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Bachelor of Science

Thyroid Hormone Receptor: Dimers, Dimers, Dimers

Senior Project

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Background

Nuclear reactors are intracellular receptors as well as transcription factors. They respond through physical interactions with their respective ligands. These ligands are small, hydrophobic signaling molecules such as steroid hormones. Once the ligand is bound, co-activators or co-repressors may be recruited that modify the nuclear receptor's effects on a gene.

Most nuclear receptors are composed of four functional domains. The N-terminus contains the A/B region, which has one or more autonomous transcriptional activation functions. Following this region is the C region, or DNA binding site. This region of the receptor contains two zinc finger motifs and several sequence elements that contribute to the recognition and binding of a response element. The ligand binding domain is located at the C-terminus. Within this region there is another activation function and also a dimerization interface. Ligand binding domain consists of twelve α-helices that form a hydrophobic pocket that can accommodate the hydrophobic ligands. Between the DNA binding domain and the ligand binding domain is a "hinge" region. This region allows the two flanking regions to adopt different conformations.

One of the nuclear receptors that we are currently working with is the thyroid hormone receptor. There are four different isoforms of the mammalian thyroid hormone receptor: alpha-1, alpha-2, beta-1, and beta-2. The thyroid hormone receptor binds to regions on DNA known as T3 response elements. These response elements contain 2 half-sites of AGGTCA.

The thyroid hormone receptor can bind to the DNA as a monomer, homodimer, or heterodimer. Forming a heterodimer with the retinoid X receptor allows for binding with the highest affinity.

The thyroid hormone receptor can bind to the T3 response elements without having T3 bound, but the presence or absence of T3 changes the activity of the thyroid hormone receptor. When bound to the DNA without being occupied by T3, the thyroid hormone receptor acts as a repressor. In contrast, binding of the thyroid hormone receptor occupied by T3 activates transcription.

We also work with the retinoid X receptor. RXRs are very important to this research because they can heterodimerize with many nuclear receptors, such as the thyroid hormone receptor. Forming heterodimers has two very important effects. First, it increases the efficiency of the receptors' binding to the DNA. It also allows for two different signaling inputs, which are the ligands for each member of the heterodimer. The goal of my research, therefore, is to produce thyroid hormone receptor dimers. First, I will attempt to form homodimers and, in the future, heterodimers with RXR.

Materials and Methods

TRα-1

pET-TRα1 in RIPL cells from a glycerol stock are plated onto Ampicillin/Chloramphenicol LB agar plates. The plates are incubated at 37°C overnight. A starter culture is then made by selecting one colony and adding it to 25mL of 2X YT media with Zn Acetate, Ampicillin, and Chloramphenicol. The starter culture is then incubated overnight in a shaker at 37° C and roughly 220 RPMs.

10mL of the starter culture (2% of final volume) are added to a flask containing 500mL of 2X YT media containing Zn Acetate, Ampicillin, and Chloramphenicol. The flask is then placed in a shaker at 37°C to allow the cells to grow.

Once the OD of the culture reaches 0.60, the flask is removed from shaker and allowed to reach room temperature. The cells are induced using 1M IPTG (250μL). The cells continue to grow in a shaker at 20°C for 20hours. The cells are then spun down at 6,000RPMs for 20 minutes. The pellets are stored at -80°C, until ready for lysis.

Cell pellets are resuspended using 40-50mL lysis buffer. Once thoroughly resuspended, the cells are lysed using the French Press. The cells are spun down at 16,000RPMs for one hour. Adjust the pH of the supernatant to 8 using 3M Tris Base.

Add 3mL (1.5mL bed volume) of Ni-NTA Agarose slurry to column. Wash column with 20mL of water. Equilibrate column with 3mL lysis buffer. Add cell lysate supernatant to column at 4°C. Allow the lysate to run through column twice (4°C). Return column to room temperature. Wash column with 20-25mL of lysis buffer. Follow by washing column with 25mL of wash buffer. To elute protein, add 1mL elution buffer to column and let sit for one minute before collecting eluent. Repeat until roughly 3mL eluent collected. Estimate the protein concentration of eluent. Then, inject sample onto an S-200 gel filtration column.

pET Sumo TRα

We used the amplicon obtained from a PCR of TRα and ligated it with the pET Sumo vector. Then Mach1-T1^{R} competent cells were transformed using the ligation product. The colonies are screened.

pET Sumo RXR

pET Sumo RXR in RIPL cells from a glycerol stock are plated onto Kanamycin/Chloramphenicol LB agar plates. The plates are incubated at 37°C overnight. A

starter culture is then made by selecting one colony and adding it to 25mL of 2X YT media with Zn Acetate, Kanamycin, and Chloramphenicol. The starter culture is then incubated overnight in a shaker at 37°C and roughly 220 RPMs.

10mL of the starter culture (2% of final volume) are added to a flask containing 500mL of 2X YT media containing Zn Acetate, Kanamycin, and Chloramphenicol. The flask is then placed in a shaker at 37°C to allow the cells to grow. Once the OD of the culture reaches 0.60, the flask is removed from shaker and allowed to reach room temperature. The cells are induced using 0.1mM IPTG (50μL). The cells continue to grow in a shaker at 10°C for 20hours. The cells are then spun down at 6,000RPMs for 20 minutes. The pellets are stored at -80°C, until ready for lysis.

Add 1mL (500μL bed volume) of Ni-NTA Agarose slurry to column. Wash column with 20mL of water. Equilibrate column with 3mL lysis buffer. Add cell lysate supernatant to column at 4°C. Allow the lysate to run through column twice (4°C). Return column to room temperature. Wash column with 20-25mL of lysis buffer. To elute protein, add 1mL elution buffer to column and let sit for one minute before collecting eluent. Repeat until roughly 2mL eluent collected. Estimate the protein concentration of eluent.

Results

pET TRα-1 Form Homodimers

After Ni-NTA purification (see methods), the protein sample was loaded onto an S-200 gel filtration column. The chromatogram (Figure 1) shows a single peak at fraction 84 in an automated fraction collector. A single peak in this region indicates the presence of pET TRα-1 homodimers. Using a protein of standard molecular weight, we found that pET TRα-1 monomers are found at fraction 40 in an automated fraction collector. Thus, pET TRα-1 homodimers should be found at around fraction 80.

pET Sumo TRα Ligation was succesful

After performing PCR of pET TRα, the amplicon was run on a 1% agarose gel. (Figure 2). The resulting band was then extracted and purified. The TRα insert was ligated with pET Sumo TRα. The ligation product was then screened. Figure 3 shows a band that indicates that the ligation was successful.

Conditions for growing pET Sumo RXR optimized

Because RXR expresses well, there is a problem with the protein aggregating and perhaps misfolding. To combat these problems, we wanted to optimize the conditions for growing cells. The goal was to grow cells at the lowest temperature and IPTG concentration. Flasks containing 500mL of cell cultures were grown as detailed in the protocol until time to induce. Then, the cells were induced using different concentrations of IPTG and grown for 20 hours at different temperatures.

After purification, the protein expression was measured by estimating the protein concentrations of each sample and running them on 12% SDS Page gel (Figure 4).

Discussion

While pET TRα-1 has successfully formed homodimers, it is not the molecule of choice to form heterodimers. It lacks the thrombin cleavage site necessary to remove the C-terminal His-tag. Removing the C-terminal His-tag is necessary to form and purify heterodimers. Thus, pET Sumo TR is a much better candidate for heterodimerization. It possesses an N-terminal His-tag and a site for cleavage using a Sumo protease. This is something I would like to pursue in the future. Work with RXR has also been very important in our attempts at dimerization. Therefore, the recent optimization of the growth protocol could result in heterodimer formation with either TR or CAR.

Forming homodimers of pET TRα is the first step in crystallizing a full-length neclear receptor. While the homodimers themselves have not been crystallized yet, it is still a possibility. If they and the TR/RXR heterodimers could be crystallized, it would allow for a comparison of the respective dimer structure. This is important because one could investigate any conformational changes that arise as a result of dimerization. This could be used to better explain why homodimers do not bind DNA with the same high affiinity as heterodimers. Purifying the TR homodimers also allows for certain kinetic experiments to be done. Binding affinity for both ligand and TR could be determined. I think it would be interesting to determine the binding affinity one TR protein has for another. Another area of interest would be to see how binding of

ligands differs between homodimers and heterodimers. This could also be explained looking at any conformation changes the occur upon dimerization and ligand binding.

Figures

Figure 1

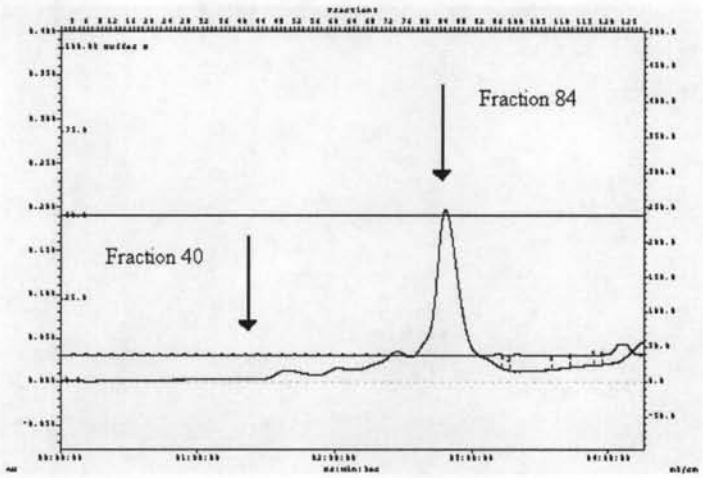
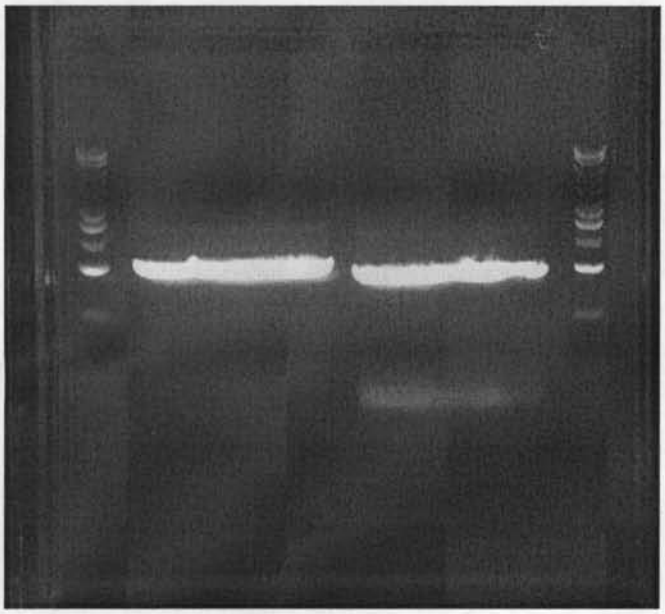


Figure 2



TRα

Figure 3

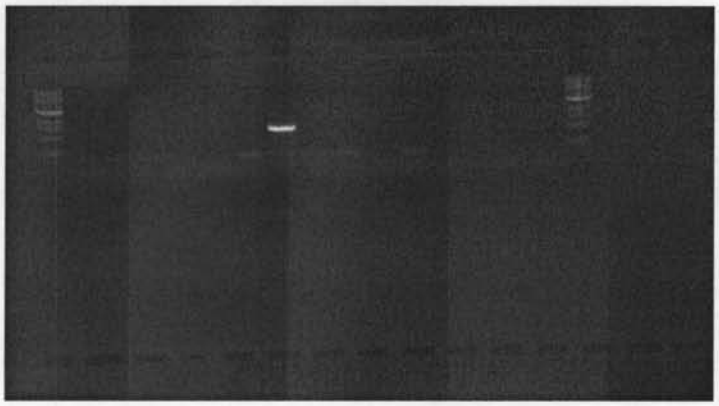
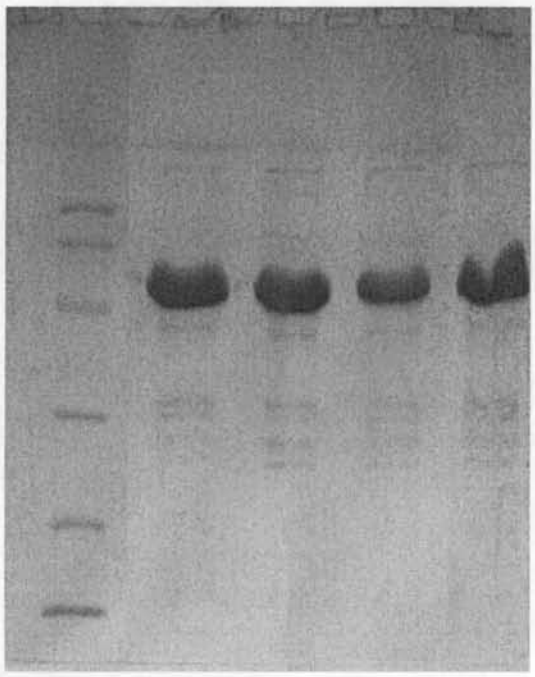


Figure 4



Lane 1 Empty
Lane 2 Marker
Lane 3 Empty
Lane 4 15°C 0.5mM IPTG
Lane 5 Empty
Lane 6 15°C 0.1mM IPTG
Lane 7 Empty
Lane 8 10°C 0.5mM IPTG
Lane 9 Empty
Lane 10 10°C 0.1mM IPTG

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