Freie Universität Berlin

Internship report

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Field of Study: Biochemistry (Bachelor)

Institution: Vanderbilt University, School of Medicine

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Scientific Part

Introduction

G-protein coupled receptors (GPCRs) are a large group of receptor proteins that are involved in many different signal transduction pathways. The name derives from an associated G-protein, that is bound to the seven transmembrane-domain receptor. Upon activation, the receptor undergoes a conformational change and acts as a guanine nucleotide exchange factor (GEF), exchanging the GDP bound by the G-proteins for a GTP. The α-subunit of the activated G-protein now dissociates from its associated β- and γ-subunits and can effect different intracellular signaling proteins.

The signaling cascade is silenced via the arrestin-pathway. First, G-protein coupled receptor kinases phosphorylate the activated GPCR. Then, arrestin proteins specifically bind to the phosphorylated receptor and trigger the internalization process. There are four different arrestin subtypes expressed in mammals: the visual arrestin, that is involved in the phototransduction pathway and three non-visual arrestins, (of which arrestin-2 is the most abundant). The visual arrestin, also named arrestin-1, specifically binds to phosphorylated, light-activated rhodopsin.

In my research internship, we focused on two projects. First, we wanted to analyze the effect of a substitution of the three native cysteines of arrestin-1 with different amino acids, on the binding of arrestin-1 to rhodopsin. For previous studies, those three native cysteins had been replaced with alanine, valine or serine. This was necessary for spin-labeling the proteins to enable EPR-based distance measurements. During the experiments, it was noticed that different combination of those three amino acids in the position of the native cysteins effected the binding of the protein to different light-activated and/or phosphorylated forms of rhodopsin. Therefore, a systematic study of the different combinations and their binding capabilities was necessary. The different combinations of cysteine-substitutions were combined with pre-activation mutations that caused binding of arrestin to the light-activated, unphosphorylated receptor. Two mutations, R175E and D296R, have been used. Both work in a similar manner, disrupting the polar core, a key structure in arrestin folding. Upon disruption of the polar core, arrestin changes its conformation, becomes more flexible and is able to bind to the unphosphorylated receptor. In the wild type protein, the negative charge of the phosphate group interacts with the polar core and partially unfolds the protein, enabling full binding to the GPCR.

The second project was aimed at a better understanding of the conformational changes in arrestin-1 upon its binding to rhodopsin. Based on a crystal structure of the arrestin-rhodopsin complex, three amino acids (valine 177, alanine 285 and isoleucine 299) were selected to be modified. The three amino acids are positioned in the N-domains of the arrestin molecule, which are believed to twist upon binding to the receptor. The twist would result in a changed position of the amino acids relative to each other, which can be detected via EPR measurement. For EPR measurements, the selected amino acid is substituted with a cysteine via mutagenesis-PCR. The translated protein is then chemically

modified, which introduces a covalently bond spin-label at the position of the cysteine. Therefore, all native cysteins need to be replaced with different amino acids.

Methods

Cloning

For the project dealing with different cysteine-substitutions, mutants containing different combinations of cysteine-substitutions had already been constructed. Therefore, either one of the two pre-activating mutations has been cloned into the vectors.

For the second project, aimed at introducing spin labels, a mutagenesis-PCR had to be performed first. The primers were designed in order to introduce a cysteine at the selected position for substitution. The PCR-products were then cut with the restriction enzymes and ligated into a pGEM-vector already containing the sequence for arrestin-1. After the successful cloning of the single cysteine substitution mutants, two double mutants were constructed, by inserting the sequence containing the A285 or I299 mutation into the V177C vector.

All clones were sequenced in order to confirm the correct sequence.

In vitro transcription and translation

10µg Plasmid DNA of each construct has been linearized with HindIII-restrictase and transcribed using SP6-polymerase.

Of the transcribed RNA, 24µg have been used for translation via the rabbit erythrocyte system. Additional ^{14}C- and ^{3}H-labeled Leucine was added, enabling the radioactive labeling of the translated arrestin-1 mutants.

Binding assay

The translated proteins were incubated with either phosphorylated, or unphosphorylated rhodopsin and subsequently exposed to light enabling activation of the photoreceptor. The samples were then loaded onto sepharose columns for gel filtration in order to separate arrestin-1-rhodopsin complexes from the unbound proteins. The fractions containing the ligand-receptor-complexes were collected and analyzed in a scintillation counter.

Protein Purification

Two of the constructed mutants of arrestin 1, the double cysteine substitution mutants V177CA285C and V177CI299C, were selected for large scale protein purification. Therefore, the arrestin-1 sequence was subcloned into a pTrcHis vector and transformed into BL21 gold cells. After the cells were grown

overnight, the translation of the arrestin-1 mutants was induced by adding IPTG. The cells were then harvested and lysed using a combination of lysozyme and sonification. To measure the expression level, the samples were analyzed via SDS-PAGE and western blotting. For the large scale preparation, the lysates were purified by multiple ion-exchange chromatography, using heparin, Q- and SP-sepharose columns. After each step, the collected fractions were analyzed via SDS-PAGE and the fractions containing the target protein were selected for further processing. After the last step, the selected fractions were combined and concentrated.

Results – Binding Assay

Both sets of pre-activated, Cys-less proteins show significantly decreased binding to the unphosphorylated form of rhodopsin, compared to pure pre-activation mutant. The affinity of the pure D296R mutant to the unphosphorylated, light-activated receptor seems to be lower than that of the pure R175E mutant, confirming previous results.

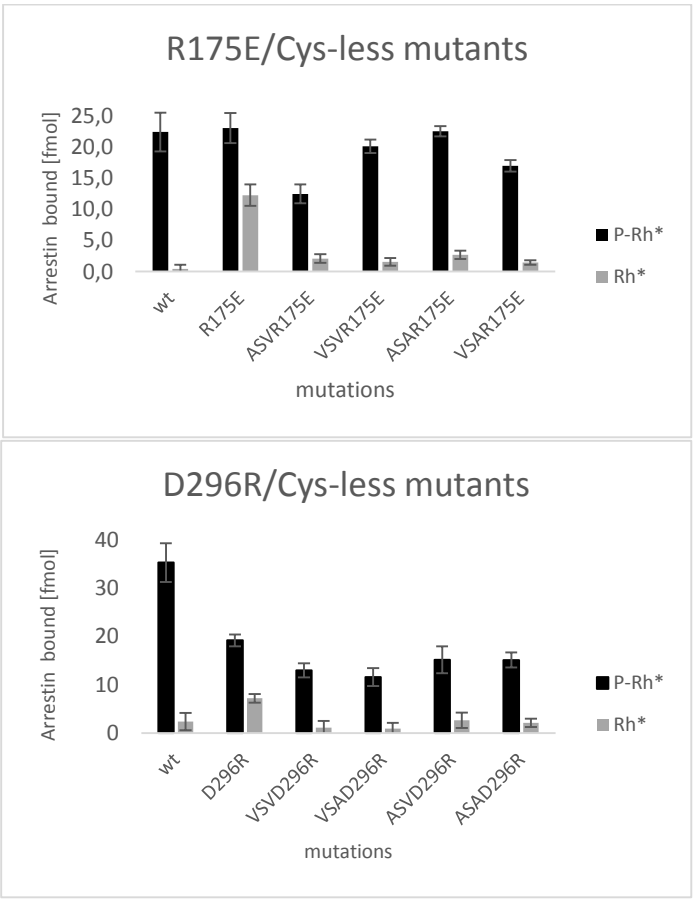

Figure 1: Bar chart displaying the results from the binding assay of the pre-activated, Cys-less mutants.
P-Rh*: phosphorylated, light-activated Rhodopsin; Rh*: unphosphorylated, light-activated rhodopsin; wt: wild type; R175E: substitution of arginine 175 with glutamic acid; D296R: substitution of aspartic acid 296 with arginine; VSV/VSA/ASV/ASA: substitution of the three native cysteine at the positions 63, 128 and 143 with either valine, serine or alanine. 0.3µg rhodopsin and 100fmol arrestin have been used for the binding assay. Errors are given as standard deviation.

Introduction

The Gurevich lab is part of the department of pharmacology and as such part of the Vanderbilt School of Medicine. Vanderbilt itself is one of the biggest universities in the southern part of the United States. According to official statistics, the university as about 12,000 students and 3300 employees. The university is part of the Association of American Universities, underlining their focus on research. The lab itself is a joined lab of Eugenia and Vsevolod V. Gurevich. As there is a high fluctuation of students and post docs, it does not make sense to give exact numbers of people working there. Generally speaking, the lab consist of the two professors, two or more post docs and a changing number of grad and undergrad students.

As this is a research group, the occupational area of the post docs and students was research. They were responsible for planning and conducting experiments, presenting them in a weekly lab-meeting and finally summarizing the data to publish them in a scientific journal.

My expectations for the internship were to get further research experience, learn new methods, improve my English skills and to experience the inner workings of a research group in the United States in general.

Application Process

I applied for this internship via the DAAD RISE worldwide internship program and was granted a scholarship. The application was conducted online and requested a letter of motivation and a commendatory letter.

Travel preparations and Visa application

When traveling to the US, a valid visa is necessary for a longer residency and a studying purpose. There are several different options, of which I choose the J-1 visa. A J-1 visa is aimed at exchange visitors, such as interns, students, short-term scholars etc. Upon applying for a J-1 visa, several documents have to be filled out and a number of fees have to be paid. The most important document, however, is the DS-2019 form. This form needs to be filled out by the intern and the hosting institution and then the original form has to be send to the intern. The form contains information necessary to apply for a visa interview at the US embassy in your home country. It was also necessary to bring the original document with you to the visa interview. In order to obtain the DS-2019 form, several other documents may be requested and a security check may be performed. Apart from acquiring the above mentioned form, the rest of the visa application process was relatively straightforward. The visa interview itself was very short and no complicated questions have been asked.

Everyday working life

As is common in science, there were no strict working hours. The internship was focused on particular projects and I was free to choose the time when I wanted to perform the necessary experiments. Generally speaking, I worked from 9am to around 6pm, sometimes 7-8pm, Monday till Friday. It was not expected to work on weekends, but sometimes the experiments required you to do so. I was closely supervised by a post-doc and he introduced me to every new method and procedure. This also ensured I would get feedback on my results, as we discussed and analyzed them together. I was able to use a lot of expertise I gained during my studies, as I was already familiar with standard methods, such as SDS-PAGE, Agarose-Gel electrophoresis, Cloning, Western Blot, etc. Therefore, we were able to focus at least in part on more advanced methods.

Conclusion

Overall, I would describe my internship as a positive and instructive experience. There were some problems during the first week, as the post doc who was supposed to be my supervisor, took up a position as an assistant professor at another institution only a few weeks before my internship started. Therefore, it took some time to figure out a new project and finally get started with it. Concerning my future professional career, I'm not yet entirely sure what to make of this internship. I got another good insight into a research group and the working life of scientists occupied in the field of research. It was inspiring to see how some of the researchers were able to mostly independently carry out their interests and plan and perform their own experiments. It was also interesting to experience a research group where the professor himself is at least in part still doing practical work in the lab. On the other hand, maybe because I was not assigned the project I actually applied for, I found it hard at first, to motivate myself for this project. As many projects, this one started with some very basic methods and procedures which are all but exciting and it took me some time to realize that I would also perform some more advanced and non-standard methods.