Mlp-dependent anchorage and stabilization of a desumoylating enzyme is required to prevent clonal lethality

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Myosin-like proteins 1 and 2 (Mlp1 and Mlp2) form filaments attached to the nucleoplasmic side of the nuclear pore complexes via interaction with the nucleoporin Nup60. Here, we show that Mlps and Nup60, but not several other nucleoporins, are required to localize and stabilize a desumoylating enzyme Ulp1. Moreover, like Mlps, Ulp1 exhibits a unique asymmetric distribution on the nuclear envelope. Consistent with a role in regulating Ulp1, removal of either or both MLPs affects the SUMO conjugate pattern. We also show that deleting MLPs or the localization domains of Ulp1 results in DNA damage sensitivity and clonal lethality, the latter of which is caused by increased levels of 2-micron circle DNA. Epistatic and dosage suppression analyses further demonstrate that Mlps function upstream of Ulp1 in 2-micron circle maintenance and the damage response. Together, our results reveal that Mlps play important roles in regulating Ulp1 and subsequently affect sumoylation stasis, growth, and DNA repair.

Introduction

The budding yeast myosin-like protein 1 (Mlp1), myosin-like protein 2 (Mlp2), and their mammalian homologue, translocated promoter region (TPR), form fibers extending from the nuclear pore complexes (NPCs) to the nucleoplasm (Cordes et al., 1997; Strambio-de-Castillia et al., 1999). Their unique localization and structural properties suggest that they play important roles in nuclear organization and transport. However, the functions of these proteins were not fully understood. Previous studies showed that lacking Mlps slightly slows the rate of protein import but does not affect mRNA or protein export, suggesting that their roles in nucleocytoplasmic transport are limited (Strambio-de-Castillia et al., 1999; Galy et al., 2004). Recently, it was found that Mlp1, but not Mlp2, is required for the nuclear retention of unspliced mRNAs (Galy et al., 2004). However, these available data do not explain the two major defects associated with deletion of MLPs. The first is that mlp1Δ, mlp2Δ, and mlp1Δ mlp2Δ are all sensitive to DNA damaging agents, such as the radiation-mimicking drug bleomycin (Galy et al., 2000; Kosova et al., 2000). Second, and most noticeably, colonies of mlp1Δ mlp2Δ, but not mlp1Δ or mlp2Δ strains, exhibit numerous indentations reflecting clonal lethality (Galy et al., 2000). This phenotype is referred to as “nibbled colony”. It has not been clear how Mlps affect cell growth and the DNA damage response.

Clonal lethality and DNA damage sensitivity are also exhibited by mutants defective in sumoylating and desumoylating enzymes (see below). Small ubiquitin-like modifier (SUMO) provides a reversible, post-translational modification mostly of nuclear proteins and regulates localization, activity, and binding properties of target proteins (Melchior, 2000; Muller et al., 2001; Seeler and Dejean, 2003). Attachment of SUMO requires the activating enzyme (E1), the conjugating enzyme (E2), and the ligases (E3s), whereas removal of SUMO requires desumoylating enzymes (Melchior, 2000). Interestingly, defects in the sumoylating E2 and E3s (unpublished data), as well as two desumoylating enzymes, Ulp1 and Ulp2 (Holm, 1982; Li and Hochstrasser, 2000; Dobson, M., personal communication), can all lead to nibbled colonies and DNA damage sensitivity. Although the underlying mechanism is not clear, these observations suggest that the proper balance of sumoylation stasis is important for growth and DNA repair. Among these enzymes, Ulp1 is localized to the NPCs (Li and Hochstrasser, 2000; Schwienhorst et al., 2000), likely in a complex with karyopherins, the transporters that carry cargo proteins through NPCs (Panse et al., 2003). However, components of NPCs that are required for the docking of Ulp1 have not been identified.
In this report, we show that Mlps and Nup60 are required to dock and stabilize Ulp1. We also show that defects in this regulation result in disruption of sumoylation stasis, clonal lethality, and DNA damage sensitivity.

**Results and discussion**

**Clonal lethality of mlp1Δ mlp2Δ is caused by increased levels of 2-micron circle**

Our initial genetic analysis revealed that the nibbled phenotype of mlp1Δ mlp2Δ is not stably inherited, suggesting that it is likely caused by extrachromosomal or epigenetic factors (see Online supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200405168/DC1). It was reported that high levels of 2-micron circle, an extrachromosomal plasmid found in most strains of *Saccharomyces cerevisiae*, can result in nibbled colonies (Broach and Volkert, 1991). 2-micron circle is packed into nucleosomes, and replicates and segregates using the same proteins as chromosomal DNA (Broach and Volkert, 1991; Mehta et al., 2002). It normally neither benefits nor harms the host when maintained at 50–100 copies per cell. Nevertheless, overproduction of 2-micron circle by overexpressing proteins unique to the plasmid’s amplification system causes clonal lethality and nibbled colonies (Reynolds et al., 1987; Rose and Broach, 1990). A plausible explanation is that increased levels of 2-micron circle can titrate out essential replication and segregation machineries because even a twofold increase can give rise to DNA equivalent to two small yeast chromosomes. To test whether the nibbled phenotype of mlp1Δ mlp2Δ is related to 2-micron circle, we first removed the plasmid and observed that mlp1Δ mlp2Δ colonies are completely smooth and uniform in size (Fig. 1 A). In fact, the growth of mlp1Δ mlp2Δ cells that lack 2-micron circle (cir0) is indistinguishable from that of wild-type cells. Next, we reintroduced 2-micron circle to mlp1Δ mlp2Δ cir0 strains. The resulting mlp1Δ mlp2Δ strains containing the plasmid (cir+) regained the nibbled colony morphology (Fig. 1 A). Finally, we found that the copy number of 2-micron circle in mlp1Δ mlp2Δ, but not in either single mutants, is ~2.5-fold higher than in the wild type (Fig. 1, B and C). These results reveal that an elevated level of 2-micron circle is the cause for the nibbled colony morphology of mlp1Δ mlp2Δ.

**Mlps and Nup60 are required to tether a desumoylating enzyme at the nuclear periphery**

To understand how Mlps affect 2-micron circle levels, we looked for other mutants that also exhibit nibbled colony morphology. As mentioned earlier, strains defective in a desumoylating enzyme, Ulp1, display a similar phenotype. The similar localization pattern of Mlps and Ulp1, as well as the common defects in colony morphology of their mutants, suggest that they may function in the same pathway. One possibility is that Mlps are required to dock Ulp1 at NPCs. Fluorescence microscopic examination of live cells showed that the localization pattern of Mlps differs from that of nucleoporins. Consistent with a recent report (Galy et al., 2004), nucleoporins are dis-
tributed all around the nuclear envelope, whereas Mlps are excluded from the region juxtaposed to the nucleolus (Fig. 2 A). We reasoned that if Mlps provide docking sites for Ulp1, Ulp1 should exhibit the same asymmetric distribution. To test this idea, Ulp1-YFP fusion protein was expressed in cells containing CFP-tagged nucleoporin Nic96 and mRFP-tagged nucleolar protein Nop1. Examination by fluorescence microscopy revealed that Ulp1-YFP is also excluded from the nucleolus-proximal region of the nuclear envelope (Fig. 2 A). Therefore, the localization pattern of Ulp1 resembles that of Mlps.

To explore further the relationship between Mlps and Ulp1, we examined the localization of Ulp1-YFP in mlp1Δ, mlp2Δ, and mlp1Δ mlp2Δ cells. We observed that the signal intensity of Ulp1-YFP at the nuclear rim decreases moderately in mlp1Δ or mlp2Δ strains compared with wild-type strains (Fig. 2 B). Because the protein level of Ulp1-YFP in these mutants is the same as in wild-type strains (Fig. 3 A), the reduction of nuclear rim signals suggests that some Ulp1 proteins are delocalized. In mlp1Δ mlp2Δ double mutants, Ulp1-YFP signals at the nuclear rim are greatly reduced (Fig. 2 B). In addition, this is associated with a fivefold reduction of Ulp1 protein levels (Fig. 3 A). These results indicate that lacking both Mlp1 and Mlp2 synergistically affects Ulp1 protein levels and localization. The simplest explanation is that severe delocalization of Ulp1 due to the lack of both Mlps leads to the reduced protein levels.

It has been shown that perturbation of Ulp1 localization by deleting its localization domain (see below) can lead to accumulation and diminution of different SUMO conjugates without affecting its SUMO precursor processing activity (Li and Hochstrasser, 2003). We suspected that loss of either or both Mlps would affect the sumoylation stasis similarly. To assess the global effect on SUMO conjugates, we examined sumoylated protein patterns in various mutants. As shown in Fig. 3 B, two sumoylated proteins accumulate in mlp1Δ mlp2Δ, but not in mlp1Δ, mlp2Δ, or wild-type strains, suggesting that severe reduction of Ulp1 at the nuclear rim impedes desumoylation. In addition, two sumoylated protein bands disappear in all three mutants, consistent with the idea that delo-
Ulp1 levels and the SUMO conjugate pattern are changed in cells lacking Mlps or Nup60. (A) Total protein was extracted from indicated strains and subjected to immunoblot analysis using anti-GFP antibody. ULP1-YFP was used to replace the endogenous ULP1 in all strains, except in WT (untagged). The same blot was stripped and reprobed with anti-PGK antibody to check loading consistency. Arrows indicate the SUMO conjugates whose levels diminish in mlp1 and nup60 strains. The asterisks mark the SUMO conjugates whose levels increase in mlp1Δ mlp2Δ and nup60Δ strains. The value for wild-type strains was considered to be 1. Error bars indicate SDs from three similar blots. (B) Immunoblot analysis of yeast lysates prepared from indicated strains was performed using anti-SUMO antibody. The same blot was stripped and reprobed with anti-PGK antibody to check loading consistency. Arrows indicate the SUMO conjugates whose levels diminish in mlp1Δ, mlp2Δ, mlp1Δ mlp2Δ, and nup60Δ strains.

Deleting the localization domains of Ulp1 can lead to elevated levels of 2-micron circle and nibbled colony morphology.

To investigate the relationship between Ulp1 and 2-micron circle directly, we tested if delocalization of Ulp1 with its anchoring proteins intact can result in elevated levels of 2-micron circle and nibbled colonies. Ulp1 contains two separate domains: the COOH-terminal catalytic domain (residues 403–612) and the NH2-terminal regulatory domain (residues 1–340), which is necessary and sufficient for NPC localization (Li and Hochstrasser, 2003; Panse et al., 2003). It was previously shown that gradual deletions of the NH2-terminal domain lead to increased delocalization of Ulp1 from the nuclear rim without affecting its enzymatic activity (Li and Hochstrasser, 2003; Panse et al., 2003). Therefore, we examined the effect of two NH2-terminal deletion constructs, ulp1NΔ210 (residues 1–209 deleted) and ulp1NΔ338 (residues 1–337 deleted), on 2-micron circle and colony morphology. Ulp1NΔ210 contains some sequences required for nuclear rim localization, whereas ulp1NΔ338 lacks all sequences for docking (Panse et al., 2003). As shown in Fig. 4 A, ulp1NΔ210 and ulp1NΔ338 colonies are nibbled, and this defect is rescued by removal of 2-micron circle. Furthermore, the copy number of 2-micron circle in ulp1NΔ210 and ulp1NΔ338 mutants increases ~2.5- and ~4-fold, respectively (Fig. 1, B and C). Therefore, delocalization of Ulp1 by deleting its NH2 terminus results in higher levels of 2-micron circle, and the severity of delocalization is positively correlated with the levels of 2-micron circle.

Ulp1 functions downstream of Mlps in 2-micron circle maintenance and DNA damage response.

The results described above raise the possibility that increased levels of 2-micron circle and the nibbled phenotype of mlp1Δ mlp2Δ strains are due to impaired function of Ulp1. However, it was formally possible that other defects of mlp1Δ mlp2Δ strains, unrelated to Ulp1, may have resulted in...
such a phenotype. To distinguish these possibilities, we took advantage of the differences in degrees of delocalization and amounts of 2-micron circle seen in mlp1Δ mlp2Δ and ulp1Δ338 mutants. If the first possibility is correct, a ulp1 mutation that completely abolishes its nuclear rim localization, such as ulp1Δ338, should be epistatic to mlp1Δ mlp2Δ; i.e., an mlp1Δ mlp2Δ ulp1Δ338 triple mutant should resemble ulp1Δ338. As shown in Fig. 1 (B and C), mlp1Δ mlp2Δ ulp1Δ338 and ulp1Δ338 strains contain similar amounts of 2-micron circle, which are higher than that of mlp1Δ mlp2Δ strains. Interestingly, a similar epistatic relationship between mlp1Δ mlp2Δ and ulp1Δ338 was also seen for the sensitivity to the DNA damaging agent bleomycin (Fig. 4 B). These results suggest that Ulp1 functions downstream of Mlps in pathways that control 2-micron copy number and DNA repair.

**Overexpression of Ulp1 suppresses clonal lethality and DNA damage sensitivity of mlp1Δ mlp2Δ**

It is noteworthy that other tethering sites for Ulp1 exist, as residual Ulp1-YFP signals were seen at the nuclear rim in cells lacking Mlps (Fig. 2 B). The fact that these sites cannot substitute for Mlps to dock Ulp1 when Ulp1 is expressed at the endogenous level suggests that they might be less efficient. Should this be the case, they might be able to dock a sufficient amount of Ulp1 when Ulp1 is overexpressed. We found that Ulp1 exhibits continuous nuclear envelope localization, including the region juxtaposed to the nucleolus when it is moderately overexpressed from the NOP1 promoter, (Fig. 5, A and B). This is different from the Mlp-dependent localization pattern, which is punctuate and excluded from the nucleolus, indicating that alternative tethering of Ulp1 occurs. Consistently, this localization pattern is independent of Mlps (Fig. 5 B). The restoration of Ulp1 localization under overexpression conditions can rescue the nibbled colony morphology and bleomycin sensitivity of mlp1Δ mlp2Δ strains (Fig. 5, C and D). This result confirms the conclusion from our epistatic analysis and strongly supports the notion that the nibbled colony morphology and bleomycin sensitivity in mlp1Δ mlp2Δ strains are due to defects in regulating Ulp1.

In summary, our results demonstrate that Mlps and Nup60 are required to anchor and stabilize Ulp1. We also show that other binding sites for Ulp1 exist and can provide sufficient anchoring when Ulp1 is overexpressed. Although the nature of these alternative sites is not clear, we speculate that they can be nucleoporins or other proteins located at the nuclear envelope. Tethering of Ulp1 could be mediated by Kap60, Kap95, and Kap123, as these karyopherins interact with nucleoporins, Mlps, and Ulp1 (Kosova et al., 2000; Allen et al., 2001; Panse et al., 2003). The last interaction is RanGTP-insensitive and different from the transient transporter–cargo interaction, suggesting that it can mediate the tethering (Panse et al., 2003). However, other forms of interaction are also possible, as the mammalian homologue of Ulp1 can bind directly to the nucleoparin Nup153 (Hang and Dasso, 2002; Zhang et al., 2002). Further work will be required to understand the biochemical interactions used in Ulp1 anchoring. Our data also show that decreased levels of Ulp1 are associated with a severe reduction of its nuclear rim signals. One possible explanation is that drastically delocalized Ulp1 desumoylates and activates proteins involved in its own degradation. This may be a means to protect cells from the catastrophic consequence of excessive and unregulated desumoylating activity.

The existence of multiple pathways for tethering Ulp1 illustrates the importance of its localization at the nuclear periphery. Ulp1 exhibits a stronger desumoylating activity in vitro than the nucleoplasmin-localized Ulp2 (Li and Hoch-
Sequestration can prevent Ulp1 from competing with Ulp2 and from desumoylating proteins in an unregulated manner. On the other hand, the localization at the NPC may also play an active role, as Ulp1 can remove SUMO from sumoylated cargo proteins during their passage through the NPC channel. Thus, releasing Ulp1 from the NPCs can perturb the sumoylation stasis by eliciting undesired desumoylation as well as abolishing normal desumoylation. This is exemplified by the reduction and accumulation of different SUMO conjugates in mlp1Δ mlp2Δ strains. Observations consistent with this view were made previously using various deletion constructs of Ulp1 (Li and Hochstrasser, 2003). As detachment of SUMO can change properties of target proteins involved in various pathways (Melchior, 2000; Muller et al., 2001; Seeler and Dejean, 2003), impairment of Ulp1 function can undermine many cellular functions, such as 2-micron circle maintenance and the DNA damage response as seen in mlp1 and mlp1Δ mlp2Δ strains. Identification of proteins whose sumoylation status is affected in mlp1Δ mlp2Δ strains will help to elucidate the molecular pathways downstream of Mlp1 and Ulp1.

Materials and methods

Removal and reintroduction of 2-micron circle

To remove 2-micron circle DNA, we used the method described by Tsai and Gartenberg (1998). To reintroduce 2-micron circle, we mated a cir− haploid to a cir+ haploid. The resulting diploid contains a normal amount of 2-micron circle due to amplification of 2-micron circle contributed from the cir+ parental strain (Broach and Volkert, 1991). Consequently, spore clones obtained from such a diploid strain inherit a normal amount of 2-micron circle.

Measurement of the level of 2-micron circle

Total yeast DNA was digested with HindIII and separated on a 0.8% agarose gel. DNA fragments were transferred to the nitrocellulose membrane using a standard genomic blot protocol. The membrane was subjected to hybridization using a 500-bp 2-micron–specific probe generated by PCR with the primer pairs “2μm D proteinR” (ATATACTATCTGTTTCAGCA) and “2μm D proteinF” (AATCTGTCATGTGGAATGCCT) and “2

Online supplemental material

Supplemental material includes the result that the nibbled colony morphology of mlp1Δ mlp2Δ mutants is not stably inherited. It also contains yeast strains, plasmids, and growth condition. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200405168/DC1.

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