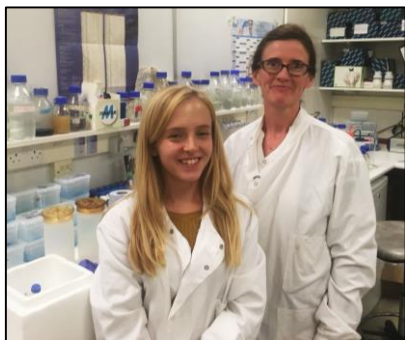


BIOCHEMICAL SOCIETY SUMMER VACATION STUDENTSHIP 2017

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Structural Studies of the Hsp90-Cdc37 Complex Using EPR Spectroscopy



Introduction

Hsp90 is a molecular chaperone, a vital protein that facilitates the folding and unfolding of a variety of proteins or 'clients'. Kinases are one such client, with over half of the

human kinome dependent on Hsp90 for activation (1). As its name suggests, Hsp90 is a heat shock protein and thus is highly expressed when the cell is under stress to ensure that proteins are folded and active. Its expression has also been found to be up to 10-fold greater in cancer cells (2), with many of Hsp90's client kinases key players in oncogenic transformation. Despite its importance, the structural changes that enable Hsp90 to activate client are still unclear.

It is known that Hsp90 is dependent on the Cdc37 cochaperone for recruitment of kinases (3), a 50 kDa protein that forms a complex with Hsp90. However, there are currently 2 alternative interfaces seen between Hsp90 and Cdc37 in the complex: a cryo-EM structure of the Hsp90-Cdc37-Cdk4 complex (4) shows an interface not previously seen in the structure obtained using X-ray crystallography in the absence of kinase (5). This suggests that some conformational cycle occurs during activation of client. Double-Electron-Resonance (DEER) spectroscopy, a type of EPR spectroscopy, can allow these dynamics to be observed. Labelling proteins by modifying the thiol groups of cysteines creates unpaired electrons, with the distances between the unpaired electrons across a protein-protein interface measured in solution. These distances can then be compared to the structural models to give an insight into what conformational changes occur and in what order. This knowledge could allow the development of small molecule inhibitors that act to regulate Hsp90-dependent kinases such as B-Raf, common drivers of oncogenic transformation.

Project Aims

The aim of my project was to express and purify the kinase B-Raf to be used in EPR experiments within the Hsp90-Cdc37 chaperone complex. Specifically, this was B-Raf V600E, a mutant with increased kinase activity. These experiments would allow new distances between labels in the complex to be measured and allow different conformations in the presence of client to be inferred, giving an insight into the client activation cycle. As a control for the DEER experiment using labelled Cdc37, I needed to assist in preparing samples for another experiment using

unlabelled Cdc37 in the complex. To confirm that the distances seen using unlabelled Cdc37 were real I aimed to then express and purify Hsp90 V391C that could be used to repeat a previously run DEER experiment as a control.

Methods

Expression of SUMO-tagged sB-Raf V600E: BL21* competent *E. coli* cells were transformed with pJ821 vector with the His-SUMO tagged B-Raf gene inserted. Small-scale and then large-scale cultures were grown with 1mM kanamycin added to select for the plasmid. 6L of large-scale cultures were incubated with shaking at 37°C until the OD reached 0.75. Cells were induced with 1mM rhamnose overnight at 20°C and were subsequently harvested by centrifugation at 4000 g for 20 minutes and resuspended in ice cold lysis buffer.

Expression and purification of the protease Ulp1: to cleave the SUMO tag from the B-Raf, Ulp1 additionally needed to be expressed and purified. BL21* competent *E. coli* cells were transformed with a pOPINE vector containing the gene for Ulp1 and harbouring kanamycin resistance. Large-scale cultures were induced with 1mM IPTG overnight at 20°C when the OD reached 0.63. Cells were harvested and resuspended in ice cold lysis buffer T500i. Resuspended cell pellets were sonicated and centrifuged at 18K RPM for 45 minutes at 4°C. Ni-NTA beads in a batch column were then used in the first purification step, binding the His tag of the Ulp1. The eluted protein was concentrated and the sample dialysed overnight in T200. The sample was centrifuged, filtered and then loaded onto an equilibrated S200 column for the second step of the purification: size exclusion.

Expression and purification of GST-B-Raf V600E: BL21* pRARE cells were transformed with a pGEX vector containing the sequence for GST-tagged B-Raf. Cells were transformed and grown as before, using both chloramphenicol and carbenicillin at 1mM concentration in plates and cultures and 1mM IPTG for induction. Purification was composed of three steps: 1) Batch column using glutathione sepharose beads and on-column cleavage to remove the GST-tag 2) Ion exchange using a Hi-Trap S column 3) Size exclusion using an S75 column.

Expression of Hsp90 V391C: the same method of expression as for B-Raf was used with BL21* pRARE cells, but here pRARE was not selected for by not adding chloramphenicol.

Results

Expression of SUMO-B-Raf was unsuccessful, as indicated by figure 1A due to the absence of a band at around 55.3 kDa (the MW of His-tagged SUMO-B-Raf) in lane 3 that would be apparent if expression was induced. Expression of Ulp1 appeared successful due to the presence of a strong band between 25 and 35 kDa in lane 5 compared to lane 4 (Figure 1A). Although the Ulp1 protein is approximately 72 kDa (in yeast), the catalytic domain is around 220 residues thus ~25 kDa. Subsequent purification was firstly carried out using Ni-NTA beads, with this ~25 kDa band evident in the elution fractions. Size exclusion chromatography using an S200

column allowed further purification of this Ulp1, seen as strong bands in fractions in peak 2 (Figure 1B). The band at ~70 kDa in fractions within peak 1 is likely an *E. coli* contaminant and not the full-length Ulp1, as expression of this sized protein was induced as seen in Figure 1A. Fractions in peak 2 were pooled and the protein concentration measured to be 1.26 mg/ml before adding 20% glycerol for storage at -80°C.

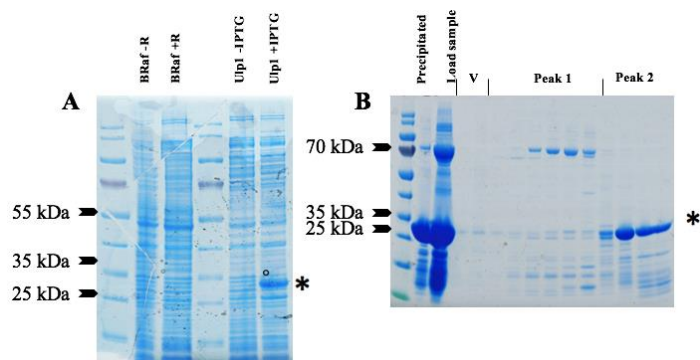


Figure 1. A) SDS-page gel showing pre- and post-induction samples for SUMO-BRaf V600E and Ulp1 protease.

There are no clear differences between the - and + Rhamnose (R) samples for BRaf. A strong band between 35 and 25 kDa for the Ulp1 +IPTG sample corresponds to the MW of the catalytic domain of Ulp1 and is not present in the -IPTG sample. B) SDS-page gel showing the final purification step for Ulp1 using gel filtration. The Ulp1 sample obtained from affinity chromatography precipitated thus was centrifuged and filtered before loading onto the S200 column. The chromatogram obtained showed 4 peaks and samples were taken from the void peak (V), peak 1 and peak 2. Peak 2 contained most of the Ulp1, indicated by the strong peaks between 25 and 35 kDa.

Due to the unsuccessful expression of the SUMO-BRaf, small scale expression tests were carried out using different *E. coli* strains for both SUMO and GST tagged B-Raf. A strong band was seen between 55 and 70 kDa when GST-B-Raf was expressed in BL21* pRARE cells so I proceeded with a large-scale expression of this (Figure 2A). This GST-B-Raf was purified, firstly using glutathione sepharose beads which successfully cleaved off the GST-tag and then by ion exchange chromatography. Size exclusion using a S75 column allowed B-Raf with a significantly greater purity to be obtained (Figure 2B). Fractions containing B-Raf were pooled and concentrated to 71 μM, aliquoted and flash frozen for storage. As only this small amount of B-Raf was obtained, GST-B-Raf was re-expressed in BL21* pRARE cells (Figure 3). In parallel, Hsp90 V391C was also successfully expressed in the same *E. coli* strain as shown by the band at ~100 kDa on the gel in figure 3, which in reality is closer to 90 kDa: the molecular weight of Hsp90.

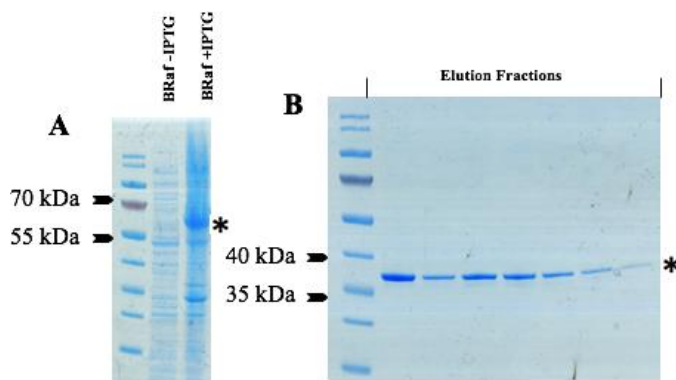


Figure 2. A) Gel showing expression of GST-B-Raf. Pre- and post-induction samples with were taken, with a clear band between 55 and 70 kDa seen in the post-induction sample. B) Final purification step for B-Raf using an S75 column. A band between 35 and 40 kDa can be seen in the 7 elution fractions and no other contaminating proteins are visible.

Future directions

The B-Raf V600E and Hsp90 V391C that I induced expression of in *E. coli* can be purified by other lab members and pooled with the B-Raf I have already purified. These can then be used in further EPR experiments to allow distances to be measured when in complex. This will improve understanding of the interactions between Hsp90, Cdc37 cochaperone and client kinase during the client activation process and what conformational changes occur to enable this. This knowledge will be beneficial in the design of new small molecule inhibitors for certain cancers by inhibiting the process and thus preventing kinase over-activation.

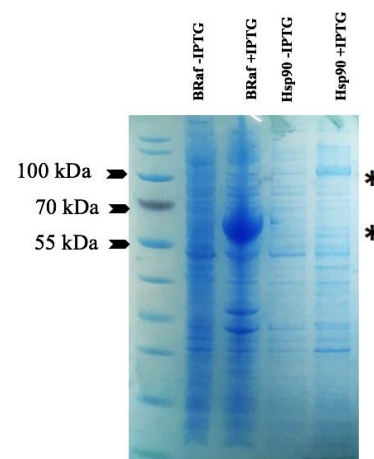


Figure 3. Gel showing induced expression of both GST-B-Raf and Hsp90 V391C. A strong band between 55 and 70 kDa in lane 3 compared to lane 2 is consistent with previous expression of GST-BRaf. A band can be seen at approximately 100 kDa in the post-induction Hsp90 sample which is not as intense in the pre-induction sample. This is therefore likely to be the Hsp90 V391C.

Departures from Original Proposal

My project deviated significantly from the original proposal due to the progression of the research group since the proposal was submitted. Originally I was to introduce cysteine mutations at positions in Hsp90, Cdc37 and a phosphomimetic Cdc37. However, this was already done and the EPR experiments already carried out for these (bar the unlabelled Cdc37 NoCys S13E, which I helped to prepare DEER samples for during my placement).

Value of Studentship

For me, the studentship was hugely beneficial and was thoroughly enjoyable. It has been a great learning experience and has enabled me to apply the theories I have learnt during my degree to a practical setting. I have developed strong skills in protein expression and purification, with learning to use an Akta for purification challenging but definitely worthwhile. Not mentioned in this report, I also gained experience in carrying out site-directed mutagenesis of protein phosphatase 5 (PP5) and in doing so gained experience setting up and running PCR experiments. The placement has additionally enabled me to see what a career in research would be like and will allow me to make an informed decision about my future path. I am very pleased to have spent my summer here.

References

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