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Does the N-terminus of BRCA1 Ubiquitinate Topoisomerase IIα?

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Introduction

Breast cancer is one of the diseases women fear most (www.mayoclinic.org) and is currently under intense study. Germline mutations in the *BRCA1* gene account for about 40-50% of all hereditary cases of breast cancer (Fan et al, 1999). BRCA1 is a 220-kDa multi-functional nuclear phosphoprotein that functions in transcription, DNA repair mechanisms, apoptosis, cell-cycle progression, ubiquitin ligase activity and maintenance of genomic integrity (Starita and Parvin, 2003; Fan et al, 2001). BRCA1 interacts with an astronomical number of proteins; therefore, determining its binding regions is important in predicting the effect of mutations in different parts of the *BRCA1* gene.

A recent study by Lou et al. showed that knocking down either BRCA1 or topoisomerase IIα in HCC1937 cells (breast cancer cell line) and HeLa cells resulted in defective chromosome condensation and lagging chromosomes during mitosis. Thus, BRCA1 plays a role in DNA decatenation. Previously, Baer and Ludwig showed that the N-terminus of BRCA1 forms a heterodimer with BARD1, and together this complex acts as an active ubiquitin polymerase. Lou et al. found that topoisomerase IIα immunoprecipitated from cells that produced endogenous BRCA1 was ubiquitinated, while topoisomerase IIα from cells lacking BRCA1 was not ubiquitinated. Furthermore, ubiquitination of topoisomerase IIα lead to an increase in its activity. However, no evidence was shown for a direct role of BRCA1 in ubiquitinating topoisomerase IIα. Is BRCA1 directly interacting with topoisomerase IIα, ubiquitinating it through its N-terminus, or is it playing a role as an upstream regulator of topoisomerase IIα?

Determining whether the N-terminus of BRCA1 is ubiquitinating topoisomerase IIα will lead to a better understanding of BRCA1's numerous functions. Furthermore, germline mutations in the N-terminus of BRCA1 can be predicted to have an adverse effect on topoisomerase IIα activity, leading to defective chromosome condensation, which could be targeted for therapy.

Specific Aims

The first goal is to make a HeLa cell line which has exons 2, 3, 4 and 5 of the *BRCA1* gene floxed. Floxing these exons will lead to the deletion of the N-terminus in the BRCA1 protein when these HeLa cells will be transfected with the retroviral vector encoding the Cre recombinase protein (Loonstra et al, 2001). It is hypothesized that deleting the N-terminus of the BRCA1 protein will make BRCA1 unable to ubiquitinate topoisomerase IIα.

The second goal of this proposal is to determine whether topoisomerase IIα is ubiquitinated in HeLa cells expressing the truncated BRCA1 protein.

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Topoisomerase IIα will be immunoprecipitated and probed for antibodies to ubiquitin. Ubiquitination of topoisomerase IIα in cells expressing normal BRCA1 and non-ubiquitinated topoisomerase IIα in cells expressing truncated BRCA1 will provide direct evidence for the role of the N-terminus of the BRCA1 protein in ubiquitinating topoisomerase IIα.

Experimental Procedure

Previous research has shown that topoisomerase IIα ubiquitination requires BRCA1. However, it is not clear if BRCA1 is directly involved in the ubiquitination of topoisomerase IIα. In order to test the hypothesis that the N-terminus of BRCA1 is directly involved in topoisomerase IIα ubiquitination, a HeLa cell line will be generated which produces a version of the BRCA1 protein lacking the N-terminus. The intron sequences flanking exon 2 on the 5' end and exon 5 on the 3' end will be obtained from the NCBI website. Then, *loxP* sites will be inserted into these intronic sequences in the genome of HeLa cells by using a modified SW137 targeting vector (Williams et al, 1994). This vector will be engineered to have regions of homology flanking the two *loxP* sites. The neomycin gene will be inserted after the 5' *loxP* site, followed by the DNA sequences of the exons and introns of the N-terminus of *BRCA1*. The second *loxP* site (3' *loxP*) will flank exon 5 of BRCA1, followed by a large region of homology and then by the thymidine kinase (TK) gene. After the HeLa cells are infected with this linearized vector by electroporation, selection will be done by adding G418 (Williams et al, 1994). Furthermore, to determine whether non-homologous recombination occurred, ganciclovir will be added to the cells selected with G418 resistance. If non-homologous recombination has occurred, the TK gene will be retained and ganciclovir addition will kill cells.

Next, since the HeLa cells do not produce the Cre recombinase enzyme, these cells will be transfected with a retroviral construct containing Cre fused to GFP. This retroviral vector is replication-defective (Loonstra et al, 2001). To infect the HeLa cells, they will be incubated with retroviral supernatant in the presence of polybrene. To determine whether the transfections have been successful, FACScalibur flowcytometer (Benton Dickinson) and CELL QUEST software analyses will be performed as transfected cells synthesize GFP (Loonstra et al, 2001).

To make sure that the loxP-Cre site-specific recombination system has indeed worked and the BRCA1 made in these cells is truncated, a western blot analysis will be done. The size of the truncated protein is expected to be about 20kDa smaller than the normal BRCA1 protein (220 kDa). Anti-tubulin will be used as the loading control. For the negative control, cells transfected with the modified SW137 targeting vector, but not transfected with the Cre recombinase enzyme will be probed for BRCA1. This band would show the size of normal BRCA1.

Finally, to determine whether this truncated BRCA1 protein is able to ubiquitinate topoisomerase IIα, immunoprecipitation will be done. Topoisomerase IIα will be immunoprecipitated from HeLa cells producing truncated BRCA1, as well as from HeLa cells producing normal BRCA1. The antibodies will be

obtained from NeoMarkers (Lou et al, 2005). Next, the protein-antibody complexes will be run on polyacrylamide gels and probed with antibodies to ubiquitin. As a positive control, the immunoprecipitate will be blotted with antibodies to topoisomerase IIα. This positive control will indicate any topoisomerase IIα immunoprecipitation from the cells.

The lane with topoisomerase IIα from HeLa cells making truncated BRCA1 would be expected not to show any band for ubiquitin; the lane with topoisomerase IIα from HeLa cells with normal BRCA1 may show a band at slightly over 220 kDa, indicating that topoisomerase IIα has been ubiquitinated. However, if topoisomerase IIα immunoprecipitated from HeLa cells making truncated BRCA1 show ubiquitination, it would suggest that the N-terminus of BRCA1 is not involved in the ubiquitination of topoisomerase IIα. BRCA1 may be regulating topoisomerase IIα by activating other proteins capable of ubiquitin ligase activity (independent of its N-terminus).

Since a negative result would support the given hypothesis, it is necessary to check that the negative result does not occur because of other factors besides the loss of the N-terminus of BRCA1. It is possible that the truncated BRCA1 protein is unstable and degrades after being expressed for only a few hours. In order to test whether BRCA1 is transiently expressed, a time-course western blot can be done. However, to do this, the HCC1937 cell line (which does not produce functional BRCA1) must be transfected with a vector containing the truncated BRCA1 under an inducible promoter.

To be able to control BRCA1 expression in HCC1937 cells, two transfections must be performed as outlined in the Complete Control® Inducible Mammalian Expression System from the Stratagene Company (www.stratagene.com). First, cells are transfected with a pVER3 plasmid, which contains the gene for a synthetic ecdysone receptor under the CMV promoter that is constitutively expressed. This plasmid also has the kanamycin resistance gene, allowing for selection of transfected cells. The second plasmid, pEGSH, contains a synthetic ecdysone response element upstream of the promoter of the gene of interest; the ecdysone receptor (transcribed from the pERV3 plasmid) binds to this element and constitutively represses the promoter. The pEGSH plasmid has the hygromycin resistance gene, allowing transfected cells to survive in the presence of hygromycin. Addition of ecdysone allows the basal transcription machinery to bind to the promoter (since the receptor can no longer repress the promoter) and for the gene of interest to be expressed. The truncated BRCA1 cDNA will be inserted downstream of the promoter in the pEGSH plasmid, and ecdysone will be added when BRCA1 expression is to be induced. Thus, the cells can be lysed and probed for BRCA1 at a number of time intervals after BRCA1 expression.

A time-course western blot may show that HCC1937 cells expressing truncated BRCA1 have the same intensity of bands when probed for BRCA1 at different time intervals post expression. This would indicate that truncated BRCA1 is stable in cells. The immunoprecipitation experiment (of topoisomerase IIα) may then be repeated in the HCC1937 cells to check for topoisomerase IIα ubiquitination.

Conclusion

These experiments will determine whether the N-terminus of BRCA1 is involved in the ubiquitination of the topoisomerase IIα enzyme. This research will add to the known functions of BRCA1 and increase our understanding of this vital protein. Furthermore, mutations in the N-terminus of BRCA1 can be predicted to cause lagging chromosomes, and therapy could be targeted at the ubiquitination of topoisomerase IIα.

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