Development of an assay to investigate functionally relevant protein mutations such as those in the DNA binding domain of IRF4.

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INTRODUCTION

Mutations in DNA are known to cause disease but it is not always apparent how a change in this DNA sequence relates to a particular disease. It may be that the DNA encodes for a protein with an important function which is lost or it could be that the mutation changes the way in which the protein interacts with other components of cells. These interactions can control how cells process information and affect signals and messages within the cells.

Interactions between protein and DNA have been implicated in a number of Neurological diseases, such as Alzheimer's and Parkinson's disease and also in cancers through deregulation of the process of converting DNA from a code to a protein. Cancer can often arise if the protein mutated is part of a group of DNA binding proteins called transcription factors which control the activity of some genes.

Methods such DNA electrophoretic mobility shift assay (EMSA), or DNA pull-down assays are often used to investigate protein-DNA interactions of transcription factors. The EMSA works on the principle that the protein can bind a labelled piece of DNA, called a probe, which will travel slower through a gel than a free piece of labelled DNA. When we image the gel, an interaction between probe and protein, is seen as a "shift" in the bands which tells us that the protein has bound to its target DNA. DNA pull-down assays work similarly, where the labelled probe binds to a protein to form a complex which sticks to coated beads. The proteins are then released and run on a gel to sort them by size. If the DNA probe bound a target protein, the protein is not eluted or washed away and will be visible on the gel at the appropriate size when imaged.

Identifying which of these techniques is most effective for investigation of DNA binding proteins can allow more efficient use of resources when working towards targeting the diseases which are caused when these proteins go wrong.

The example used here is IRF4, a transcription factor implicated in Multiple Myeloma. If it could be identified that mutations of the DNA binding domain are inactivating, disrupting IRF4 DNA binding and signalling, we may better understand the role of the mutation in disease and help to find ways to treat it.

METHODS

Electrophoretic Mobility Shift Assay

EMSA were carried out following protocol described for the Thermo Scientific Lightshift Chemiluminescent EMSA kit. The Control Reactions were conducted when using the kit for the first time to ensure components and procedure worked efficiently. Binding reactions are then conducted using protein of interest and relevant biotinylated oligonucleotides (Biotin-Oligo). Optimisation of the protocol incorporated additional components: 50% Glycerol, 1% NP-40, 100mM $MgCL_2$ and 200mM EDTA The 4-6% polyacrylamide gel was pre run with 0.5% TBE and the wells flushed before protein loading, and the gel run at 100V. The protein and DNA were transferred to a nylon membrane which was crosslinked for 15 minutes on a transilluminator equipped with 312nm bulbs. The Biotin-labelled DNA, was detected by chemiluminescence.

DNA Affinity Precipitation Assay

DAPA buffer was prepared using 10mM Tris HCL pH 7.5, 50mM NaCl, 1mM DTT, 1mM EDTA, 5% Glycerol and water and chilled. 50-100 µg of recombinant protein was made up to 500µl with DAPA buffer. A volume of Biotin-Oligo +/- a volume of competitor oligo was added to 15 µl of agarose beads and incubated at room temperature for 2 hours on a rotator. Beads were then washed 3 times in DAPA buffer, resuspended in 20µl of buffer. 25µl of 2X SDS Loading Dye added and the samples boiled for 5 minutes at 95°C. 20µl of supernatant was loaded onto an SDS PAGE gel, separated by electrophoresis and detection immunoblotting takes place for the IRF4 mutant using an antibody against the proteins histidine tag.

RESULTS

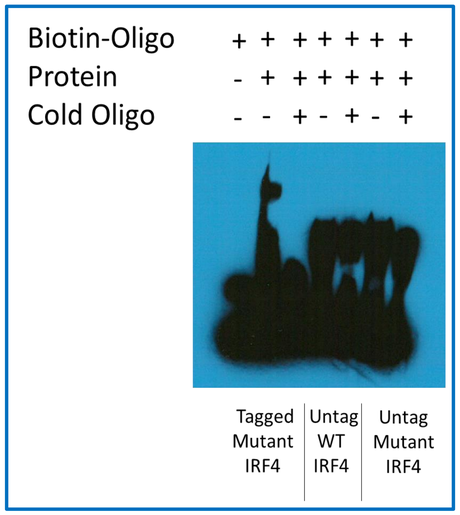

Figure 1. Scan following a DNA electrophoretic mobility shift assay (EMSA), on 3 proteins. Lanes 1-3 contain Tagged IRF4 mutant protein, 4-5 Wild Type (WT) IRF4 protein, untagged. And lanes 6-7 contain untagged IRF4 mutant protein.

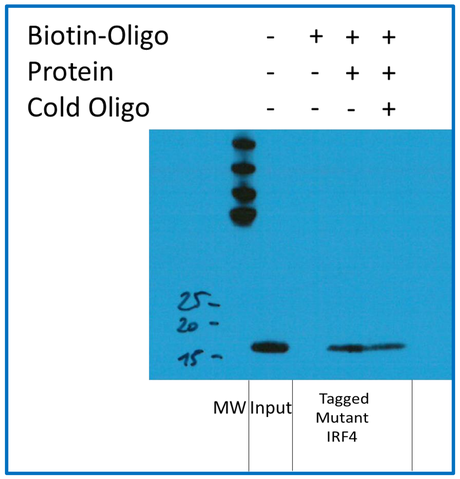

Figure 2. Scan following a DNA Affinity Precipitation Assay (DAPA) on tagged mutant protein. Lanes 1 contains a molecular weight ladder to confirm bands are the protein of interest, lane 2 is an input control to show what the protein of interest looks like when run on the gel, lane 3 contains biotinylated oligo alone, lane 4 contains biotinylated oligo and protein alone, and lane 5 contains biotinylated oligo, protein and a competitor oligo.

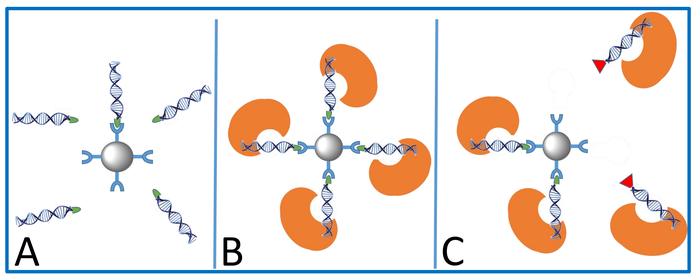

Figure 3 . The principles of a DNA Affinity Precipitation Assay. **A.** represents lane 3 of Figure 2. Here there are latex beads present which can bind to the biotinylated DNA oligo, however as no protein is present, no band will appear when what had attached to the beads is run on a gel**. B.** Represents Biotinylated Oligo, as in lane 4, which has bound to the binding site on the protein, and the biotin has bound to the beads. **C.** represents lane 5 where some protein like in **B.** which has bound to the beads, but some protein cannot bind the beads as it has bound competitor non-biotinylated oligo instead.

RESULTS AND DISCUSSION

Results

Figure 1. EMSA data show that when biotinylated oligo is added alone to the mutant IRF4 protein, the expected shift can be seen, between lanes 1 and 2. When a competitor oligo is introduced in lane 3 the shift is reversed. No shift however can be identified between lanes 1 and 4, and 1 and 5 respectively, with the Wild Type (WT) IRF4 protein and the untagged.

Figure 2, DAPA data show, in the second lane (input control), more protein is present when no beads are added to pull down the biotinylated sample. In lane 3, no protein was added and there is no band. In lane 4, there is reduced signal compared to input. Lane 5 shows reduced signal again compared to lane 4. The first lane is a molecular weight ladder (marker used to size the proteins).

Discussion

The protein shift in Figure 1 shows that despite the mutation, the biotinylated oligo and competitor oligo can still bind to the protein, implying that the mutation in this case is not enough to prevent DNA binding to the DNA binding domain. It is difficult to see whether there is a shift with the WT IRF4 or the untagged protein. Further repeats on other mutated versions of the IRF4 protein showed that only the tagged mutant could clearly be shown to shift following EMSA. The limitations of the EMSA mean that it is very difficult to achieve a clear image, and thus identify shifts in the protein. In order to see the shifted band, the film is often required to be exposed for longer than usual, meaning it becomes overexposed and difficult to interpret. The EMSA is also quite inconsistent, susceptible to even the smallest deviation from a very specific methodology, often the same test on the same protein can produce different results.

Figure 2 shows that the DAPA works to separate protein bound to the labelled DNA probe. In lane 3, no protein is present and so there is no band. In lane 4, some protein has bound to the beads via the Biotin-Oligo, therefore the signal is reduced but in lane 5, the signal is further reduced, as the competitor oligo binds to the protein and thus less protein is captured on the beads, eluted off and run on the gel to be imaged (as in Figure 3c). The technique is much more consistent than the EMSA, and on the whole, produces cleaner, easier to interpret images. The DAPA assay does however show some variation in results as well, amongst repeats.

CONCLUSIONS

While the EMSA can be conducted more rapidly than the DAPA, the EMSA requires a complex set of binding reactions for each protein, involving numerous reagents sensitive to errors in pipetting. The DAPA introduces less error and also provides much cleaner images than the EMSA. The DAPA data is easier to interpret and thus can be better used when investigating the interactions between a protein of interest and the DNA upon which it binds. Further investigation of the DAPA technique on other proteins would be required before it could fully be accepted as the technique of choice, and of course, some techniques may suit some interactions better than others. There is a need to evaluate the correct choice on a protein to protein basis, depending on the information required.

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