MODELING CELL CYCLE EFFECTS OF HUMAN 14-3-3 TUMOR PROMOTING PROTEINS IN SACCHAROMYCES CEREVISIAE

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MODELING CELL CYCLE EFFECTS OF HUMAN 14-3-3 TUMOR PROMOTING PROTEINS IN SACCHAROMYCES CEREVISIAE

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Abstract

In this study, we used budding yeast as a model organism to examine the effects of overexpression of Bmh1, a yeast homolog of 14-3-3γ. We found that in the presence of modest DNA damage, Bmh1 overexpression had its most prominent effect during G2/M-phase of the cell cycle. We also observed that overexpression of Bmh1 concurrent with the induction of DNA damage partially rescued the G2/M arrest defect caused by the absence of Rad9, a key component of the G2/M DNA damage checkpoint pathway. When RAD53, a gene in the “Rad53 pathway” of the G2/M checkpoint, was deleted, overexpression of Bmh1 had no effect. However, overexpression of Bmh1 in a strain bearing the rad53-11 mutation partially rescued the arrest. Additionally, Bmh1 overexpression had a minimal effect on the G2/M arrest response with deletion of Chk1, a key component of the parallel G2/M checkpoint pathway. This led us to hypothesize that overexpression of Bmh1 in the absence of Rad9 modulates the Rad53 pathway. We propose a model in which the rescue of Rad9’s otherwise obligatory role in the DNA damage checkpoint is the consequence of Bmh1 subserving the adaptor function of Rad9 by bringing Mec1 and Rad53 together.

Introduction

The 14-3-3 proteins, a highly conserved family of scaffold proteins which bind phosphoproteins, regulate a variety of cellular processes. In humans, there are seven different isoforms of 14-3-3, one of which, 14-3-3γ, is overexpressed in many lung and colon cancers. Previous studies in our laboratory have shown that mammalian cells overexpressing human 14-3-3γ exhibit evidence of transformation and generate significantly increased numbers of polyploid cells; it has been suggested that transformation and/or polyploidy, which may lead to genomic instability, contribute to the development of cancer. However, the mechanisms by which overexpressed 14-3-3 proteins produce these phenotypes remain poorly defined. Consequently, we chose to examine 14-3-3 overexpression in the genetically tractable model organism Saccharomyces cerevisiae. Budding yeast have two 14-3-3 homologs, Bmh1 and Bmh2. Bmh1 is considered the major yeast homolog of human 14-3-3γ; thus, we chose to focus our studies on Bmh1 to model the effects of 14-3-3γ overexpression.

Recent studies have suggested that one mechanism by which Bmh1 regulates the cell cycle is by modulating the G2/M DNA damage checkpoint, thus potentially leading to polyploidy. In this study, we examined the effects of Bmh1 overexpression on responses to DNA damage in yeast, in order to determine the mechanisms by which 14-3-3 overexpression might lead to genomic instability. Because the 14-3-3 family is so highly conserved amongst all eukaryotes, what we learn regarding the effects of Bmh1 in yeast is likely applicable to the mechanisms of 14-3-3γ in mammalian cells.
Results

Overexpression of Bmh1 in the Presence of Modest DNA Damage

In our studies, we used two main strain backgrounds: the wildtype (WT) strain and the rad9 (9) strains. The WT strains have a cdc13 cdc15 RAD9+ background, while rad9 (9) strains are isologous except for the deletion of RAD9. When naming strains, V indicates that the strain contains the empty vector (thus serving as a control) while B indicates that the strain contains a TET repressible overexpression construct of Bmh1, the pBmh1 vector. The cdc13 mutation causes DNA damage at the restrictive temperature; the cdc15 mutation causes cells to arrest at exit from mitosis.

We were curious how Bmh1 overexpression affected the kinetics of the cell cycle. Thus, Bmh1 was overexpressed in both WT and 9 strains; strains were also transformed with V as a control. When growing the strains at 23°C, below the restrictive temperatures for both the cdc13 and cdc15 mutations, we saw that there was a delay in S-phase with rad9 deletion in both vector and pBmh1 strains. The most prominent effects of Bmh1 overexpression, however, were seen in G2/M. WTB had the longest G2/M phase (Figure 1), followed by 9B. When comparing WT strains, Bmh1 overexpression resulted in longer G2/M. Similarly, when comparing 9 strains, Bmh1 overexpression also resulted in longer G2/M. It should be noted that although the strains were grown at the permissive temperature, the cdc13 mutation is “leaky”; as a result, these strains, in all likelihood, suffer mild DNA damage. Thus, we concluded that in the presence of mild DNA damage, Bmh1 overexpression results in a prolonged G2/M phase, regardless of the presence of Rad9, a protein considered essential for the G2/M DNA damage checkpoint.

![Figure 1. Overexpression of Bmh1 has its most prominent effects in G2/M, indicating that Bmh1 plays a role in G2/M kinetics.](image)

Overexpression of Bmh1 in Wildtype Cells in the Presence of DNA Damage

In light of our observations that overexpression of Bmh1 has its most prominent cell cycle effects in G2/M phase (Figure 1), we reasoned that Bmh1 overexpression might affect the arrest response initiated by the G2/M DNA damage checkpoint. WT and 9 strains containing either vector or pBmh1 were grown at 37°C, well above the restrictive temperatures, thus ensuring that the cdc13 and cdc15 mutations were expressed. We then first looked at the wildtype cells: comparing WTV and WTB strains, we saw a very modest G2/M arrest defect in cells overexpressing Bmh1 (Figure 2). This modest arrest defect is most apparent when looking at the
progression of cells through G2/M. Because of the massive DNA damage inflicted by the cdc13 mutation, WT cells ordinarily completely arrest at the G2/M DNA damage checkpoint. Indeed, in WTV, close to 100% of cells arrested at the G2/M checkpoint and remained fully arrested for nearly eight hours. In WTB, the strain with Bmh1 overexpression, only about 85% of cells arrested at the checkpoint, thus indicating a modest arrest defect caused by Bmh1 overexpression. Thus, we conclude that Bmh1 overexpression in the presence of massive DNA damage results in a modest G2/M arrest defect, suggesting that Bmh1 somehow modulates the G2/M checkpoint.

Figure 2. In wildtype (RAD9+) cells, overexpression of Bmh1 induces a modest G2/M arrest defect.

Overexpression of Bmh1 in rad9 (9) Cells in the Presence of DNA Damage

Rad9 is an essential component in the DNA damage G2/M checkpoint pathway. Consequently, we also looked at overexpression of Bmh1 in a rad9 strain (9). As expected, RAD9 deleted cells exhibited a complete arrest defect (Figure 3); at 180 minutes, only about 10% of cells remained in G2/M, despite the presence of DNA damage due to the CDC13 mutation. Quite surprisingly, when Bmh1 was overexpressed in the 9 strain, we saw a partial rescue of the arrest defect: at 180 minutes, 30% of 9B cells remained arrested in G2/M. Thus, we conclude that Bmh1 overexpression partially circumvents the complete arrest defect caused by deletion of RAD9.
Figure 3. Deletion of RAD9 completely abolishes the G2/M DNA damage checkpoint. However, strains deleted for RAD9 are partially rescued by overexpression of Bmh1.

Overexpression of Bmh1 in the Absence of DNA Damage

In the experiments described so far, we looked at overexpression of Bmh1 in the presence of cdc13-induced DNA damage, whether it be modest (Figure 1) or severe (Figures 2 and 3). To exclude a cell cycle effect independent of DNA damage caused by Bmh1 overexpression, we examined cell cycle progression at G2/M in a CDC13+ strain transformed with either vector or pBmh1; the CDC13+ strain also has cdc15 mutation and the RAD9 deletion. Because Bmh1 overexpression caused a partial rescue of the rad9 arrest defect, we chose to look at CDC13+ cdc15 rad9 strains in order to determine whether the augmented G2/M delay occurred in the absence of DNA damage. In the CDC13+ strains, however, we saw no different in the G2/M progression between the Bmh1 and vector strains (Figure 4), thus indicating that the effect of Bmh1 overexpression on the rad9 arrest defect is DNA damage-dependent.

Figure 4. The effect of Bmh1 overexpression on the rad9 arrest defect is damage dependent. In the absence of DNA damage (cdc13 induced DNA damage), overexpression of Bmh1 has no effect on the G2/M phase of the cell cycle.
Overexpression of Bmh1 in mec1 Cells
The central component of the G2/M DNA damage checkpoint is Mec1, a kinase belonging to the PIKK family; Mec1 acts in conjunction with Rad9 to initiate downstream signaling. Because our previous experiments indicated that Bmh1 plays a role in the G2/M DNA damage checkpoint, we tested strains with mec1 deletion. The mec1 deletion resulted in a complete arrest defect after cdc13-induced DNA damage, as expected (Figure 5). Here, Bmh1 overexpression had no effect on the arrest defect; the vector and Bmh1 curves were nearly identical. Because Bmh1 overexpression had no effect upon G2/M progression in the mec1 strain, we conclude that Mec1 is required for the Bmh1 effect on G2/M progression in the rad9 strains, and that Bmh1 is likely acting downstream of Mec1.

Figure 5. Overexpression of Bmh1 does not rescue the arrest defect in mec1 strains.

Overexpression of Bmh1 in rad9 chk1 Cells
Downstream of Mec1/Rad9, two main signaling pathways control the G2/M DNA damage checkpoint. One involves RAD53 and the other requires CHK1; we deem these the “Rad53 pathway” and “Chk1 pathway”, respectively. Because the results just described suggest that overexpression of Bmh1 is acting at the level of downstream signal transduction, we wanted to determine in which pathway(s) Bmh1 acts. We first tested to see whether Bmh1 influenced the Chk1 pathway. In rad9 mutated strains, chk1 was deleted and strains were transformed with either vector or pBmh1. When looking at progression of rad9 chk1 mutants through G2/M, we first observed that deletion of CHK1 had no significant effect upon the rad9 arrest defect, an observation consistent with the two pathway model (Figure 6). However, superimposition of the CHK1 deletion diminished the effect of Bmh1 overexpression upon the rad9 arrest defect; fewer than 25% of cells arrested in G2/M for both V and B strains. Nevertheless, deletion of CHK1 did not completely abolish the effect of Bmh1 overexpression on the arrest response, suggesting a minor contribution of the Chk1 pathway to Bmh1’s effect on the rad9 arrest defect.
Overexpression of Bmh1 does not significantly alter the G2/M arrest response in the *chk1* strain, suggesting that Bmh1 acts in the Rad53 pathway, not the Chk1 pathway.

**Overexpression of Bmh1 in rad53-11 Mutants**

Because we detected only a minor contribution by the Chk1 pathway to the rescue of the *rad9* arrest defect by overexpression of Bmh1, we sought to test the prediction that Bmh1 acted predominantly via the Rad53 pathway, the other parallel pathway responsible for the G2/M DNA damage checkpoint. In *cdc13 cdc15* strains, we introduced the *rad53-11* mutation; this mutation is a partial loss-of-function mutation and, by analysis of secondary structure, is predicted to affect the binding of Rad53 to Rad9. As anticipated, the *rad53-11* mutation caused a partial G2/M arrest defect (Figure 7). Interestingly, we observed that overexpression of Bmh1 also partially rescues the *rad53-11* arrest defect; the Bmh1 overexpression curve remains consistently above the vector curve from 135 minutes onward, indicating that overexpression of Bmh1 results in a greater percentage of cells remaining in G2/M, despite the inherent arrest defect of *rad53-11*. Because Bmh1 overexpression partially rescued the arrest defect due to the *rad53-11* mutation, we can conclude that Bmh1 acts in the Rad53 pathway.

**Figure 6.** Overexpression of Bmh1 does not significantly alter the G2/M arrest response in the *chk1* strain, suggesting that Bmh1 acts in the Rad53 pathway, not the Chk1 pathway.

**Figure 7.** In strains with the *rad53-11* mutation, overexpression of Bmh1 partially rescues the arrest defect, thus indicating that Bmh1 plays a role in the Rad53 pathway of the G2/M DNA damage checkpoint.
Overexpression of Bmh1 in rad53Δ Mutants

The partial rescue of the rad53-11 strain by the overexpression of Bmh1 implies that Bmh1 is somehow interacting with Rad53, even in the absence of Rad9. If this supposition is correct, then deleting RAD53 would abolish the checkpoint-rescue phenotype of Bmh1 overexpression. We therefore tested the effect of Bmh1 overexpression when RAD53 was deleted. Although RAD53 is an essential gene, deleting the gene SML1 complements the essential function of Rad53, allowing the later to be deleted (deleting SML1 alone has no effect on the DNA damage G2/M checkpoint response; data not shown). In the absence of Rad53, Bmh1 overexpression had no effect upon the G2/M arrest in response to DNA damage (Figure 8). This observation, along with the rad53-11 data, allows us to conclude that Bmh1 works in conjunction with Rad53. When the checkpoint function of Rad53 is abolished by the rad53-11 point mutation, Bmh1 apparently can interact with the mutated Rad53 protein; when Rad53 is deleted, however, Bmh1 is unable to interact with checkpoint proteins to restore partial function.

Figure 8. Overexpression of Bmh1 has no effect on the G2/M arrest defect caused by the deletion of RAD53 in rad53Δ mutants.

Discussion

The series of experiments in our study allow us to reach several conclusions regarding the role of yeast 14-3-3 proteins in the DNA damage checkpoint response. Initially, we tested overexpression of Bmh1 in the presence of very modest DNA damage due to a “leaky” cdc13 mutation. We saw that Bmh1 caused a prolonged G2/M phase in strains which were either RAD9+ or rad9-. This led us to focus specifically on progression of cells through G2/M in order to determine the mechanism of action of Bmh1.

In the presence of far more substantial DNA damage caused by cdc13 at the restrictive temperature, we observed that Bmh1 overexpression partially rescued the G2/M checkpoint arrest defect caused by deletion of rad9. To ensure that this response is in fact damage-related, we looked at Bmh1 overexpression in CDC13+ strains; we saw no effect of Bmh1 overexpression in
these cells, indicating that the effects of Bmh1 overexpression are DNA damage-dependent. Rad9, along with Mec1, plays a central role in the G2/M DNA damage checkpoint, thus leading us to conclude that Bmh1 acted in one of the two pathways governing the checkpoint. Loss of Mec1 kinase function by mutation, however, was not rescued by Bmh1, leading us to conclude that Bmh1 likely acted in either or both of the two downstream pathways governing the checkpoint.

Moving further downstream of Mec1/Rad9 in the checkpoint signaling pathway, there are two main parallel pathways: the Rad53 pathway and the Chk1 pathway (Figure 9). We first tested the Chk1 pathway. Deleting \( \text{CHK1} \) diminished the effect of Bmh1 overexpression on the \( \text{rad9} \) arrest defect, indicating that Bmh1 may initiate a modest activation of the Chk1 kinase but that this effect is not sufficient to account for the Bmh1 effect in \( \text{rad9} \) strains. Accordingly, we examined the role of the other parallel pathway, first looking at a strain with a mutant Rad53 protein, the \( \text{rad53-11} \) mutated strain. The \( \text{rad53-11} \) is a point mutation which is positioned to disrupt the ability of Rad53 to bind with Rad9. In these strains, Bmh1 overexpression also partially rescued an arrest defect. However, in strains in which Rad53 was completely knocked out, there was no effect of Bmh1 overexpression on the arrest defect. This led us to conclude that Bmh1 acts in conjunction with Rad53, even when mutated, perhaps by bridging Rad53 to Mec1, even in the absence of Rad9.

Figure 9. Pathways of the G2/M DNA damage checkpoint.

From our observations, we suggest the following model (Figure 10): in the absence of Rad9, Bmh1 substitutes for the scaffold function of Rad9, bringing Rad53 into apposition with Mec1, allowing Mec1 kinase to phosphorylate Rad53. This results in at least partial activation of Rad53 (and perhaps allows slight activity of the Chk1 pathway by unknown mechanisms). Thus, the Rad53 pathway of the G2/M DNA damage checkpoint can be partially activated in \( \text{rad9} \)
strains by Bmh1 overexpression, a novel observation; previously, it had been assumed that Rad9 is absolutely required for activity of the DNA damage-dependent, G2/M checkpoint.

**Figure 10.** We propose that in the absence of Rad9, Bmh1 dimers substitute for the scaffold function of Rad9, bringing Rad53 into apposition with Mec1. This then allows Mec1 kinase to phosphorylate Rad53 and activate a checkpoint response, even in the absence of Rad9.

**Materials and Methods**

**Growth curve assay**
Haploid yeast cells were cultured in rich medium at 23°C, a permissive temperature for the *cdc13* and *cdc15* mutations. Once strains were determined to be in mid-log phase, samples were collected every hour for 8 hours. Samples were analyzed by flow cytometry and for cell density using spectrophotometry. Cell concentrations were plotted on a logarithmic scale in order to determine doubling time.

**Flow cytometry**
Samples from the growth curve assay were stained for nuclear DNA with Sytox Green and sorted using fluorescence-activated cell sorting. Flow cytometry data was then used to determine the percentage of cells in each phase of the cell cycle at each time point; because the strains were in mid-log phase, the percentage of cells in each phase of the cell cycle remains constant. Flow cytometry data were used in conjunction with doubling times to calculate the duration of each phase of the cell cycle.

**Synchronized cell cycle assay**
Haploid yeast cells were synchronized in G1 phase with the mating pheromone α-factor at 23°C, released by washing, and cultured in rich medium at 37°C. Samples were collected every 15 to 20 minutes for up to 240 minutes, depending on the strain background.

**Cell cycle analysis**
Samples from synchronized cell cycle assays were fixed in ethanol and stained with DAPI. Using fluorescent microscopy, at least 100 cells were counted at each time point and scored as G1, S-phase, G2/M, or post-anaphase. Because we focused specifically on G2/M
progression, the percentage of cells in G2/M at each time point was plotted to examine how strain background affected progression through G2/M and the G2/M DNA damage checkpoint arrest.

**Further Experimentation**

Although we have learned a great deal from this study, we also have discovered much more we wish to determine. We will examine the effect of Bmh1 overexpression on the activity of Rad53 in the absence of Rad9 through western blotting. We have constructed a FLAG-tagged RAD53 strain, allowing us to examine Rad53 activity with overexpression of Bmh1. The FLAG-tagged RAD53 strain will allow us to visualize protein concentration and phosphorylation of Rad53. We also plan to study the effects of Bmh1 overexpression on cell survival after the induction of cdc13 DNA damage; improved G2/M arrest may not be synonymous with increased survival.

Moreover, preliminary results suggest that Bmh1 may also play a role in regulating Sml1, a negative regulator of the dNTP synthesis pathway. We plan to study the effects of Bmh1 in strains manipulating Sml1, along with other key proteins in related pathways.

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**References**
