Jonathan Prescott Biochemistry Society Summer Project 2009

Introduction

REST (Repressor Element 1 (RE-1) Transcription Factor), also known as NRSF (Neuron-Restrictive Silencer Factor) is a zinc-finger DNA binding protein that associates with many different co-factors, and has a large number of target genes. As a consequence, REST has a variety of roles in healthy cells including repression of neuronal differentiation in neuronal stem cells and non-neuronal cells. The protein is not present at normal levels however, in terminally differentiated neurones. REST has also been implicated in a number of diseases, most notably cancer. Different types of cancer cell display opposing roles for REST, with the protein having an oncogenic role in medulloblastoma [1], whilst seemingly acting as a tumour suppressor in epithelial cancers [2]. Dysregulation of REST is found in small cell lung cancer (SCLC) [3], an aggressive form of lung carcinoma strongly linked to the long term effects of smoking and often carrying a poor prognosis. SCLC cells have a neuroendocrine phenotype and express little REST. In contrast, non-small cell lung cancer (NSCLC) cells do express REST and therefore serve as a useful comparison against SCLC cells.

Like many cellular proteins, levels of REST within the cell are regulated by polyubiquitination. The E3 ligase complex $SCF^{\beta TrCP}$ is responsible for REST polyubiquitination and initiating its proteosomal degradation [4]. As with phosphate groups, it is now understood that ubiquitin can be removed from proteins as well as added. Deubiquitinating enzymes (DUBs) [5] fulfil this function and therefore specific DUBs may have a role in the stabilisation of cellular REST. From previous experiments, USP15 (Ubiquitin Specific Protease 15) was identified as a potential DUB for REST. The specificity of DUBs and their involvement in stabilisation of proteins dysregulated in cancer, could make them a potential drug target that is less toxic than current proteosome inhibitors such as bortezomib [6], making this area of research all the more important. Antagonists could be designed to target REST specific DUBs in cancers where REST is over expressed, such as medulloblastoma, allowing REST to be degraded more rapidly by $SCF^{\beta TrCP}$, to restore normal levels.

Project Aims and Objectives

The experimental aims of this project were to build on the previous research performed by Dr. Coulson's laboratory and investigate whether over expression of USP15, or the structurally similar DUBs USP4 and USP11, can stabilise co-transfected REST levels in deficient SCLC cells and stabilise endogenous REST levels in NSCLC cells. To achieve these goals, I learnt a variety of experimental techniques including: cell culture and transfection, fluorescence microscopy, preparation of protein extracts, protein assays and western blotting.

My personal goals, as well as fulfilling the above objectives, were to improve my biochemical and physiological knowledge in order to aid my academic studies, to learn some of the experimental skills and techniques necessary for scientific research and to experience working in a successful laboratory environment under the guidance of dedicated scientists.

Experiment Summary and Project Outcomes

The project ran over a 7 week period, initially focusing on an introduction to the laboratory and preliminary experiments in order to learn experimental protocols and techniques that would be required in later investigations. The remaining five weeks focused on refining newly acquired skills through a series of investigations designed to fulfil the aims of the project.

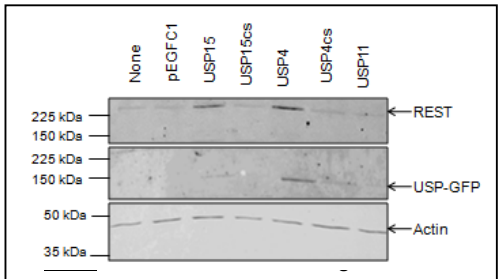
We first determined the most efficient method of transfecting NSCLC cells (NCI-H460) with GFP (green fluorescent protein)-tagged DNA plasmid in order to over-express USP15. Transfection efficiency was assessed for Lipofectamine 2000 and electroporation using 10 µg pEGFP-USP15. Fluorescence microscopy indicated a maximum transfection efficiency of approximately 50%; quantitative western blot analysis showed 9.3 fold more USP15 expression by electroporation compared to Lipofectamine 2000. Therefore, electroporation was selected as the most suitable transfection process.

In order to quantify the influence of each DUB on the stability of REST, titration experiments were required to ensure that each was expressed at a similar level. The plasmids transfected were GFP-tagged USP15, USP15cs ("-cs" indicating a cysteine to serine mutation in the catalytic site of the DUB), USP4, USP4cs and USP11. NCI-H460 cells were transfected with 7.5, 10 and 15 µg of each GFP-tagged plasmid using the established protocol. Untransfected cells or cells transfected with pEGFPC1 (10% relative to USP plasmids) were used as controls. Cells were observed by fluorescent microscopy then harvested 48 hours post-transfection for analysis of whole cell lysates by western blotting. We determined that, relative to 10 µg pEGFP–USP15, relatively comparable expression was achieved by transfection with 15 µg USP15cs, 7.5 µg USP4, 15 µg USP4cs and 7.5 µg USP11. Whereas similar amounts of USP15 and USP4 were expressed, we found 1.4 fold more REST in the USP15 transfected cells.

NCI-H460 cells were transfected with optimised quantities of plasmid, i.e. 0.5 µg pEGFPC1, 10 µg pUSP15-EGFP, 20 µg pUSP15cs-EGFP, 5 µg pUSP4-EGFP, 20 µg pUSP4cs-EGFP, 12.5 µg pUSP11-EGFP, 20 µg pUSP11cs-EGFP with a mock transfection and an untransfected sample as controls. Excluding USP4, approximately equal expression was achieved between the individual transfected USP plasmids and their mutant pairs, but expression was not equal between different USPs. Samples were harvested

at 24 hours and 48 hours post-transfection to observe the difference in expression over time. Fluorescence microscopy signified that the transfection efficiency of pUSP15-EGFP (approximately 50%) was lower than other transfections (70-90%) at both 24 and 48 hrs. USP11 and USP11cs were expressed in the region of 50% higher than USP4 after 24 hours, which in turn was expressed at more than 4 fold the level of USP15. At 48 hours, western data showed that all USP-GFP expression levels had fallen. Despite these changes in expression, no significant change in the relative expression of REST was observed in the transfected NCI-H460 cells.

To test the effect of specific USPs on co-transfected REST (RE-EX1), Lu 165 (SCLC) cells were transfected with pEGFPC1, pUSP15-EGFP, pUSP15cs-EGFP, pUSP4-EGFP, pUSP4cs-EGFP and pUSP11-EGFP with an untransfected control. Nuclear and cytoplasmic samples were harvested at 72 hours, with only the nuclear samples showing significant REST expression by western blotting. Cytoplasmic expression of USPs was also much lower than nuclear expression. Samples transfected with USP15 and USP4 showed the highest levels of REST with 4 and 5 fold higher expression relative to the untransfected control, USP11 or the catalytically inactive mutants [Fig. 1]. However, the relative nuclear expression of USP4 was 6 fold higher than USP15 and 100 fold higher than USP11.



expression of REST in Lu-165 cell nuclear extracts. Cells were co-transfected with RE-EX1 and indicated USPs/USP mutants.

This experiment was then repeated so that Lu 165 cells were co-transfected with 5 µg RE-EX1 and either 10 µg pEGFPC1, 20 µg pEUSP15-GFP, 5 µg pEUSP15cs-GFP, 10 µg pEUSP4-GFP, 10 µg pEUSP4cs-GFP, 10 µg pEUSP11-GFP or 20 µg pEUSP11cs-GFP to achieve more equivalent USP expression. Untransfected cells and cells transfected with 5 µg RE-EX1 were used as controls. Cells were harvested at 72 hours and whole cell extracts were analysed using western blots. USP11 and USP4 were expressed at 4 and 2 fold the level of USP15 respectively. pUSP4-EGFP transfected cells displayed double the level of REST observed in pUSP15-EGFP transfected cells. pUSP11-EGFP transfected cells exhibited 3 times the level of REST relative to the pUSP15-EGFP cells (data not shown). Thus it appears that each of the related USPs could affect REST in this model.

Conclusions

The project has yielded some results that help to address the questions posed in the project aims and provide the basis for further work. Although over expression of USP4 and USP11 in NCI-H460 cells did not reproducibly alter the stability of REST, results from the initial experiment may indicate a role for USP15 in these cells. These experiments should be repeated to optimise the amounts of plasmid transfected to achieve similar expression levels. Alternative NSCLC cell lines (e.g. A549) could also be investigated to confirm observed changes in REST levels. In Lu 165 SCLC cells, the expression of transfected REST, USP15, 4 and 11, appear to be mainly nuclear. The degree of co-transfected REST stabilisation appears to be correlated with the level of expression of USP15, USP4 and USP11. This could indicate that each of these DUBs have some specificity for REST in Lu 165 cells and play a role in its stabilisation. From the Lu 165 nuclear extract western blot data [Fig. 1], USP15, despite being expressed at lower levels than USP4, had a relatively similar effect on co-transfected REST stabilisation. This may indicate that USP15 has a higher specificity for REST than USP4 or 11. Repeat experiments should be carried out to investigate these possibilities and include less closely related DUBs as additional negative controls. Statistical analysis following three or more experimental replicates would be necessary in order to confirm any significant differences in expression level.

In the original aims for this project, the analysis of DUBs in relation to REST during the cell cycle was highlighted as an area of interest. During the project, our experiments took an alternative path but these have helped to inform future studies in synchronised cells to understand the role specific DUBs play in relation to REST at different cell cycle time points.

Personal Progress

This project has been a challenging and enjoyable learning experience, which has provided me with invaluable practice of scientific study and knowledge of an important area of cancer research. In addition to fulfilling all of my personal goals, I was pleased to increasingly be able to contribute to discussions concerning my experiments and possible future directions of investigation. I would like to extend my thanks to Dr. Judy Coulson for supervising this project and to the Biochemistry Society for funding my studentship.

References

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