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Chymotrypsin Digestion Analysis to Characterize Site-Specific Incorporation of an Extrinsic Fluorescent Probe on a Ras-related Protein.

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Chymotrypsin Digestion Analysis to Characterize Site-Specific Incorporation of an Extrinsic Fluorescent Probe on a Ras-related Protein.

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By

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Abstract:

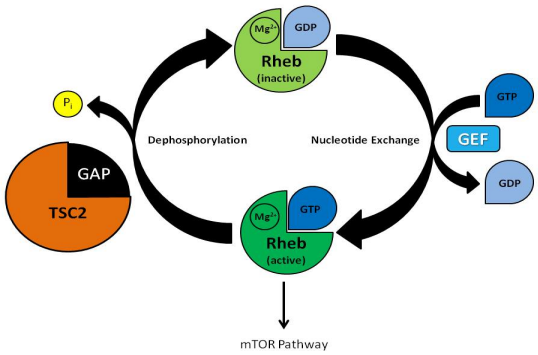
Ras Homolog Enriched in Brain (RHEB) is a member of Ras GTPase family and plays an important role in regulation of cell growth and cell cycle proliferation. RHEB is regulated by cycling between active (GTP-bound) and inactive (GDP-bound) state. Guanidine Nucleotide Exchange Factors (GEFs) and GTPase-activationg proteins (GAPs) are the key regulators of RHEB, and it is of interest to closely study their interactions with RHEB. Towards that end, this project was designed to label RHEB with an environmentally sensitive extrinsic fluorophore, succinimidyl 6-(N-(7-nitrobenz-2-oxa-1.3-diazol-4-yl)amino)hexanoate (sNBD), to be used to monitor RHEB-protein interactions. Due to its extreme sensitivity to the surrounding environment, sNBD is expected to show changes in fluorescence when labeled RHEB reacts with other protein effectors. Histidine tagged RHEB was expressed using E. coli bacteria as host cells and was purified using Histidine affinity column chromatography. Time-dependent chymotrypsin digestion experiments were performed in order to compare digestion patterns of RHEB and sNBD-labeled RHEB. Using the purified protein, RHEB-sNBD binding procedure was optimized. A modified purification procedure had to be designed in order to meet the buffer environment and concentration requirement for the RHEB-sNBD binding procedure. The RHEB-sNBD binding experiment was determined to work best with 1:24 molar ratio of RHEB to sNBD. Successful RHEB-sNBD binding was performed using PBS buffer as the solvent condition for protein, and DMF as the solvent for sNBD.

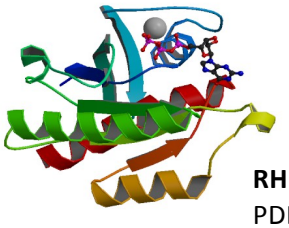
I) Introduction:

Ras-related GTP-binding proteins are a superfamily, whose members are conserved across all eukaryotic organisms. Their extraordinary evolutionary conservation suggests that they have essential cellular functions (Santos, 1989). Ras proteins have been found to be involved in a variety of different biological pathways including the regulation of cell growth and differentiation, vesicular transport, and cytoskeletal organization. Deregulation of these proteins has been associated with multiple disease conditions. The structural, functional, and biochemical similarities of Ras proteins with other G-proteins suggest that they are involved in signal transduction from cell surface (Santos, 1989). In all cases, these GTP-binding proteins act as molecular switches by cycling between GTP-bound active state and GDP-bound inactive state (Nomanbhoy 1996). This GTP cycling is closely regulated by Guanidine Nucleotide Exchange Factors (GEFs) and GTPase-activationg proteins (GAPs), which are specific for each GTP-binding protein (Marshall 2012). GTPase activating proteins (GAPs) promote hydrolysis of GTP, thus impeding the biological activity of GTPases, whereas guanine nucleotide exchange factors (GEFs) promote exchange of GDP for GTP and activate GTPase proteins (Figure-1).

Ras Homolog Enriched in Brain (RHEB) is a member of the superfamily of Ras-related GTP-binding proteins and has been linked with regulation of cell growth and cell cycle proliferation. The biological activity of RHEB is controlled in part by an important protein interaction with a GTPase activating protein (GAP) Tumor Suppressor Complex (TSC2), which stimulates the GTPase activity of RHEB (Figure-1) (Marshall 2009).

A. B.





RHEB
PDB ID: 1XTS

Figure – 1: (A.) GTPase activity of RHEB and functions of GAP, GEF. (B.) RHEB

RHEB plays an important role in the regulation of cell growth via the mammalian Target Of Rapamycin (mTOR) pathway (Marshall 2009). mTOR is a signaling pathway, which transcends signal from external messengers such as growth factors and hormones into appropriate cellular processes such as proliferation and cell growth. When activated by TSC-2 in response to an upstream signal, RHEB binds to and activates mTOR Comlex-1, which in turn activates protein synthesis and growth (Figure-2).

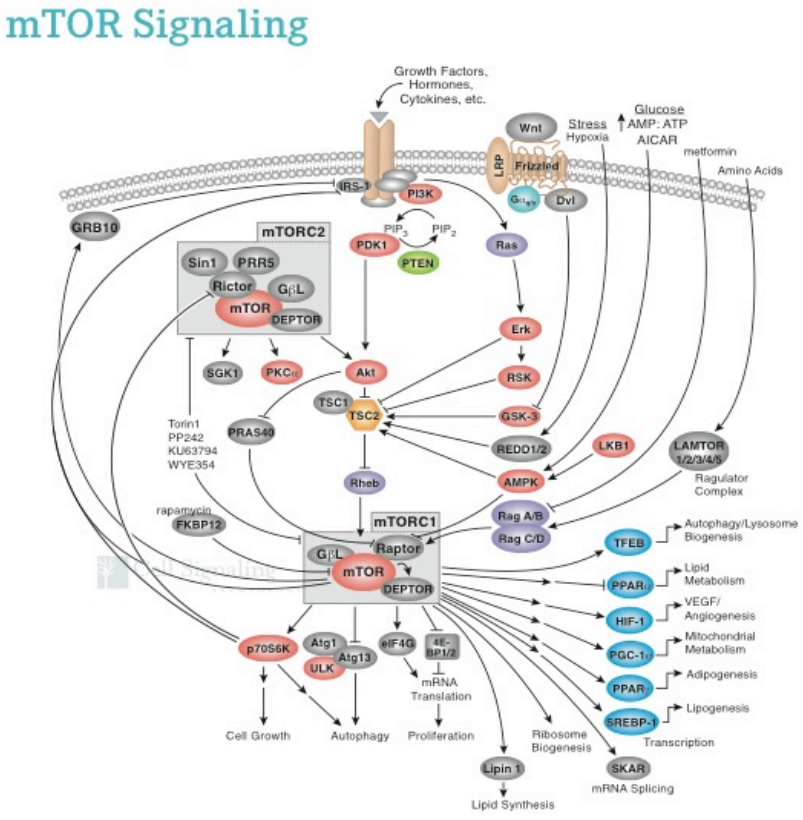


Figure-2: mammalian Target Of Rapamycin (mTOR) pathway (Cell Signaling Technology Inc.)

The mammalian target of rapamycin (mTOR) signaling pathway is implicated in the pathogenesis of a number of cancers and inherited hamartoma syndromes (Dunlop, 2009). GTP hydrolysis of RHEB by TSC2 (GAP) has been an area of particular research interest in recent years, in part because a mutant of TSC2 has been found in Tuberous Sclerosis patients, which could lead to tumor development (Marshall 2009). A 218 amino acid long derivative of TSC2 has been successfully shown to bind to RHEB and increase its GTP hydrolysis activity (Marshall 2009). It has also been shown that the mutant of TSC2 found in Tuberous Sclerosis patients, TSC2-218 K114A, does not bind to RHEB to increase its GTPase activity (Morris 2013). While this provides valuable insight about the effect of wild-type vs. mutant TSC2-218 on RHEB, the actual mechanism for the interaction between RHEB and TSC2 has not yet been characterized. This is partly due to the lack of knowledge about the exact structure of TSC2 protein. One possible way to characterize this interaction might be by using aromatic amino acid tryptophan (Trp) as an intrinsic fluorophore and performing binding assay between RHEB and TSC2-218. However, a major drawback of this approach is that it would be nearly impossible to differentiate between fluorescence from the intrinsic Trp fluorophore on RHEB and from that on TSC2-218 as they both contain Trp.

This project focuses on using extrinsic fluorescence to study RHEB-protein interactions. An environmentally sensitive extrinsic fluorophore succinimidyl 6-(N-(7-nitrobenz-2-oxa-1.3-diazol-4-yl)amino)hexanoate (sNBD) was used for this purpose. sNBD is a commonly used extrinsic fluorophore, which is selective towards lysine residues, and shows fluorescence at 488 nm (Figure-3).

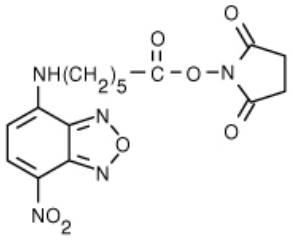


Figure-3: sNBD: *NBD-X, SE, Succinimidyl 6-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)Hexanoate*. Digital image. *Life Technologies*. N.p., n.d. Web. 30 Jan. 2014.

Because of its extreme sensitivity to the external environment, once bound to RHEB, sNBD can provide highly detailed and accurate information about the changes in configuration of RHEB as it interacts with TSC2-218, and very small concentrations of protein can be used to acquire valid results (Nomanbhoy 1996). First step towards this was to express Histidine tagged RHEB using E.coli bacteria as host cells. Next, the protein was purified using affinity chromatography. This purified protein was then used to optimize binding between RHEB and sNBD. Next, time-dependent chymotrypsin digestions were used to analyze digestion of wild-type RHEB and sNBD-labeled RHEB; these two digestions were compared with each other to study changes in conformation of labeled RHEB.

II) Experimental Methods:

The first procedure in this experiment was the overexpression of RHEB. Second step was purification of the protein. Next, limited chymotrypsin digestion was performed on the purified RHEB. Purified RHEB was then used to optimize RHEB-sNBD binding. For this experiment, the purification procedure was modified to suit the experimental conditions.

RHEB Expression:

E.coli bacteria were used as host organisms to overexpress proteins. The bacterial cells were genetically modified to contain genes for histidine tagged RHEB. The his-tag includes six histidine residues at the end of the RHEB gene. His-tag allows for purification of protein by affinity chromatography since imidazole groups on histidine residues have high affinity towards Ni^{2+} column. The vector carrying the gene of interest and an Ampicillin (antibiotic) resistant region selectively allows the growth of RHEB only in a medium with ampicillin.

A seed culture was made by adding the genetically modified E.coli bacteria in LB (Lysogeny Broth) medium with 0.1 mg/mL ampicillin. The culture was allowed to grown overnight at 37^0C. This overnight culture was used to inoculate 1-2 liters of fresh LB and that was allowed to grow while shaking at 37^0C until the optical density (OD_{600}) reached 0.6-0.8. The optical density (measured at 600nm) was optimized to be 0.6-0.8 for RHEB as per previous experiments (Yu, Y et al 2014, Morris 2013). Next, the cells were induced with final concentration of IPTG (isopropyl-β-D-thioglactopyranoside) and allowed to express at 37^0C for 4 hours. IPTG inhibits lac-repressor, allowing for

transcription and translation in bacterial cells (Chou 2008). The cells were harvested by centrifugation at 6500 rpm for 20 minutes at 4^0C. The pellet was resuspended in 10mM Tris (pH 8) and centrifuged again. The cell pellets were stored at -80^0C.

RHEB Purification:

Affinity chromatography was utilized to purify RHEB. The histidine residues in the His-tag attached to RHEB include imidazole groups, which have high affinity towards Ni^{2+}. Therefore, Ni^{2+} column was used to purify RHEB. Buffers containing increasing concentrations of imidazole (Figure - 4) were used to load and elute RHEB from the column.

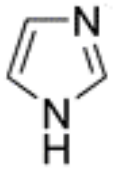


Figure-4: Imidazole, competitive binder of His tag (Chou, 2015)

To begin the purification process, each 1-liter pellet of cells was thawed on ice and resuspended in lysis buffer (PBS Ni^{2+} binding (PBS pH 8.0, 50 mM $MgCl_2$, and 25 mM imidazole), 25 mg lysozyme, Halt protease inhibitor cocktail to a final concentration of 0.5x, DNAse, and 1 mM PMSF). Lysis buffer helps break up cell components. Cells were then lysed via sonication on ice (3 second pulse with 5 second pause, 40 repetitions). The cell debris and the soluble fraction were separated by centrifugation at 18,000 rpm for 25 minutes at 4°C (Morris 2013). The cell lysate as well as PBS Ni^{2+} binding buffer and PBS Ni^{2+} elution buffer (PBS pH 8.0, 50 mM $MgCl_2$, and 400 mM imidazole) were filtered before purification on FPLC using a 5-mL prepacked Ni_{2+} affinity column (GE Healthcare HiTrap His Column) (Figure-5). After the column was equilibrated with 5 CV (column volume) PBS Ni^{2+} binding buffer, the cell lysate was loaded over the column. A linear gradient was used to increase the

imidazole concentration for elution of the purified protein. The protein was either dialyzed overnight at 4°C against the required buffer for the next experiment or lyophilized for storage (Morris 2013). Samples of lysate, flow through, and purified RHEB were collected during the purification procedure and later run on an SDS-PAGE gel (Figure-9).

Figure-5: Ni^{2+} affinity (HisTrap columns)

GE Healthcare Life Sciences. GE Healthcare, n.d. Web. 13 Feb. 2014.

To activate the purified RHEB, a nucleotide exchange was performed using GMPPNP (Guanosine 5′-[β,γ-imido]triphosphate trisodium salt hydrate), a non-hydrolysable analog of GTP (figure-6). His-tagged RHEB was first incubated with 5 mM EDTA and 0.5 mM GMPPNP for 1 hour at room temperature. Following incubation, EDTA and excess nucleotide were removed with an equilibrated desalting column (GE Healthcare PD-10). After desalting, 10 mM Mg^{2+} was replaced.

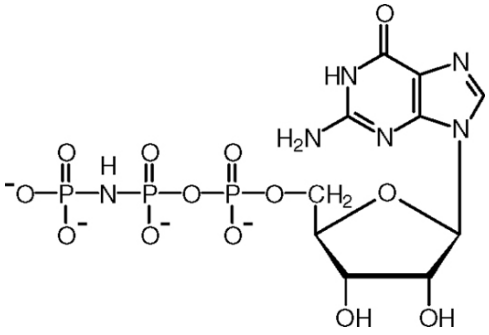


Figure-6: GMPPNP (Guanosine 5′-[β,γ-imido]triphosphate trisodium salt hydrate)

(CAS Number 148892-91-5)

Limited Chymotrypsin Digestion:

Chymotrypsin is a digestive enzyme that cleaves at C-terminal ends of tyrosine, tryptophan, and phenylalanine (Berg 2007). Time dependent chymotrypsin digestion was utilized in order to study digestion patterns of RHEB and use the digested fragments for

further experiments. Based on the known sequence of RHEB and digestion sites of chymotrypsin, digestion sites can be predicted for RHEB (Figure-7).

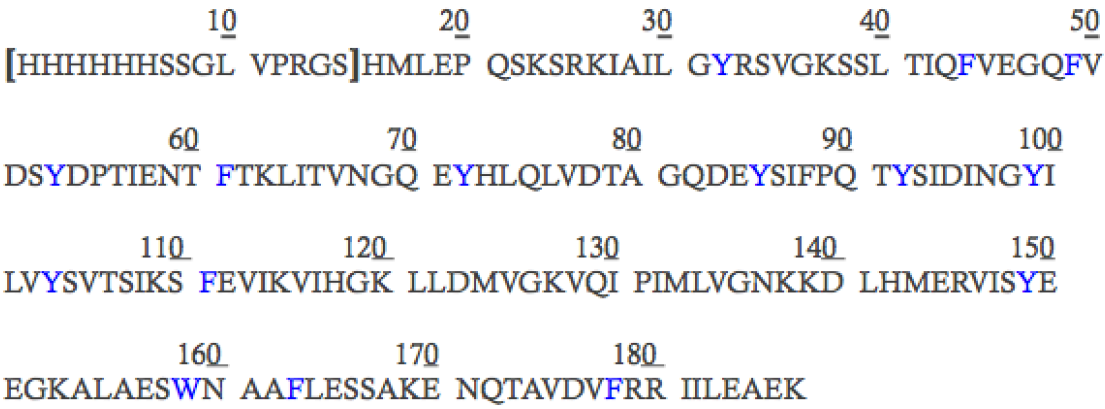


Figure-7: Chymotrypsin digestion sites on RHEB

Multiple chymotrypsin digestion experiments were performed using varying enzyme to protein molar ratio, and the optimized ratio was determined to be 1:10 (enzyme to protein). This ratio facilitates gradual digestion of protein, allowing for time dependent collection of digestion data. The digestion experiment was performed at constant temperature of 25^0C. Samples of the reaction mixture were collected at 0, 5, 10, 15, 30, 45, and 60 minutes. To 50 µL of each of these samples, 5 µL of 10% SDS was added, the mixture was boiled for 5 minutes, and stored to be run on an SDS PAGE later. All of these samples were run on an SDS PAGE gel along with pure RHEB and pure chymotrypsin to observe the digestion pattern (Figure-11).

sNBD incorporation:

RHEB-sNBD binding procedure was derived from previous experiment performed by Dr. Paul Adams, and from a similar experiment performed on other Ras proteins (Nomanbhoy 1996, Adams, unpublished data). This procedure was also

optimized after performing it multiple times using varying parameters. The major parameters that were optimized were RHEB to sNBD molar ratio, and buffer conditions. In terms of RHEB to sNBD ratio, 1:12 and 1:24 were two ratios that were performed. In terms of the buffer conditions for the protein, 2 different buffers were used. One of them was the PBS Ni^{2+} binding buffer (PBS pH 8.0, 50 mM $MgCl_2$, and 25 mM imidazole) used in the purification procedure. The second buffer was called NMR buffer (25mM NaCl, 5mM NaH_2PO_4, 5mM $MgCl_2$, 1mM NaN_3, pH 7.2).

Knowing the amino acid sequence of RHEB, and that sNBD is selective for lysine residues, possible sNBD binding sites on RHEB can be predicted (Figure-8). However, from previous experiment, it was expected that sNBD would occupy only occupy one binding site on each RHEB (Adams, unpublished data).

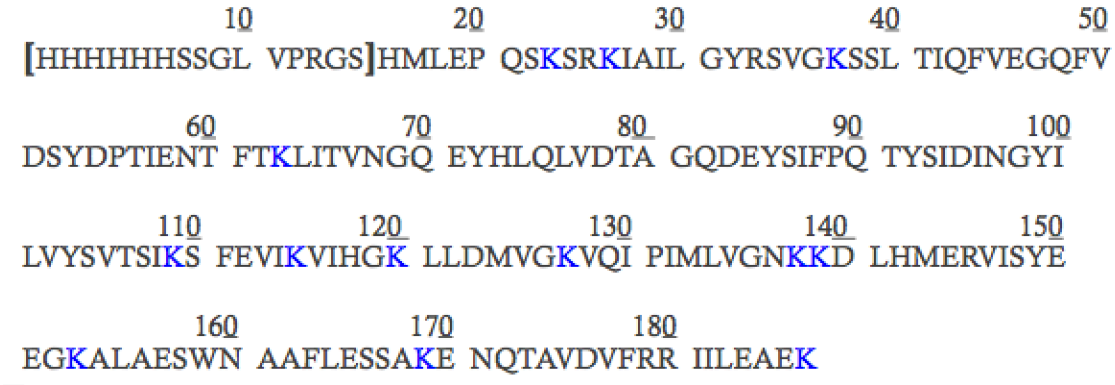


Figure – 8: sNBD binding sites on RHEB

As a first step in the binding experiment, purified RHEB was resuspended and/or dialyzed into either PBS or NMR buffer. Next, appropriate amount of sNBD was dissolved in DMF (dimethylformamide) based on either 1:12 or 1:24 ratio of RHEB to sNBD. DMF provides ideal condition to dissolve sNBD. The protein and sNBD solutions were mixed and allowed to incubate at room temperature for 1 hour. The reaction mixture

was then centrifuged (18,000 rpm, 20mins) to remove white precipitate and unreacted sNBD. At this point, the mixture contained both bound as well as some unbound sNBD. To clear of the unbound sNBD, the mixture was vigorously dialyzed overnight in NMR buffer using 6000-8000 Da dialysis bags. Due to sNBD being extremely small (391 Da) in comparison to RHEB (21 kDa), any unbound sNBD would move out of the solution and into the NMR buffer. After overnight dialysis, the solution was analyzed for presence of sNBD (absorbance at 488 nm) using UV-Vis spectrophotometer. The UV spectrum (200 – 550 nm) for this solution was plotted along with the same spectra for blank (water), pure protein, pure sNBD, and the mixture before dialysis (Figure-12).

Modified purification procedure:

As mentioned earlier, some variations of the RHEB-sNBD binding experiment required protein to be in NMR buffer. However, according to the purification procedure, the protein was in PBS buffer at the end of the purification. One option to transfer protein from PBS to NMR buffer was to dialyze in NMR buffer overnight. However, multiple overnight dialyses tended to decrease protein concentration, and inadequate protein concentrations did not provide clear results for binding experiments. Therefore, the purification procedure was modified to incorporate the NMR buffer from the beginning of the procedure. The modified purification procedure included NMR binding buffer (NMR buffer + 40mM imidazole) and NMR elution buffer (NMR buffer + 250 mM imidazole). This modification allowed for sNBD binding experiment directly after purification, without having to dialyze and risk the chances of loosing protein concentration. An SDS PAGE gel showing results for this modified purification procedure is included in results section (Figure-10).

III) Results and Discussion:

Purification:

SDS PAGE is an electrophoresis technique that is widely used to separate molecules based on their molecular weight (Berg 2007). Figure-9 represents an SDS PAGE showing samples collected at different stages of the purification process. Lane-1 is the molecular weight ladder, accompanied by weights corresponding to each band. Lane-2 represents lysate, which is the mixture of proteins before purification. Lane-3 shows the flow-through. This is the solution collected as lysate is being loaded onto the affinity column. This lane does not show a band at 21 kDa, because RHEB was bound to the column at this point due to its affinity towards the column. Lane-4 represents the final product of the purification process.

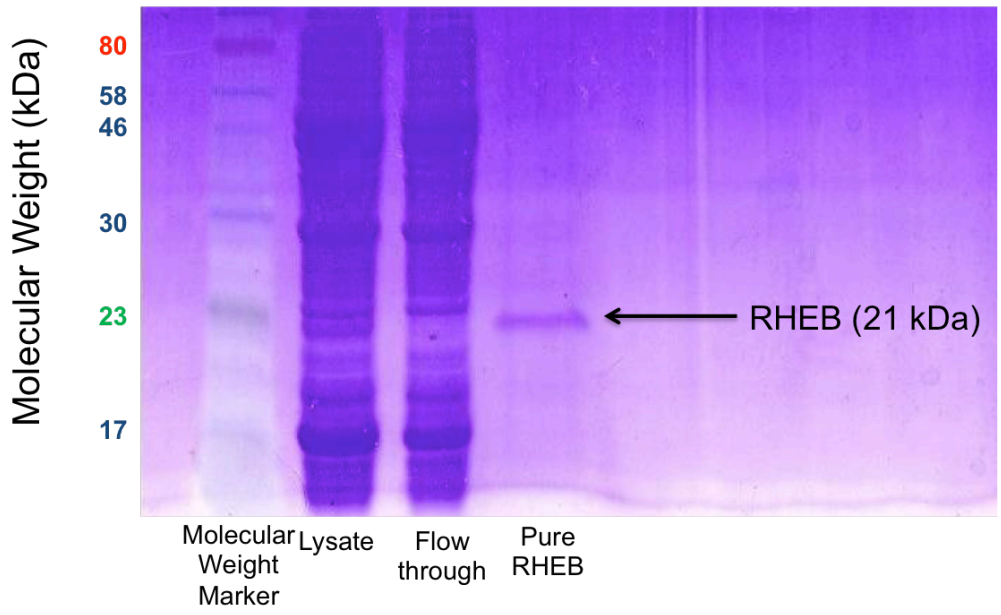


Figure – 9: RHEB purification gel.

Modified Purification:

As the use of NMR buffer in RHEB-sNBD binding experiment and excessive loss of protein due to multiple dialyses required the use of modified procedure, NMR binding and NMR elution buffers were used to purify RHEB while keeping rest of the purification procedure the same. Figure-10 represents an SDS PAGE with lysate, flow through, washout, purified RHEB, and RHEB after nucleotide exchange. While bands for RHEB are apparent, there are at least four other bands that might be other proteins. For the sake of moving forward using this RHEB, these other bands or impurities were in significant concentration that they would interfere in the sNBD binding experiment. Therefore, the sNBD binding experiment was carried forward only using RHEB purified using regular procedure. One way to utilize the protein seen in the gel blow would be to perform multiple stepwise purifications on the products of this experiment. However, that option did not seem to be very efficient in terms of time consumption and product yield as compared to the regular purification procedure.

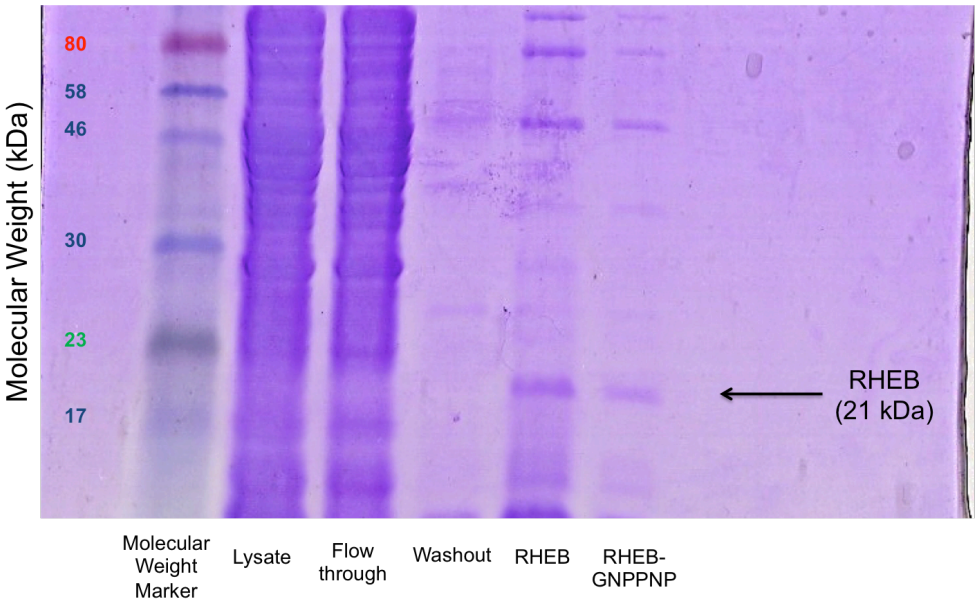


Figure – 10: RHEB purification gel for modified procedure

Chymotrypsin Digestion:

Figure-11 represents the SDS PAGE for time-dependent chymotrypsin digestion (1:10 RHEB to Chymotrypsin ratio). Lanes 2 and 3 represents pure RHEB and pure Chymotrypsin respectively. Lanes 4-10 represent samples collected at different time intervals. As we follow the 21 kDa RHEB band through the gel, it decreases with time, representing gradual digestion. At the same time, bands of much lower molecular weights appear and get thicker moving across the gel. These bands represent the digested fragments of the protein. Other bands observed on the gel are from chymotrypsin.

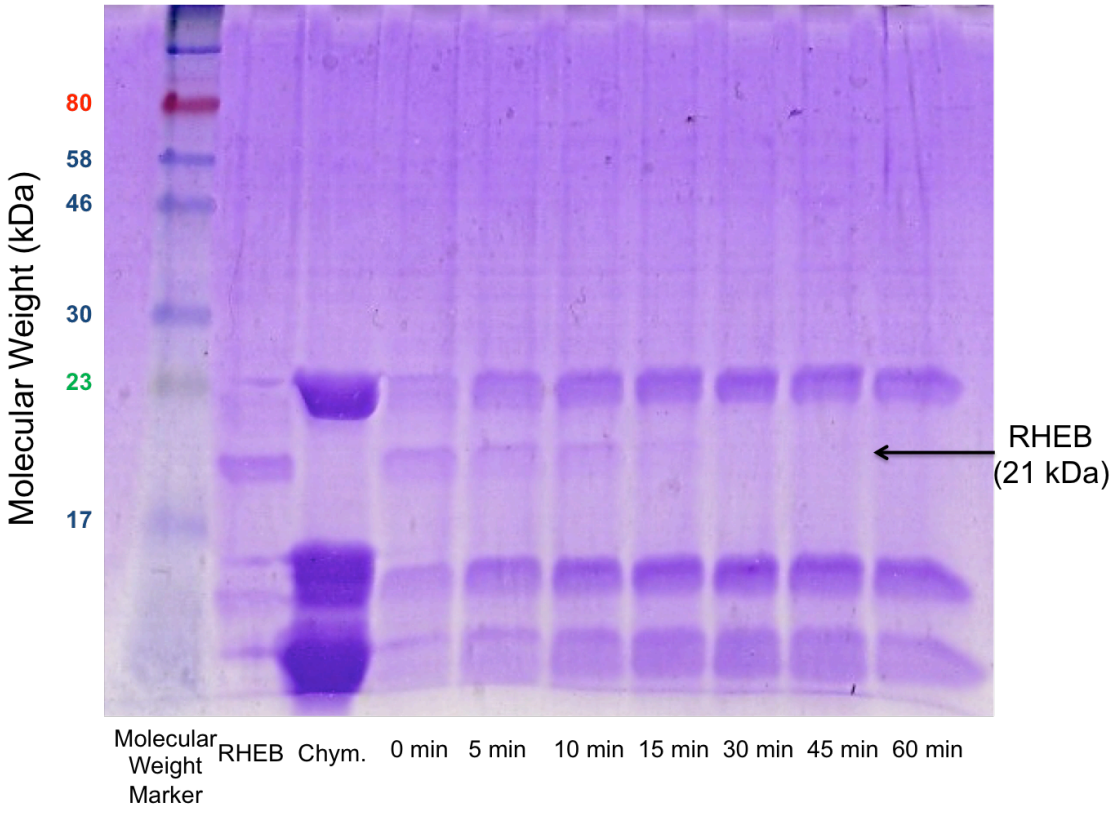


Figure – 11: Time-dependent chymotrypsin digestion of RHEB

RHEB-sNBD binding:

The binding experiment was performed using two different RHEB to sNBD ratios (1:12 and 1:24) and using two different buffers (NMR and PBS). However, for the reasons described in the previous sections and complications with the modified purification procedure, the binding could not be performed using NMR buffer. Also, RHEB to sNBD ratio of 1:12 was not enough to be able to detect any sNBD (488nm peak) in the solution after dialysis.

The Figure-12 represents the UV-Vis spectrum for binding experiment performed using the RHEB in PBS buffer (original purification procedure) and sNBD in DMF (dimethyl formamide) with 1:24 ration of RHEB to sNBD. Water was used as the blank for this procedure. Compared to the blank, the absorbance spectrum for RHEB shows a peak around 280 nm (peak for intrinsic flurophore tryptophan), showing the presence of protein. The spectrum for pure sNBD shows multiple peaks, with one around 488 nm being sNBD, and other peaks being components of the solvents. While these other peaks are not identified, they stay constant for all of the solutions that contain sNBD. Therefore, it is safe to assume that they are from some component(s) of sNBD solution, and do not affect the reaction at hand. The spectrum labeled "RHEB-sNBD before dialysis" represents the solution after centrifugation, but before dialysis. At this point, there is both bound and unbound sNBD present in the solution. This is consistent with the fact that sNBD peak in the spectrum is of high intensity compared to the peak for protein. Next, the spectrum labeled "RHEB-sNBD after dialysis" represents the solution after it is dialyzed vigorously overnight. This spectrum shows peaks for both protein (280 nm) and sNBD (488 nm). Assuming that overnight dialysis cleared all of the unbound sNBD, this

spectrum shows successful binding between RHEB and sNBD. Also, since the peak height for both protein and sNBD are about the same, it is also consistent with the information known from previous experiment that there should be only 1 sNBD bound per RHEB (one to one ratio). However, further experimentation is needed to verify this information.

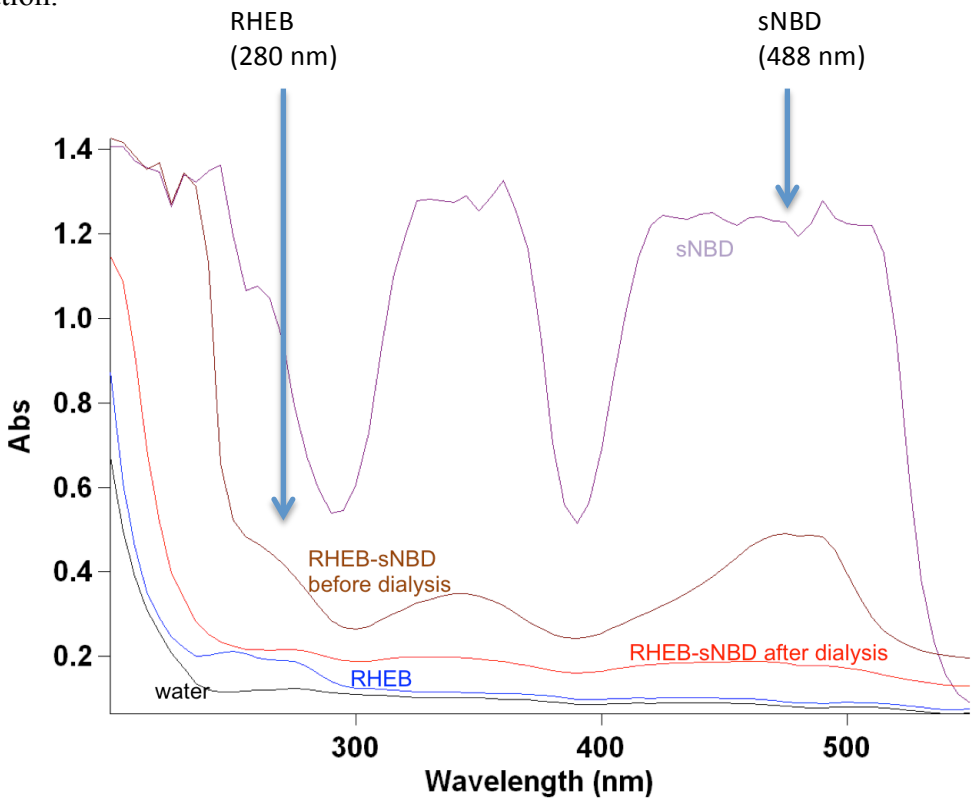


Figure – 12: RHEB-sNBD binding (1:24) absorbance spectrum

IV) Conclusion:

Ras Homolog Enriched in Brain (RHEB) is a Ras GTPase protein that plays a critical role in regulation and cell proliferation. Disturbances in interactions of RHEB with effectors could lead to detrimental effects. The expression and purification procedure used for RHEB have been well developed, and optimized. They give excellent results in terms of purified RHEB. While modified purification procedure using NMR binding and elution buffer seems to have potential to make RHEB-sNBD binding more efficient, the procedure still needs to be optimized.

Optimization of time-dependent chymotrypsin digestion revealed that the procedure works well with 1:10 ratio of enzyme to protein. Chymotrypsin digestion analysis was originally designed to be able to compare the digestion patterns of pure and sNBD-labeled RHEB. However, time constraint did not allow for a successful chymotrypsin digestion experiment with labeled RHEB. That is something that remains to be carried out. In addition, once chymotrypsin digestion can be successfully performed on labeled RHEB, next step would be to separate the digestion fragments using HPLC and analyze individual fragments to locate sNBD label on RHEB.

Based on the experimental results, RHEB-sNBD binding procedure seems to work best using 1:24 ratio of RHEB to sNBD. This binding experiment was successfully performed using PBS buffer as the solvent for RHEB, and DMF as the solvent for sNBD. An assumption that allowed us to conclude successful binding between RHEB and sNBD was that all of unbound sNBD was removed from the reaction mixture after a vigorous overnight dialysis. Looking at the spectrum for RHEB-sNBD after dialysis, it seems like

there is 1:1 binding between RHEB and sNBD. However, further experiments need to be performed in order to be able to confirm that.

Future directions with this project will include determination of binding site of sNBD on RHEB, GTP hydrolysis using labeled RHEB, and RHEB-TSC2 binding experiments using labeled RHEB in order to gain insight into the reaction mechanism of these two proteins.

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