BamHI and 3' EcoRI restriction sites. Following the PCR reaction the DNA was double digested with BamHI and EcoRI. The digested DNA was separated on a 1% agarose gel and the band containing the desired PCR product was excised and purified. This purified fragment was then ligated into a pGEX-6P1 vector using T4 ligase. This vector contains a PreScission protease cut site.

Following ligation, a small amount of the plasmid was digested with BamHI and EcoRI to ensure it contained the desired insert. Once the insert's presence was confirmed,

the plasmid was transformed into DH5 α cells. DH5 α is a strain of *E. coli* that lacks *recA* and *endA* making them useful primarily for the expression of plasmid DNA. Once the cells had taken up the DNA they were plated on Luria broth (LB) agar plates that contained the antibiotic carbenicillin. The following day individual colonies were picked and grown up into 5 mL cultures. These cell cultures were pelleted, lysed and their DNA purified using a QIAGEN miniprep kit. The purified DNA was again checked for presence of the desired insert and sequenced by the Nucleic Acid Facility at UMass Medical School.

Rac1 Expression

Protein expression was carried out in BL21 cells. BL21's are a strain of *E. coli* that lacks *lon* and *ompT* making them useful for expression of protein. The purified plasmid from the DH5 α s was transformed into BL21 cells. Colonies were allowed to grow overnight on an LB agar plate containing carbenicillin. Individual colonies were picked and grown into 5 mL cultures. The next day, 1 L of 2xYT (yeast tryptone) was inoculated with the BL21 cells from one of the 5 mL cultures. The cells were allowed to grow at 37° C until they reached their exponential growth phase. This was determined by measuring the cultures optical density at a wavelength of 600 nm (O.D. 600). Once the O.D. 600 was between the values of 0.8 and 1.0 (indicating exponential growth) protein expression was induced using 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The culture was allowed to grow at 20°C for another 4 hours. The cells were then centrifuged down, separated from the supernatant, flash frozen in liquid nitrogen and stored at -80°C.

Protein Purification

Purification of the protein was accomplished using immobilized glutathione beads and PreScission protease. The frozen cell pellet was thawed and resuspended in 25 mL of lysis buffer (50 mM Tris, 0.5 M NaCl, 0.1% Tween 20). To inhibit general protease activity 250 μ L of 1 M PMSF was added to the resuspended cells. The cells were sonicated for 10 seconds five times to lyse the cells. 250 μ L of 1 M DTT was added to the lysate as a reducing agent. The cells were then centrifuged for 1 hour at 35,000 rpm and 4°C. The clarified supernatant was then incubated for 2 hours with immobilized glutathione beads. After 2 hours the beads were filtered, washed once using lysis buffer and once using wash buffer #2 (50 mM Tris, 250 mM NaCl, 5 mM MgCl₂, pH 8.1). The beads were then washed with PreScission Protease buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.4) twice. Beads were then incubated overnight with 960 μ L of PreScission Protease buffer and 40 μ L of PreScission Protease. After incubation the beads were filtered and washed twice with PreScission Protease buffer. Following cleavage, samples of every step of the purification process were run on a 12% SDS-PAGE gel in order to determine where Rac1 ended up. Aliquots of the protein were made, flash frozen and stored at -80° C for later use.

Peptide Synthesis and Purification

Synthesis of the inhibitory peptide was done using solid phase peptide synthesis (SPPS). SPPS is the sequential addition of amino acids to a chain tethered to a solid

support. The amino acid chain can then be cleaved from the support. The support used during the course of this project was Rink Amide MBHA resin obtained from Novabiochem.

First the resin was deprotected of its Fmoc group using 20% piperidine (Pip) in *N,N'*-dimethylformamide (DMF) 2x 2 minutes. Following deprotection 4 equivalents of amino acid and 4 equivalents of coupling agent (HBTU or HATU) were dissolved in 0.4 M *N*-methylmorpholine (NMM). HBTU was used when coupling to a normal amino acid or to the resin; HATU was used when coupling to an *N*-methylated amino acid. This mixture was added to the resin and allowed to couple for 1.5 hours for regular coupling and 3 hours when coupling to *N*-methylated amino acids. This process was repeated until the desired amino acid sequence was made. Following the addition of the final amino acid, the Fmoc group is removed and the product is cleaved from the resin. The cleavage process was done using 1 mL of the cleavage cocktail [95:2.5:2.5 trifluoroacetic acid (TFA):H₂O:triisopropylsilane (TIS)] for 1 hour. The resin was washed with 1 mL of TFA. Precipitation into ether was used for quick clean up of the peptides. Adding the cleavage mixture to cold diethyl ether causes the formation of the TFA salt which precipitates out of solution. When the peptide was cleaved with the Fmoc group still attached or when dealing with *N*-methylated peptides the cleavage mixture was removed using a rotary evaporator equipped with an acid trap that would collect the TFA. The dried peptide in either case was dissolved into acetonitrile (ACN)/H₂O 0.1% TFA, frozen and finally lyophilized to give a powdery product.

N-Methylation of Asp on Solid Support

Some *N*-methylated amino acids, for example (Me)Leu and (Me)Ala, are commercially available. For *N*-methylated amino acids that were not commercially available a three step methylation process was used. This process was originally described by Stephen Miller and Thomas Scanlan [6]. First the amino acid was deprotected using 20% Pip in DMF 2x 2 minutes. The residue was then reprotected using 133 mg of nitrobenzylsulfonyl chloride (NBS-Cl) and 133 μ L collidine. The amino acid was then methylated using 174 mg methyl *p*-nitrobenzylsulfonate (Me-NBS) and 86 μ L of 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD). Finally the NBS-Cl was removed using 140 mL of β -mercaptoethanol and 151 mL of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The product is the *N*-methylated amino acid that can be coupled to the next amino acid using HATU.

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) was used for both analysis and purification during the course of this project. The type of HPLC used during this project was RP-HPLC. An Atlantis C-18 column was used from Waters. Mobile phases used were 0.1% TFA in H₂O and 0.1% TFA in acetonitrile (ACN). A gradient of Solvent A (0.1% TFA in H₂O) to Solvent B (0.1% TFA in ACN) was used to elute the samples. A typical gradient was 15-85% Solvent B over the course of 20 minutes. Detection of compounds was accomplished using a Waters 2996 photodiode array detector which is able to measure reflected light from 200nm-600nm. A Waters 474 scanning fluorescence detector was also used for detection of fluorescent compounds.

Matrix Assisted Laser Desorption/Ionization (MALDI)

Mass analysis of the peptides was done using matrix assisted laser desorption/ionization (MALDI). MALDI was performed by mixing the sample with a matrix of α -cyano-4-hydroxycinnamic acid in 1:1 ACN:water. The mixture was then spotted on a metal plate and placed in a dessicator to allow the solvent to dry leaving only the matrix and sample. A laser was then fired at the spot which ionizes the matrix allowing it to transfer its charge to the sample. Once the sample was charged, time of flight (TOF) mass spectrometry was used. This uses retention times based on how long a compound takes to travel a known distance to determine mass.

Cell Permeability Tests

Purified K(MeL)V(MeF)F was labeled with N-methylantranilic acid (MANT) and was tested for cell permeability in HeLa cells. Cells were incubated for 1 hour with 100 μ M and 10 μ M MANT-K(MeL)V(MeF)F in Leibovitz L-15 media. Following incubation, the cells were washed three times with media to remove extracellular fluorophore. The cells were then examined by fluorescence microscopy.

Binding Assays

25 μ L of GST-GEF-H1 fusion protein bound to immobilized glutathione beads was incubated for 1 hour with 100 μ g of nucleotide free Rac1 in a buffer containing PBS, 50 mM NaCl and 1 mM TCEP. This assay was done in presence or absence of inhibitory peptides. Peptides tested were the Gao *et al* W56 peptide (synthesized by the Peptide Core Facility), Ac-NLGLWDTAG-NH₂, Ac-WDTAG-NH₂, and Ac-W(MeD)T(MeA)G-

NH₂. All peptides were added at a concentration of 200 μM. After incubation, the beads were filtered from the liquid and washed twice with buffer containing PBS, 2 mM TCEP and 0.1% Tween 20. A 12% SDS-PAGE gel was run of the samples to view bound versus unbound protein.

RESULTS

Rac1 Expression and Purification

Rac1 expression using *E. coli* cells was successful. The polymerase chain reaction gave one major product with a few truncated products (Fig. 10). The major product was sequenced after it had been purified and expressed in DH5 α cells.

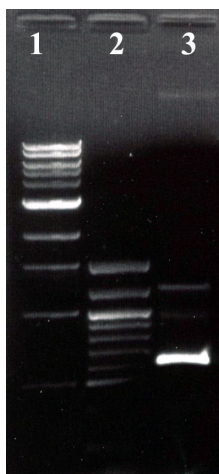


Figure 10: Agarose gel of PCR product. Lane 1: 1 kB Ladder. Lane 2: 100 bp Ladder. Lane 3: Rac1 PCR Product.

The sequence obtained was compared to known sequences of Rac1 using New England Biolabs NEBcutter and NCBI BLAST. The sequence matched up with full length, wild type Rac1.

Purification of Rac1 done with immobilized glutathione beads and PreScission Protease was also successful. SDS-PAGE gels were run to show the cleavage of the Rac1 from the beads. The gel (Fig, 11) that was run shows all steps of the purification process from left to right: molecular weight standard, cell lysate, cell pellet, clarified supernatant, supernatant after incubation with beads, wash #1, wash #2, wash #3, wash #4, unbound protein, cleavage wash #1, cleavage wash #2, bound protein.

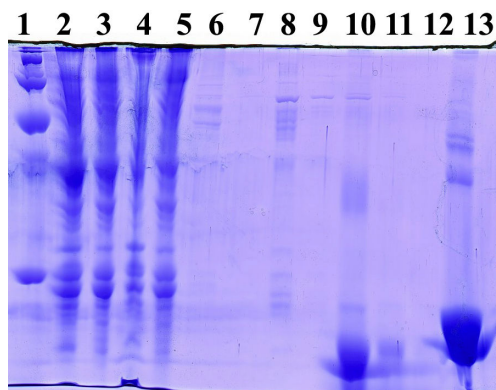


Figure 11: SDS-PAGE gel of Rac1 purification. Lane 10 shows purified Rac1, Lane 13 shows GST that is still bound to the immobilized glutathione.

Peptide Synthesis

The first peptides synthesized were the same as the β -amyloid peptides described in the Gordon *et al* paper. Their sequences were K(MeL)V(MeF)F and KLVFF.

Fluorescently tagged versions of these peptides were also made. The purpose of making these peptides was to practice peptide synthesis skills with N-methylated peptides known previously to work and to test membrane permeability of these peptides. Following this, inhibitory peptides were synthesized using the described techniques. These were to be used in any functional or inhibitory assays.

β -Amyloid Peptide

The first batch of K(MeL)V(MeF)F that was made was labeled with N-methylantranilic acid (MANT). It was analyzed by HPLC and was found to be of low purity. Low concentration of peptide prevented analysis by electrospray mass spectroscopy. The second batch of MANT-K(MeL)V(MeF)F behaved much more

reliably on the HPLC yet still had several impurities as can be seen from its HPLC chromatogram (Fig. 12).

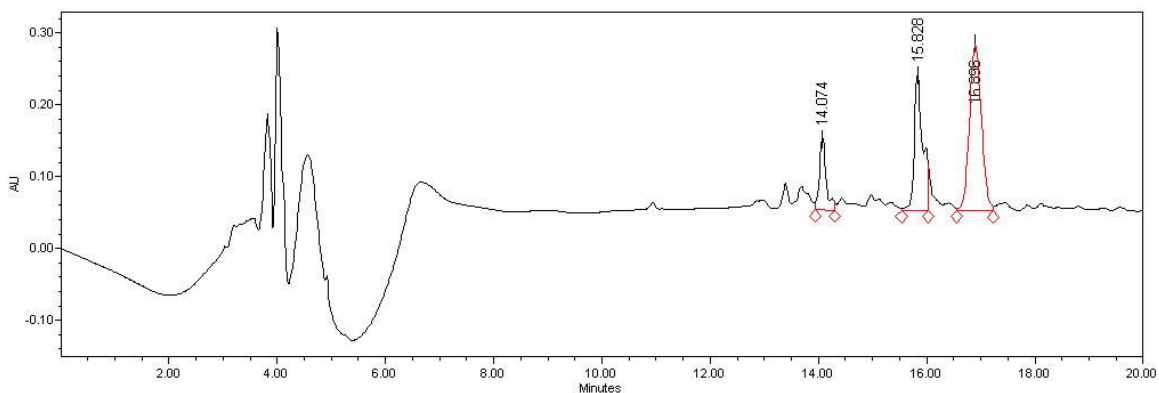


Figure 12: HPLC chromatogram of MANT-K(MeL)V(MeF)F batch 2. Product peak highlighted in red.

Impurity Analysis

The largest impurity, seen at 15.946 min in Fig. 12 (batch 2) and the proposed product peak, seen at 17.021 min in Fig. 12, were analyzed by MALDI. The mass analysis showed that the product peak was of the correct mass and that the impurity peak was off by 10 mass units. Professor Miller proposed that this change in mass would coincide with DMF (a solvent used for washing the resin) cyclizing between MANT and the N-terminal amine in the presence of TFA (Fig 13).

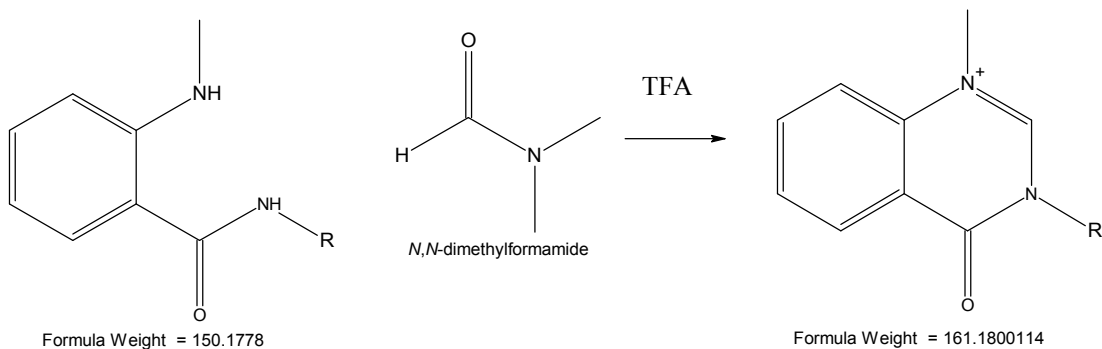
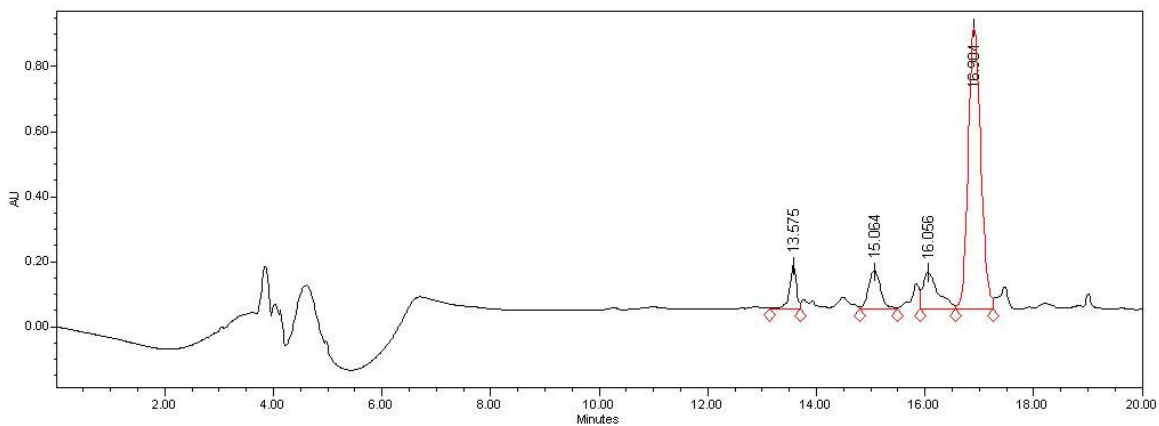


Figure 13: MANT attached to a peptide plus DMF in the presence of TFA forms the cyclized compound seen at the right.

After revising the procedure to exclude washing with DMF and synthesizing batch 3 of MANT-K(MeL)V(MeF)F there was a large reduction in the amount of impurity at that retention time. There was still, however, a significant impurity with a retention time of around 14 min. This impurity was thought to be a deletion peptide caused by incomplete couplings. To remedy this a fourth and final batch of MANT-K(MeL)V(MeF)F was prepared using acetic anhydride to cap after each step. This capping step would prevent any unreacted amines from reacting at some point later during the synthesis. The result was a much cleaner peptide with fewer impurities (Fig. 14) and the correct mass as determined by MALDI (Fig. 15). In the data from the MALDI there are other noticeable peaks after the product peak. These are due to the fact that the sample was not desalted before analysis.



**Figure 14: HPLC chromatogram of MANT-K(MeL)V(MeF)F batch 4.
Product peak highlighted in red.**

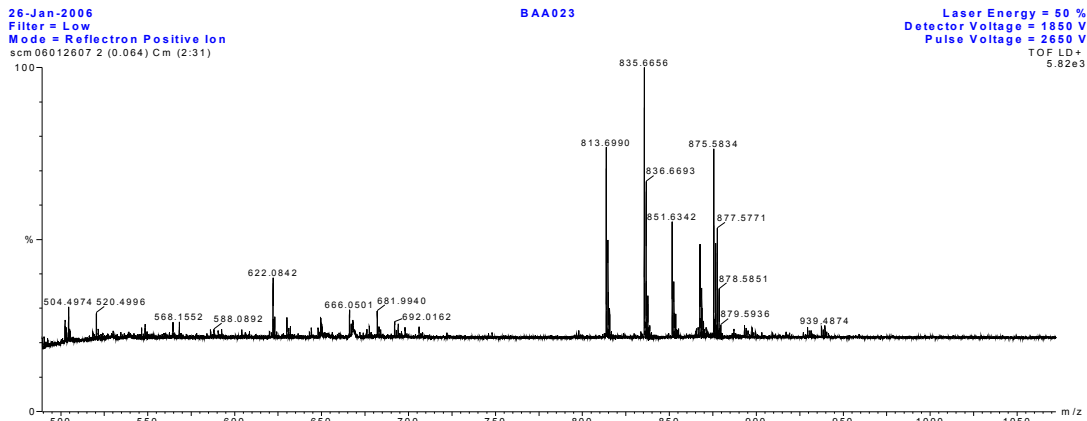


Figure 15: MALDI of MANT-K(MeL)V(MeF)F. Correct mass peak (M) at 813. 835 is M+ sodium (Na), 851 is M+ potassium (K), 875 is M+ copper (Cu).

Inhibitory peptides

Truncated versions of the Gao *et al* peptide were synthesized using the methods described. Peptides that were successfully synthesized were Ac-NLGLWDTAG-NH₂, Ac-WDTAG-NH₂ and Ac-W(MeD)T(MeA)G-NH₂. Difficulty optimizing HPLC for the peptides led to no chromatograms for the inhibitory peptides. The peptides were analyzed by MALDI prior to their acetylation. MALDI analysis (Fig. 16) showed that there was some amount of the correct peptide sequence for all inhibitory peptides, though the amount is difficult to quantify given the lack of HPLC data.

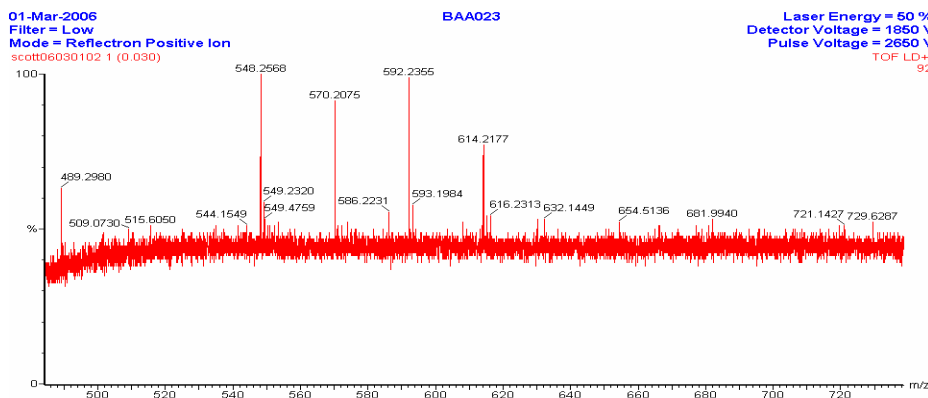


Figure 16: MALDI of unacetylated WDTAG. Correct mass peak at 548, other large peaks are salts.

Purification of Inhibitory Peptides

Purification of the inhibitory peptides was left at precipitation into cold ether. This would remove the bulk of non-peptide impurities. Purification of the β -amyloid peptides was initially removal of TFA by rotary evaporation followed by the collection of individual peaks from the HPLC for analysis by MALDI.

Cell Permeability Test

MANT-K(MeL)V(MeF)F was tested for cell permeability on HeLa cells as described previously. The excitation and emission wavelengths of MANT are similar to that of autofluorescence of the cells, but Fig. 17 shows that there is a definite increase in fluorescence dependant on concentration of MANT-K(MeL)V(MeF)F (Fig.17). Figure 17 also shows effective nuclear exclusion as can be seen by the darker spots in the middle of the cells.

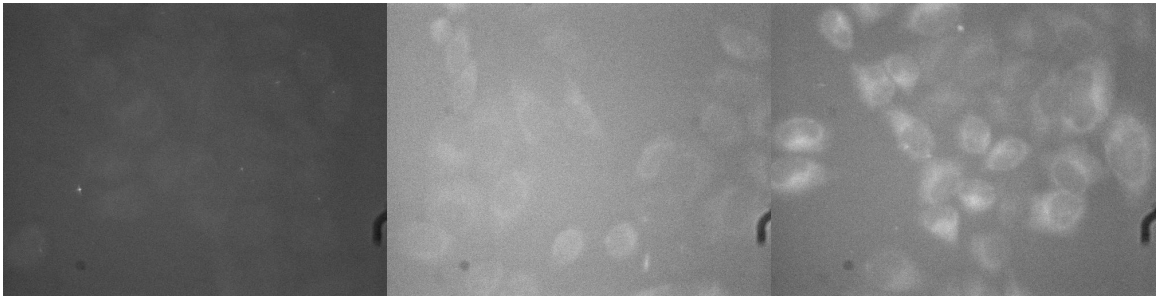


Figure 17: Cell permeability test using MANT-K(MeL)V(MeF)F. Left is untreated HeLa cells. Middle panel is cells treated with 10 μ M peptide. Right panel is cells treated with 100 μ M peptide.

Binding Assay

The binding assay that was performed was inconclusive. As can be seen from Fig. 18 there is no binding of GEF-H1 to Rac1 when there was no inhibitory peptide present. There also appeared to be no binding in the lanes treated with peptide. The untreated

protein was supposed to act as a positive control and without it there is no way to tell if there was any inhibition caused by the inhibitory peptides.



Figure 18: Preliminary inhibition assay using synthesized peptides. Lanes were loaded as follows. 1: Benchmark Ladder 2: Untreated Unbound (UB) 3: Untreated Bound (B) 4: Gao *et al* Peptide UB 5: Gao *et al* Peptide B 6: NLGLWDTAG UB 7: NLGLWDTAG B 8: WDTAG UB 9: WDTAG B 10: W(MeD)T(MeA)G UB 11: W(MeD)T(MeA)G B 12: Rac1 13: GEF-H1 14: Benchmark Ladder

DISCUSSION

Proteins

Initial attempts to purify Rac1 using a pGEX-4T vector, which contains a thrombin cleavage site, were ineffective. Thrombin has to be purified using benzamidine sepharose beads which experience a lot of unspecific binding. As a result of this, there was marked loss of Rac1 during purification. The switch to a pGEX-6P1 vector which contains a PreScission Protease site proved to be much more effective. The reason for this being that PreScission Protease is also expressed as a GST fusion protein. This allows it to cleave Rac1 from the glutathione beads then attach to the beads itself, effectively cleaving and purifying itself out in one step. The Rac1 that was expressed and purified during the course of this project is the right construct and has shown to be active in experiments done by other members of the lab for different purposes. The protein is in frozen aliquots and ready for use in any number of inhibition assays. The limiting factor that prevented assays from being done was active GEF-H1. The GEF-H1 was expressed and had the right sequence. The problem came when attempting to remove it from the immobilized glutathione beads. The PreScission Protease that was used to cut it from the beads was found to be inactive, therefore the GEF-H1 remained stuck to the beads. In the time spent trying multiple times to cleave the protein from the beads there was a significant amount of degradation of the protein. This explains the lack of activity seen in the GEF-H1/Rac1 pulldown assay (Fig. 18).

Peptides

Based on previous studies it is known that an inhibitory peptide can block the interaction between Rac1 and GEF-H1. The crystal structure of Rac1 and TiamI, which interacts in the same way as GEF-H1, shows that there is a portion of the inhibitory peptide made by Gao *et al* that does not come in contact with the GEF at all. This would lead us to believe that the Ac-NLGLWDTAG-NH₂ peptide would block the interaction just as well. There is also a significant chance that the shorter truncation, Ac-WDTAG-NH₂, would work also. This is because the most significant attachment points to the GEF seem to be at N52, W56 and G60. N-methylation should also increase activity of the inhibitor because it will reduce the entropic cost of forming an extended conformation.

The problem with the N-methylation and with the sequences of the peptides is that synthesis, purification, and analysis become more difficult. Synthesis of N-methylated peptides requires longer coupling times which still do not necessarily go to completion because of the nature of the secondary amine found on N-methyl amino acids. When an amino acid is not commercially available with the N-methyl substitution the problem of methylating it arises. These problems are easily fixed, but the fix can be time consuming.

Synthetic obstacles aside, the purification and analysis of these peptides has become the largest problem. The inhibitory peptides would not run reliably on the HPLC making it difficult to assess the purity of the compounds. This was a problem due more likely to the sequence than the N-methylation because the N-methylated β -amyloid peptide was easy to optimize on HPLC. Furthermore, the inhibitory peptides had to be analyzed by MALDI before acetylation. After acetylation the peptides were more

difficult to ionize and as a result would not show as easily by MALDI. Though these problems have potential solutions there was not enough time in the scope of this project to optimize them.

Cell Permeability

Testing of MANT-K(MeL)V(MeF)F on HeLa cells showed that the peptide is cell permeable. The data showed an increase in fluorescence, but that is difficult to quantify given the similarity between background autofluorescence of the cells and the fluorescent spectra of MANT. What is important is the nuclear exclusion that was seen. This shows that the peptide is in the cells as opposed to attached to the surface. Had it not been permeable, there would be no dark spot where it was left out of the nucleus. Attempts were made to couple K(MeL)V(MeF)F to the bright green fluorophore BODIPY-FL (Invitrogen) and a bright near-IR oxazine derivative prepared by a member of the lab. Coupling to BODIPY-FL was successful, but the treatment with TFA to cleave the peptide from the resin inactivated its fluorescence (data not shown). The oxazine derivative was stable to TFA, but there was little coupling to the peptide sequence (data not shown). No attempts were made to label the inhibitory peptides due to inability to purify and analyze properly.

Future Research

The proteins and peptides made during this project will be useful to future experiments. The Rac1 is functional and the inhibitory peptides are of the correct sequence. The peptides will require some equipment optimization to get them to a desired

purity and to analyze their mass after acetylation. Provided that functional GEF-H1 can be obtained, the peptides will be very useful in binding inhibition assays as well as assays to test for GEF-H1 functionality. Given the proper amount of time one would be able to determine whether N-methylated peptides are good candidates for blocking these types of interactions.

BIBLIOGRAPHY

1. Bishop, Anne L., and Alan Hall. Rho GTPases and Their Effector Proteins. *Journal of Biochemistry* (2000) 348: 241-255.
2. Burridge, K. and K. Wennerberg. Rho and Rac Take Center Stage. *Cell* (2004) 116(2):167-179.
3. Gao, Yuan et al. Trp56 of Rac1 Specifies Interaction with a Subset of Guanine Nucleotide Exchange Factors. *The Journal of Biological Chemistry* (2001) 276, 50: 47530-47541.
4. Gordon, D. J., R. Tappe, S. C. Meredith. Design and Characterization of a Membrane Permeable N-Methyl Amino Acid-Containing Peptide that Inhibits A β 1-40 Fibrillogenesis. *Journal of Peptide Research* (2002) 60: 37-55.
5. Guzzetta, Andrew. "Reverse Phase HPLC Basics for LC/MS." Ionsource (Last updated March 10, 2006)
<http://www.ionsource.com/tutorial/chromatography/rphpc.htm> (Accessed April 20, 2006).
6. Miller, Stephen C. and Thomas S. Scanlan. Site-Selective N-Methylation of Peptides on Solid Support. *Journal of the American Chemical Society* (1997) 119:2301-2302.
7. Mott, Helen R. et al. Structure of the Small G protein Cdc42 Bound to the GTPase Binding Domain of ACK. *Nature* (1999) 399: 384-388.
8. Owen, Darerca et al. Residues in Cdc42 That Specify Binding to Individual CRIB Effector Proteins. *Biochemistry* (2000) 39: 1243-1250.
9. Rizo, J. and L. M. Gierasch. Constrained Peptides: Models of Bioactive Peptides and Protein Substructures. *Annual Review of Biochemistry* (1992) 61: 387-416.
10. Rossman, Kent L., and John Sondek. Larger than Dbl: New Insights Into RhoA Activation. *Trends in Biochemical Science* (2005) 30(4):163-165.
11. Sprang, Stephen R., David E. Coleman. Invasion of the Nucleotide Snatchers: Structural Insights into the Mechanism of G Protein GEFs. *Cell* (1998) 95: 155-158.
12. Wikipedia contributors, "Lamellipodia," Wikipedia, The Free Encyclopedia, <http://en.wikipedia.org/w/index.php?title=Lamellipodia&oldid=40709319> (accessed February 28, 2006).

13. Worthylake, David K., Kent L. Rossman and John Sondek. Crystal Structure of Rac1 in Complex With the Guanine Nucleotide Exchange Region of Tiam1. *Nature* (2000) 408: 682-688.
14. Yamazaki, D., S. Kurisu and T. Takenawa. Regulation of Cancer Cell Motility Through Actin Reorganization. *Cancer Science* (2005) 96(7):379-386.
15. Zheng Y. Dbl Family Guanine Nucleotide Exchange Factors. *Trends in Biochemical Science* (2001) 26(12):724-732.