1 Investigating the effects of ADAR RNA editing activity and editing-independent ADAR effects on the functions of dsRNA sensors.

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1.1 SUMMARY

We will use ADARs to 1) identify endogenous RNA substrates for common dsRNA sensors and 2) to study the role of ADAR1 in PKR activation. For the first part, we will fuse the dsRNA sensors MDA5 and PKR to the deaminase domain of ADAR2. Transcripts bound to these sensors will be identified by high-throughput RNA sequencing, as they will undergo editing (A to G transitions). In the second part, we will investigate how ADAR1 inhibits PKR activation; whether it is editing dependent or independent.

1.2 SCIENTIFIC BACKGROUND

The RNA editing enzymes, ADAR1 and ADAR2 can convert adenosine to inosine in dsRNA (for review (Sinigaglia, Wiatrek et al., 2019)). In humans most of the dsRNAs that are edited by ADAR1 are generated by Alu elements embedded in inverse orientations in longer transcripts (Bazak, Haviv et al., 2014). Inosine in dsRNA marks the dsRNA as being self and prevents activation of the innate immune response by the dsRNA sensors (Mannion, Greenwood et al., 2014). We hypothesize that not all dsRNAs formed from inverted Alus are highly immune-inducing as they must be extremely duplex in nature. However, it is difficult to isolate these endogenous, highly immune-inducing dsRNAs using standard experimental approaches based on immunoprecipitation techniques, as the dsRNA sensors MDA5 and PKR are very 'sticky', bind many types of dsRNAs and form oligomers, thus decreasing the yield of immunopurified dsRNA. Therefore, by using a tethering approach with the dsRNA sensors fused to ADAR2 deaminase domain, we hope to identify a subclass of highly immunogenic dsRNAs and to determine if they are the same dsRNAs binding to both dsRNA sensors, MDA5 and PKR.

We have unpublished data that reveal that removing PKR can help rescue the *Adar1* mutant phenotype in mice (Maurano, Snyder et al., 2021). ADAR1 has an unknown editing independent activity and one possibility is that it interacts with PKR and prevents its activation (Liddicoat, Piskol et al., 2015). Therefore, we plan to investigate this by studying their interaction to determine if it requires editing activity. If the interaction between ADAR1 and PKR is protein-protein, we will utilize our stable cell lines expressing a deletion series of ADAR1 to delineate which region of ADAR1 is essential for this interaction.

1.3 AIMS

The first aim of the project is to generate fusions between the deaminase domain of ADAR2 and the dsRNA sensors MDA5 and PKR. Stable cell lines will be generated expressing these fusion proteins under doxycycline promotor control so the level of induction of the fusion proteins can be tightly regulated. The dsRNAs that bind PKR and MDA5 will be identified by RNA Seq as they will have an increased A to G signature. Analysis of these dsRNAs will reveal if the same dsRNAs bind preferentially to both PKR and MDA5.

The second aim is to investigate the interaction between PKR and ADAR1, to elucidate how ADAR1 inhibits PKR activation.

1.4 WORK PROGRAMME

We presume that the tethered ADAR2 protein will have the ability to edit dsRNA as previously described. MDA5 oligomerizes on dsRNA so it is possible that it may be difficult for the tethered ADAR2 deaminase domain to edit the dsRNA. PKR has to dimerize on dsRNA for activation by self-phosphorylation (Patel & Sen, 1998) so it is unlikely that this will interfere with RNA editing. However, we will generate an additional control of DGCR8-ADAR2 deaminase domain fusions in cells that are deficient of DGCR8 so as to determine if the technique is sensitive enough to detect low-abundance interactions. The DGCR8 experiment would be a control, but potentially could also develop in a parallel project, as it is still very debatable if it binds RNAs others than pri-miRNAs.

1.4.1 The experimental plan

We will fuse the deaminase domain of ADAR2 as previously described. We will use the published ADAR2 deaminase mutants that have increased activity (Katrekar, Xiang et al., 2022). We will fuse the ADAR2 deaminase domain to the carboxy termini of MDA5, PKR and DGCR8. All of these proteins will have a FLAG epitope tag at the amino terminus for ease of detection. We will generate stable stem cell lines with these constructs under the doxycycline promoter. We will visualize expression of the fusion proteins by immunoblot. We will select two sets of three stable cell lines that have either high and low expression of the fusion protein.

We will perform RNA Seq on the stable ADAR2 fusion cell lines versus WT control and look for A to G conversion which is the hallmark of RNA editing by ADAR2. We will only use short-time points of dox induction to identify primary editing sites and avoid identification of other sites resulting from long-term dox treatment and/or overexpression. Next, we will compare the RNA editing levels of transcripts at low and high levels of the fusion proteins. We will determine if it is the same or different transcripts that bind to both dsRNA sensors.

For the second aim we will make plasmids to over-express an epitope tagged PKR. We will test its interaction with both active and inactive over-expressed full-length ADAR1 in A549 cell line that has a basal level of interferon induction. This will elucidate if RNA editing activity is required for ADAR1 to prevent activation of PKR as it is possible that by editing the dsRNA it reduces the quantity of dsRNA for PKR to bind to. We will perform an immunoblot to determine if PKR is autophosphorylated which is associated with kinase domain dimerization

and activation. We will also check for PKR activation by performing RT-qPCR on downstream transcripts that are activated by PKR such as *Trib3*, *Atf5*, and *Eif4ebp1* (Wong, LeBon et al., 2019). If the inhibition of PKR activation by ADAR1 requires protein-protein interaction we will over-express PKR in HEK 293 cells. We already have stable cell lines expressing a deletion series of ADAR1 in HEK 293 so we will be able to immunoprecipitated ADAR1 and determine which domain of PKR interacts with.

The generation of the constructs and stable cell lines will be performed at Edinburgh University and the bioinformatics will be performed at CEITEC. This will be synergistic for both groups as the CEITEC group have the expertise working with ADAR proteins whereas the Edinburgh group have expertise with protein fusions and generation of stable cell lines in challenging cell lines, such as embryonic stem cells.

This project will be hugely beneficial for the CEITEC group as it addresses a major question in the field: what is the dsRNA that triggers the innate immune response in human cells? It also tackles another question how does ADAR1 prevent the activation of PKR.
If this project is successful then it would form the preliminary results for future joint application for funding.

1.5 REFERENCES

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1.6 BUDGET

Sequencing of fusion proteins to ADAR2: 4 biological replicates of the following samples: -Dox, +Dox, +Dox MDA5-ADAR2, +Dox PKR-ADAR2, + Dox DGCR8-ADAR2. Total of 20 samples NextSeq 2000 at the Wellcome Trust Genetics Core Service (Edinburgh). Each sample is around £165 for ribodepletion and library preparation (20 x £165 = £3,300). Samples will be pooled in a single sequencing lane (NextSeq 2000 P3 flowcell), generating a total of 1 billion 2x100bp reads, which will generate around 50 million reads per sample (cost of sequencing: £4,200). Total cost: £3,300 + £4,200 = £7,500).

We plan to apply for a secondment to cover the stay of the PhD student in Edinburgh.