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13. ABSTRACT (Maximum 200 Words) Abnormal chromosome number is a phenotype characteristic for most of the cancer cells. Thus, it may be a direct cause of human cancer including breast cancer. In this research project, we aim to test this hypothesis by abrogating the spindle checkpoint that is a major surveillance mechanism responsible for maintenance of the normal chromosome number. p55CDC serves as a target of the checkpoint. If it is unable to bind to a spindle checkpoint protein, Mad2, it abrogates the checkpoint in a dominant manner. In Year 1 of this project, we have carried out a large scale mutagenesis to generate mutants of p55CDC that are defective in binding to Mad2. Approximately 70 mutants have been generated and integrated into the genome of human cultured cells under control of an inducible promoter. Biochemical and physiological analysis to evaluate these mutants will be performed. Promising mutants would be an excellent reagent to abrogate the function of the checkpoint and can be used to test if the chromosome instability is a direct cause of breast cancer in the following years.				
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FOREWORD

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Toshiyuki Hal 7/17/00
PI - Signature Date

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Introduction: Abnormal karyotype is a hallmark of cancer cells, including breast cancer. Therefore, it has been postulated that chromosome instability may be a cause of the genesis/stage progression of cancer. The spindle checkpoint, that delays the onset of sister chromatid until all the kinetochores capture the mitotic spindle, plays an important role for maintenance of the normal chromosome number. In the last 3 years, several genes involved in the spindle checkpoint function have been found defective in cancer cells, suggesting that a loss of the checkpoint may be a cause of cancer. In this research project, we aim to abrogate the function of the spindle checkpoint to test directly if 1) a loss of the spindle checkpoint results in the genesis of breast cancer in mice and 2) cells lacking the functional spindle checkpoint are more sensitive to spindle poisons such as taxol.

Body: Our strategy to abrogate the spindle checkpoint stems from the previous study in the fission yeast model. Slp1 is a fission yeast protein that serves as a final target of the spindle checkpoint [1]. A spindle checkpoint protein, Mad2, binds to Slp1 and inhibits its activity to promote sister chromatid separation. We have generated a mutant of Slp1 that no longer interacts with Mad2 and shown that the mutant slp1 can abrogate the checkpoint in a dominant manner. Budding yeast CDC20 is a homolog of Slp1. By a similar strategy, it has been shown that CDC20 is also a target of the spindle checkpoint [2].

p55CDC is a structural homolog of Slp1/CDC20 in human, mouse and rat. Not only the similarity in structure, but also its physical interaction with Mad2 strongly suggests that p55CDC is a target of the spindle checkpoint [3, 4 and 5]. As shown in Figure 1, the Mad2 binding site of Slp1/CDC20 shares a similarity with the corresponding region of p55CDC. We believe that a mutation in this region would make p55CDC defective in binding to Mad2 and that such a mutant may abrogate the function of the checkpoint just like the yeast slp1 mutant.

For Year 1 (July 1999 to June 2000) of this research project, we have proposed the following tasks;

Task 1: to introduce mutations on p55CDC gene.

Task 2: to transform them into cultured cells

Task 3: to analyze the mutants biochemically

Task 4: to test the mutants for sensitivity to a spindle poison.

As shown in Figure 1, through the model study in the two yeasts, mutants that abrogate the checkpoint in a dominant manner have been isolated by mutagenesis of particular segments (segment 6 of fission yeast and segments 8 and 9 of budding yeast). It should be noted that these mutants are not only defective in binding to Mad2, but also otherwise biologically active. If a mutation affects the function of Slp1 to promote sister chromatid separation, it would not cause a dominant phenotype.

We have originally proposed to generate several mutants of p55CDC, which are analogous to yeast *slp1/cdc20* mutants. The panel of the reviewers of this grant proposal felt that success of this project heavily depends on whether or not we can isolate a dominant mutant of p55CDC. As we agreed this criticism, we have decided to perform a mutagenesis experiment in a much larger scale. The Mad2 binding domain of p55CDC was divided into 19 segments each of which was a subject to extensive mutagenesis. Each mutant was introduced into the yeast two-hybrid system and tested for its activity to interact with Mad2. As summarized in Figure 1, we have found a hot spot (segments 4, 5 and 6 of p55CDC) that yields mutants defective in binding to Mad2 more frequently than other segments. This hot spot locates on or near the mutation sites which were identified in the yeast model study.

Approximately 70 mutants which have lost the binding activity to Mad2 have been cloned into a tetracycline-inducible expression vector and integrated in the genome of cultured cells (a derivative of HeLa that allows the use of tet-inducible system). Therefore, Tasks 1 and 2 have been completed.

Because we have scaled up the mutagenesis experiment, the project has been slightly delayed. Tasks 3 and 4 are to be completed at this time. However, we strongly feel that the scale-up was necessary to generate a dominant mutant of p55CDC successfully. Among the 70 mutants which have lost the activity to bind to Mad2, we expect that some of them still restore the activity of p55CDC to promote sister chromatid separation. Such mutants are a good candidate for a dominant mutant to abrogate the function of the spindle checkpoint. The stable integrants that would induce the expression of the p55CDC mutants will be tested for their sensitivity to a spindle poison, nocodazole. If the function of the checkpoint is abrogated, the integrants would not be arrested at

metaphase. We will also test the activity of the mutants to bind to Mad2 in the cultured cells. We estimate that Tasks 3 and 4 can be completed in a few months and the delay will not negatively impact the progress of the project. Once we find promising mutants, they will be introduced in mouse as proposed originally.

It has very recently been reported that deletion of a gene involved in the spindle checkpoint function causes cell lethality [6, 7]. The results indicate that deletion strategy is not ideal to test if loss of the checkpoint results in the genesis/stage progression of cancer. Our strategy employs ectopic expression of a dominant mutant whose expression level can be controlled. Under a certain condition, the activity of the checkpoint could be maintained partially and the cells would be able to propagate for a long term (which would be necessary for neoplastic transformation).

Key Research Accomplishment:

1. Generation of p53CDC mutants with less activity to bind to Mad2.
2. Construction of cell lines which induce expression of the p53CDC mutant.

Reportable Outcomes:

None

Conclusion: We have generated approximately 70 mutants of p53CDC which have lost the activity to bind to Mad2. Because they are supposed not to be under control of Mad2, some of them are expected to promote sister chromatid separation even when the spindle is not attached to kinetochores correctly. Such mutants would be excellent reagents to test if loss of the checkpoint caused breast cancer.

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Appendix: Figure 1 is attached in the next page.

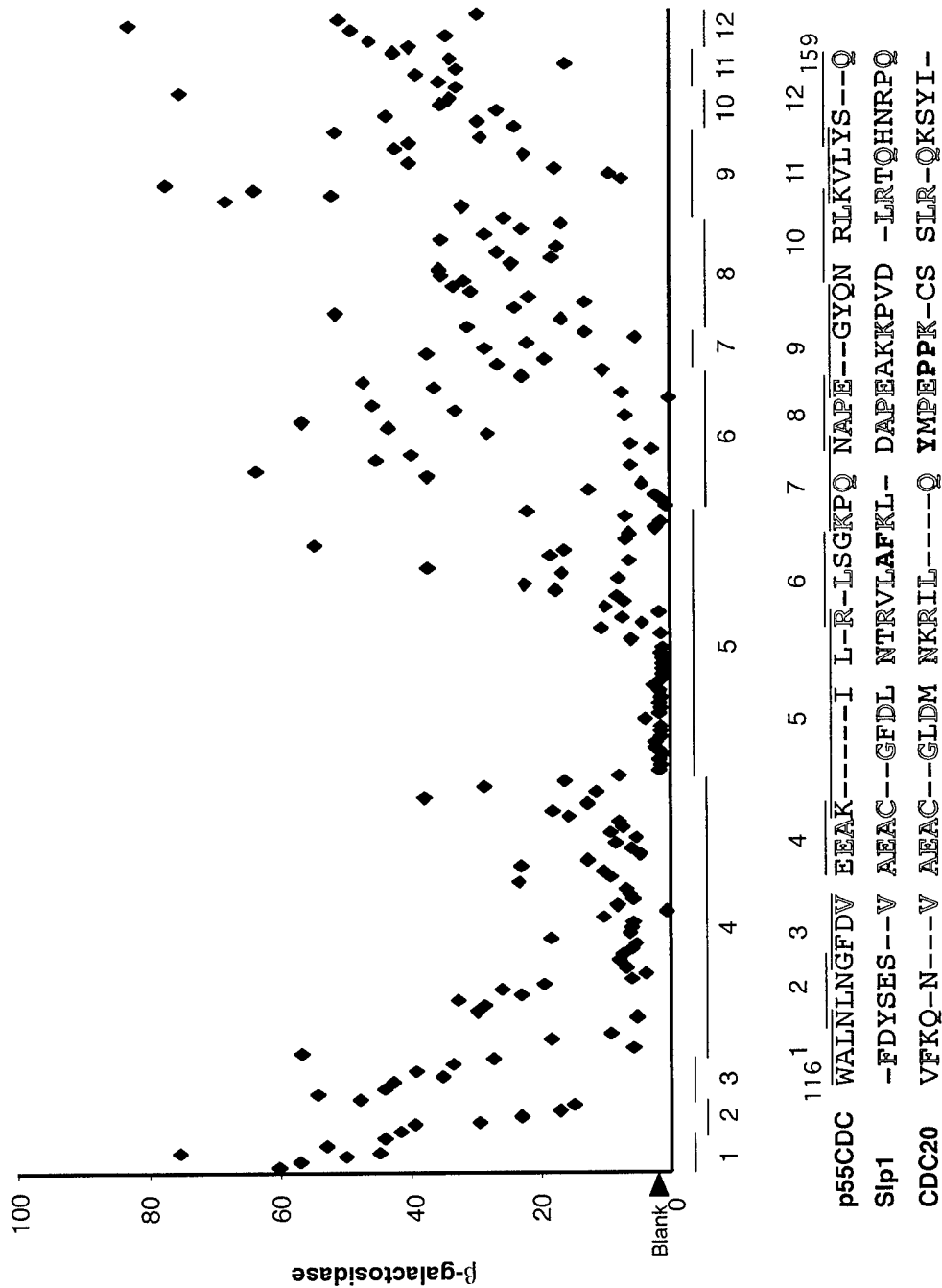


Figure 1. Mutational analysis of p55CDC. The Mad2 binding site was divided into 19 segments (indicated by numbered bars) of which 12 are shown here. Diamonds represent individual mutants obtained through random mutagenesis. The vertical axis is for the activity of beta-galactosidase that indicates the strength of the physical interaction between each mutant of p55CDC and Mad2 in the yeast two hybrid system. The horizontal axis is to indicate the amino acid sequences of each segment. The amino acid sequences of homologous proteins are shown in the conventional one letter mode. Other seven segments outside of this region did not produce any significant mutants. The amino acids shown by bold style indicate the positions of mutations that have been isolated in the two yeasts.