95 4 30

DNA

Ndt80

Ndt80   Ndt80

Ndt80

NDT80-bc   Ndt80-bc

Ndt80-bc

Ndt80-bc

Ndt80

zip1   Ndt80

Ndt80-bc

Ndt80

two-hybrid   Ndt80

Abstract

Keywords   meiosis, cell cycle, pachytene checkpoint, budding yeast

Meiosis is essential for sexual reproduction. Single round of DNA replication with two successive nuclear divisions produce haploid gametes; and therefore, organisms maintain a constant genetic content from generation to generation after fertilization of two gametes. To ensure the success of meiosis, meiotic checkpoints operate to coordinate the proper order of meiotic events. In particular, the pachytene checkpoint prevents exit from the pachytene stage of meiotic prophase when meiotic recombination and chromosome synapsis are incomplete. Our research is planned to study the molecular mechanism of the pachytene checkpoint in detail.

In budding yeast *Saccharomyces cerevisiae*, the *NDT80* gene encodes a meiosis-specific transcription activator that is required for progression from pachytene into meiosis I. We have proposed that Ndt80 is a direct target of the pachytene checkpoint. In our previous studies on defining Ndt80 functional domains, we have isolated a dominant deletion mutation, *NDT80-bc*. This Ndt80-bc protein is resistant to the control of the pachytene checkpoint and becomes constitutively active. The *NDT80-bc* mutant provides a strong evidence for our hypothesis and a powerful tool for the studies on the mechanism of the pachytene checkpoint.

This research project is to study the molecular interaction between Ndt80-bc and the pachytene checkpoint. The Ndt80-interacting protein of the pachytene checkpoint machinery will be isolated. Our data showed that Ndt80 was retained in cytoplasm in the *zip1* mutant, while Ndt80-bc was localized into the nucleus of the same cell. These results suggest that nuclear localization of Ndt80 is regulated by the pachytene checkpoint. In the pachytene-arrest cells, the Ndt80 protein is retained in the cytoplasm by an inhibitor interacting with the bc target domain of Ndt80.The information learned from this project will provide valuable information for the understanding of meiotic cell-cycle control. It is also useful and applicable to researches on mitotic cell cycle, human genetic diseases, and cancers.

Introduction

In eukaryotic organisms, the integrity of genetic information is maintained through the operation of cell-cycle checkpoints. Checkpoints ensure the proper order of events in mitotic cell cycle by arresting or delaying in response to defects in cellular process. Checkpoint machinery also operates in meiosis. In particular, there is a checkpoint prevents exit from the pachytene stage of meiotic prophase when meiotic recombination and chromosome synapsis are incomplete. This "pachytene checkpoint" prevents chromosome damage or chromosome missegregation that would lead to the production of inviable or aneuploid gametes (Roeder 1997, Roeder and Bailis 2000). The pachytene checkpoint requires meiosis-specific proteins, in addition to a subset of mitotic DNA damage checkpoint. In the budding yeast *Saccharomyces cerevisiae*, mutants that confer defects in recombination (e.g., *zip1, dmc1, hop2*) cause cells to arrest at the pachytene stage of prophase (Bishop et al. 1992, Sym et al. 1993, Storlazzi et al. 1996, Tung and Roeder 1998, Leu and Roeder 1998). This arrest is triggered by defective meiotic recombination intermediates and is dependent on checkpoint genes, e.g., *RAD17, MEK1*, and *PCH2* (Lydall et al. 1996, Xu et al. 1997, San-Segundo and Roeder 1999). Mutations in these checkpoint genes allow *zip1* and *dmc1* mutants to exit pachytene, to complete meiosis, and to produce inviable spores. However, the molecular mechanism for the pachytene checkpoint control is not yet clear.

It is well demonstrated that the progression from pachytene into meiosis I is a critical step for meiosis. It involves highly regulated protein activation and gene expression. There are two important genes, *CDC28* and *NDT80*, play essential roles for this transition. Cells with mutations in *CDC28* or *NDT80* are able to complete recombination, but arrest at the end of pachytene (Shuster and Byers 1989, Xu et al. 1995). *NDT80* is a meiosis-specific gene. It was independently identified from screens for mutants defective in sporulation (Xu et al. 1995, Hepworth et al. 1998), and from a screen for multicopy suppressors of *zip1* sporulation defects (Tung et al. 2000). Ndt80 is a meiosis-specific transcription factor, it binds to middle sporulation elements (MSEs) and activates transcription of genes required for both meiotic nuclear divisions and spore formation, including *NDT80* itself and most B-type cyclins (Chu and Herskowitz 1998, Hepworth et al. 1998).

The essential role of *NDT80* suggests that Ndt80 would be a central target of meiotic checkpoint machinery. It was suggested that the pachytene checkpoint controls the activation of Ndt80 at transcription (Lindgren et al. 2000, Pak and Segall 2002a, 2002b, Pierce et al. 2003). In this model, the expression of *NDT80* is repressed by the Sum1 transcription repressor. The Sum1 binds to MSEs and represses middle sporulation gene expression during vegetative growth (Xie et.al. 1999). The level of Sum1 decreases as meiosis progresses, and on activation of the pachytene checkpoint, Sum1 is stabilized and some MSE-containing genes, including *NDT80*, is repressed (Lindgren et al. 2000, Pak and Segall 2002a).

Recently, among a set of in-frame *ndt80* deletion mutations, we have isolated a very interesting dominant allele, *NDT80-bc* (*NDT80-*bypass checkpoint). This allele completely suppresses all the pachytene-arrest mutants that we have tested, such as *zip1*, *dmc1*, and *hop2*. This exciting result provides strong evidence that Ndt80 protein itself is a direct target to the pachytene checkpoint. It points out a new direction in studying the molecular mechanism of the pachytene checkpoint control.

Aims

The detail mechanisms that control cell cycle progression and coordinate chromosomes segregation during meiosis are not clear. However, as described above, Ndt80 plays a central role in coordinating these procedures. Exploring the detail of Ndt80 function and its regulation will be essential for understanding meiotic cell cycle control. Our previous studies have suggested that Ndt80 itself is the regulatory target for meiotic checkpoint machinery. In this study, we have further analyzed the suppression by the Ndt80-bc protein, and to study the controlling step on Ndt80 activity by the pachytene checkpoint (e.g., whether it occurs at nuclear import or thereafter?). This should uncover the checkpoint control mechanism in more detail. In addition, we have performed a two-hybrid screen using the deleted segment from Ndt80-bc as the bait to isolate the checkpoint component that directly interacts with Ndt80.The answers for these questions provide key points for the unique control of meiotic cell cycle and chromosome behavior.

Research Design

A). Characterizing the control step of Ndt80-bc suppression
During our studies of *NDT80-bc* suppression, it was noticed that the *NDT80/ NDT80-bc* heterozygous status delayed but not reduced the suppression of *zip1* mutants in nuclear divisions and sporulation, based on repeated time course experiments. We also clarified that this delay is due to the presence of the wild-type *NDT80* allele, but is not due to dosage effect of single copy of *NDT80-bc*. This interesting result suggests that in *zip1* mutants, the inactive wild-type Ndt80 protein might compete with the active mutant Ndt80-bc protein for some unidentified step or interaction site that is essential for its normal function. This unidentified step or site, in fact, might be the controlling step of pachytene checkpoint. Nuclear import and MSE binding are two possible candidate steps for the control. The answer for this hypothesis will be essential for understanding the molecular mechanism of the pachytene checkpoint.

1). To address this question, we will fuse Ndt80 and Ndt80-bc with different epitope tags (e.g., HA and Myc) and analyze their expression and localization in *zip1* mutant cells (to activate the

pachytene checkpoint). Using different antibodies against HA and myc, respectively, we shall be able to distinguish between these two versions of Ndt80 protein for their localization within a cell. This experiment will tell us whether the control step is before or after nuclear import.

2). Furthermore, using different tagged Ndt80 and Ndt80-bc, chromatin immunoprecipitation (ChIP) experiments will be performed in *zip1* mutant cells to analyzed the *in vivo* DNA binding ability of these two protein. This experiment will tell us whether the control step is before or after Ndt80 binding to MSE.

B). Screening for Ndt80-interacting checkpoint proteins by the two-hybrid method
According to our working model, the pachytene checkpoint protein directly interacts with Ndt80 at the region that is deleted from the Ndt80-bc, and thus, controls the activity of Ndt80. Without this "target site", Ndt80-bc is able to bypass the control by the checkpoint protein and to allow those pachytene-arrest mutants to complete meiosis and sporulation. Therefore, to identify the Ndt80-interacting checkpoint protein will be the top-priority goal in this study.
The yeast two-hybrid protein system has been shown to be a powerful tool in isolation of interacting proteins. However, due to the DNA-binding and transcription-activation ability of Ndt80, full length of Ndt80 did not work properly in the system. Our experiment to dissect the function domains of Ndt80 provides information for the alternative strategy

Since the deleted region from Ndt80-bc is very likely to be the interacting site to the unidentified checkpoint protein. This potential interacting fragment, instead of the full-length Ndt80 will be a good choice for the bait in the two-hybrid screening. The candidates obtained from the two-hybrid screening will be further studied for their function in meiotic cell cycle control.

Results and Discussion

A). Nuclear localization of Ndt80, but not Ndt80-bc is inhibited in *zip1* cells
We examined the expression of Ndt80 and Ndt80-bc in the *zip1/zip1 NDT80/NDT80*, *zip1/zip1 NDT80-bc/NDT80-bc*, and *zip1/zip1 NDT80/NDT80-bc* cells throughout meiosis by Western blot analysis with the tubulin protein as an internal control. The accumulation of Ndt80 was inhibited in the *zip1/zip1 NDT80/NDT80* cells. On the contrast, Ndt80-bc was quickly accumulated. The induction pattern of Ndt80 appeared to be similar to that of Ndt80-bc in the *zip1/zip1 NDT80/NDT80-bc* cells. The ratio in protein amount of Ndt80 or Ndt80-bc to the corresponding tubulin control in each time point was also plotted for the quantitative comparison. The result showed that the kinetics of protein accumulation was equivalent between Ndt80 and Ndt80-bc in the *zip1/zip1 NDT80/NDT80-bc* cells, suggesting that the expression and accumulation of Ndt80 is not the control target by the pachytene checkpoint.

The *zip1/zip1 NDT80/NDT80-bc* cells used in the Western blot analysis were examined for

subcellular localization of Ndt80 and Ndt80-bc before meiosis I. Cells were collected at 11 h, 12 h, 13 h, and 14h after inoculation into the sporulation medium. The Ndt80 and Ndt80-bc proteins were detected by the rabbit anti-HA, and mouse anti-myc antibodies, respectively. To avoid any bias due to the failure of staining and for the purpose of comparison between Ndt80 and Ndt80-bc, only cells with both signals were scored. Three major categories of subcellular localization of Ndt80 and Ndt80-bc within a single cell were observed in the analysis. In category I, both the Ndt80 signal and Ndt80-bc signal were in the cytoplasm. In category II, the Ndt80 signal was in the cytoplasm, while the Ndt80-bc signal was concentrated in the nucleus. In category III, both the Ndt80 signal and Ndt80-bc signal were concentrated in the nucleus. Cells with nuclear Ndt80 signal and cytoplasmic Ndt80-bc signal were hardly observed. In the *zip1/zip1 NDT80/NDT80-b*c strain, most cells were belonged to the category II, in which the Ndt80 signal was cytoplasmic and the Ndt80-c signal was nuclear. The proportion of category I was decreased and category II was increased as meiosis progressed. The sum total of Ndt80 nuclear localization was significantly less than that of the Ndt80-bc in the *zip1/zip1 NDT80/NDT80-b*c cells. These results indicated that the nuclear localization of Ndt80, but not Ndt80-bc was severely affected by the *zip1* mutation.

These results suggest that nuclear localization of Ndt80 is regulated by the pachytene checkpoint. In the pachytene-arrest cells, the Ndt80 protein is retained in the cytoplasm by an inhibitor interacting with the target domain of Ndt80. It is unlikely that the regulation is at the export of Ndt80, since that there is no need for Ndt80 to shuttle in and out of the nucleus.

B). Screening for Ndt80-interacting checkpoint proteins by the two-hybrid method
We have performed a yeast two-hybrid screen for Ndt80-interacting proteins. So far, we have obtained only one consistently positive candidate. Its function in meiosis, especially to the pachytene checkpoint is currently under analysis.

Significance and Evaluation

Meiosis plays a central role in the sexual reproduction of eukaryotes. Regulation of cell cycle progression and control of chromosome segregation is critical for producing normal chromosome set in gametes. Defects in meiotic chromosome segregation are the primary causes of aneuploidy, which contributes significantly to infertility, reproductive failure, birth defects, and has been associated with various cancers. Dawn's syndrome (trisomy 21) is one of the well-known diseases that are caused by aneuploidy. Studies of meiotic cell cycle control and chromosome segregation can provide insight into the molecular basis of human disease due to chromosome abnormality.
In this study, we analyzed a very interesting mutation, the *NDT80-bc*. This deletion causes Ndt80 to become resistant to the control of the pachytene checkpoint. More importantly, our data

suggests that nuclear import of Ndt80 is regulated by the pachytene checkpoint. This is different from the model proposed by several research groups. Our finding opens a new direction in studying the molecular mechanism of meiotic cell cycle control.

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