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Aggregation Characterization of Wild-Type p53 and Six Common p53 Mutants

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ABSTRACT: P53 is a tumor suppressor protein, which functions in maintaining the cell cycle. When p53 loses its function, cells may multiply at an uncontrolled rate and form tumors. This loss of function is linked to over fifty percent of human cancers. This investigation aims to explore the possible link between p53 aggregation and tumorigenesis. There is a possibility that p53, especially in mutant form, will aggregate beyond its normal tetrameric conformation and lose its function, leading to tumor formation. Wild-type p53 and six mutants, R175H, R175C, R248Q, R248W, R273C, and R273H (six of the most common mutations found in human cancers), were purified from *E. coli* using Ni-NTA agarose resin. Aggregation rates were monitored for the wild-type and each mutant by Thioflavin-T binding assays. Atomic force microscopy was used to visualize each of the p53 mutants pre-incubation and at time points that Thioflavin-T binding suggested the presence of aggregates. Utilizing this data, each mutant was characterized for its relative speed of aggregation; this suggests which mutants are more aggregation-prone. Combined with cell viability results, our data indicates that p53 aggregation of mutated variants is a contributing factor to tumorigensis. Those mutants with the greatest propensity to aggregate also appear to be those with the greatest ability to become immortal.

INTRODUCTION

P53 is a critical tumor suppressor that regulates the cell cycle.[1] When the cell cycle is not regulated, cells multiply rapidly and may become cancerous. The loss of p53 function, or cell cycle regulation, is linked to over 50% of human cancers.[1] In particular, there are known mutations of p53 that can lead to tumor formation. The mutation of p53 may cause mutant p53 products to improperly fold;[2] as protein function is vitally dependent on the folded shape that proteins take, misfolding of proteins causes them to be dysfunctional.

Generally, when proteins fold incorrectly, cellular machinery breaks them down and gets rid of them as waste. However, in some diseases, such as Type II diabetes and Alzheimer's, specific improperly folded proteins clump together instead of getting degraded. These clumps, or aggregates, are toxic to cells and are known as amyloid.[3] As the toxic amyloid is found in these diseases, though it is not the definitive cause, the diseases are known as amyloid diseases.

The question posed is whether or not the mutant strains of p53 aggregate to form toxic amyloid. Furthermore, by association to p53 function, is tumor formation linked to the formation of amyloid? Could cancer be a type of amyloid disease, when it is linked to loss of p53 function? The implications of this research are far-reaching; it links the fields of cancer and amyloid research and explores an aspect of tumor-suppression that has little research currently published. If successful, this research could identify causes for tumor formation.

As p53 has become a source of excitement in the scientific community, headlining for the March 2014 edition of Nature Reviews, it seems a fruitful protein to explore. In the last five years, aggregation of specific p53 domains have been characterized, and it has been shown that p53 can gain toxicity in an aggregated state. P53 aggregates have even been found in breast tumors.[3,4,5] What is missing is specific characterization of mutants of p53 that are commonly linked to cancers. This would enable a correlation between particular mutations and the process by which they cause tumor formation.

This investigation aims to explore the possible link between p53 aggregation and tumorigenesis. There is a possibility that p53, especially in mutant form, will aggregate beyond its normal tetrameric conformation and lose its function, leading to tumor formation. Wild-type p53 and six mutants, R175H, R175C, R248Q, R248W, R273C, and R273H (six of the most common mutations found in human cancers), were purified from *E. coli*. Thioflavin-T binding assays were used to monitor the aggregation rates of the wild-type and each mutant. Atomic force microscopy was used to visualize the p53 mutants pre-incubation and at time points that Thioflavin-T binding suggested the presence of aggregates. Utilizing this data, each mutant was characterized for its relative speed of aggregation; this suggests which mutants are more aggregation-prone.

METHODS

2mL samples of *Escherichia coli* in which the p53 protein is expressed were grown in 500mL of LB Media in autoclaved Erlenmeyer flasks with 1 mL of Kanamycin. The cultures were grown in a shaking incubator at 37 ˚C to an optical density of A_{600} = ~0.6-0.8. They were then rapidly cooled on ice for 5 minutes and inoculated with 5 mL of sterile filtered 10mM IPTG in DI water. The flasks were then grown overnight in the shaking incubator at room temperature (approx. 25˚C). Following the overnight incubation, the cultures were pelleted in 500mL centrifuge bottles by spinning at 5000rpm for 15 minutes. The pellets were resuspended in 5 mL 50mM NaH_2PO_4 1M NaCl Buffer 1 and 1 mL B-PER. Each sample was then sonicated for four intervals of 30 seconds, followed by centrifugation at 5000rpm for 15 minutes. The pellets were resuspended in 10 mL Buffer 1 with 0.5% Triton X-100 and sonicated for two intervals of 30 seconds. After 15 minutes of centrifugation at 5000rpm, the pellets were resuspended in an inclusion body purification buffer of 8M urea, 0.1M Tris, 1mM glycine, 1mM EDTA, 10mM DTT, 1mM reduced glutathione, 10mM 2-mercaptoethanol, and 0.1% SDS. The samples were then sonicated for three intervals of 30 seconds and centrifuged at 5000rpm for 15 minutes. Finally, the supernatants were desalted into the original buffer. The samples were run on an SDS-PAGE gel and sterile filtered. Then a Thioflavin-T assay was performed for each sample at 15 and 105 minutes post-filtration, with shaking and incubation at 37˚C between time points. Samples were also visualized by atomic force microcroscopy at the 15 minute time point.

RESULTS

The wild-type and mutant p53 samples were visualized for relative purity on an SDS-PAGE gel, shown in Figure 1. Though each lane contains a number of protein bands, suggesting impurity, it is visually clear that the majority of the protein present in solution is p53, suggested by the large, thick bands near 50 kDa band on the protein standard ladder.

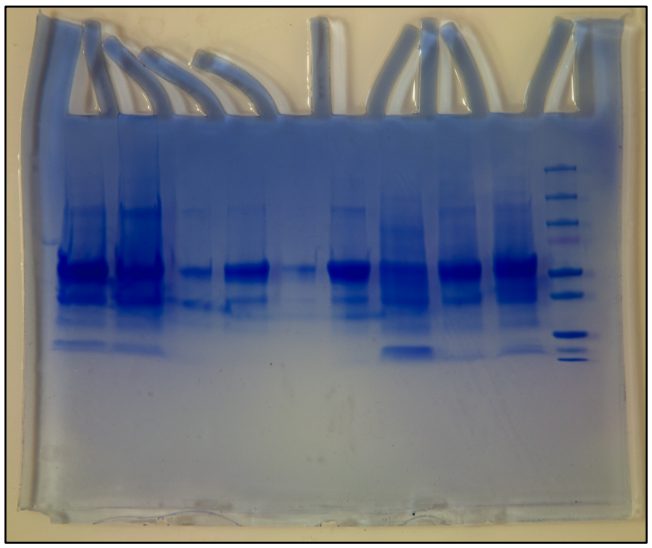

Figure 1. The SDS-PAGE gel shows the presence of high levels of p53 protein of relative purity. The lanes, from right to left: (1) Protein standard ladder (2) Wild-type p53 (3) R175C (4) R175H (5) R248Q (7) R248W (9) R273C (10) R273H.

The results of the Thioflavin-T fluorescence assay are shown in Figure 2. High fluorescence indicates the presence of aggregates, and all p53 mutants showed higher fluorescence than the wild-type, with the exception of the R248W mutant. This suggests that the mutations to p53 do promote an increase in aggregation propensity. The higher fluorescence for each of the mutants at the first time point than the second time point suggests that the aggregation time point occurs between 15 minutes and 105 minutes post-filtration. Compared to the wild-type, which showed increased fluorescence at the 105 minute time point, the mutants reached the aggregation point more quickly, suggesting that the mutants form aggregates more quickly than the wild-type. Finally, from the varied maximum fluorescence values, it is clear that some mutants, specifically R273C, R273H, and R175H, have a higher propensity for aggregation than others.

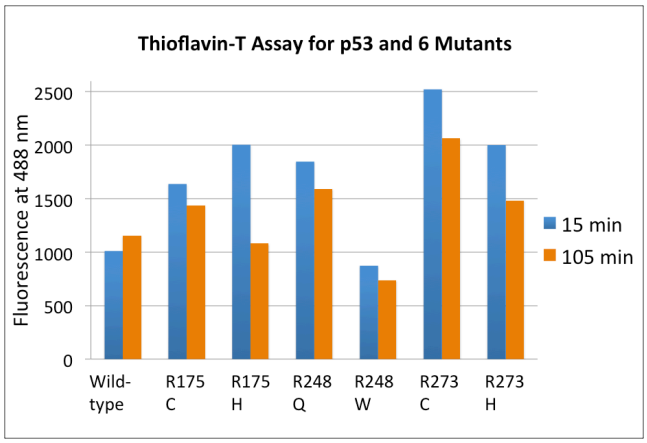

Figure 2. Thioflavin-T Fluorescence was measured at 488 nm at 15 and 105 minutes post-filtration, with shaking and incubation at 37˚C between time trials. High absorbance suggests the presence of aggregates. Note that all p53 mutants except R248W indicate higher propensity for aggregation than the wild-type p53.

To confirm the results of the Thioflavin-T assays, atomic force microscopy was used to visualize each of the samples at the 15 minute time point. The images for the wild-type and the mutant R248Q are shown in Figure 3. The presence of white aggregates on the mutant slide confirms that the mutant has a higher propensity for aggregation than the wild-type, and also that Thioflavin-T accurately characterizes p53 aggregate formation.

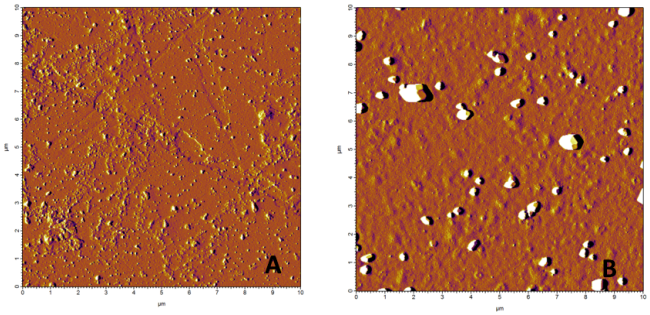

Figure 3. Each panel shows the p53 at 15 minutes post filtration. Each sample was deposited onto a freshly cleaved mica slide and analyzed using tapping mode AFM in air. (A) is wild-type p53. (B) is the p53 mutant R248Q.

DISCUSSION

The observed data from the purification and the analysis procedures are significant in several ways. The initial attempts to purify the protein were unsuccessful. It was determined that this was most likely due to the formation of inclusion bodies during the purification steps. The selection of the reagents in the purification buffer reflect this. The buffer is designed to keep aggregation prone proteins soluble. When the buffer was removed during the desalting steps, the solution visibly clouded, possibly due to the quick aggregation of the protein. This evidence was supported by the high Thioflavin-T fluorescence, even on initial readings. Aggregation was also suggested by the visible aggregates on the AFM slides. Combined with cell viability results, the collected data indicates that p53 aggregation of mutated variants is a contributing factor to tumorigenesis. Those mutants with the greatest propensity to aggregate also appear to be those with the greatest ability to become immortal.

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