Optimization of Expression and Purification of the NEMO Fragment Crucial for Interaction with IkBα

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Abstract
NEMO, the regulatory subunit of the IKK complex, helps activate the NF-κB cascade through its interaction with IkBα. The NF-κB cascade regulates hundreds of genes with roles in major cellular activities, including cell death and proliferation. The main signaling pathway of NF-κB involves the activation of the IKK complex. When this complex is activated, NF-κB dimers are able to translocate to the nucleus where they can bind to DNA and induce transcription. The NF-κB cascade’s main focus has been to obtain a pure and significant amount of NEMO to use in future assays. To do this, NEMO genes were subcloned into the pET15b(+1) vector. This vector was transformed into the T7 Express I Competent E. coli. The E. coli were grown in LB broth at 37°C overnight. A new culture was obtained from the overnight culture at a 1:200 dilution and grown to an OD600 of about 0.6. 1 mM IPTG was used to induce protein expression and the cells were grown for another 6 hours. The cells were pelleted and stored at -80°C. The pellets were dissolved in 100 mL of 20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole wash buffer along with a protease inhibitor tablet and 0.1 µL/mL of nuclease. The cells were lysed by microfluidization at 20 kpsi and then 100 mL of wash buffer containing 8 M urea was added. The cells were incubated for one hour at 37°C. The lysate was clarified by using a centrifuge at 38000 rpm for 30 minutes and then sonicated at 65% efficiency for 5 minutes on and 5 minutes off, 20 times. The lysate was filtered with a 0.2 µm filter. It was then applied to a 5 mL His nickel column, followed by 5 column volumes of 20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, and 6 M urea (pH 7.4). The protein was then refolded on the column using a 20 column volume urea gradient from 6 to 0 M. The protein was eluted with a step gradient of an increasing concentration of 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole (pH 7.4). The purpose of denaturing and refolding the protein in this way is to remove impurities. NEMO that eluted from the nickel-NTA column was concentrated and applied to a size exclusion column equilibrated with 20 mM sodium phosphate and 500 mM NaCl (pH 7.4). The elution samples were run on a SDS-Page gel to determine which of the NEMO containing fractions to pool, concentrate, aliquot, and store at -20°C. This general procedure was repeated with slight variations, including using a zinc column instead of the nickel column, in order to optimize the purification yield and purity.

Introduction
The NF-κB is composed of multiple proteins which play important roles in cell activities such as inflammation, immunity, cell proliferation, differentiation, and survival. The main signaling pathway of NF-κB involves the activation of the IKK complex. When this complex is activated, NF-κB dimers are able to translocate to the nucleus where they can bind to DNA and induce transcription (Figure 1). There are also other signaling pathways and complexes that lead to the release of different NF-κB dimers that affect cells differently. Through transcription, NF-κB is able to influence various cell activities.

Activation of IKK Complex

Although the NF-κB signaling pathway is necessary for various activities essential to life, it is also linked to multiple diseases. For example, it has been found that in some cases, extensive activity of NF-κB is seen in diseases related to inflammation. On the other hand, too little activity of NF-κB has been seen in diseases dealing with immunodeficiencies. Having more control of the regulation of NF-κB can help in the treatment of such diseases. One part of the NF-κB that has not yet been extensively studied is IkBα’s interaction with NEMO. Inhibiting this interaction has the potential to modify the effects of the NF-κB by limiting or amplifying the release of certain transcription factors into the nucleus. Thus, learning more about IkBα and NEMO’s interaction can contribute to the treatment of a number of diseases.

Methods
The main focus has been to obtain a pure and significant amount of NEMO to use in future assays. To do this, NEMO genes were subcloned into the pET15b(+1) vector. This vector was transformed into the T7 Express I Competent E. coli. The E. coli were grown in LB broth at 37°C overnight. A new culture was obtained from the overnight culture at a 1:200 dilution and grown to an OD600 of about 0.6. 1 mM IPTG was used to induce protein expression and the cells were grown for another 6 hours. The cells were pelleted and stored at -80°C. The pellets were dissolved in 100 mL of 20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole wash buffer along with a protease inhibitor tablet and 0.1 µL/mL of nuclease. The cells were lysed by microfluidization at 20 kpsi and then 100 mL of wash buffer containing 8 M urea was added. The cells were incubated for one hour at 37°C. The lysate was clarified by using a centrifuge at 38000 rpm for 30 minutes and then sonicated at 65% efficiency for 5 minutes on and 5 minutes off, 20 times. The lysate was filtered with a 0.2 µm filter. It was then applied to a 5 mL His nickel column, followed by 5 column volumes of 20 mM sodium phosphate, 500 mM NaCl, 40 mM imidazole, and 6 M urea (pH 7.4). The protein was then refolded on the column using a 20 column volume urea gradient from 6 to 0 M. The protein was eluted with a step gradient of an increasing concentration of 20 mM sodium phosphate, 500 mM NaCl, and 500 mM imidazole (pH 7.4). The purpose of denaturing and refolding the protein in this way is to remove impurities. NEMO that eluted from the nickel-NTA column was concentrated and applied to a size exclusion column equilibrated with 20 mM sodium phosphate and 500 mM NaCl (pH 7.4). The elution samples were run on a SDS-Page gel to determine which of the NEMO containing fractions to pool, concentrate, aliquot, and store at -20°C. This general procedure was repeated with slight variations, including using a zinc column instead of the nickel column, in order to optimize the purification yield and purity.

Results
Gel of Pre and Post Induction NEMO

Gel Comparing NEMO Purified Using a Nickel Column vs a Zinc Column

Discussion
In each gel, the NEMO protein can be found around 30 kDa. The strength of the band with NEMO protein relative to the strength of the other bands in the same gel can be used to determine how pure a particular fraction is. Figure 2 shows the results from the optimized procedure of protein induction. The second well shows the protein segment pre-induction while the third well shows the protein segment post-induction. The pre-induction well shows the effect that NEMO was run through a nickel column. The fractions resulting from this procedure are seen in the first 11 fractions of Figure 3. Although NEMO is present in some of these wells, it is not very pure, as seen by the presence of other bands. To accommodate for this, the procedure was changed so that NEMO would be run through a zinc column, another element that NEMO has a high affinity for, instead of a nickel column. This is reflected by Figure 3. In the fraction run in the zinc column, NEMO is much more pure compared to the fractions run with a nickel column. The band containing NEMO is very strong and all the other bands are weak. However, the quantity of NEMO yielded with this method was still less than optimal for use in experiments. The procedure was once again changed to try and get a higher yield of NEMO specifically while using the zinc column. The step where NEMO is denatured was taken out because it reduced the quantity of NEMO and was found unnecessary for purification with the zinc column (Figure 4). Although the quantity of NEMO that came from the procedure with the nickel column was higher, using the procedure with the zinc column and without denaturation still yielded enough pure NEMO to be used to start conducting experiments.

Conclusion
The expression of NEMO was successfully optimized so that enough was present for purification. Also, enough NEMO of a high enough purity was successfully purified to begin some experiments including SAXS, thermofluor buffer optimization, and crystallization screening. However, to improve the results yielded from these assays, and to begin more future assays, the process of purifying NEMO needs to be further optimized. It would be ideal if NEMO of a high enough purity could be collected in large quantities. Thus, the improvement of the purification procedure will remain a focus of the project. After this is done, the focus of the project can shift to finding crystal hits on a larger scale and working to optimize crystallization. Doing this will help further characterize the region of NEMO that binds to IkBα. Another step for the future of the project would be to perform SAXS and fluorescence anisotropy assays to conduct landing assays with NEMO and IkBα. Carrying out and optimizing such assays will contribute to the overall goal of learning more about NEMO’s interaction with IkBα.

References
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