Expression and Structural Characterization of Rab8 Protein

Thomas Kenneth Spain Jr.

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Expression and Structural Characterization of Rab8 Protein

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May 1, 2006

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Date: 4 May 2006
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Abstract

Rab8 is a member of a class of small GTPase proteins involved in the trafficking of vesicles within eukaryotic cells. Rab8 has been identified as a key protein in the transport of vesicles from the trans-Golgi network to the plasma membrane. The Laboratory for Structural Biology is primarily interested in the role of Rab8 as a binding partner for the protein FIP-2, also called optineurin. Optineurin functions as an effector protein for Rab8 transport and links Rab8 to other cellular proteins, including huntingtin. Huntington has been identified as the culprit in the hereditary neurodegenerative disorder Huntington's Disease. The role of Rab8 in the process of Huntington's Disease is unknown. The major goal of this research was to obtain information on the structure of Rab8, revealing information about its function. Previous efforts to express and purify Rab8 protein were complicated by difficulties in obtaining soluble protein. This is an important step in the determination of Rab8 structure. Several techniques were previously tried, without great success, in an attempt to improve this yield. Multiple bacterial expression vectors and Rab8 gene constructs were used to address this issue, including the development of a fusion protein combining Rab8 with a second, more soluble protein, glutathione-s-transferase. A minimal amount of soluble Rab8 protein was obtained and analyzed using circular dichroism spectroscopy. Future research will focus on improving protein yields and additional structural analysis.
Introduction

The protein Rab8 belongs to the Rab family of GTPase proteins, the largest family of the Ras superfamily. The Rab proteins serve a regulatory role in the intracellular transportation of cellular vesicles. Specifically, Rab8 regulates polarized membrane transport pathways through structural reorganization of actin and microtubules, thus affecting cellular morphogenesis. Previous studies identify Rab8 as a participant in biochemical processes potentially related to multiple diseases, including Huntington Disease (HD), glaucoma, and melanoma. The involvement of Rab8 in the HD process is of primary interest in this research. Extended length repeats of a GAC trinucleotide sequence in the gene for Huntingtin protein, resulting in a poly-glutamine region, have been linked to the development of HD. There exists a negative correlation between the length of this poly-glutamine region and the age of onset in HD patients. The protein optineurin (FIP-2) has been shown to function as a binding partner for Huntingtin, Rab8, and the motor protein myosin VI, linking these proteins in Golgi complex-related vesicle transportation. Structural characterization of Rab8 protein may allow for a better understanding of its activities and binding partner interactions. The goal of this research was to characterize the secondary structure of Rab8 using circular dichroism (CD) spectroscopy.

To structurally characterize Rab8, soluble protein must be expressed. Previous research involving full-length (207 a.a.) Rab8 protein expression in E. coli was
severely complicated by issues of protein solubility. For this research, two different approaches were tried for obtaining soluble protein using E. coli expression hosts: the use of a Rab8 fusion protein and the use of a truncated Rab8 protein construct. A fusion protein—designated Rab8b, indicating the presence of a blunt 5’ end on the DNA insert—was designed using full-length Rab8 and glutathione-S-transferase (GST). This fusion method is experimentally known to increase the solubility of some proteins during prokaryotic expression. Following initial purification, the GST protein can be cleaved enzymatically in an attempt to yield soluble Rab8. In addition to the fusion protein, four different truncated versions of Rab8, designated Rab8T1-4, were also designed. When expressed in vivo in eukaryotes, Rab proteins are known to be post-translationally modified by the attachment of lipid moieties at the C-terminus end, creating a lipid “tail” and consequently decreasing solubility. Specifically, this involves isoprenylation with 20 carbon geranylgeranyl moieties at C-terminus cysteine residues by geranylgeranyl transferases. These truncated constructs remove the cysteine at residue 204 in an attempt to prevent solubility-reducing reactions at the exposed sulfhydryl group.

Additional possible approaches for increasing the soluble yield of these proteins from prokaryotic expression techniques include slowed expression at cold temperatures using the pCold plasmid and denaturation and refolding of insolubly expressed protein using urea buffers. Both of these additional approaches were utilized, with varying degrees of success.
Methods

Three different methods for obtaining Rab8 protein were used in this research: full-length Rab8b was expressed from pET-42b plasmid, truncated Rab8T2 was expressed from pCold plasmid, and truncated Rab8T2 previously expressed from pET-28 was purified. The amino acid sequence of full-length Rab8 is shown in Figure 1. In addition, Table 1 displays the modifications for each truncated Rab8 construct.

```
5'- MAKTYDYLFK LLLIGDSGVG KTCVLFRFSE
  DAFNSTFIST IGIDFKIRTI ELDGKRIKLQ
IWDTAGQERF RTTITAYYRG AMGIMLVDI
  TNEKSFDNIR NWIRNIEEHA SADVEMILG
NKCDVNDKQRQ VKSERGEKLA LDYGKVMET
  SAKANINVEN AFFTAARDIK AKMDKKLEGN
  SPQGSNQQVYK ITPDQPKRSS FFRCVLL -3'
```

Figure 1 – Rab8 Sequence (207 Amino Acids)

<table>
<thead>
<tr>
<th>Construct</th>
<th>Amino Acids</th>
<th>(M_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB8 (full-length)</td>
<td>1 – 207</td>
<td>23668.2</td>
</tr>
<tr>
<td>RAB8T1</td>
<td>1 – 170 ((\Delta c37))</td>
<td>19549.4</td>
</tr>
<tr>
<td>RAB8T2</td>
<td>1 – 172 ((\Delta c35))</td>
<td>19748.7</td>
</tr>
<tr>
<td>RAB8T3</td>
<td>1 – 182 ((\Delta c25))</td>
<td>20848.9</td>
</tr>
<tr>
<td>RAB8T4</td>
<td>1 – 197 ((\Delta c10))</td>
<td>22458.7</td>
</tr>
</tbody>
</table>

The basic methods used to work with each protein fall into four general categories: subcloning, expression, purification, and structural analysis. Data describing the results of this research is included in the results and discussion section.
Full Length Rab8b from pET-42b

Subcloning:

The Rab8b DNA sequence, a full-length Rab8 gene insert with a blunt 5'-end, was generated by PCR from a Rab8 template using the primers indicated in Table 2 and Stratagene PfuTurbo DNA polymerase at annealing temperatures of 45°C, 55°C, and 65°C. The DNA was purified using the Qiagen QIAquick PCR purification kit microcentrifuge protocol at 13,000rpm. Unpurified and purified samples from each of the three PCR scenarios were analyzed by agarose gel electrophoresis. The 65°C PCR product was digested overnight with restriction enzyme BamHI using Promega buffer E to form the insert and purified by the QIAquick PCR microcentrifuge protocol. Novagen pET-42b vector, containing sequences for an internal His-Tag and the 220 amino acid N-terminal GST-Tag for the fusion protein, was double digested overnight with restriction enzymes BamHI and PshAI using New England BioLabs NEBuffer 4 and purified by the QIAquick PCR microcentrifuge protocol. The insert and vector were incubated with New England BioLabs T4 DNA Ligase at room temperature for 15 minutes. Plasmid from the ligation was transformed into Novagen NovaBlue E. coli competent cells (CaCl2 method). Transformed NovaBlue cells were plated onto 20 mL LB/agar with 20µL of 35mg/mL kanamycin and incubated overnight at 37°C. A round of 6 colonies was grown in 5mL LB/kanamycin overnight at 37°C and screened for Rab8 insert using

<table>
<thead>
<tr>
<th>Table 2 – Oligonucleotides Used for Rab8b</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAB8FWD:  5'--ATG GCG AAG ACC TAC GAT TAC CTG-3'</td>
</tr>
<tr>
<td>RAB8REV:  5'--CGC GGA TCC CTA CAG AAG AAC ACA TCG GAA AAA GC-3'</td>
</tr>
</tbody>
</table>
the Qiagen QIAprep Spin Miniprep Kit microcentrifuge protocol at 13,000rpm and room temperature. The purified plasmid was double digested with PshAI and BamHI for 1.5 hours at 37°C and analyzed by agarose gel electrophoresis. No insert was present. An additional 6 colonies were screened by the same methods, and no insert was present. The ligation step was repeated using new T4 DNA Ligase, with no insert detected in the resulting plasmid. New digestions were performed and subsequent 2 hour ligations and 15 minute ligations were performed. No clones ever indicated the presence of Rab8b insert. No expression was attempted and new primers for a Rab8 construct with two sticky ends were ordered.

**Truncated Rab8T2 from pCold**

**Subcloning:**

Rab8T2 PCR product from a previous preparation was digested as above and ligated into the Takara pCold plasmid vector using the same protocols as above. The Rab8T2/pCold plasmid was transformed into BL21(DE3) competent *E. coli* cells. The cells were incubated on LB/agar plates with 20μL of 50mg/mL ampicillin and screened for clones as above.

**Expression:**

A starter culture from a colony of BL21(DE3) clones identified to contain the Rab8T2 insert was grown overnight in LB/ampicillin. The culture was transferred to a flask containing 2 L of LB and 2mL of 50mg/mL ampicillin and grown at 37°C to an OD₆₀₀ of 0.59. The BL21(DE3) cells were induced with 2 mL IPTG and incubated
at 15°C with shaking for 24 hours. The BL21(DE3) cells were pelleted by centrifugation at 6000xg and 4°C for 25 minutes.

**Purification:**

For nickel-metal column purification of Rab8T2 by Fast Protein Liquid Chromatography (FPLC), a binding buffer (Buffer A) and elution buffer (Buffer B) were made. Buffer A was 20mM Tris base, 20mM HCl, 500mM NaCl and 5mM imidazole, adjusted to pH 8.0. Buffer B was 20mM Tris base, 20mM HCl, 500mM NaCl and 500mM imidazole, adjusted to pH 8.0. The BL21(DE3) pellet was resuspended in Buffer A and centrifuged at 20,000xg for 20 minutes to collect the soluble protein. The supernatant was filtered through a .45µm filter and washed onto a charged Ni-column. FPLC was performed using the Amersham Pharmacia Biotech ÄKTA explorer and Buffers A and B. Fractions 3, 4, 14, 15, 16, 28, 29, 31, 34, and 35 were collected and analyzed using SDS-gel electrophoresis. No appreciable yield of Rab8T2 was detected. The insoluble fraction from centrifugation of the BL21(DE3) cells was resuspended in Buffer A containing 6 M urea. FPLC was performed as above, using Buffers A and B with each containing 6 M urea. Fractions 46-51 were combined. The amount of Rab8T2 protein obtained was not useful for structural analysis.
Truncated Rab8T2 from pET-28

Purification:
The insoluble cell fractions from frozen, previously expressed BL21(DE3) cell pellets containing Rab8T2 coded in a pET-28 vector were denatured using 6 M urea buffers as above. FPLC was conducted on the denatured Rab8T2 with urea buffers as above, and fractions 47-51 were combined. The purified protein was dialyzed in 20mM sodium phosphate buffer (pH = 5.7) for refolding in the cold room. This protein precipitated during refolding. The above denaturation and purification process was repeated with another insoluble cell fraction, and the purified Rab8T2 was dialyzed for refolding in 10mM sodium acetate buffer (pH = 4.5) in the cold room.

Structural Analysis:
Purified, refolded Rab8T2 in sodium acetate buffer was prepared for CD spectroscopy using a 6/32 dilution with distilled water, yielding a 4.106 µM CD sample. The CD blank was prepared using a 6/32 dilution of the equilibrated dialysis buffer with distilled water. Circular dichroism spectroscopy was performed using an Olis Rapid-Scanning Monochromator and Circular Dichroism Module. A series of 150 CD readings were taken in the wavelength band 195nm – 260nm. The measurements at each point consisted of three, 1-second readings. These three readings were averaged to give a list of ordered pairs describing measured ellipticity as a function of wavelength. The data was exported to Microsoft Excel and transferred to Mathematica for manipulation and graphing. The list was interpolated using a Bezier spline function to provide a smooth curve fit of the spectral data.
**Results and Discussion**

**Rab8b Insert was not Successfully Incorporated into the Expression Vector.**

Analysis of clones transformed using Rab8b/pET-42b in NovaBlue competent cells indicated that attempts to introduce Rab8b into the pET-42b vector were unsuccessful. The DNA gel electrophoresis results from screening two trials of six clones for Rab8b are displayed in Figure 2. A series of troubleshooting steps revealed two possible sources of this difficulty. First, test single digestions of pET-42b using each restriction endonuclease from the double digest indicated that
PshAI, the enzyme responsible for the blunt end digestion, had impaired activity. This is indicated in the DNA gel in Figure 3. The presence of a second band in the PshAI digest lane indicates undigested circular vector, most likely experiencing supercoiling. This impaired activity was attributed to enzyme age, and fresh PshAI was ordered to address the problem. The second difficulty, evident after the introduction of fresh PshAI, appeared to involve the blunt end ligation. As indicated in Figure 4, the Rab8b insert was still not noticeably introduced into the expression vector.

A new full length Rab8 insert has been designed, incorporating two sticky end digestion sites into its DNA sequence. Future research should reveal whether this new insert overcomes the difficulties associated with a Rab8b.
No Significant Yield of Soluble Rab8T2 Protein was Obtained from pCold Expression.

Using a Ni-metal affinity column, FPLC analysis of Rab8T2 containing a His-tag from pCold plasmid expressed in BL21(DE3) competent cells indicated a minimal yield of Rab8T2 in the purified soluble cell fractions. The FPLC chromatogram is included in Figure 5.

Additionally, the insoluble cellular fraction was re-suspended in a 6 M urea denaturing buffer and FPLC was performed again. A minimal yield of Rab8T2 protein was present from the insoluble fraction. These FPLC purification results suggest that very little protein was produced during cold expression, and of the Rab8T2 that was expressed, most was misfolded and retained in the insoluble cellular debris.
Soluble Rab8T2 was Obtained Using Protein Denaturation and Renaturation Methods.

Using a Ni-metal affinity column, FPLC analysis of denatured Rab8T2 containing a His-tag from pET-28a plasmid expressed in BL21(DE3) competent cells indicated the presence of Rab8T2 protein. The FPLC chromatogram is shown in Figure 6.

Dialysis of the unfolded protein into 0.02 M sodium phosphate buffer at pH 5.7 resulted in significant precipitation of the protein. Dialysis of the unfolded protein into 0.01 M sodium acetate buffer at pH 4.5 did not result in noticeable protein precipitation. Approximately 20mL, yielding 1.82g, of soluble Rab8T2 was obtained.
Data from CD Spectroscopy of Rab8T2 Indicates Secondary Structure with a High $\beta$-Sheet Composition.

The data from the CD analysis of Rab8T2 refolded into 0.01 M sodium acetate buffer is represented graphically in Figure 7. Because the Rab8T2 was in an acetate containing buffer, some buffer noise is present in the lower wavelength end of the scanned spectrum.

![Figure 7 - Circular Dichroism Spectroscopy of Refolded Rab8T2](image)

This CD data provides useful information about Rab8T2 in a couple of ways. First, the presence of a recognizable CD signature indicates that a regular secondary structure was obtained during the refolding dialysis. Second, the CD signature indicates that Rab8T2 has $\beta$-sheet secondary structural characteristics. This is consistent with known structural information for other proteins in the Rab family. It also supports the possibility that the correct secondary structure was regained during the process of refolding. The presence of consistent and expected secondary structure
in refolded Rab8T2 lends support to the usefulness of denaturation/renaturation methods for obtaining soluble Rab8 protein.

For reference, the raw CD spectroscopy data for Rab8T2 refolded by dialysis in 0.01M sodium acetate buffer is included in Table 3.

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<th>Wavelength (nm)</th>
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Table 3 – Circular Dichroism Spectroscopy Data for Refolded Rab8T2
Conclusions

Difficulties in characterizing Rab8 protein were encountered at multiple steps, including subcloning, expression, and purification. Many of these difficulties can be attributed to the tendency of Rab8 to express insolubly in *E. coli*. However, some success was gained by denaturing insoluble truncated Rab8 protein, Rab8T2, and slowly refolding by dialysis with a moderately acidic refolding buffer. Initial studies using circular dichroism and comparison with other Rabs indicate that regular secondary structure was regained during the refolding process. This suggests that denaturing and refolding insoluble Rab8 may be an effective way to obtain soluble protein for structural analysis. Through this method, and utilization of a newly designed Rab8-GST fusion protein, future research will focus on improving the yield of soluble Rab8 protein. This will potentially allow future structural studies on Rab8 and Rab8/binding partner complexes using x-ray crystallography, circular dichroism spectroscopy, and fluorescence spectroscopy. It is worth noting that after the completion of this research, some x-ray crystal structure data for the complex of a Rab8 fragment with the protein MSS4 was published. Future structural studies of Rab8, concentrating on its binding activity with optineurin and related huntingtin interactions may provide information on the regulation of these proteins and the roles they play in the biochemical disease processes of Huntington Disease.
Acknowledgments

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Director – Laboratory for Structural Biology, Professor of Chemistry, University of Alabama in Huntsville.

Randall Wilson
Talitha Holmes
Chris James
Graduate Students, colleagues, and lifesavers – Laboratory for Structural Biology, University of Alabama in Huntsville

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Tadeusz Ciszak
Undergraduate students at Vanderbilt University and Columbia University, respectively. Their work on Rab8 in the Laboratory for Structural Biology during the summer of 2005 was foundational for my research project. Notably, the Rab8T2 I analyzed by CD spectroscopy was obtained from their saved insoluble cell fractions.

Dr. Jerry Mebane
Director – Honors Program, Professor of English, University of Alabama in Huntsville.

Dr. Mebane, I will forever appreciate the compassion and understanding you displayed throughout my difficult transition to this research.

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Literature Cited


Complete Bibliography


