Isolation of cGAS-bound DNA for Sequencing

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Background

In August and September 2018, I worked in Professor J. Rehwinkel's laboratory at the Weatherall Institute of Molecular Medicine, as a recipient of a Summer Studentship from the Lister Institute. I had the opportunity to carry out experiments with cGAS, a critical protein in the immune response, which has meant so much more than simply understanding the role and function of the signalling intermediate.

I was mentored by Dr Natalia Sampaio, under whose guidance I developed research skills in areas such as basic tissue culturing, where I learnt how to split cells and carry out cell transfections, as well as discovering the importance of aseptic conditions and rigorous methodology in order to minimise contamination.

I also performed molecular restriction cloning of a plasmid, ran the fragments on a gel and followed a gel extraction protocol to obtain isolated and purified LacZ. I carried out multiple PCR and agarose gel runs and learnt to analyse the results, whilst practising careful PCR preparation to avoid non-reliable gel products. Furthermore, I gained experience in protein gel running and Western blotting. When commencing the project, immunoprecipitation was a novel concept to me, but I successfully carried out several IPs, using both coupled beads and protein G Dynabeads to isolate cGAS-bound DNA.

I am grateful to Professor Rehwinkel for the opportunity to have worked in his laboratory at WIMM and for permission to use figure 1 in my report which follows. I also truly appreciate having been able to stay in residence for the duration of the project as a beneficiary of Merton College's Summer Project's Scheme.

Introduction

The innate immune response is a non-specific immune strategy, which is employed within hours of infection and is crucial to the survival of the organism. Pathogen detection via the innate immune response is a fundamental step in the activation of the much more permanent and specific responses involved with the adaptive immune system. The innate immune system primarily consists of physical barriers (such as skin), defence mechanisms (such as secretions and bile), and general immune responses. The latter group includes inflammation and non-specific cellular responses, such as the activation of transduction pathways resulting from the detection of cytosolic nucleic acids.

Much research is being undertaken to better understand the modes of initiation of these pathways, the proteins and cytokines involved, and the cellular response achieved by the cell following pathway activation. In particular, Professor Rehwinkel's group at the Weatherall Institute of Molecular Medicine is conducting ground-breaking research into the activation and regulation of cytosolic innate immune receptors that recognise and bind to nucleic acids and stimulate a signal cascade, resulting in the release of interferons. DNA in the cytosol indicates either degradation of the nuclear/mitochondrial membrane due to cellular stress or a viral infection, and when DNA is so detected the interferons are released extracellularly and signal neighbouring cells to heighten their antiviral defences.

Nucleic acids in the cytosol are detected by pattern recognition receptors (PRR), the activation of which stimulates a type 1 interferon (T1IFN) response, and subsequently upregulates genes crucial to pathogen defence. RIG-1-like receptors (RLR) and cytosolic DNA receptors (CDR) are PRRs that bind to and recognise RNA and DNA respectively. The protein cGAS is an example of a CDR, and is increasingly recognised as a crucial player in the detection of cytosolic DNA, and thus infecting DNA viruses such as Herpes Simplex Virus (HSV).

Following activation of cGAS, a second messenger, cyclic GMP-AMP (cGAMP) is synthesised, which subsequently binds to STING (stimulator of interferon genes) and induces a signal transduction cascade, eventually resulting in the release of T1IFN (figure 1).

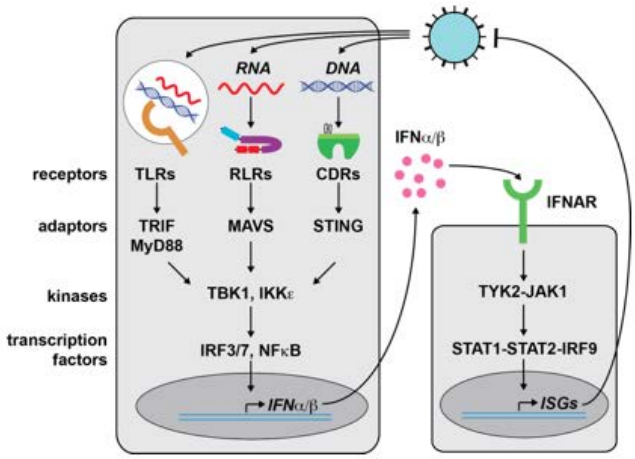


Figure 1: Schematic showing the key steps in viral infection detection, commencing with RNA/DNA detection via PRRs, and resulting in the release of interferons. [1]

The three-dimensional structure of cGAS, with and without a DNA ligand bound is shown in figures 2a and 2b.

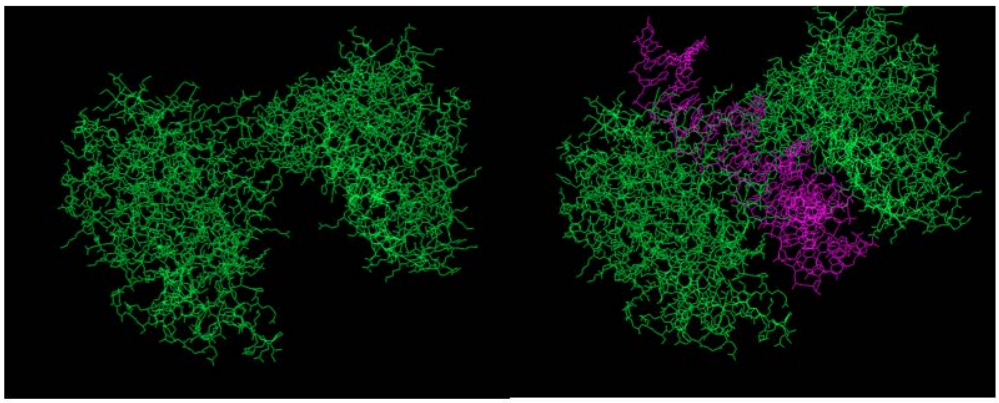


Figure 2a: Three-dimensional structure cGAS without a ligand bound, showing the ligand binding region.
Figure 2b: Three-dimensional structure of cGAS with a ligand bound. [2]

The aim of this project was to isolate DNA bound to cGAS and sequence the ligand to determine the origin of the stimulatory DNA. The experimental design was to begin by isolating LacZ bound cGAS and continue with optimisations until isolation of HSV-1 DNA bound to overexpressed FLAG-tagged-cGAS in knock-in MEF cells was successful and efficient.

Methods and Results

Experiment 1

For the initial optimisations of the method to isolate DNA bound to cGAS, LacZ DNA was used due to its length and lack of expression under normal cellular conditions. The LacZ was isolated from the plasmid shown in figure 3, using restriction enzymes XbaI and Sal1-HF. PCR for LacZ was then optimised by testing 3 different temperatures (55, 57, and 60°) as well as primer pairs 86,87 and 88,89. Changes in temperature showed little effect and primers 86,87 gave the strongest bands.

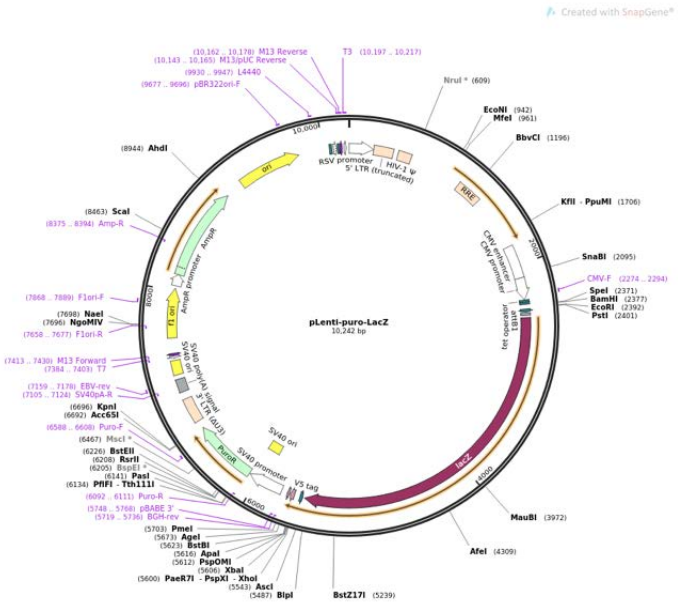
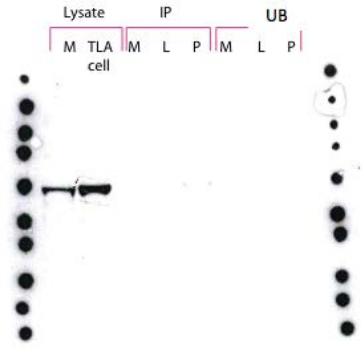


Figure 3: LacZ containing plasmid used in experiment 1. [3]

Post PCR optimisation, both HEK 293T cells and TLA cells were transfected with 10μg cGAS-FLAG plasmid (133-pcDNA3.1-mcGAS-FLAG) using Lipofectamine 2000. Following an overnight incubation, the cells were transfected with 1μg/ml LacZ plasmid and 0.5μg/ml isolated LacZ DNA. After a further two-hour incubation, the cells were lysed using a lysis buffer and protein concentrations were determined via a BCA assay. Mouse M2 anti-FLAG monoclonal antibodies were conjugated with magnetic Dynabeads, and 10μl of the conjugated beads was incubated with the lysate. After incubation, the supernatant was removed to give unbound (UB) samples, and immunoprecipitation (IP) samples were extracted post-washing and subsequently treated with 2-mercaptoethanol and heat to give single polypeptide chains. Lysate (extracted prior to IP), UB, and IP samples were run on SDS-PAGE for a cGAS FLAG Western Blot using HRP FLAG directly conjugated antibodies, as shown in figure 4.

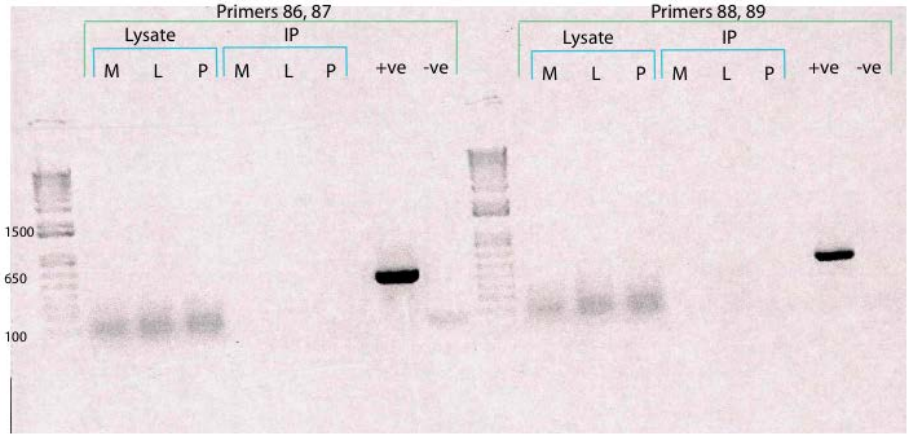
Following washing, IP samples were treated with Proteinase K to break peptide bonds, and a phenol-chloroform DNA extraction was carried out. These IP samples, as well as lysate samples, underwent PCR for LacZ and the products were run on a 1% agarose gel (figure 5).



M = mock
L = LacZ
P = plasmid
IP = immunoprecipitation samples

Figure 4 (left): Western Blot of lysate, IP and UB samples of mock, LacZ and plasmid transfections into HEK cells, and lysate sample of LacZ transfected into TLA cells.

Figure 5 (below): Gel of PCR products of lysate and IP samples from mock, LacZ and plasmid transfections into HEK cells.



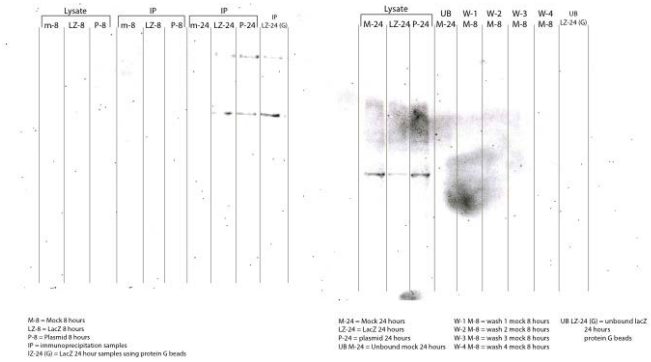
IP = immunoprecipitation samples
M = mock
L = LacZ
P = plasmid
+ve = positive control (LacZ)
-ve = negative control (water)

As evident from figure 4, cGAS was transfected into the cells successfully as the protein is in the lysates. However, bands for cGAS are very weak in the IP samples (the strongest being in the plasmid sample), but non-existent in the unbound samples, thus suggesting despite an efficient IP, cGAS was lost during the washing of the IP samples. It is interesting and useful to note the stronger band for the TLA sample, suggesting TLA cells transfect more successfully than HEK cells.

Figure 5 also shows a distinct lack of bands and LacZ was not detected in the lysate or IP samples at all. The feint bands in the lysate lanes are most likely due to other oligonucleotides present in the cells' cytosols, as they are not present post IP. These results suggest that the LacZ and plasmid transfections were not successful, and possible reasoning is insufficient DNA transfection, or even insufficient incubation time between transfection and lysis.

Experiment 2

To investigate the previous experiment's disappointing results, and to determine whether the incubation time before lysis is a limiting factor, a time course experiment was carried using the same protocol as before. DNA was transfected into HEK cells and either an 8-hour or 24-hour incubation was allowed before lysis. Two plasmid spike-ins were run on the gel as well, one at 2.5µg LacZ and the other at 30ng LacZ, to hopefully compare band strengths more quantitively and to determine if DNA is lost during the extraction and purification steps. The same amounts of plasmid and LacZ were transfected into the cells as in experiment 1. To determine the extent of cGAS loss in the washing steps after the IP, samples were extracted after each wash and underwent Western Blotting as well. Lastly, Protein G beads were tested separately as an alternative IP method, as they may prove to have a higher efficacy than the M2 FLAG-conjugated Dynabeads used previously.



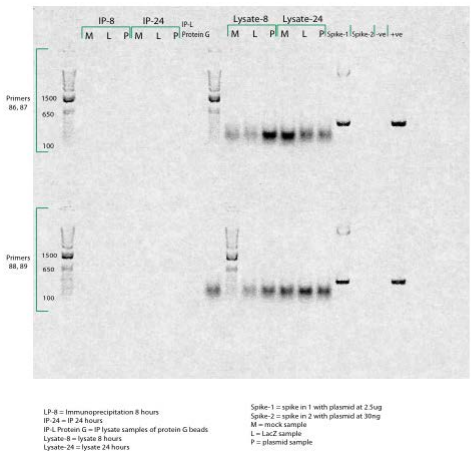
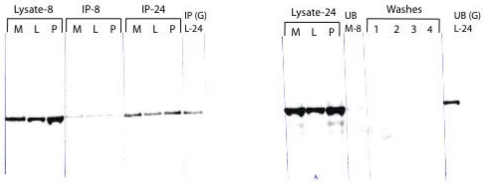


Figure 6 (above): Western Blot of lysate, UB, and IP samples for post transfection incubation times of 8 and 24 hours
Figure 7 (left): PCR products of 8- and 24-hour IP and lysate samples run on gel, using both primer pairs.

The PCR gel run once again showed no bands, indicating that no transfected DNA was present in the lysate. The fact spike-in 1 has strong signal strength, suggests no DNA was lost during the extraction and purification steps, and thus the lack of bands may be due to an insufficient quantity of DNA transfected into the cells.

In terms of the Western Blot, both the 8-hour lysate and IP samples show no cGAS present. In contrast to this, however, the 24-hour IP and lysate samples show strong signals, which is what one would expect as the cells had thrice as long to synthesise the protein as the 8-hour samples. Interestingly, no cGAS is present in the washes, and thus the protein does not seem to be lost in these steps. Due to the weak signal strength of the WB, it was stripped and reprobed with rabbit anti-cGAS as the primary antibody (figure 8).

Figure 8: Stripped and reprobed Western Blot with rabbit anti-cGAS and HRP conjugated goat antibodies.

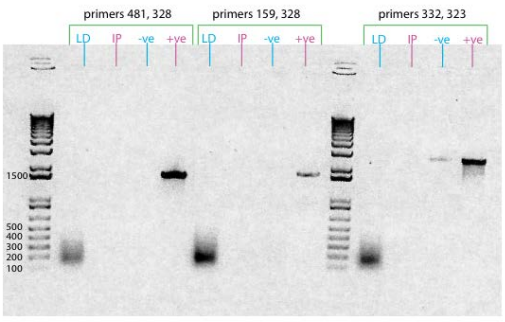


Lysate-8 = lysate 8 hours
IP-8 = immunoprecipitation samples at 8 hours
IP-24 = immunoprecipitation samples at 24 hours
IP (G) = IP samples with protein G beads
Lysate-24 = lysate 24 hours
UB M-8 = unbound mock samples 8 hours
M = mock
L = LacZ
P = plasmid
UB (G) L-24 = unbound LacZ samples with protein G beads at 24 hours

The reprobed WB shows bands in all lysate and IP samples. It should be noted, however, that twice the amount of protein was run in the IP samples, and therefore one would have expected stronger bands for the IPs; however, this was not the actual result. There was also a signal in the unbound sample, thus proving cGAS was lost during the IP, suggesting the beads appeared to have low efficacy binding.

Experiment 3

Although LacZ was not detected in the PCR gel runs, LacZ is only being used as a stimulant for cGAS, and cGAS should bind to any DNA present in the cytosol. Therefore, the protein should also bind to its own plasmid that was transfected into the cell. To test this hypothesis, unbound and IP samples from experiment 2 underwent PCR and subsequent electrophoresis using primers for the cGAS plasmid (as oppose to LacZ). The results are shown in figure 9.



LD = lysate DNA sample
IP = Immunoprecipitation samples
-ve = negative control (water)
+ve = positive control (plasmid)

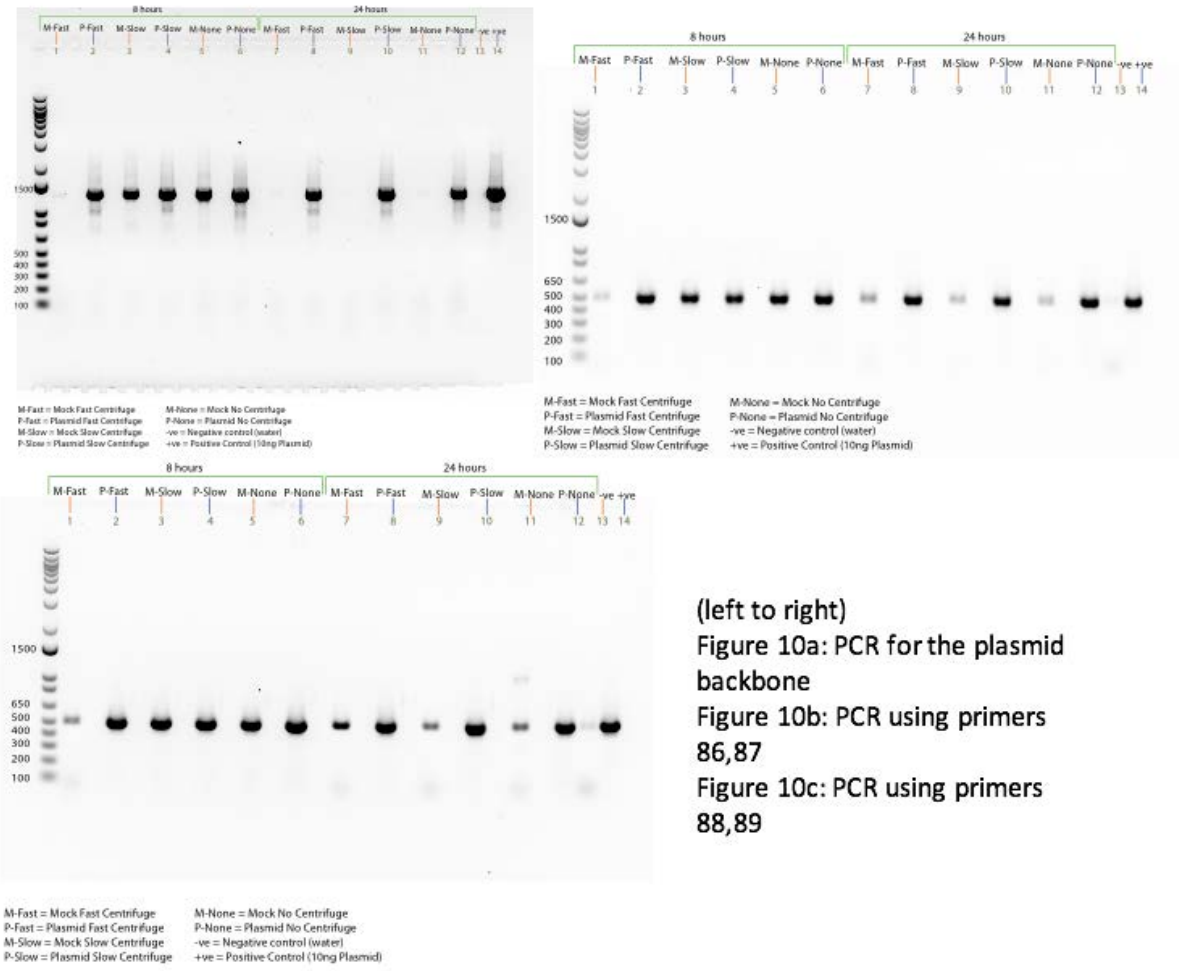
Figure 9: Gel run of PCR products with 3 primer pairs against the cGAS containing plasmid

The expected result was bands for IP and lysate samples mirroring the positive controls. There were, however, none, indicating that either cGAS does not actually bind to the plasmid, or the plasmid DNA is lost in the method. The bands at 200bp are stray DNA fragments.

Experiment 4

A plausible explanation for not having yet detected LacZ was that an insufficient concentration of DNA had been transfected into the cells. Therefore, experiment 2 was repeated using a higher concentration of LacZ plasmid (2.5μg/ml) and no isolated DNA, as oppose to the previous 0.5μg/ml LacZ and 1μg/ml plasmid. TLA cells were also transfected rather than the HEK cells, as TLAs previously showed better transfection results.

In addition to this, the method of collecting the cell lysate may not have been appropriate. Until now the cells were lysed with lysis buffer, and the lysate was transferred to microtubes that were subsequently centrifuged for 10 minutes at 10000xg and 4 degrees. Therefore, experiment 4 used three different centrifugation conditions to try and optimise the amount of recoverable DNA: 10 minutes at 10000xg, 10 minutes at 2000xg, and no centrifugation. IP was excluded, and extraction was tested from whole cell lysates.



(left to right)
Figure 10a: PCR for the plasmid backbone
Figure 10b: PCR using primers 86,87
Figure 10c: PCR using primers 88,89

Figure 10a, b, and c all show the plasmid was present in the plasmid samples, but unfortunately also present in the mock samples, suggesting that cross contamination had occurred between samples. The results were therefore non-reliable. Contamination was actually less in the 24-hour samples, and in analysis, the products of the three centrifugation conditions differed little, strongly suggesting that this is not the step in which the DNA was lost.

Regardless of the contaminated mock samples, the band signals on the gels were substantial, thus indicating 2.5μg/ml of transfected DNA was sufficient for post lysis recovery. Therefore, the primary contributor to the lack of bands in previous gels was most likely insufficient quantities of transfected DNA.

Experiment 5

Having concluded 2.5μg/ml of DNA was sufficient, it needed to be determined if there was a lower limit of DNA that could be transfected into the TLA cells and then recovered post lysis. A DNA titration was therefore carried out in which 2.5, 2, 1.5, 1, 0.5, or 0 μg/ml of plasmid DNA was transfected into the cells, followed by lysis and the usual DNA extraction and purification steps. For this experiment, the lysate was collected via centrifugation at 2000xg for 10 minutes, as it was felt that a less harsh method would help detect cGAS-bound DNA if the complexes formed higher order molecular structures.

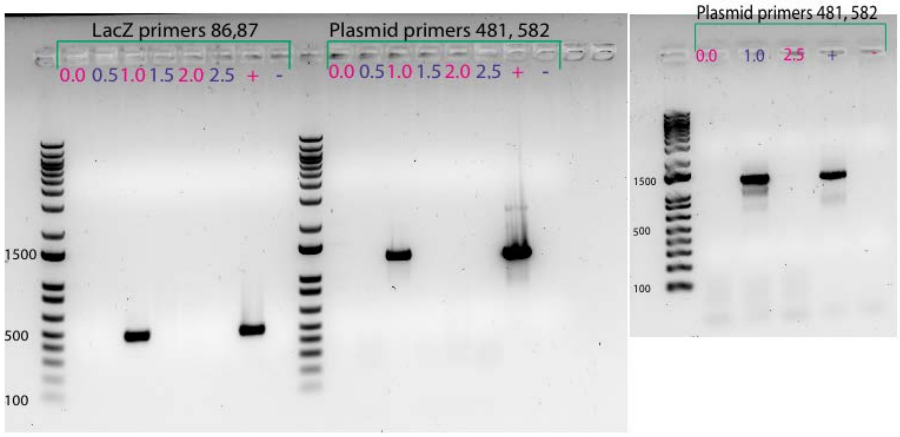


Figure 11a (left): PCR gel run of lysates with DNA titration transfection
Figure 11b (right): Repeat PCR gel run to confirm the PCR in 11a was carried out correctly

The only band present was at 1μg/ml transfected plasmid. One would have expected to observe either a gradient of signal strength in the bands or a strong band flanked by less strong signals, with the lightest band on the left to represent the minimum amount of transfected recoverable DNA. However, the observed results were very different to the expected. The PCR was therefore repeated in case of error in this step, but the same results were achieved. Owing to time constraints this experiment was not continued.

Experiment 6

The cGAS-FLAG IP using M2 antibodies in experiment 2 proved that the IP was not efficient, as the lysate samples showed much stronger WB bands that the IP samples at both 8 and 24 hours, despite the fact more protein was used in the IP samples. The 'unbound' band confirmed low efficacy binding of the antibody to cGAS. One reason for this could be the possible degradation of the antibodies over time, and therefore new antibody was purchased, and the IP repeated using both old and new antibody for comparison.

Protein G Dynabeads were used, as they are more convenient for small sample numbers. Experiment 2 had shown that the highest cGAS-FLAG expression occurred after 48 hours of transfection, therefore the IP was carried out at this time point. Furthermore, two elutions of the beads were implemented. No LacZ was transfected in as only the IP conditions were being tested. Results are shown in figure 12.

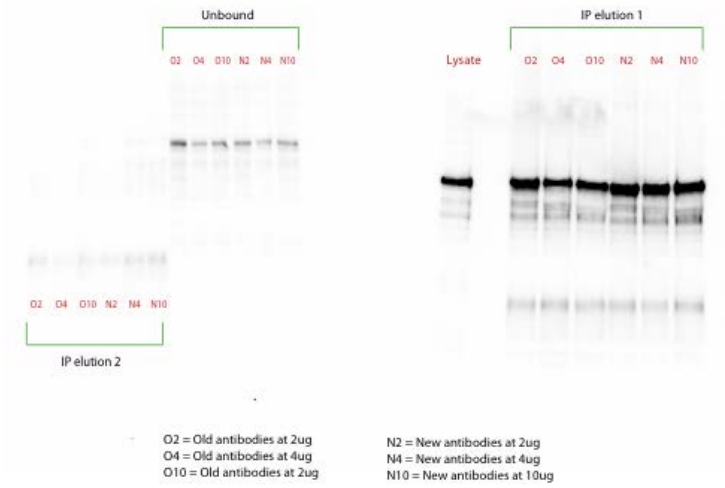


Figure 12: Western blot comparing binding efficacy of old and new antibodies used at different masses

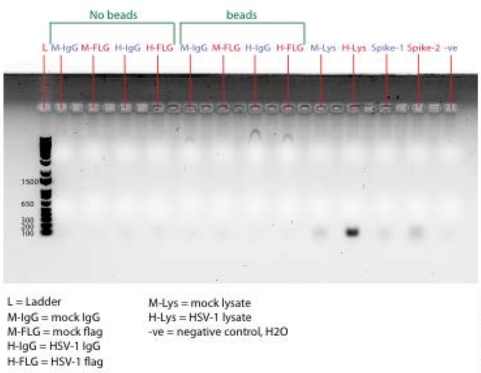
Although both the old and new antibodies were present and gave strong signals, the new antibodies did provide stronger bands than the old, which was expected. The second elution had much weaker signals than the first, indicating the majority of cGAS left the beads during the first mercaptoethanol treatment. Unfortunately, however, the unbound samples still had relatively strong bands, but those of the old antibodies were stronger than those of the new. Interestingly, there was no observable difference between the band strengths for the different masses of antibody used, which suggested that 50µl beads are already saturated at 2µg antibody. In terms of the IP these were encouraging results.

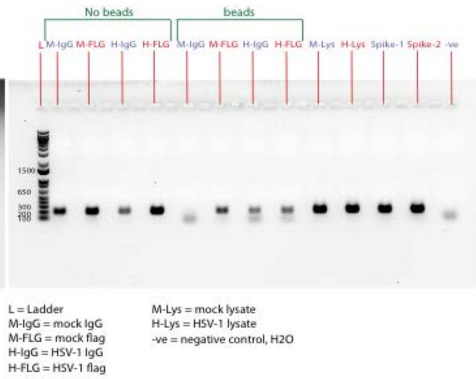
Experiment 7

Previous experiments proved that recovering the DNA post lysis is difficult, and the positioning of the one band achieved in experiment 5 was somewhat unexpected. A recent paper (Lian et al[4]) describes an experiment in which a cGAS IP and DNA extraction was performed using Herpes Simplex Virus 1 (HSV-1) infected cells and managed to detect the

DNA via qPCR. This method was therefore adapted to utilise this lab's cGAS-FLAG system, and the cells were transfected with cGAS-FLAG for 48 hours, followed by infection with HSV-1 at MOI 100. A virus infection should, in theory, provide much greater quantities of DNA in the cytosol as the DNA is actively replicated, as oppose to transfecting in a set amount.

During the IP, separate samples of lysate were used for a FLAG IP and an IgG IP. The IgG antibodies do not specifically bind to cGAS and thus serve as a negative control. Should they bind to other proteins, those Western Blot bands could be discarded from the analysis of the FLAG IP. Furthermore, after the IP and washing, the beads were kept as a separate sample to run on PCR as to determine the extent to which cGAS remains on the beads relative the amount present in the normal 'no beads' IP samples. Also, two spike ins were used in the PCR, the first containing mock IgG + mock lysate samples of the 'no bead' group, and the second containing the same samples but of the 'beads' group. These were included as there was a lot of salt in the pellet post IP, and the spike ins would help determine the salt's effect on the PCR. Lastly, the PCR was carried out with HSV-1 primers as well as mitochondrial DNA primers. Due to viral infection and the lysis of the cells, the mitochondrial DNA should be present in the lysate, and thus cGAS should bind to it. The PCR and WB results are shown in figures 13 and 14.





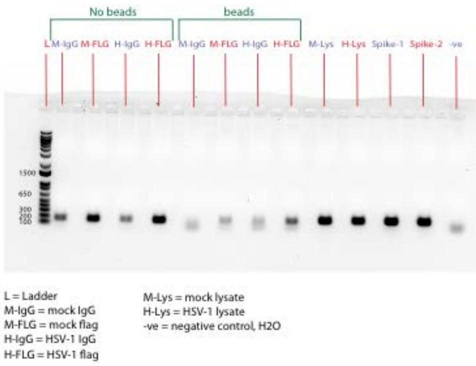


Figure 13a (above left): PCR products with HSV-1 DNA primers

Figure 13b (above right): PCR products with mitochondrial primer pair mt14841, mt14620.

Figure 13c (left): PCR products with mitochondrial primer pair mt14841, mt146663

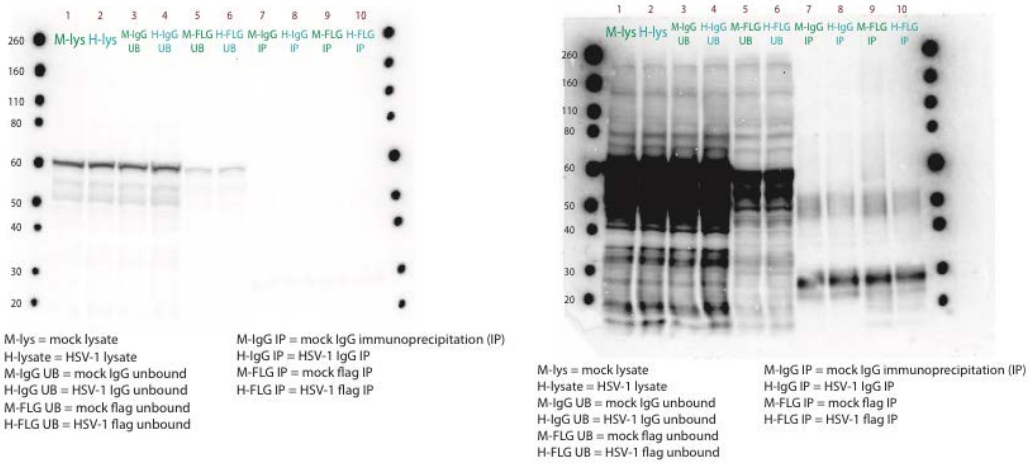


Figure 14a and 14b (above left and right): Western blot for cGAS with mock and HSV-1 infected samples

The PCR for HSV-1 DNA gave no bands except one strong signal in the HSV-1 lysate sample, which was unfortunate. As there was no DNA present in the IP samples, but some existed in the lysate samples, it could be concluded that it was lost either during or post IP. However, after the beads were incubated with the lysates, the samples were washed with a high salt buffer that contained 1M NaCl, which in hindsight was a very high concentration to treat DNA with. It seems likely that DNA was degraded and/or lost in the step.

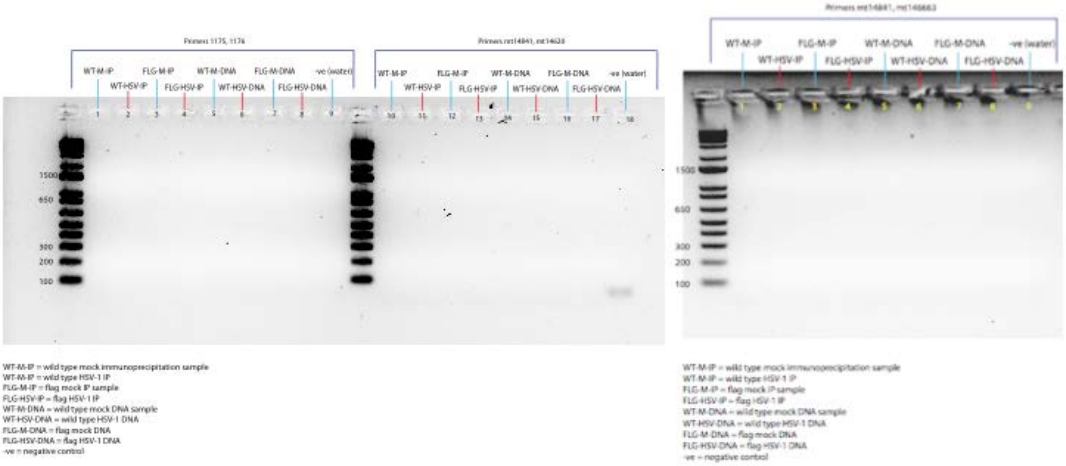
Figures 13b and 13c are very similar. The IgG samples for both 'beads' and 'no beads' were lighter than the FLAG samples, which was encouraging, as the IgG antibodies should not have bound cGAS to the extent that FLAG antibodies did. The 'bead' samples also contained weaker signals than those of the samples excluding the beads, suggesting less cGAS is remaining on the beads than is present in the IP supernatant, which is expected. The lysate samples gave very strong bands.

In terms of the Western blot (figure 14), strong bands were present in the lysate and IgG unbound sample, which was expected. There was also a faint band present in the FLAG unbound samples, which proved most cGAS did bind to the antibody. Despite this, all IP samples showed no bands for cGAS, which was most surprising. The protein may have been lost in the high salt wash mentioned in the PCR analysis.

Experiment 8

Due to the lack of signal for HSV-1 DNA in experiment 7, the experiment was repeated with a lower concentration of salt buffer (0.2M NaCl) used to treat the IP samples. Furthermore, knock-in cGAS-FLAG MEF cells were used, as these are more biologically realistic and relevant. Having an overexpression of cGAS-FLAG also reduced the need to transfect in the cGAS plasmid, although, Interferon A/D (final concentration 30 U/ml) was added to the cells prior to infection in order to stimulate their antiviral properties and closer simulate an in-vivo

infection. The same primer pairs as in experiment 8 were used. Results are shown in figures 15 and 16.



Figures 15a and 15b (above left and right): PCR products against HSV-1 (1175, 1176) and mitochondrial DNA in cGAS-FLAG MEF cells.

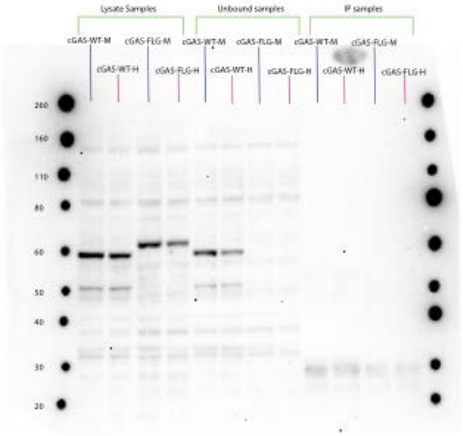


Figure 16 (right): Western Blot for cGAS from cGAS-FLAG MEFs.

The PCR gels unfortunately showed no bands at all, for which no clear explanation could be found. The DNA may have once again been lost in the post IP washes. Therefore, the PCR was repeated (figure 17) to ensure no error occurred during this stage. In the repeats, the HSV-1 lysate sample was included as a positive control, as well as a different ladder for the lower number of base pairs.

cGAS was present in both the lysate samples for the cGAS-Flag knock ins. No protein was present in the knock-in unbound samples, which was encouraging, confirming the IP therefore worked and was efficient for those samples. However, the IP samples once again had no bands at all, indicating cGAS was lost in the post IP stages, and possibly the salt wash again, despite the decrease in NaCl concentration. It should be noted that due to an error in execution of the experiment, an acetate precipitation was completed to recover protein diluted in incorrectly added wash buffer. It was also likely that cGAS was lost during this precipitation step.

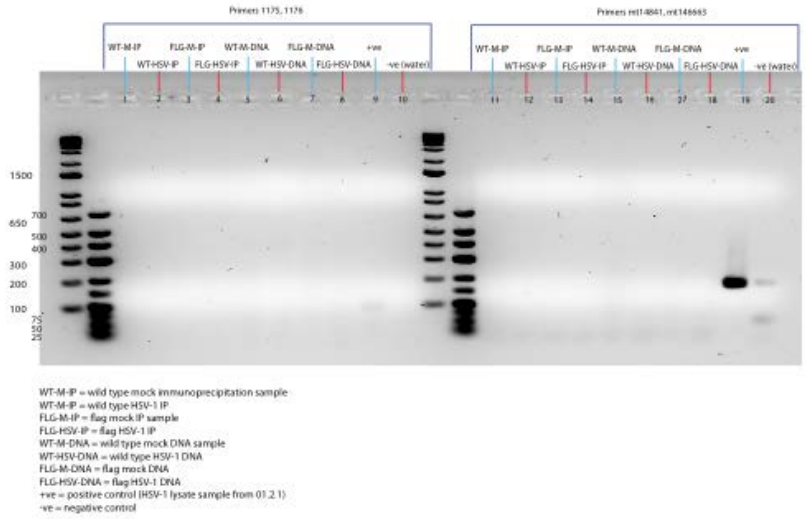


Figure 17: Products of repeat PCR

As is evident in figure 17, no bands were obtained at all, which seemed inexplicable. Due to time constraints, no further work was carried out on this project to resolve the major difficulties experienced in recovering either LacZ or HSV-1 DNA.

Conclusion

Throughout this project, it proved very difficult to isolate and recover cGAS bound DNA, whether the cells were HEK cells, TLAs, or MEFs, and regardless of whether LacZ was transfected in or an HSV-1 infection was carried. One of the reasons behind the lack of DNA observed in experiment 1 and 2 was resolved in experiment 4, where a higher concentration of DNA was transfected into the cells. Unfortunately, on further investigation of this in experiment 5, when a DNA titration was performed to determine the minimum amount of DNA that can be transfected in and recovered, unexpected results were received that did not mirror the results of the previous experiment. Therefore experiments 7 and 8 sought to use a viral infection as appose to a LacZ transfection, thereby dramatically increasing the amount of DNA in the cytosol and thus the lysates. Despite this, no DNA was recovered post lysis using primers against the viral DNA. There may have been issues with the infection, or specific steps post IP lost the DNA, but time unfortunately ran out. However, cGAS-bound mitochondrial DNA was at least recovered, indicating that upon further optimisation and testing, the method in experiment 8 has potential to recover viral DNA.

In terms of isolating cGAS, although the results in experiment 1 were disappointing, experiment 2 aided in the correction of this by increasing the incubation time post transfection. Upon re-probing of the nitrocellulose sheet, however, it became evident that binding of the beads to cGAS was inefficient. Inexplicably, relatively strong bands for cGAS were achieved in experiment 6 using the same antibodies, although the newly purchased antibodies were more effective and thus gave stronger Western Blot signals. Experiment 7 indicated the protein was possibly lost in the 1M NaCl salt wash; however, cGAS did bind to the antibody, and therefore the IP seemed to be a success. Similar results were obtained in experiment 8, regardless of the decrease in concentration of NaCl, although a necessary acetate precipitation may have been to be to blame for this.

The methods to isolate cGAS-bound DNA show potential, as many of the optimisations did improve the results, especially with regard to recovering cGAS. However, it does seem that carrying out an IP, certainly with regard to this project, is difficult and finicky. Given more time, however, I believe it can be achieved successfully.

Bibliography

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