ABSTRACT

I produce a mutant type of p53 that has an aspartic acid residue instead of tyrosine residue of the wild type at the 42nd amino acid position from the N-terminus. The mutant protein has been produced and purified. The same mutant protein was also expressed in H1299 human lung cancer cells by transient transfection. This report discusses improved steps in genetic manipulation and recombinant protein production. Other p53-related researches in apoptosis have been reviewed and new insights into future studies on p53-related cancer research are proposed.

INTRODUCTION

Apoptosis is a form of controlled cell death as a result of cellular stress such as DNA damage or lack of growth factor. Apoptosis proceeds through a group of intracellular enzymes called cysteine-aspartic acid proteases (caspases).

Mutation in the p53 gene has strong relationship with tumor development. Evidence comes from that over 50% of human cancers overexpress one type of mutant p53 protein encoded by missense mutation in the genome. Not only has it been discovered that mutant p53 loses the transcriptional function, gain of function has also been suggested (Michalovitz, 1991 and Dittmer, 1993). The key in studying the effect of p53 mutation lies in elucidating a comprehensive picture of p53 activities.

It is currently known that the p53 transcriptionally upregulates the Bcl-2 family proteins PUMA, NOXA and Bax in the intrinsic pathway (Schuler, 2001). However, doubt is raised over if p53 only regulates the activity of Bcl-2 family proteins at the transcription level. The intermediate protein interaction between p53 and Bcl-2 family proteins leading to caspase activation is unclear (Schuler, 2001). Supporting the prospect over p53’s non-transcriptional role, however, in revealed in the discovery that p53 directly induces mitochondrial permeabilization by binding to anti-apoptotic Bcl-2 proteins (Mihara, 2003).

I have produced the p53 protein with mutation of a single amino acid (from aspartic acid to tyrosine) at the 42nd codon. I provide the refolded protein at a high recovery rate by careful optimizing each step. The genetics in protein expression and the biochemistry in protein extraction and recovery were carefully reviewed through this project. The p53MutD42Y (denoting an amino acid change from aspartic acid to tyrosine at the 42nd codon) was also expressed in H1299 human lung cancer cells.

MATERIALS AND METHODS

1 Student
2 Supervisor
Wild type pET32a-p53 plasmid was transformed using a reverse primer p53 C42 of sequence 3’- T CGT TAC CTA ATA AAC TA - 5’ and amplified in PCR. The mutated plasmid was cycle sequenced using PCR. *E. coli* BL21 (DE3) competent cells were transformed with the mutant plasmid. Bacterial cells were overgrown with induction by IPTG. Recombinant protein p53D42Y was harvested as inclusion bodies by precipitation in Tris buffer (pH 7.9) containing salt, imidazole, protease inhibitor and DNase. Inclusion bodies were solubilized in high concentration of urea and stored at 4°C. Protein was rapidly diluted and refolded in renaturation buffer (pH 8.0) containing L-arginine and DTT at 15°C, followed by dialysis against sodium pyrophosphate with DTT and glycerol (pH 8.0). H1299 human lung cancer cells were transfected with mutant plasmid pXJ40-p53C42. After incubation, cell free lysate was harvested. Western blot confirmed expression of the mutant p53 protein.

RESULTS AND DISCUSSIONS

Reference against NCBI genome database (Figure 1) confirmed mutagenesis of the wild type plasmid. Protein concentration of inclusion bodies solubilized in 10 ml urea was determined at 5.5 mg/ml. High purity of the inclusion bodies was shown by SDS-Polyacrylamide Gel Electrophoresis (Figure 2). Western blot shows expression of p53D42Y in H1299 cells (Figure 3).

**Figure 1:** (Partially shown)

Matching between Sequencing Result and Genome Database (from 5’ to 3’)

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<th>Query</th>
<th>CATCAAAATATCCATTGGCTGACGGCAAGGGGGACAGAACGTTGTTTTCAGGAAGTAG</th>
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<tr>
<td>Subject</td>
<td>CATCAAAATATCCATTGGCTGACGGCAAGGGGGACAGAACGTTGTTTTCAGGAAGTAG</td>
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**Figure 2:** SDS-PAGE to detect p53

(S1: solubilized portion of the bacterial cell lysate after the first sonication; IB 1: inclusion bodies)

**Figure 3:** Western Blot Showing Expression of p53mutC42 in H1299

Empty mt pXip53 Ladder
Initial growth in 50 ml selective medium was tightly controlled by hourly observation of turbidity. The reason is that β-lactamase (the product of ampicillin resistant gene) degrades ampicillin and the selective pressure will be lost after a few generation of cell division (Jonasson, 2002). Overcrowding will lead to reduction of viable cells and make the whole culture unsuitable for further growth.

Inclusion body (IB) formation is the easiest and also the most ideal way of protein production in this case. Many advantages have been expounded by Jonasson et al. (2002). In brief, high yield can be achieved due to IB’s inaccessibility to host cell proteases and inert character which makes IB-unharmful to host cells. The strain of *E. coli* used was DH5α which has a strong T7 promoter. A strong promoter also enhances IB formation because rapid formation of polypeptide accelerates protein aggregation (Jonasson, 2002).

It was crucial to achieve exhaustive resuspension of inclusion body in urea because if the protein was trapped in cell debris and inaccessible to urea, it would only be solubilized to a small extent and the yield would be low. Resuspension was done by grinding the pellet from previous centrifugation in the urea-added buffer with a glass rod until the suspension looked as much homogenized as it was from the sonicator. This was followed by a short vortex to disrupt the colloid inclusion bodies further. To avoid heat destruction to the protein, sonicator was not used.

Previous researchers have timed the ice incubation of the urea-solubilized inclusion bodies to one hour. In my opinion, since the protein was in a denatured state right from the inclusion bodies inside the bacterial cells to the stage of solubilization in urea, this incubation period can be extended for more exhaustive solubilization, as long as we keep the temperature low. Therefore, I incubated the protein in urea for eight hours on ice. As the result shows, this contributed to the high yield of the protein of interest.

In this project a high concentration of urea, a chaotropic agent, has been used to solubilize the inclusion bodies. Chaotropic agents destroy the secondary structures in proteins and cause formation of random coils which makes the denatured protein more prone to aggregation during refolding process. Researchers have found that the proteins in inclusion bodies have already formed some secondary structures and mild solubilization (using denaturants at lower concentrations) has been recommended for increase of bioactivity in recovered proteins (Singh and Panda, 2005).

A deeper look into the H1299 cell line gave rise to controversy. According to Xiong and Li (2004), the mechanism behind H1299’s p53-null phenotype is due to the transcription of the wild type p53 sequence into a non-functional strand of mRNA. Considering this finding, the ability of
H1299 cells to express mutant types of the synthesized p53 gene and not the wild type p53 gene in its own genome is intriguing. The successful expression of mutant p53 indicates that H1299 cells are able to transcribe the mutant p53 gene into a functional mRNA. However, given the in vitro condition, it is not certain if the ability to transcribe the mutant p53 gene is due to the artificial promoter within the plasmid or to other sequences in the plasmid that may secure correct transcription or to the mutation in the gene.

The structure of wild type p53 was studied through literature search. It can be deduced that a change from an acidic amino acid residue (aspartic acid) to a nearly neutral one (tyrosine) will cause a structural change in the N-terminus and subsequent alteration in transcription activity and dimerization. The mutant protein, p53mutD42Y, may have structural changes and lose transcriptional activity. It may not be able to rescue the cell on occasions of cellular stress. As the residue change is very close to the Mdm-2 binding domain (at the N-terminus), it may also disrupt the regulation of the mutant type p53 by Mdm-2 and cause abnormally high activity of the mutant protein.

ACKNOWLEDGEMENTS

I would like to thank the Developmental Biology Lab for supports.

REFERENCES