主　論　文　の　要　約

論文題目： Functional analysis of P53 negative regulators in *Bombyx mori*

(カイコにおける P53 抑制制御因子の機能解析)

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Chapter 1 General introduction

Apoptosis serves as one of the crucial intracellular antiviral immune responses in insects that lack the adaptive immune systems mediated by B- and T-cells found in vertebrates. Apoptosis is induced in several insect cell lines following infection with various species of nucleopolyhedroviruses (NPVs), which are insect-pathogenic, large DNA viruses that belong to the family *Baculoviridae*. A recent study from our laboratory demonstrated that apoptosis induction is mediated by the *Bombyx mori* ortholog of *p53* (*bm-p53*) in *B. mori* cells infected with vBmΔp35, a recombinant B. mori NPV (BmNPV) lacking the functional anti-apoptotic gene *p35*. In mammals and *Drosophila melanogaster*, the tumor suppressor P53 plays a central role in the cellular stress responses. It is responsible for the transcriptional activation of downstream genes such as those involved in cell cycle arrest, senescence, autophagy, global protein synthesis shutdown, and apoptosis. In normal cells, the P53 protein is tightly regulated by multiple post-translational modifications, including ubiquitination. In this study, to provide a basis for the Bm-P53 regulation mechanism in normal and NPV-infected cells, I identified and characterized P53 negative regulators in *B. mori*.

Chapter 2 Cloning and characterization of putative P53 negative regulators in *B. mori*

In this chapter, *B. mori* homologs of negative regulators of P53 in mammals and/or *D. melanogaster*, Bonus, Corp, Mdm2, Rad6, Sce, and Synoviolin, which has a direct interaction with P53, was searched using a tblastn search (search translated nucleotide databases using a protein query) against the NCBI nucleotide

collection (nr/nt) database. The amino acid sequences of the *Drosophila* E3 ligases Bonus (NCBI Acc. No. AAF19646), SCE (NCBI Acc. No. NP_477509), and Synoviolin (NCBI Acc. No. Q95SP2), E2 conjugating enzyme Rad6 (NCBI Acc. No. P25153), and Corp (NCBI Acc. No. NP_001245578), and mouse sequence of the mammalian E3 ligase Mdm2 (NCBI Acc. No. Q00987) were used as queries. The search revealed that *B. mori* possessed homologs of the mammalian and *Drosophila* E2 ligase Rad6 (Bm-Rad6), and E3 ligases Bonus (Bm-Bonus), Mdm2 (Bm-Mdm2), Rad6, SCE (Bm-SCE), and a longer Synoviolin isoform (Bm-SynoviolinX1), while not that of the *Drosophila* regulator, Corp. The cloned ORFs of Bm-Bonus, Bm-Mdm2, Bm-Rad6, Bm-SCE, and Bm-SynoviolinX1 were deposited in DDBJ/EMBL/GenBank database under the accession numbers LC598229, LC598230, LC598231, LC598232, and LC598233, respectively.

To determine whether Bm-Bonus, Bm-Mdm2, Bm-Drad6, Bm-Sce, and Bm-SynoviolinX1 contributed to Bm-P53 negative regulation and apoptosis induction, RNAi-mediated knockdown and transient expression of these proteins were carried out. Among *B. mori* homologs examined here, only Bm-Mdm2 is clearly involved in the negative regulation of Bm-P53 levels. RNAi-mediated knockdown of *bm-mdm2* resulted in increased endogenous Bm-P53 accumulation in *B. mori* cells, whereas transient overexpression of Bm-Mdm2 significantly reduced Bm-P53 accumulation. Furthermore, co-expression assay in *B. mori* cells demonstrated that Bm-Mdm2 attenuated Bm-P53-induced apoptosis and effector caspase activity. Moreover, the immunoblot of the Bm-Mdm2 and Bm-p53 co-transfected cells also showed two additional bands above the original Bm-P53 bands, and these were stronger than those found in the EGFP co-expressing cells. These bands were assumed to be ubiquitinated Bm-P53.

Interestingly, despite its capacity for ubiquitylation of Bm-P53 and attenuation of Bm-P53-induced apoptosis, Bm-Mdm2 lost the N-terminal P53-binding domain and underwent several extensive deletions of the acidic region (AR), both of which are crucial for P53 binding and subsequent ubiquitylation of P53 in mammals. Furthermore, overexpressed Bm-Mdm2 was localized in the nucleolus, which differs from mammalian Mdm2 that is localized in the nucleus and cytoplasm. These results suggest that *B. mori* has a different P53 negative regulation mechanism from mammals and from *D. melanogaster*, which has Corp instead of Mdm2.

Chapter 3 Regulation mechanism of Bm-P53 by Bm-Mdm2

Motif alignment of Mdm2 and Corp from various organisms identified five highly conserved motifs, M1, M2, M3, M5, and M6 (aa270–328, 125-151, 102-124, 39-58, and 18-37 of Bm-Mdm2, respectively), among insects and invertebrates. M1 and M2 corresponded to RING and zinc finger (Zn-F) of Bm-Mdm2, respectively, M3 and M5 included a portion of the predicted acidic region (AR), and M6 was found in the N-terminal of Bm-Mdm2. To gain knowledge on the mechanism of Bm-Mdm2-mediated regulation of Bm-P53 via ubiquitination, transient co-expression analyses of Bm-P53 and Bm-Mdm2 motif deletion mutants were conducted. As a result, all Bm-Mdm2 motif deletion mutants could not inhibit apoptosis induced in *B. mori* cells transfected with Bm-P53, indicating that all the deleted regions of Bm-Mdm2 contained the functional region for Bm-P53 negative regulation. Furthermore, none of the motif deletion mutants was able to ubiquitinate Bm-P53 like that of the full-length Bm-Mdm2 effectively. This analysis also demonstrates that M1 includes the signal responsible for Bm-Mdm2 localization to the nucleolus.

To determine the ubiquitinated residues of Bm-P53, Bm-P53 protein bearing C-terminal deletion, which initially contains the oligomerization domain (OD) and regulatory domain (RD), was examined for ubiquitination by Bm-Mdm2. I found that *B. mori* cells co-expressing Bm-Mdm2 and C-terminal deletion mutant of Bm-P53 resulted in apoptosis, and ubiquitinated Bm-P53 was not observed. These results suggest that Bm-Mdm2 inhibits Bm-P53-triggered apoptosis through functional interaction with the Bm-P53 C-terminal region. This Bm-P53 C-terminal region has five lysine residues which are suspected to be ubiquitinated by Bm-Mdm2. However, single or double lysines to arginine mutation within each domain did not affect ubiquitylation of Bm-P53 by overexpressing Bm-Mdm2. These results suggest that Bm-Mdm2 can alternatively use other available lysine residues, including those found in the DNA binding domain, and it will take complete mutations of all possible attachment sites to stop ubiquitination as reported in mammalian P53.

Chapter 4 Analysis of Bm-Mdm2 response in *B. mori* cells infected with NPV

The previous study demonstrated that RNAi-mediated knockdown of *bm-p53* expression abolishes apoptosis of *B. mori* cells induced by infection with vBmΔp35 and that levels of both *bm-p53* mRNA and Bm-P53 protein were maintained at a basal level during BmNPV infection. These results suggest the complexity of Bm-P53-mediated apoptosis induction in NPV-infected *B. mori* cells. In this chapter, to understand the function of Bm-Mdm2 in BmNPV-infected *B. mori* cells, changes in endogenous Bm-Mdm2 mRNA and protein levels were examined during NPV infection. The infection of *B. mori* cells with either BmNPV or vBmΔp35 resulted in a drop of *bm-mdm2* mRNA levels. In contrast, endogenous Bm-Mdm2 protein levels started to accumulate from 6 to 24 h post-infection (p.i.). and seemed to be degraded at 48 h p.i.

Further analyses using fluorescence microscopy and a knockdown assay of viral genes suggest that this increase of Bm-Mdm2 protein levels was not due to localization change of Bm-Mdm2 during infection and was triggered by the onset of viral early gene expression and viral DNA replication. However, overexpression of Bm-Mdm2 has no influence on apoptosis induction, as shown by the formation of apoptotic cells in vBmΔp35-infected cells. Furthermore, overexpressed Bm-Mdm2 also do not affect virus protein expression as reflected by comparable levels of viral late and very late proteins, VP39 and Polyhedrin, in *B. mori* cells infected with BmNPV or vBmΔp35. Therefore, I could not elucidate the function of Bm-Mdm2 in NPV-infected *B. mori* cells in this study.

Chapter 5 Conclusion

In this study, I identified and characterized the *B. mori* homologs of Bonus, Mdm2, Rad6, Sce, and Synoviolin, which are crucial for the ubiquitylation and negative regulation of P53 in *D. melanogaster* and/or mammals. Among the identified homologs, Bm-Mdm2 is the prime negative regulator for Bm-P53, and this regulation is mediated through ubiquitination of Bm-P53 and is suggested to occur in the nucleolus. Mdm2, which is a primary negative regulator of P53 levels in mammals, is also widely conserved in the arthropods, including insects, but is not encoded by the *D. melanogaster* genome. This study is the first demonstration of the functionality of an insect Mdm2 using the Mdm2 homolog of the lepidopteran insect *B. mori*. I have shown that Bm-Mdm2 also serves as a negative regulator of P53 though Bm-Mdm2 possessed many differences with

the mammalian Mdm2, including its localization and conserved domains. Taken together, these findings suggest that lepidopteran insects use a different P53 regulation mechanism from those used in dipteran insects, such as *D. melanogaster*, which lost Mdm2 and acquired a new regulator, Corp, and those used in mammals. Further analyses using P53 and its negative regulators from various insects will give insight into the conservation and diversification of the P53 negative regulation mechanism.