The Binding Site Between the Adenoviral Protein E4 11k and the Cellular Protein Ddx6

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ABSTRACT

Adenovirus is a nonenveloped virus that consists of an icosahedral capsid that contains double-stranded DNA. Viruses of this type can cause the common cold, conjunctivitis, and diarrhea. An important gene of adenoviruses is an early viral gene called E4 ORF3, which encodes the protein, E4 11k. This gene is responsible for changing the cellular environment, which aids in viral replication and prevents the host cell's synthesis of proteins. E4 11k binds to Ddx6, a cellular protein located in P-bodies, and disrupts its localization. P-bodies are granules in the cytoplasm that contain mRNAs that have been repressed and proteins that are related to the degradation of mRNA. Overall, the purpose of this experiment is to determine the binding site of E4 11k and its interaction with Ddx6. This research will be conducted by transfecting cells with Ddx6 and E4 11k mutants and determining their binding ability. To begin, the transfection efficiency must be optimized. This was performed by observing protein concentrations of the transfected genes by immunoblotting. In addition, the single cell concentrations were examined through immunofluorescence. This innovative research will eventually aid in learning about the effect of the interaction between E4 11k and Ddx6 on the viral life cycle.

INTRODUCTION

Adenovirus is a family of double-stranded DNA viruses that has an icosahedral capsid that is not surrounded by an envelope. An icosahedral capsid has 20 sides and 12 vertices. There are two important structures that are common within this family of viruses, which are the outer capsid and the inner core. The inner core is what contains the dsDNA, which is wrapped around histone-like viral proteins. These viruses can cause upper respiratory tract infections, such as the common cold, within the human host. It can also result in conjunctivitis and diarrhea. This family of viruses are usually self-limiting in the immunocompetent host, meaning the patient recovers without medical intervention. There have been over 60 human serotypes detected by viral neutralization assays. However, this research focuses primarily on adenovirus 5 (Ad5) and adenovirus 9 (Ad9).

These viruses have two phases in their life cycles: an early and late phase. The early phase consists of the virus entering the host and beginning to express its own genes. Here the virus genome travels to the nucleus where early gene transcription occurs. The late phase is the production of gene products that allow the formation of capsid proteins. In this lab, the early gene that is being studied is E4 ORF3, which stands for early transcription region 4 open reading frame 3. Some of this gene's functions are to inhibit the host cell's stress response and promotes late viral protein synthesis over the host cell's protein synthesis. E4 ORF3 encodes for a protein called E4 11k. In Ad5, this E4 11k protein binds to Ddx6, which is a cellular protein that is found in P-bodies. When this attachment occurs, then localization is disrupted. P-bodies, which stands for processing bodies, are small particles that are found in the cytoplasm that contain mRNAs, that have been repressed. Proteins that can degrade mRNAs are also located in P-bodies. While the Ad5 E4 11k protein binds to Ddx6, the Ad9 E4 11k protein does not. The purpose of this experiment is to locate the binding site of Ddx6 and E4 11k and determine the effect of their binding on the viral life cycle. We hypothesize that this binding of Ad5 E4 11k and Ddx6 is the mechanism by which Ad5 E4 11k controls late protein synthesis.

MATERIALS AND METHODS

Transfections: The cell line used in this experiment are HeLa cells. These cells were grown in medium that consists of 15% calf serum. In order to begin this research the HeLa cells were transfected with an HA-tagged E4 ORF3-expressing plasmid. FuGene 6 was used as the transfection reagent according to the manufacturer's protocol. This commercial transfection reagent was used to increase the efficiency of this transfection. Complexes were made at varying ratios with the plasmid DNA and FuGene reagent. These complexes were added to several plates of cells and incubated for 24 hours (Figure 1).

Immunoblot: An immunoblot was performed to identify specific proteins of interest within the sample. First, lysates were made by breaking open the cell using a RIPA buffer. The lysates were subjected to SDS-PAGE to separate the proteins based on their mass. Finally, the proteins were identified by transferring to a membrane and probing with mouse anti-gamma tubulin antibody and mouse anti-HA antibody. An infrared-conjugated secondary antibody was used and visualized using the LICOR Odyssey machine.

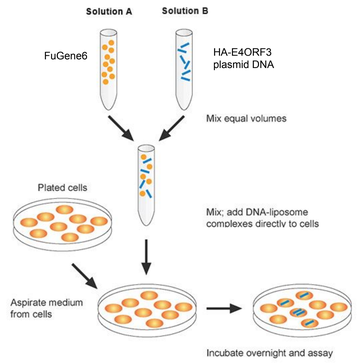

Figure 1. Transfection Protocol. Mix the FuGene6 and plasmid DNA at varying ratios. Incubate at room temperature for 5-10 minutes and add to plates of cells. Incubate for 24 hours at 37˚C.

RESULTS

In order to determine the optimal transfection conditions, several ratios of FuGene 6 to plasmid DNA were used. We started with the recommended ratios of 3:1 and 1.5:1 with 6 µg of E4 ORF3 plasmid DNA. The first experiment did not show the loading control, gamma-tubulin, at approximately at 48 kDa, nor did E4 11k appear approximately at 11 kDa when the immunoblot was done. Upon repeating the experiment, the immunoblot displayed the loading control but no E4 11k (Figure 2A). For the third experiment, the amount of DNA was increased to 13µg and the dilution of secondary antibody decreased from 1:10,000 to 1:7,500. This created a clearer band for the loading control; however, E4 11k still did not appear (Figure 2B). In the last experiment, the transfection agent to DNA ratio was increased to 3:1, 4:1, and 6:1. In this experiment, gamma-tubulin and E4 11k did not appear in the immunoblot.

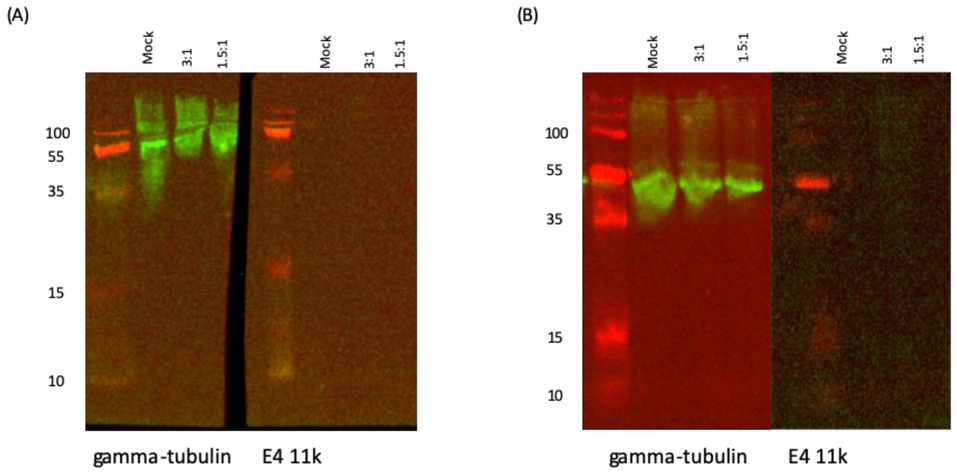

Figure 2. Optimization of transfection protocol. HeLa cells were transfected with HA-E4 ORF3 using FuGene6 for 24 hours. Lysates were subjected to SDS-PAGE and immunoblotted using gamma tubulin and HA antibodies to visualize the results. (A) Transfection results using 6 µg of HA-E4 ORF3 plasmid at various ratios of FuGene 6. (B) Transfection results using 13 µg of HA-E4 ORF3 plasmid at the same ratios of FuGene6 with an increased concentration of the secondary antibody.

CONCLUSIONS

Due to the research in the lab being minimized by the pandemic of COVID-19, this project is still incomplete. We improved our immunoblot technique, but have further optimization experiments to perform. Future directions in this lab would be to obtain a transfection efficiency that would allow higher levels of E4 11k expression as observed by the immunoblot. This will be achieved by continuing to change the amount of DNA and the ratio of FuGene 6 to the plasmid DNA. The co-immunoprecipitation would then be completed using hybrids of both Ad5 and Ad9 to narrow down the binding site between E4 11k and Ddx6 (Figure 3). Then site-directed mutagenesis can be performed to determine which amino acid(s) are required for E4 11k to bind Ddx6. Eventually, the role of this interaction during an infection can be observed.

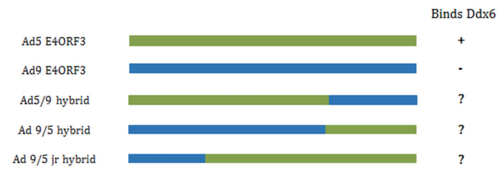

Figure 3. Map of Ad5, Ad9, and hybrid E4 ORF3 genes. Ad5 E4 ORF3 binds to Ddx6 while Ad9 E4 ORF3 does not. Hybrid proteins will be used in co-immunoprecipitation experiments to determine which region of Ad5 E4 ORF3 is required for binding to Ddx6.

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