Engineering an improved expression vector for the Nupl53 RNA binding domain

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Engineering an improved expression vector for the Nup153 RNA binding domain

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Natural Sciences at Carroll College, Helena, Montana

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Abstract

The nucleoporin Nup153 has a critical role in both import and export of cellular substances across the nuclear envelope. A unique RNA binding domain has been revealed in Nup153 (Nup153-RBD). In order to further understand the interaction between the RNA binding domain (RBD) of Nup153 and RNA, a crystal structure of the domain would be invaluable. This can be accomplished only if a significant quantity of purified RBD is isolated. Several RBD constructs have been produced to aid in purification of this protein. In most cases, recombinant RBD preparations are fairly heterogeneous and thus unsuitable for structural studies. However, a GFP-RBD construct showed robust expression in *E. coli* and can be purified without degradation. Although crucial to production and purification, additional domains and tags are not desirable in the final protein sample used for X-ray crystallography. Thus, my goal was to improve the GFP-RBD construct by inserting a protease site that will enable cleavage of the protein once purification is complete, resulting in isolated, pure RBD. Using first QuikChange® and then a PCR based cloning strategy, a TEV protease site was inserted into the GFP-RBD construct. In the future, use of this construct could aide in elucidating how the Nup153 RNA binding domain recognizes its targets. This could allow us to better understand, and ultimately control, nuclear pore function in the context of normal and disease states.
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Introduction

Compartmentalization of cellular functions through membrane bound organelles makes nucleocytoplasmic transport essential for the success of eukaryotic cells. The membranous structure separating the nucleus from the cytoplasm is known as the nuclear envelope. Transport of cellular materials across the nuclear envelope is facilitated by embedded macromolecules known as nuclear pore complexes (NPCs). A NPC is a large structure, about 125 MDa, consisting of about 30 identified and characterized repeating proteins known as nucleoporins. It is structurally organized by way of eight fold symmetry with a central framework penetrating the membrane and protein moieties extending into both the cytoplasm and nucleus (Koser et al., 2005). Expanding into the nucleus is the nuclear basket, an arrangement of fibers that is anchored to form a distal ring (Ball and Ullman, 2005). The NPC is an integrative macromolecular complex with functional roles in the cellular processes of transport, division, differentiation, and apoptosis (Koser et al., 2005).

Understanding the mechanisms of transport at the NPC begins with the characterization of the nucleoporins. Transport is initiated when importin receptor molecules interact with specific domains on cargo molecules, either nuclear import signals (NIS) or nuclear export signals (NES), thus forming a receptor-cargo complex (Lim et al., 2006). Hydrophobic interactions between the FG-rich region of the nucleoporin and hydrophobic residues on the receptor surface guide the cargo through the central pore of the NPC (Lim et al., 2006). Nucleoporins with FG domains are natively unfolded and thus innately hydrophobic and capable of many binding interactions at once.
(Lim et al., 2006). This provides the nucleoporin with an increased level of mobility and greater transport efficiency.

One such FG-nucleoporin that has received extensive study is Nup153, conventionally named for its molecular weight (Ball and Ullman, 2005). Nup153 appears to be primarily located on the nuclear basket with three distinguishable domains: a highly mobile C-terminal FG-rich region, a central zinc-finger, and the N-terminus anchored in the nuclear ring (Dimaano et al., 2001). This N-terminal domain includes a nuclear pore associating region that places Nup153 in the nuclear basket as well as an RNA binding domain (Ball and Ullman, 2005). The presence of this domain and capability to directly interact with RNA is unique amongst vertebrate nucleoporins (Dimaano et al., 2001). It has been shown that the RBD tends to bind to single-stranded RNA, and more specifically mRNA (Ball et al., 2004).

Nup153 has also been shown to be dynamically associated with the nuclear pore and this mobility along with its unique RNA binding domain may imply an early role of Nup153 in the synthesis and recruitment of RNA to the NPC (Ball and Ullman, 2005). Such a function can be made clear with an improved understanding of the interaction between RNA and Nup153, ideally with the elucidation of a crystal structure of the RBD. This would allow very specific mutations to be designed in order to selectively disrupt RNA binding and assess the impact on RNA metabolism. Although there are several current constructs of Nup153 RBD, the most stable and well expressed appears to be one with both a 6-histidine tag and green fluorescent protein, known as GFP-RBD. These fused proteins make purifying the RBD possible, but they are not desirable in the final sample. Inserting a protease site between the GFP and RBD sequences in the stable
construct will enable isolation of the RBD by cleavage in the final steps without interfering with purification. The Nuclear Inclusion A (NIA) protein encoded by the tobacco etch virus (TEV) has a stringently specific catalytic site. The protease TEV is both efficient and commercially available, and at only 21 base pairs is a good candidate for mutagenesis.

Fusing a protease site between two protein sequences of a plasmid construct requires a cloning strategy. QuikChange® (Stratagene) is one method that makes the insertion of a desired sequence into a construct possible. A plasmid with the GFP-RBD construct is prepared and then used in a PCR reaction. During the temperature cycling of PCR the plasmid is denatured, and primers containing the TEV site flanked by GFP and RBD sequences anneal to the plasmid. The Pfu DNA polymerase extends and incorporates the primers with the TEV site into the plasmid. By not displacing the strands during this extension, the PCR results in unmethylated strands. The next step is to digest the nonmutated plasmid with the restriction enzyme DpnI, which recognizes only the methylated parental strands. Now only the undigested, nicked, mutated strands are available to be transformed into ultracompetent E. coli cells. Once the plasmid has been successfully transformed, the cells repair the nicks.

Another cloning strategy that could effectively insert the TEV site is that of PCR “sewing”. First, two separate PCR reactions are prepared; the first with a SalI restriction site in the forward primer and a TEV site flag on the reverse, the second with a forward primer containing the TEV site flag and a NotI restriction site on the reverse. Annealing the products of the two reactions results in the insertion of the TEV site. This GFP-TEV-RBD construct can then be inserted into a TOPO TA® (Invitrogen) vector. TOPO TA®
is an efficient, one-step cloning strategy that enables the direct insertion of the PCR product into a plasmid vector. *Taq* polymerase has the ability to add a single deoxyadenosine residue to the 3' end of PCR products regardless of the sequence of the product. The TOPO vector has a single overhanging 3' deoxythymidine residue, thus enabling successful annealing between vector and insert. Moreover, topoisomerase, which has ligase activity, is covalently attached to the vector, ensuring successful ligation with the insert. This ligated plasmid can now be transformed into ultracompetent cells. Subsequent digestion of the TOPO plasmid with restriction enzymes (*NotI* and *SalI*) releases the GFP-TEV-RBD insert with sticky ends, making it ready for insertion into another vector for transformation and expression. The *SalI* and *NotI* sites were chosen because they are present in the pET28a vector and for their buffer compatibility, thus facilitating a double digest. The pET28a vector is well expressed in *E. coli* and contains convenient kanamycin resistance as well as a T7 promoter and terminator; thus insertion of the GFP-TEV-RBD construct is favorable.

I hypothesize that a cloning strategy will successfully complete the objective of inserting the TEV protease site into the GFP-RBD Nup153 construct.
Materials and Methods

Isolation of pET-28a GFP-RBD DNA from bacterial prep.

Fifty milliliters of LB solution was added to a 250 mL beveled Erlenmeyer flask. A sterile swab of the pET28a GFP-RBD #16 E. coli glycerol stock was placed in the flask. The vector had a kanamycin resistance gene, and to ensure that only cells with the vector proliferate 30 μg/mL of kanamycin were added to the culture flask. The culture was incubated overnight at 37°C with shaking at 220 rpm. The DNA was then purified the following day using a Qiagen® Midi kit.

Primer design to use in Quickchange mutagenesis

Primer design was facilitated with the computer program VectorNTI®. The forward and reverse primers were made to be complementary, with 10 nucleotides corresponding to the GFP sequence preceding the TEV protease sequence and 10 nucleotides corresponding to the RBD following the TEV protease sequence, thus resulting in a 41 base oligonucleotide (5’-

CAAAGAATTCGAAAACCTGTATTTCCAGTCCAACCGTCAGC -3’ and 5’-

GCTGACGGTTGGACTGAAATACAGGTTTTCGAATTCTTTG-3’). These primers were then prepared by the peptide facility at the University of Utah.

Quickchange® site-directed mutagenesis

The Quickchange® (Stratagene) protocol of PCR, digest, and transformation were carried out and then the following modifications were made in an attempt to obtain the desired product:

- Increased the extension time in the PCR cycle to 20 minutes instead of 6.
- Used 100 ng, 50 ng, and 20 ng plasmid template DNA instead of 10 ng.
- Added 4 µL of DpnI digested DNA to ultracompetent cells instead of 2 µL.
- Precipitated digested DNA to use in transformation by adding 0.1x volume of sodium acetate, 2x volume of 95% EtOH, and 1 µL seeDNA to digest reaction sample in 4° C for 30 min, spun at 4° C for 30 min., poured off EtOH and added 300 µL of 70% EtOH, spun for 5 min. Poured off EtOH and used gel pipette tip to remove liquid. Resuspended DNA in 5 µL ddH₂O.
- Heat competent Top 10 cells were used for transformation instead of the included heat competent XL-10 Gold cells.
- Purchased a new kit and followed the original protocol.
- Primers were found to be at incorrect concentration; protocol called for 125 ng and instead only about 25 ng was being used, PCR was repeated using the correct primer concentration.

Colony PCR was performed on any transformants. The colony dilutions were made by using a sterile pipette tip to transfer an isolate colony to 20 µL sterile dH₂O. The PCR reaction included final concentrations of 1x Taq buffer, 1.5 mM MgCl₂, 0.25mM dNTP, 1 unit Taq DNA polymerase, 1.0 µM of T7 and T7 terminator, 2 µL of colony dilution, and a final volume of ddH₂O to 20 µL. An internal primer that annealed to the TEV site was also used with the T7 terminator to screen for the presence of the TEV site in the construct (5’-GAATTCGAAAAACCTGTATTTCCAGTCC-3’).
Primer design to use in cloning

Primer design was facilitated by the program VectorNTI®. Two sets of primers were needed, one for each of the two PCR reactions that added the TEV site. The external primers also had to include a restriction site so the resulting GFP-TEV-RBD segment could be inserted back into the vector. The first reaction consisted of a forward primer with the SalI restriction site that annealed to the GFP segment, and a reverse with a TEV tag following a sequence that annealed to the EcoRI site and GFP (5’-CCGTCGACATGAGTAAAGGAGAAGAACTTTTCACTGG-3’ and 5’-GGACTGGAAATACAGGTTTTCGAATTCTTTGTATAGGTTTCATCC-3’). The next reaction used a forward primer with a TEV tag preceding the sequence that annealed to the RBD, and a reverse with a NotI restriction site (5’-GAAAAACCTGTATTTCCAGTCCAACCGTCAGCTTGGTGATTC-3’ and 5’-CGAGTGCGGCCGCTGGCTGTATTCTGCGACTG-3’). These primers were then prepared by the peptide facility at the University of Utah.

Amplification of TEV tagged construct segments

Two PCR reactions using the Pfx DNA polymerase and touchdown thermocycler program were prepared. The annealing segment of touchdown began at 95°C and decreased 0.1°C every 5 seconds until 4°C, ensuring the proper annealing temperature of the primers was reached. Both reactions consisted of final concentrations of 2x Pfx buffer, 0.25 mM dNTP, 1 mM MgSO₄, 0.25 mM of each primer, 10 ng dsDNA template, 1 unit of Pfx DNA polymerase, 1 unit of Pfx enhancer, and ddH₂O to 50 µL. The first reaction used the SalI forward and TEV flag reverse primers; the second reaction used the TEV flag forward, and NotI reverse primer. A negative control without any dsDNA
template and a positive control using DHFR plasmid and DHFR forward and reverse primers were also used.

*Overlap and Extension*

Next the amplification products were annealed together to create a plasmid insert with the TEV site. This was accomplished by preparing a PCR reaction with 10 ng of both GFP-TEV and TEV-RBD dsDNA fragments, 1x of 10x *Pfx* amplification buffer, 0.3 mM dNTP, 1 mM of MgSO₄, 1 unit of *Pfx* DNA polymerase, and ddH₂O to a volume of 50 μL. The reaction then went through 5 PCR cycles with an annealing temperature of 50 °C before the primers were added; the external primers SalI-GFP For and NotI-RBD Rev were used. The reaction then proceeded with the Touchdown program. The product was analyzed using gel electrophoresis and the DNA was purified with a Qiagen® gel extraction kit.

*Double Digest with SalI and NotI restriction enzymes*

Digest of both the pET-28a Nup288-611 parental vector and the GFP-TEV-RBD fragment facilitated the insertion of the fragment through directional cloning. The gel purified DNA from the annealing step amounted to 450 μg and was all digested. Therefore for the insert a volume of 30 μL DNA, 3.2 μL 10x Buffer O (50 mM TRIS-HCl pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 0.1 ng/ml BSA), and 1 μL of both NotI and SalI were used. For the parental pET-28a Nup228-611, 16 μL of DNA was added to 2 μL of 10x Buffer O and 1 μL each of NotI and SalI. A control of linear pET28a NotI, SalI was also digested with the same conditions as the vector. The reactions were incubated at 37 °C for one hour, and then at 65 °C for ten minutes to inactivate the restriction
enzymes. The reactions were analyzed by gel electrophoresis and the DNA was gel purified.

**Ligation**

For the ligation to produce optimal yields of the desired clone the insert must be in about five fold molar excess to the vector. Due to the discrepancy in size (at about 6000 bp the pET28a vector is five times the size of the 1200 bp GFP-TEV-RBD insert) 1 µg of insert is about five times the amount of 1 µg of vector. Three reactions were all prepared with 4 µL of T4 buffer, 1 µL T4 DNA ligase, and ddH₂O up to 20 µL, with different vector to insert ratios. The first was a 1:5 vector to insert, with 50 ng of both the vector and insert used. The following ratios were also used, 1:1, 1:3, 1:10, 1:15, 5:1, and 10:1. Total DNA in each reaction was kept around 100 ng. Reactions were incubated both in the thermocycler overnight at 16° C and at room temperature for two hours.

**TOPO TA® cloning**

A TOPO TA® cloning reaction was performed in parallel to the digest and ligation. The kit protocol was directly followed, and once the GFP-RBD-TEV construct was inserted into the TOPO® vector and transformed into *E. coli*, the plasmid was digested with *NotI* and *SalI* to release the construct. The digested insert was then gel purified for ligation back into the pET-28a vector using the aforementioned protocol.

**Transformation into ultracompetent cells**

All ligation reactions were used in the transformation in addition to a negative control and positive control with pUC19 (ampicillin resistant). Two different cell lines with two different protocols were used in an attempt to successfully transform the plasmid. First, Top 10 heat ultracompetent cells were gently thawed on ice and 2 µL of
the ligated DNA was added to the cell aliquot. The cells were incubated on ice for 30 min before a heat flash was administered at 42 °C for 30 seconds, and then were returned to ice for 2 minutes. 250 µL SOC media was added to the cells and they were incubated at 37 °C with 220 rpm shaking for 1 hr. The cells were then spread on kanamyacin agar plates and left to incubate overnight at 37 °C. Next XL-1 chemically ultracompetent cells were used. The cells were thawed on ice, then 5 µL of ligated DNA, 15 µL ddH₂O, and 5 µL KCM (sterile filtered 0.5 M KCl 0.15 M CaCl₂ and 0.25 M MgCl₂) were mixed and added to 25 µL of cells. They were incubated on ice for 20 min and then at room temperature for 20 min. 200 µL SOC media was added, and then the cells were recovered for 1 hr with shaking at 37 °C. The cells were then spread on kanamyacin agar plates and left to incubate overnight at 37 °C.
Results

**QuikChange® Strategy**

The first QuikChange® attempt produced no colonies. Transformation efficiency was tested using the pUC 18 plasmid and was found to be $10^6$ transformants/µg indicating that the transformation protocol was effective. As modifications to the protocol were made transformants were observed, but they contained only parental plasmid DNA and lacked the TEV insert as confirmed by both colony PCR (Fig. 1) and automated DNA sequencing.

![Gel electrophoresis of colony PCR of QuikChange® transformants. A) PCR with EcoRI TEV internal primer that screens for the presence of the TEV site. The primers amplify a 624 bp region of the plasmid. No observable bands indicate the lack of the insert in the plasmid DNA. B) PCR with T7 promoter and T7 terminator. Bands corresponding to the GFP-RBD parental indicate that the colonies contain parental plasmid DNA and the mutagenesis was unsuccessful.](image)

**PCR Sewing Strategy**

The first step of the PCR sewing strategy was to produce two DNA fragments using specific primers with TEV tags in a PCR cycle, each with one strand containing the TEV site. The first fragment produced was the 747 bp GFP-TEV, the second was the 483 bp TEV-RBD. Gel electrophoresis of the DNA from the PCR illustrates the successful
amplification of these fragments (Fig. 2). Annealing the GFP-TEV and TEV-RBD fragments resulted in assembling the complete GFP-TEV-RBD construct. This was confirmed by both gel electrophoresis (Fig. 2) and automated DNA sequencing.

The resulting GFP-TEV-RBD construct was then effectively ligated into the TOPO® vector and transformed into an ultracompetent E. coli cell line (Fig. 3). Use of the internal *EcoRI* TEV primer assured the presence of the TEV site in the insert (Fig. 3). To achieve the ultimate goal of inserting the construct into the versatile pET-28a vector it was necessary to next digest the GFP-TEV-RBD insert out of the TOPO® vector. This digest resulted in vector and insert fragments analyzed by gel electrophoresis (Fig. 4), and also ensured that the construct was indeed cut, resulting in sticky *SalI* and *NotI* ends that can be productively ligated. The pET 28a vector was also digested in parallel, resulting in an approximate 6000 bp vector with sticky *NotI* and *SalI* ends, and an 1100 bp Nup228-611 insert.
Fig 3. 1% agarose gel of TOPO® transformants. A) Colony PCR of TOPO® transformants. Bands near 1200 bp in the second and third colony lanes correspond to the GFP-TEV-RBD insert amplified by PCR using the M13 forward and reverse primers on TOPO® vector. B) Colony PCR of transformants 2 and 3 with EcoRI TEV internal primer to screen for presence of TEV site. The ligation of the insert to the TOPO vector is nondirectional and therefore reactions with both the M13 forward and reverse primers had to be conducted to effectively screen for the insert. All lanes contain bands near 650 bp, indicating the presence of the TEV site. The more intense bands seen in the lanes with the M13 forward primer reveal the more prominent insertion conformation of RBD-TEV-GFP.
Fig 4. 1% agarose gel analysis of NotI and SalI double digest. A) Digest of parental pET 28a Nup228-611 vector results in two fragments, the vector is near 6000 bp and the Nup228-611 insert is represented by the band near 1100 bp. A linear form of pET 28a with the same digest is also shown, as well as a digested GFP-TEV-RBD insert. Gel purified sample of the pET 28a vector is then used for ligation. B) Digest of the TOPO® vector gives two fragments, of which the gel purified sample of the GFP-TEV-RBD insert is used for ligation with pET 28a.

Once ligation was complete I attempted to transform the pET28a GFP-TEV-RBD plasmid into ultracompetent E. coli cells. Any colonies that did arise were tested for the plasmid using the T7 promoter and T7 terminator (Fig. 5). No transformants containing the ligated plasmid were obtained. Transformation efficiency was tested using the positive control pUC19 plasmid and was found to be $10^7$ transformants/μg.
Fig 5. 1% agarose gel electrophoresis of colony PCR of pET 28a GFP-TEV-RBD transformants. The plasmid DHFR was used as a positive PCR control. T7 promoter and T7 terminator were used for the PCR reaction. No bands are observable in either colony lane, indicating that they were simply contaminate colonies and did not harbor the desired ligated DNA.
Discussion

In order to better understand the unique interaction between RNA and Nup153, I sought to isolate the Nup153 RBD. The objective of this project was to produce a construct of the Nup153 RNA binding domain that would facilitate continued characterization of the Nup153-RNA cargo interface. By producing a large enough amount of pure RBD, structural studies such as x-ray crystallography become possible. Obtaining an x-ray crystallography data of Nup153 will provide information about its cargo interfaces, binding interactions, and overall functional role in nuclear transport. The fused proteins in an existing construct are essential to the stable expression and purification of the Nup153 RBD, but the RBD cannot be further isolated. Insertion of a protease site into the construct enables stable expression and purification as well as isolation of the RBD by means of cleavage. As hypothesized, a cloning technique successfully inserted the TEV site into the existing GFP-RBD construct.

Two different protocols were used in an attempt to insert a TEV site into the GFP-RBD construct. The first strategy utilized a site-directed mutagenesis cloning protocol. Although seemingly simple and convenient, this method proved to be ineffective and as shown in my results, no transformants were obtained. There are several explanations for these observations. The ultracompetency of the cells used for transformation could have been compromised in the serial freezing/thawing that occurred. Given the complementary nature of the designed oligonucleotide primers, primer coupling may have interfered with proper annealing to the DNA. Although a new QuikChange® kit was purchased in order to address the possibility of expired enzymes, still no mutagenized transformants were
obtained. Perhaps the system still did not work with new enzymes because at 21 bp the TEV site was simply too large for effectual site-directed mutagenesis.

The second strategy utilized PCR sewing, and through a double restriction enzyme digest and T4 DNA ligation successfully inserted the TEV sequence. Although more complicated than a straightforward retail kit, the basic cloning technique proved to be the most efficient and productive method to produce the desired construct.

Although the created GFP-TEV-RBD construct has yet to be expressed in bacteria, the parental version (lacking the TEV site) was robustly expressed and thus we have a good starting point from which to put the current construct to use. Using similar conditions to previous purifications, the GFP-TEV-RBD fusion protein will be produced and purified. Once this is accomplished, the fusion protein will be cleaved with TEV protease and the RBD will be used for further elucidation of the interaction between RNA and Nup153.

There are many venues of study at the nuclear pore complex, and understanding the interaction between RNA and Nup153 is just one piece of the puzzle to comprehending nucleocytoplasmic transport. Other unique domains of both Nup153 and other nucleoporins can also provide information. For example, the role of the FG domains is under current debate. One model describes FG domains as forming a "polymer brush" with a general exclusionary function to transport (Lim et al., 2006). However another model has been proposed implicating FG domains as forming a protein meshwork that requires selective interaction for transport (Frey et al., 2006). Obtaining a comprehensive picture of how all the nucleoporin domains interact to accomplish successful transport is essential to understanding the function of the NPC. Elucidation of
the unique RNA-Nup153 cargo interface will contribute to the overall understanding of the function of Nup153 at the NPC, and how nucleocytoplasmic transport contributes to cell growth. With more information in hand, we hope to understand how pore function affects the physiology of cancer cells and whether manipulating pore function can be used to control cell growth.
Literatura Cited


