Genetic Requirements and Meiotic Function of Phosphorylation of the Yeast Axial Element Protein Red1^{▽†}

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Received 2 August 2010/Returned for modification 7 September 2010/Accepted 9 December 2010

The synaptonemal complex (SC) is a meiosis-specific tripartite structure that forms between two homologous chromosomes; it consists of a central region and two parallel lateral elements. Lateral elements also are called axial elements prior to synapsis. In *Saccharomyces cerevisiae*, Red1, Hop1, and Mek1 are structural components of axial/lateral elements. The *red1/mek1/hop1* mutants all exhibit reduced levels of interhomolog recombination and produce no viable spores. Red1 is a phosphoprotein. Several earlier reports proposed that phosphorylated Red1 plays important roles in meiosis, including in signaling meiotic DNA damage or in preventing exit from the pachytene chromosomes. We report here that the phosphorylation of Red1 is carried out in *CDC28*-dependent and *CDC28*-independent manners. In contrast to previous results, we found Red1 phosphorylation to be independent of meiotic DNA recombination, the Mec1/Tel1 DNA damage checkpoint kinases, and the Mek1 kinase. To functionally validate the phosphorylation of Red1, we mapped the phosphorylation sites on this protein. A *red1*^{14A} mutant showing no detectable Red1 phosphorylation did not exhibit decreased sporulation efficiency, defects in viable spore production, or defects in meiotic DNA damage checkpoints. Thus, our results suggest that the phosphorylation of Red1 is not essential for its functions in meiosis.

Meiosis is a critical component in the cycle of sexual reproduction, because it reduces the chromosome complement to haploidy in preparation for fertilization. This event is achieved by a single round of premeiotic DNA replication followed by two successive rounds of chromosome segregation to produce four haploid gametes. The first nuclear division (MI) is reductional, separating the newly recombined homologs from one another while leaving sister chromatids attached. The second nuclear division (MII), in which the sister chromatids segregate, is more typical of mitotic division and is called equational division. A prominent feature of meiosis is that pairing and recombination must occur between homologous chromosomes during the meiotic prophase. In contrast, these events rarely happen in mitosis. Meiotic DNA recombination plays a crucial role in meiosis, not only providing a potent source of genetic variation but also playing a mechanical role during MI. Specifically, crossover recombination results in a physical connection (i.e., chiasmata) between homologous chromosomes that allows them to orient properly on the spindle (for a review, see reference 59).

Meiotic DNA recombination is initiated by the formation of DNA double-strand breaks (DSBs). Spo11, a meiosis-specific type II topoisomerase, generates DSBs together with several other factors in a cell cycle-programmed manner (26). The Mre11-Rad50-Xrs2 nuclease complex then resects these DSBs

to generate 3' single-stranded tails that invade the intact DNA duplexes used for DNA repair (35). Most of these events use homologous chromosomes, not sister chromatids, as the templates for DNA repair to yield crossover and noncrossover products (for a review, see reference 6). DNA repair is facilitated by Dmc1, a meiosis-specific RecA-like protein that promotes interhomolog (IH) recombination (5, 42).

To promote MI in many eukaryotic organisms (e.g., *Saccharomyces cerevisiae* and humans), crossovers often occur in the context of the synaptonemal complex (SC), a zipper-like proteinaceous structure that connects a pair of homologous chromosomes along their entire length. The SC consists of a central region and two dense lateral elements. Each lateral element constitutes the rod-like homolog axis, also called an axial element (AE), prior to synapsis, along which the chromatin loops of sister chromatids are organized. The central region contains transverse filaments, oriented perpendicularly to the longitudinal axis of the SC, resulting in the striated, zipper-like appearance of the SC (20). The components of the yeast AE include sister chromatid cohesin proteins (e.g., Rec8), DNA topoisomerase II (Top2), and a few meiosis-specific chromosomal proteins (e.g., Mek1, Hop1, and Red1) (7, 22, 29, 39, 44). Mek1 is a protein kinase that functions together with Red1 and Hop1 to ensure *DMC1*-dependent IH recombination by preventing the use of a sister chromatid as the template for DNA repair (2, 23, 36, 42, 52, 57). Rad51, also a RecA-like recombinase, is utilized primarily for sister chromatid recombination in vegetative and meiotic cells (9, 48). Mek1 phosphorylates Rad54, a dsDNA-dependent ATPase required for Rad51 recombinase activity. Phosphorylated Rad54 acts synergistically with the meiosis-specific protein Hed1 to suppress the recombinase activity of Rad51 (37). The activation of

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† Supplemental material for this article may be found at <http://mcb.asm.org/>.

[▽] Published ahead of print on 20 December 2010.

Mek1 kinase depends on Hop1 phosphorylation, which is mediated by Mec1 and Tel1, the yeast homologs of mammalian DNA damage checkpoint kinases ATR and ATM (10).

Red1 interacts with itself and with Hop1 (2, 14, 23). Recently, we reported that Red1 associates with the polymeric chains of the small ubiquitin-like modifier (SUMO) to promote Mec1/Tel1-dependent Hop1 phosphorylation as well as SC assembly (30). Furthermore, the binding of Red1 to the 9-1-1 checkpoint complex (Ddc1/Mec3/Rad17) has been reported to be crucial for meiotic checkpoint activation (16).

Red1 is also a phosphoprotein (1, 2, 14). Mek1 has been reported as the kinase phosphorylating Red1 (1, 2, 14), while the protein phosphatase type I (Glc7) was found to dephosphorylate Red1 (1). Phosphorylated Red1 is thought to prevent exit from pachytene, and the completion of meiotic recombination is believed to trigger the Glc7-mediated dephosphorylation of Red1 (1, 40). It also was reported that essential components of the Mec1/Tel1 DNA damage checkpoint pathway, including Rad24 and the 9-1-1 complex, promote both the stable association of Mek1 with chromosomes and Mek1-dependent Red1 phosphorylation (24). However, in those earlier reports the amino acid residues responsible for Red1 phosphorylation were neither distinctly mapped nor functionally validated. Moreover, some of the biochemical studies of those earlier reports, particularly the immunoprecipitation and Western blotting experiments, were not designed to avoid high levels of vacuolar protease activities in the meiotic total cell lysates. Vacuolar proteases (e.g., Pep4, Prb1, and Prc1), which are not only highly induced upon starvation but also essential for sporulation (60), are extremely problematic for the biochemical analysis of yeast meiosis. Although infrequently cited, it was reported more than 30 years ago that vacuolar proteases are activated upon cell lysis in native and in SDS-containing sample buffers, leading to the widespread proteolysis of yeast proteins (38).

To overcome this problem, we applied a trichloroacetic acid (TCA) precipitation method to prepare meiotic total cell lysates (see Materials and Methods). We found that total cell lysates prepared from 0.1 ml sporulating cell culture (optical density at 600 nm [OD₆₀₀], ~1.4) were sufficient for the Western blot analysis of most proteins. In contrast, in two earlier papers (1, 2), 3 liters of sporulating cell cultures was used to prepare, in a native lysis buffer, meiotic cell lysates for subsequent immunoprecipitation and Western blotting experiments. Because the amounts of cells used at the beginning of the experimental procedure in those two studies were excessively high, most proteins must have been degraded in their lysates. In addition, a third paper also reported a mysterious phenomenon that, to reproducibly detect Red1-3HA, total cell lysates must be prepared the same day as the cells were sporulated, i.e., the cells could not be frozen first (14). Interestingly, these three papers reported that Red1 is a *MEK1*-dependent phosphoprotein.

Later on, two laboratories independently demonstrated that Red1 is not a target of Mek1 kinase (21, 52) but acts upstream of Mek1 (52). It was proposed then that phosphorylated Red1 interacts with the FHA domain of Mek1 to recruit Mek1 to DSB sites where Mek1 is activated to prevent *DMC1*-independent DSB repair (52). Although the identity of the kinase involved in the phosphorylation of Red1 remained unknown

and the importance of Red1 phosphorylation for meiosis had not been demonstrated, it still was proposed that phosphorylated Red1 plays important roles in meiosis (21, 25, 36). In the present study, we provide strong evidence suggesting that the phosphorylation of Red1 is not required for its function in meiosis.

MATERIALS AND METHODS

Yeast strains, media, Western blot analysis, and custom gene synthesis. All strains used here were derived from the *SK1* background and are described in Table S1 in the supplemental material. Yeast culture, sporulation medium techniques, and Western blot analysis were performed as described previously (13, 30, 55). Details regarding strains and plasmid construction are available on request. The custom gene synthesis of the *V5-red1¹⁵* allele was performed by Epoch Biolabs (Missouri City, TX).

Inhibition of the analogue-sensitive *cdc28-as1* mutant. 4-Amino-1-tert-butyl-3(1'-naphthylmethyl)pyrazolo[3,4-*d*]pyrimidine (1-NM-PP1) was generously provided by Cellular Genomics (New Haven, CT). To assay for sensitivity to the inhibitor, sporulating cell culture was first treated with 5 μ M 1-NM-PP1 (3) at the indicated time points. The culture was returned to undergo sporulation until the 8-h time point and harvested for functional analysis.

Preparation of total yeast cell extracts. Yeast total extracts were prepared by a trichloroacetic acid (TCA) precipitation method (13). Sporulating cell culture (3.5 ml; OD₆₀₀ ~1.4) was harvested and washed once with ice-cold water. Cells were resuspended in 1 ml ice-cold water and mixed with 150 μ l of freshly prepared buffer D (1.85 M NaOH, 7.5% β -mercaptoethanol). The samples then were incubated on ice for 15 min and mixed with 150 μ l of 55% TCA for 10 min on ice. The cell suspension was centrifuged at 10,000 $\times$ g for 10 min. The protein pellets were resuspended with 250 μ l of buffer H (200 mM Tris-HCl [pH 6.5], 8 M urea, 5% SDS, 1 mM EDTA, 0.02% bromophenol blue, and 5% β -mercaptoethanol). Protein samples were denatured for 10 min at 65°C and then separated by SDS-PAGE. To detect V5-Red1, monoclonal anti-V5 antibody (Invitrogen) and peroxidase-conjugated anti-mouse IgG were used for Western blot analysis.

Dephosphorylation assay. Whole-cell extracts were prepared by the TCA precipitation method as described above. The protein pellet obtained from 3 ml sporulating cell culture was resuspended in 250 μ l of buffer A (50 mM Tris-HCl [pH 8.9], 1 mM MgCl₂, containing EDTA-free protease inhibitor cocktail [Roche]), and then 4 μ l of 2 M Tris-Base was added to adjust the pH to 7.5. Dephosphorylation reactions were carried out by mixing 37.5 μ l whole-cell extracts with 90 U of calf intestinal alkaline phosphatase (CIAP; New England BioLabs), followed by incubation for 4 h at 37°C. In some reactions, the phosphatase inhibitor β -glycerophosphate (Sigma) was added to a final concentration of 16 μ M. Dephosphorylation reactions were stopped by adding an equal volume of 3 $\times$ Laemmli sample buffer (188 mM Tris-HCl [pH 6.8], 3% SDS, 15% β -mercaptoethanol, 30% glycerol, 0.01% bromophenol blue), followed by incubation for 6 min at 95°C. Alternatively, some dephosphorylation reactions were stopped by the addition of 7% TCA. After the removal of supernatant, the pellet was resuspended in buffer H and then incubated for 10 min at 65°C.

Phos-tag affinity method. Phos-tag acrylamide (28) was purchased as a lyophilized powder from the Phos-tag Consortium (Tokyo, Japan). Samples were resolved on 6% (wt/vol) polyacrylamide running gels (37.5:1 [wt/wt] acrylamide: *N,N'*-methylene-bisacrylamide) containing 375 mM Tris (pH 8.8), 0.1% (wt/vol) SDS, and either 50 μ M phos-tag and 100 μ M MnCl₂ or 100 μ M phos-tag and 200 μ M MnCl₂ (27, 28). Stacking gels contained 4.5% (wt/vol) polyacrylamide (37.5:1 [wt/wt] acrylamide:*N,N'*-methylene bisacrylamide), 125 mM Tris-HCl (pH 6.8), and 0.1% (wt/vol) SDS. Yeast total cell lysates were prepared by the TCA precipitation method and then treated with CIAP or left untreated as described above. The samples then were precipitated again by TCA. After the removal of the supernatant, the pellet was resuspended in buffer H (without EDTA) and incubated at 65°C for 10 min. The phos-tag acrylamide gels were run at 4°C under constant voltage (4.4 V/cm) for 4 h.

FACS. Samples from meiotic or mitotic cultures (1 ml each) were pelleted and resuspended in 40% ethanol containing 0.1 M sorbitol (12). Samples were stored at -20°C until fluorescence-activated cell sorting (FACS) analysis. One day before FACS analysis, cells were pelleted and resuspended in 0.3 ml of 50 mM Tris-HCl (pH 7.5) containing 100 μ g/ml RNase, incubated overnight at room temperature, and then mixed with 0.3 ml of SYTOX green solution (1 mM SYTOX green in 50 mM Tris-HCl, pH 7.5). Samples were sonicated briefly and analyzed on a FACScan analyzer (Becton-Dickinson). The FL1 detector with a

standard 530/30 band-pass filter was used in the acquisition of SYTOX green fluorescence (19).

RESULTS

Red1 phosphorylation is independent of *SPO11*. To facilitate the detection of Red1, a *V5-RED1* diploid strain expressing V5-tagged Red1 was constructed. The addition of the V5 epitope resulted in no apparent defects in sporulation; the *V5-RED1* cells carried out meiosis normally and produced as many viable spores (96%) as the *RED1* cells. The *V5-RED1* cells then were induced to undergo relatively synchronous sporulation. At the indicated time points, cells were harvested for the preparation of total cell lysates according to the TCA precipitation protocol described previously (13). In short, first NaOH was used to break the yeast cell walls, then TCA was employed for protein precipitation, and finally buffer containing SDS and urea (8 M) was added for the SDS-PAGE analysis of samples. This protocol not only efficiently extracted proteins from yeast cells grown on various media but also prevented proteolysis or changes in protein posttranslational modifications (e.g., dephosphorylation, desumoylation, or deubiquitination).

The Western blotting time course analysis of proteins separated by SDS-PAGE, carried out with an antibody specific to the V5 epitope, revealed that V5-Red1 first appeared as a fast-migrating band approximately 1 h after the cells were transferred to sporulation medium (SPM). A slower migrating form could be observed after the 2-h time point (Fig. 1A). A dephosphorylation assay using CIAP confirmed that the faster and slower migrating species represent nonphosphorylated and phosphorylated V5-Red1, respectively (Fig. 1B). In the samples without CIAP, we observed two discrete bands (left lane). With the addition of CIAP, the band with lower mobility was no longer visible (middle lane). The addition of β -glycerophosphate, a general phosphatase inhibitor, to the sample prior to its incubation with CIAP effectively prevented the conversion of the slower migrating fraction to the faster migrating product (right lane). These results are consistent with previous studies, in which a significant fraction of Red1 was found to be phosphorylated *in vivo* during meiosis (2, 14).

The phosphorylation of Red1 also occurred in the sporulating *mek1 Δ* diploid (Fig. 1C, left, and D, left), confirming that Red1 is not a *MEK1*-dependent phosphoprotein (see reviews in references 21 and 52). In contrast to previous reports (1, 2, 14, 24), we observed that V5-Red1 still was phosphorylated in sporulating *spo11 Δ* diploid cells (Fig. 1C, right). The extent of Red1 phosphorylation was similar in both *mek1 Δ* and *spo11 Δ* diploid cells compared to that of the wild type. These results suggest that Red1 phosphorylation is independent of meiotic DNA recombination and the DNA damage checkpoint.

Red1 phosphorylation is not required for exit from pachytene. Phosphorylated Red1 is thought to prevent exit from pachytene, and the completion of meiotic recombination is considered to trigger the Glc7-dependent dephosphorylation of Red1. Glc7 comprises the catalytic subunit of the type 1 protein phosphatase (1). It was reported that Red1 was degraded shortly after the release of *NDT80-IN* cells from a reversible pachytene arrest (25). In the absence of inducer (β -estradiol), *NDT80* is not expressed, and cells accumulate in pachytene with persis-

FIG. 1. Detection of V5-Red1. Shown is a Western blotting time course analysis with an anti-V5 antibody. Total cell lysates of wild-type (A) and *mek1 Δ* and *spo11 Δ* (C) meiotic cells were prepared at the indicated sporulating time point. (B and D) Gel mobility shift analysis of V5-Red1 proteins. Total cell lysates of various meiotic cells at the 10-h time point were treated with calf intestinal alkaline phosphatase (CIAP) in the absence or presence of a general phosphatase inhibitor, β -glycerophosphate (see Materials and Methods). (E to G) Total cell lysates of *NDT80-IN* (E), *clb5 Δ clb6 Δ* (F), and *ime2 Δ* (G) meiotic cells were prepared at the indicated sporulating time point. β -Estradiol (1 μ M) was added at the 6-h time point to release the *NDT80-IN* cells from the pachytene arrest (E).

tent joint molecules and full-length SC. β -Estradiol addition induces *NDT80* expression, and cells synchronously exit from pachytene and undergo meiotic nuclear divisions (11).

To examine whether Red1 phosphorylation affects Red1 degradation after pachytene release, a pachytene arrest-and-release experiment was performed using *V5-RED1 NDT80-IN* cells. Meiotic cells first were arrested at pachytene and then released from the arrest by the induction of Ndt80 expression via the addition of β -estradiol. Using this system, more than 75% of nuclei from the *V5-RED1* strain showed full SCs prior to release (data not shown). Western blotting time course

FIG. 2. Expression patterns and meiotic functions of *V5-RED1*, *V5-RED1*^{7A}, *V5-red1*^{I758R}, and *V5-red1*^{I758R,7A} strains. (A) Western blot time course analysis of these four strains was carried out as described in the legend to Fig. 1. Arp7 was used as a loading control. (B) For the direct comparison of proteins by gel mobility shift analysis, total cell lysates of each strain at the 6-h time point were analyzed by Western blotting with an anti-V5 antibody on the same gel. Arp7 was used as a loading control (data not shown). (C) The ability of these four strains to produce viable spores was determined by tetrad analysis. *V5-red1*^{I758R} and *V5-red1*^{I758R,7A}, like the *red1Δ* null mutant, produce very few viable spores.

analysis revealed that both phosphorylated and nonphosphorylated Red1 disappeared slowly with similar kinetics. Moreover, the steady-state level of phosphorylated Red1 was always higher than that of nonphosphorylated Red1 (Fig. 1E), indicating that dephosphorylation is not required for exit from the pachytene chromosomes. Unlike previous reports (1, 2, 14, 25), we prepared yeast total cell lysates only by TCA precipitation and performed all experimental procedures on ice or at 4°C but not at room temperature.

Since our results differed from those of previous reports (1, 2, 14, 24, 25, 52), we reinvestigated in this study the genetic requirements for the phosphorylation of Red1 and also examined whether such modification of Red1 is essential for its function in meiosis.

Loss of Cdc28/Clb5,6 or Ime2 kinase activity leads to Red1 hypophosphorylation and ubiquitination. Clb5 and Clb6 are B-type cyclins that activate Cdc28 kinase to promote premeiotic DNA replication (15, 46), the initiation of meiotic DNA recombination (41, 45, 53), and SC assembly (3, 45). Ime2 is a meiosis-specific serine/threonine kinase (34) that controls meiotic G₁-S transition by decreasing the level of Sic1 protein, an inhibitor of Cdc28/Clb5,6 kinase (3, 15, 17). Our results indicate that V5-Red1 proteins were hypophosphorylated in *ime2Δ* and *clb5Δ clb6Δ* mutants, as the phosphorylated V5-Red1 in these mutants possessed a higher electrophoretic mobility than the wild-type cells (Fig. 1D, F, and G).

The two mutants also accumulated a ladder of high-molec-

ular-weight (HMW) V5-Red1 that migrated much more slowly than phosphorylated V5-Red1 (Fig. 1F and G). These HMW proteins were barely detected in the wild-type meiotic cells (Fig. 1A). After treatment with CIAP, they migrated slightly faster as two sharper bands (marked by white arrows in Fig. 1D, right) in the SDS-PAGE gel, indicating that they also were phosphoproteins. The HMW phosphoproteins may have been ubiquitinated by the anaphase-promoting complex (APC/C), a ubiquitin ligase, for the following reasons. First, early Clb kinases (e.g., Clb5 and Clb6 kinases) have been implicated in the inactivation of APC/C during mitotic S phase to allow Clb2 accumulation (59); second, Ime2 also has a critical role in switching off the APC/C ubiquitin ligase activity during early meiosis (8). Notably, Ime2 decreases the level of Sic1 to activate the Cdc28/Clb5,6 kinase activity in meiosis (3, 15, 17).

Dual modes of Red1 phosphorylation. Red1 has seven S/T-P or S-X-K-K motifs, which comprise putative target sites of Cdc28 kinase (49). We mutated each of these seven serine/threonine residues (Thr395, Thr417, Ser469, Ser550, Ser631, Thr698, and Ser723) to alanine to generate a *V5-RED1*^{7A} strain. Western blotting time course analysis revealed that V5-Red1^{7A} was hypophosphorylated compared to the phosphorylation of V5-Red1 (Fig. 2A and B). Intriguingly, the *V5-RED1*^{7A} cells could sporulate and produce as many viable spores as the *V5-RED1* cells (Fig. 2C), indicating that the phosphorylation of these seven serine/threonine residues is dispensable for meiosis.

FIG. 3. Phosphorylation of V5-Red1^{I758R} and V5-Red1 is Cdc28 dependent. (A) Progression of premeiotic DNA replication. Execution point analysis was carried out by the addition of 5 μ M 1-NM-PP1 at the indicated time points, and the sporulating cells were harvested 8 h after transfer into sporulation medium. The cells then were stained with SYTOX green, and the DNA content was measured by flow cytometry (see Materials and Methods). (B) Western blotting time course analysis of V5-Red1^{I758R} proteins in the sporulating *V5-red1^{I758R} cdc28-as1* cells (upper). Execution point analysis was carried out as described for panel A (lower). Total proteins were separated by SDS-PAGE gel and then analyzed by Western blotting with an anti-V5 antibody. (C) Execution point analysis also was carried

out to examine the *V5-RED1 cdc28-as1* cells as described for panel B. (D) Western blotting time course analyses of V5-Red1^{7A} (upper) and V5-Red1^{I758R} (lower) in sporulating *ime2Δ* cells, respectively. Total cell lysates of *V5-RED1* and *red1Δ* cells were used as positive and negative controls for Western blotting with an anti-V5 antibody, respectively.

Red1 contains an essential Smt3-interacting motif at its C terminus (i.e., amino acid residues 758 to 761; ISII). The V5-Red1^{I758R} mutant, in which the critical Ile758 residue was converted into Arg, is defective in binding to SUMO chains and in the production of viable spores (30). Like V5-Red1^{7A}, the V5-Red1^{I758R} mutant also was hypophosphorylated compared to the phosphorylation of V5-Red1 (Fig. 2A and B).

We then constructed *V5-red1^{I758R,7A}*, a synthetic mutant of *V5-RED1^{7A}* and *V5-red1^{I758R}* genes. This synthetic mutant, like *V5-red1^{I758R}*, generated very few viable spores (Fig. 2C). Intriguingly, V5-Red1^{I758R,7A} migrated at the same speed as the nonphosphorylated V5-Red1 in the SDS-PAGE gel throughout the sporulation time course (Fig. 2A and B), indicating that V5-Red1^{I758R,7A} is not a phosphoprotein.

In summary, our results suggest that two different pathways are independently responsible for the phosphorylation of V5-Red1^{7A} and V5-Red1^{I758R}, respectively. V5-Red1^{I758R} phosphorylation appears to be *CDC28* dependent, whereas V5-Red1^{7A} phosphorylation likely is *CDC28* independent but dependent on Red1-SUMO chain interaction (30).

Phosphorylation of Red1^{I758R} is dependent on Cdc28 protein kinase. To substantiate the hypothesis that Cdc28 kinase activity is responsible for V5-Red1^{I758R} phosphorylation, we constructed a *V5-red1^{I758R} cdc28-as1* strain. It was reported that the treatment of *cdc28-as1* diploid cells with a high concentration of 1-NM-PP1 (5 μ M) blocked meiotic DNA replication so that the cellular DNA content remained at 2N for more than 8.5 h (3). 1-NM-PP1 is a cell-permeable inhibitor of kinases that have been mutated, by a single base substitution, to become analog sensitive (*as*) compared to the wild-type kinase (4). In contrast, in the absence of 1-NM-PP1, almost all *cdc28-as1* diploid cells accumulated 4N DNA content by 5 h. This arrest was caused by the specific inhibition of Cdc28-as1 kinase, because DNA replication was blocked only by the combination of *cdc28-as1* mutation and 5 μ M 1-NM-PP1 and not by either condition alone (3).

Using the ability of 1-NM-PP1 to inhibit *cdc28-as1*, we performed execution point analyses to determine if and when during meiosis Cdc28 kinase activity is required for V5-Red1^{I758R} phosphorylation. At the indicated meiotic time points (0 to 8 h), 1-NM-PP1 (5 μ M) was added to 5 ml SPM aliquots, and then the cultures were returned to undergo sporulation. The cells all were harvested at the 8-h time point for Western blot analysis. As expected, the addition of 1-NM-PP1 at the time points between 0 and 4 h prevented both DNA replication (Fig. 3A) and V5-Red1^{I758R} phosphorylation (Fig. 3B) until the 8-h time point. In contrast, DNA replication (Fig. 3A) and phosphorylated V5-Red1^{I758R} (Fig. 3B) began to appear if 1-NM-PP1 was added after the 5-h time point. These results not only define ~4 h as the endpoint for the requirement of Cdc28 kinase activity in the formation of phosphory-

FIG. 4. Time course analyses of V5-Red1 and V5-Red1^{7A} proteins in *P_{TET}-DBF4-6MYC* cells treated with doxycycline (right) or left untreated (left). Doxycycline was added 3 h before shifting the cells to sporulation medium to achieve full Dbf4 depletion at the beginning of meiosis (50). The V5-Red1, V5-Red1^{7A}, and Dbf4-6myc proteins were detected by Western blotting with anti-V5 and anti-Myc antibodies, respectively. Arp7 was used as a loading control (data not shown).

lated V5-Red1^{1758R} but also confirm that Cdc28 itself or Cdc28-dependent kinase(s) is responsible for V5-Red1^{1758R} phosphorylation. We also used the same execution point experiment to examine the *V5-RED1 cdc28-as1* cells. Unlike V5-Red1^{1758R}, V5-Red1 is hypophosphorylated upon the addition of 1-NM-PP1, which inactivates the Cdc28 kinase activity (Fig. 3C). Taken together with the findings that the V5-Red1^{1758R,7A} mutant is not phosphorylated in meiosis (Fig. 2A), we conclude from these results that V5-Red1 is phosphorylated in both CDC28-dependent and -independent manners.

As in the *clb5Δ clb6Δ* and *ime2Δ* mutants, a large portion of V5-Red1 apparently was ubiquitinated when the *cdc28-as1* diploid cells were treated with 1-NM-PP1 (Fig. 3C). In contrast, V5-Red1^{1758R} was neither phosphorylated nor ubiquitinated when the Cdc28-as1 kinase was inhibited by 1-NM-PP1 (Fig. 3B). Intriguingly, V5-Red1^{7A} also was ubiquitinated in *ime2Δ* cells (Fig. 3D, upper), whereas V5-Red1^{1758R} was not ubiquitinated in the same mutant (Fig. 3D, lower). The ubiquitinated forms of V5-Red1 (Fig. 1A) or V5-Red1^{7A} (Fig. 2B) were rarely detected in the wild-type cells (Fig. 1A). As described above, this outcome may be the result of the roles of Ime2 and Cdc28 in switching off the APC/C ubiquitin E3 ligase activity during early meiotic prophase (8, 58).

Cdc7 and the DNA damage checkpoint kinases are not involved in CDC28-independent Red1 phosphorylation. To further dissect the genetic requirement(s) for CDC28-independent Red1 phosphorylation, we compared the expression patterns of V5-Red1^{7A} in various meiotic mutant cells. CDC7 encodes a Dbf4-dependent kinase that is essential for premeiotic DNA replication and for the formation of DSBs (41, 50, 53, 54). It was reported before that Cdc7 kinase activity could be inactivated through the depletion of its unstable regulatory subunit, Dbf4, by shutting off its transcription from a

FIG. 5. Genetic requirements of CDC28-independent Red1 phosphorylation. Western blotting time course analyses of sporulating *mek1Δ* (A), *spo11Δ* (B), *rad24Δ tel1Δ* (C), and *hop1Δ* (D). Hop1 phosphorylation did not occur in the *rad24Δ tel1Δ* cells. Arp7 was used as a loading control (data not shown).

repressible tetracycline promoter (*P_{TET}-off*) (50). Because Dbf4 degradation occurs gradually after transcriptional shut-off, doxycycline was added 3 h before shifting the cells to sporulation medium to achieve full Dbf4 depletion at the beginning of meiosis (50). Our results indicate that both V5-Red1 and V5-Red1^{7A} still were phosphorylated in the homozygous *P_{TET}-DBF4* diploid strains treated with doxycycline (Fig. 4), suggesting that V5-Red1^{7A} phosphorylation is independent of Cdc7 kinase activity.

Like V5-Red1 (Fig. 1C), V5-Red1^{7A} still was phosphorylated in the *mek1Δ* (Fig. 5A), *spo11Δ* (Fig. 5B), and *rad24Δ tel1Δ* cells (Fig. 5C, lower). Rad24 is a checkpoint protein involved in the activation of Mec1 kinase (18, 31, 32, 43). We also confirmed that Hop1 was phosphorylated in the *V5-RED1^{7A}* and *V5-RED1* cells (Fig. 5C, upper, and data not shown). In contrast, Hop1 was not phosphorylated in the *V5-RED1^{7A} rad24Δ tel1Δ* meiotic cells (Fig. 5C, middle) or in the *V5-RED1 rad24Δ tel1Δ* meiotic cells (data not shown), thus confirming that Hop1 is a Mec1/Tel1-dependent phosphoprotein (10). We conclude that V5-Red1^{7A} and V5-Red1 are Mec1/Tel1-independent phosphoproteins.

Hop1 is required for CDC28-independent Red1 phosphorylation. Because *RED1* and *HOP1* act together to promote interhomolog recombination by repressing intersister recombination (2, 23, 30, 56), we then examined if *HOP1* itself is

FIG. 6. Expression patterns of V5-Red1 and V5-Red1^{7A} in sporulating *rim11Δ* (A and C) and *rim15Δ* (B and D) cells. Because Rim11 and Rim15 are essential for the induction of several early meiosis-specific proteins (e.g., Hop1 and Red1), the *V5-RED1*, *V5-RED1*^{7A}, or *HOP1* gene was engineered under the control of the *GAL1* promoter in *rim11Δ* and *rim15Δ* strains, respectively. Galactose (0.03%) was added to sporulating cultures at the zero time point to induce V5-Red1 (A and B), V5-Red1^{7A} (A to D), or Hop1 (C and D), respectively. The addition of 0.03% galactose led to no apparent sporulation defect of the *V5-RED1* or *V5-RED1*^{7A} diploid cells (data not shown).

essential for the phosphorylation of Red1. Our results indicate that V5-Red1^{7A} was not phosphorylated in a *hop1Δ* null mutant, whereas V5-Red1 was phosphorylated in the same mutant (Fig. 5D). Therefore, *HOP1* is specifically required for CDC28-independent Red1 phosphorylation but not for CDC28-dependent Red1 phosphorylation.

CDC28-independent Red1 phosphorylation does not occur in the absence of Rim11 or Rim15. CDC28-independent Red1 phosphorylation likely is meiosis specific. When the V5-Red1 and V5-Red1^{7A} proteins were ectopically expressed in vegetatively growing wild-type yeast cells under the control of the *GAL1* promoter, V5-Red1 was phosphorylated, whereas V5-Red1^{7A} was not (data not shown). We then examined if two important early meiotic protein kinase genes, *RIM11* and *RIM15*, are responsible for CDC28-independent phosphorylation. *RIM11* encodes a glycogen synthase kinase 3 protein kinase required for signal transduction during entry into meiosis (33). *RIM15* encodes a glucose-repressible protein kinase involved in signal transduction during cell proliferation in response to nutrients (51). In brief, both Rim11 and Rim15 protein kinases promote the formation of the Ime1-Ume6 complex, which acts as a transcriptional activator for early meiotic genes. Rim11 directly phosphorylates Ime1 to promote the formation of this complex, whereas Rim15 stimulates the interaction between Ime1 and Ume6 (51).

Because Red1 was not expressed in the *rim11Δ* and *rim15Δ* mutants, V5-Red1 and V5-Red1^{7A}, respectively, were ectopically expressed in these two mutants under the control of the *GAL1* promoter. Both proteins were induced by the addition of 0.03% galactose at the time cells were transferred into SPM. The addition of 0.03% galactose into SPM resulted in no apparent sporulation or Red1 phosphorylation defect in *V5-RED1* or *V5-RED1*^{7A} cells (data not shown). We found that V5-Red1 was phosphorylated in these two mutants, whereas

V5-Red1^{7A} was not phosphorylated (Fig. 6A and B). Because both Rim11 and Rim15 are required for the expression of early meiotic proteins (e.g., Hop1) (47) and Hop1 was lacking in these two mutants, we could not ascertain whether Rim11 or Rim15 is responsible for V5-Red1^{7A} phosphorylation. To address this issue, we simultaneously expressed both Hop1 and V5-Red1^{7A} in the *rim11Δ* and *rim15Δ* mutants, respectively. Our results revealed that V5-Red1^{7A} still was not phosphorylated in the presence of Hop1 (Fig. 6C). Taken together, these results suggest that both Rim11 and Rim15 are required for CDC28-independent Red1 phosphorylation. These two kinases may act directly to stimulate Red1 phosphorylation or indirectly affect the interaction between Red1 and Hop1. Nevertheless, our results confirm that CDC28-independent Red1 phosphorylation only occurs in meiosis.

Determination of V5-Red1^{7A} phosphorylation sites during meiosis. Although we did not identify the protein kinase(s) responsible for the CDC28-independent phosphorylation of Red1 in this study, mapping the sites and validating the biological significance of Red1 phosphorylation still are important. We adopted a domain-swapping approach to map the phosphorylation sites of V5-Red1^{7A}. The wild-type Red1 contains 71 serine and 48 threonine residues. We first synthesized an artificial gene encoding a V5-Red1^{1S} protein. This mutant contained only 1 serine residue (i.e., Ser759) of the wild-type V5-Red1, while the other 70 serine and 48 threonine residues were mutated to alanines. Ser759 was preserved to retain the essential SUMO-interacting motif (ISII; residues 758 to 761) (30). The coding sequence of the *V5-RED1*^{7A} gene then was divided into six segments (A to F), and each segment then was replaced by that of the *V5-red1*^{1S} gene to generate six chimeric genes, *V5-red1*^{CHA} to *V5-red1*^{CHF}, respectively (Fig. 7A). Western blotting time course analyses were carried out to determine which segments carried sites for V5-Red1^{7A} phosphorylation.

FIG. 7. (A) Schematic showing the chimeric V5-Red1 proteins constructed and investigated in this study. (B) Sequences between amino acid residues 401 and 439 of various chimera proteins. All serine and threonine residues are underlined. The capability of these chimera proteins to be phosphorylated or to produce viable spores, respectively, is given on the right. Protein phosphorylation was determined by gel mobility shift assay on polyacrylamide gels containing SDS (see Fig. 8), whereas spore viability was determined by tetrad dissection analysis. Arp7 was used as a loading control (data not shown).

A potential pitfall of this approach is that the lack of the phosphorylation of a chimeric protein (e.g., V5-Red1^{CHA}, V5-Red1^{CHB}, or V5-Red1^{CHC}) might simply derive from improper protein folding due to multiple substitutions of Ser/Thr to Ala. Fortunately, the approach worked for the V5-Red1^{CHD} mutant, which showed no detectable phosphorylation. Moreover, the V5-RED1^{CHD} homozygous mutant could produce as many viable spores (99% spore viability) as the V5-RED1 or V5-RED1^{7A} strains. The V5-Red1^{CHE} and V5-Red1^{CHF} proteins are phosphorylated, and the V5-RED1^{CHE} and V5-RED1^{CHF} strains produced 80 and 31% viable spores, respectively (Fig. 7 and 8). These results indicate that segment D (amino acid residues 401 to 504) likely contains the CDC28-independent phosphorylation sites.

Segment D then was further divided into three smaller segments (D1 to D3) to generate yeast strains expressing V5-Red1^{CHD1}, V5-Red1^{CHD2}, or V5-Red1^{CHD3} protein, respectively (Fig. 7A). All three yeast strains, like the V5-RED1^{CHD} mutant, exhibited no apparent defect in sporulation efficiency or the production of viable spores. Western blotting time course analysis revealed that V5-Red1^{CHD1}, in contrast to the other two mutants, showed no detectable phosphorylation. Therefore, the segment D1 (amino acid residues 401 to 439) contains the sites for V5-Red1^{7A} phosphorylation. Compared to V5-Red1^{7A}, V5-Red1^{CHD1} contains seven additional serine/

threonine-to-alanine substitutions (S403A, S404A, S421A, S423A, S426A, S433A, and T435A). V5-Red1^{CHD1} subsequently was designated V5-Red1^{14A}.

To confirm that these seven serine/threonine residues indeed represent CDC28-independent phosphorylation sites, one, two, or four of these seven alanines in V5-Red1^{14A} then were reverted to serine/threonine to yield six yeast strains expressing V5-Red1^{12A-1}, V5-Red1^{12A-2}, V5-Red1^{10A}, V5-Red1^{13A-1}, V5-Red1^{13A-2}, or V5-Red1^{13A-3} protein, respectively (Fig. 7B). V5-Red1^{12A-1}, V5-Red1^{12A-2}, and V5-Red1^{10A} became phosphorylated in meiosis (Fig. 8C), whereas V5-Red1^{13A-1}, V5-Red1^{13A-2}, and V5-Red1^{13A-3} showed no detectable phosphorylation (Fig. 8D). Taken together, the results indicate that at least four residues (Ser403, Ser404, Ser433, and Thr435) are sites of CDC28-independent Red1 phosphorylation. These four residues also may contain priming sites that could trigger more phosphorylation elsewhere. Finally, our results also indicate that the mobility of V5-Red1 in SDS-PAGE gels is highly sensitive to protein phosphorylation, since only one or two phosphorylation events sufficed to generate a notable mobility shift.

To further confirm that V5-Red1^{14A} is not a phosphoprotein, we used the phos-tag affinity method (28) as an alternative way to separate phosphorylated and nonphosphorylated proteins. The phos-tag molecule is thought to preferentially cap-

FIG. 8. (A) Western blotting time course analyses of the various chimeric proteins. (B to D) Side-by-side comparison of different chimeras. Total cell lysates prepared at the 6-h time point were resolved by SDS-PAGE and then analyzed by Western blotting with an anti-V5 antibody. Arp7 was used as a loading control (data not shown).

ture anionic substituents such as phosphomonoester dianions present in phosphoproteins under alkaline conditions. Presumably, phosphorylated proteins can be detected as slower migrating species by electrophoresis and Western blotting using an SDS-PAGE gel containing appropriate amounts of phos-tag molecule and Mn^{2+} (28). Here, we followed the standard phos-tag-affinity SDS-PAGE (or Mn^{2+} -phos-tag SDS-PAGE) protocol (27, 28) and have successfully separated phosphorylated Hop1 from nonphosphorylated Hop1. Previously, it was reported that Hop1 was phosphorylated by Tel1/Mec1 DNA damage checkpoint kinases (10) and that Hop1 phosphorylation did not occur in the absence of Red1 (30). Therefore, the total cell lysate of sporulating *red1Δ* mutant was used as a control to detect nonphosphorylated Hop1 (Fig. 9A, left). Our results revealed that phosphorylated Hop1 in the wild-type sporulating cells migrated more slowly than nonphosphorylated Hop1 in the SDS-PAGE gel containing 100 μ M phos-tag (Fig. 9A, right).

We then applied the same approach to compare V5-Red1 and V5-Red1^{14A} before and after treatment with CIAP. Before treatment, V5-Red1 migrated as two bands in a regular SDS-PAGE, whereas it migrated mostly as a single band after CIAP treatment, indicating that CIAP effectively dephosphorylated V5-Red1. In contrast, V5-Red1^{14A}, before and after CIAP treatment, migrated as a single band in a regular SDS-PAGE gel (Fig. 9B, left). The migrating patterns of V5-Red1 and V5-Red1^{14A} in the Mn^{2+} -phos-tag SDS-PAGE gel are

unexpectedly complicated. First, in the SDS-PAGE gel containing 50 μ M phos-tag, V5-Red1 migrated as one major and one minor band without CIAP. Unlike the case in the regular SDS-PAGE gel, treatment with CIAP results in an increase in the slower migrating band in the Mn^{2+} -phos-tag SDS-PAGE gel. In contrast, V5-Red1^{14A}, with or without CIAP treatment, migrated essentially as a single band (Fig. 9B, middle). When the concentration of phos-tag in the SDS-PAGE gel was increased from 50 to 100 μ M, not only V5-Red1 but also V5-Red1^{14A} migrated as multiple bands. Generally speaking, CIAP treatment affects significantly the overall migrating patterns of V5-Red1 in Mn^{2+} -phos-tag SDS-PAGE and in regular SDS-PAGE gels but exhibits nearly no effect on those of V5-Red1^{14A}. These results support our conclusion that V5-Red1^{14A} is not a phosphoprotein.

The results shown in Fig. 9B may be explained as follows. First, nonphosphorylated V5-Red1 (at 50 and 100 μ M phos-tag) or nonphosphorylated V5-Red1^{14A} (at 100 μ M phos-tag) could stably bind to Mn^{2+} -phos-tag, resulting in complexes migrating more slowly than V5-Red1 alone or V5-Red1^{14A} alone in the SDS-PAGE gel. In addition, V5-Red1 may have a greater tendency than V5-Red1^{14A} to stably associate with the phos-tag. Because V5-Red1 contains 14 serine or threonine residues more than V5-Red1^{14A}, it is intriguing to consider that these residues (at least partly) are responsible for binding to the phos-tag molecules. This assumption is reasonable from a chemical viewpoint, because an Mn^{2+} -phos-tag molecule

FIG. 9. (A) Separation and detection of phosphorylated Hop1 by the phos-tag affinity approach. Total cell lysates of sporulating wild-type, *hop1Δ*, and *red1Δ* cells were prepared at the 5-h time point. The proteins were separated by a regular SDS-PAGE (left) and by Mn^{2+} -phos-tag SDS-PAGE at 100 μM phos-tag and 200 μM $MnCl_2$ (right) and then analyzed by Western blot analysis with anti-Hop1 sera (30). Previously, it was reported that Hop1 is phosphorylated in the sporulating wild-type cells but not in the sporulating *red1Δ* mutant cells (10, 30). (B) Separation of V5-Red1 and V5-Red1^{14A} with or without CIAP treatment by the phos-tag affinity approach. Total cell lysates of sporulating *V5-RED1* and *V5-red1^{14A}* were prepared at the 5-h time point by TCA precipitation and then treated with CIAP or left untreated (see Materials and Methods). The samples again were precipitated by TCA, and the proteins were dissolved in buffer H (without EDTA) and then separated on either a regular SDS-PAGE gel (left) and on a Mn^{2+} -phos-tag SDS-PAGE gel containing 50 μM phos-tag and 100 μM $MnCl_2$ (middle) or 100 μM phos-tag and 200 μM $MnCl_2$ (right). V5-Red1 and V5-Red1^{14A} were visualized by Western blot analysis with an anti-V5 antibody.

could bind to the hydroxyl groups of two serine and/or threonine residues in the V5-Red1 polypeptide that was denatured by SDS. Second, phosphorylated V5-Red1 also could form a complex with a Mn^{2+} -phos-tag molecule. Judging from the differences between the migrating patterns of V5-Red1 before and after CIAP treatment (Fig. 9B), we believe that the phosphorylated-V5-Red1- Mn^{2+} -phos-tag complexes migrated faster than the nonphosphorylated V5-Red1- Mn^{2+} -phos-tag complexes in the SDS-PAGE gel. Third, because V5-Red1^{14A} is not phosphorylated, its migrating patterns were virtually identical before and after CIAP treatment at 0, 50, or 100 μM phos-tag.

Red1 phosphorylation is not essential for meiosis. We found that all of the six new mutant strains generated in this study could undergo meiosis and produce as many viable spores as wild-type *V5-RED1* cells (Fig. 8C and D). We then performed 4',6'-diamidino-2-phenylindole (DAPI) staining to monitor if meiotic progression in terms of MI nuclear division was de-

FIG. 10. Timing of nuclear division (MI). The number of DAPI-stained foci per cell ($n = 200$) was counted for each time point after the transfer of cells to sporulation medium (SPM).

layed when Red1 was not phosphorylated. The completion of MI nuclear division took about 6.5 and 8 h for 50% *V5-RED1* cells and 50% *V5-RED1^{14A}* cells, respectively. Eventually, almost all *V5-RED1* and *V5-RED1^{14A}* cells (>95%) completed MI and MII at the 24-h time point (Fig. 10). We also found that the *V5-RED1^{14A} dmc1Δ* double mutant, like the *dmc1Δ* mutant, exhibited a cell cycle arrest before MI (Fig. 10), further confirming that *V5-RED1^{14A}* is phenotypically similar to *V5-RED1*. Taken together, these results indicate that the phosphorylation of Red1 is not essential for meiosis. This conclusion is inconsistent with those of previous reports, which suggested that phosphorylated Red1 has critical roles in the DNA damage checkpoint, Mek1 activation, or exit from the pachytene checkpoint (1, 2, 14, 24, 25, 36, 52).

DISCUSSION

Not all posttranslational modifications of a protein are functionally essential. In this report, we show that the phosphorylation of Red1 is carried out in both *CDC28*-dependent and *CDC28*-independent manners in meiosis. Red1 phosphorylation not only occurs in the absence of meiotic DNA recombination but also is independent of Mek1 and Mec1/Tel1 kinase activities. We mapped the putative Red1 phosphorylation sites and demonstrated that corresponding mutant proteins (*V5-Red1^{14A}*, *V5-Red1^{13A-1}*, *V5-Red1^{13A-2}*, and *V5-Red1^{13A-3}*) were not phosphorylated in meiosis. However, these phosphorylation-deficient mutants not only could carry out sporulation but also produced as many viable spores as the wild-type *V5-RED* cells, suggesting that the phosphorylation of Red1 is not essential for its function in meiosis.

Although the kinase (or kinases) responsible for *CDC28*-independent Red1 phosphorylation remains unidentified, our results indicate that *CDC28*-independent Red1 phosphorylation does not occur in a *hop1Δ* mutant or when Red1 (i.e., *V5-Red1^{1758R}*) cannot bind to SUMO chains. In a separate study, we have shown that Hop1 is not phosphorylated in a *V5-RED1^{1758R}* or *red1Δ* mutant (30). Intriguingly, *V5-Red1^{1758R}* not only forms a stable complex with Hop1 but also is localized to meiotic chromosomes (30). Taken together with the current findings, these results lead to the interesting possibility that an integral ensemble of a Hop1-Red1-SUMO chain supercomplex is a prerequisite for both Red1 and Hop1

phosphorylation. Further investigations may show how this supercomplex activates the Tel1/Mec1 kinases for Hop1 phosphorylation and determine if Rim11, Rim15, or other novel protein kinases are responsible for SUMO-dependent and CDC28-independent Red1 phosphorylation.

Progress in biochemical studies of yeast meiosis has been significantly impeded because of high levels of vacuolar protease activities in the sporulating yeast cells. These vacuolar proteases (e.g., Pep4, Prb1, and Prc1) are highly induced upon starvation and also are essential for sporulation (60). To our knowledge, most commercially available protease inhibitors are ineffective against these proteases. Moreover, detergents commonly used in the preparation of total meiotic cell lysates, including SDS (14, 38), Triton X-100, deoxycholate (14), and NP-40 (1, 2, 24), could induce the widespread proteolysis of yeast proteins. Our results suggest that more caution should be taken in biochemical and cytological experiments of yeast meiosis, particularly when native lysis buffers are used to prepare total meiotic cell lysates. Because the sporulating yeast cells exhibit extremely high vacuolar protease and phosphatase activities, the majority of phosphorylated V5-Red1 proteins were either degraded or dephosphorylated in the native lysis buffer immediately upon cell lysis or during immunoprecipitation experiments (data not shown). We could not isolate intact phosphorylated V5-Red1 proteins and then directly identify the phosphorylation sites.

In general, it is extremely difficult to prove that phosphorylation plays no role. Although we have applied both regular SDS-PAGE and Mn^{2+} -phos-tag SDS-PAGE methods to demonstrate that V5-Red1^{14A} is likely not a phosphoprotein, our results do not exclude entirely the possibility that V5-Red1^{14A} is phosphorylated but does not yield mobility shifts. In view of the opposite results published before (1, 2, 14, 24, 25, 36, 40, 52), our negative results in this report not only are unexpected but also pose a serious challenge to the hypothesis that “visible” phosphorylated Red1 proteins play important roles in meiosis (1, 2, 14, 24, 25, 36, 40, 52). We believe, however, that it is important to provide scientists with responsible and balanced information in the context of current tenets.

In contrast to those earlier reports (1, 2, 14, 24, 25, 36, 40, 52), two recent papers have reported new meiotic functions of Red1 (16, 30). First, we reported that Red1 associates with the polymeric chains of SUMO to promote Mec1/Tel1-dependent Hop1 phosphorylation as well as SC assembly. Hop1 is not phosphorylated in *V5-RED1*^{1758R} during meiosis, because V5-Red1^{1758R} mutant is defective in binding to SUMO chains (30). We also showed that Red1 and Zip1, the major structural component of the SC central element, noncovalently sandwich SUMO polymeric chains to mediate SC assembly (30). Second, Red1 also interacts with the 9-1-1 checkpoint complex (Ddc1/Mec3/Rad17), and this interaction is essential for the induction of histone H2A serine-129 phosphorylation (equivalent to mammalian γ -H2AX) (16). However, it is not clear whether the 9-1-1-Red1 interaction also is required for Hop1 phosphorylation. Finally, a very small portion of Red1 is covalently modified by SUMO during meiosis (16, 30); however, yeast mutant *red1*^{KR} that expresses only the sumoylation-defective Red1 still could form the SC as well as wild-type cells (16). Therefore, SC assembly is mediated by the noncovalent asso-

ciation of Red1 and Zip1 with polymeric SUMO chains but not with sumoylated Red1.

Phos-tag has been widely applied to separate and detect phosphoproteins. We demonstrated that the Mn^{2+} -phos-tag SDS-PAGE method indeed could separate phosphorylated and nonphosphorylated Hop1, and that nonphosphorylated Hop1 apparently did not associate with Mn^{2+} -phos-tag. Surprisingly, in this report we also found that the Mn^{2+} -phos-tag could stably associate with nonphosphorylated Red1 in the SDS-PAGE gel. Our results indicate that both phosphorylated V5-Red1 and nonphosphorylated V5-Red1 could form stable complexes with the Mn^{2+} -phos-tag molecules in the SDS-PAGE gel. We believe that 14 Ser/Thr residues of nonphosphorylated V5-Red1 are responsible for its high binding affinity to the phos-tag molecules. Further studies will be performed to verify this intriguing possibility. The results should be important for those who are interested in using the phos-tag affinity approach to identify phosphoproteins.

ACKNOWLEDGMENTS

We thank Guido Guidotti (Harvard University) for his wise advice about John Pringle's work on yeast vacuolar proteases, Franz Klein (University of Vienna, Austria) and Nancy Hollingsworth (Stony Brook University) for the anti-Hop1 antibodies, Breck Byers (University of Washington) for the *clb5Δ clb6Δ* strain, Aaron P. Mitchell (Columbia University, New York, NY) for the *rim11Δ* strain, Kirsten Benjamin (University of California, San Francisco, CA) for the *cdc28-as1* strain, Etienne Schwob (CNRS, France) for the *P_{TET}-DBF4* strain, Douglas Bishop (University of Chicago) for the *rad24Δ* strain, Angelika Amon (Howard Hughes Medical Institute & Massachusetts Institute of Technology) for the *NDT80-IN* strain, Heiko Kuhn (Institute of Molecular Biology, Academia Sinica) for manuscript editing, and an anonymous reviewer for suggestions concerning the phos-tag SDS-PAGE experiments.

We gratefully acknowledge support from Academia Sinica and the National Science Council, Taiwan.

We have no conflicts of interest.

ADDENDUM IN PROOF

We recently confirmed that Red1^{14A} also shows no detectable phosphorylation in sporulating *pph3Δ*, *psy2Δ*, or *P_{CLB2}-GLC7* cells. *PPH3* and *PSY2* encode the catalytic subunit and the regulatory subunit of protein phosphatase 4 (PP4), respectively. *GLC7* encodes the catalytic subunit of protein phosphatase 1 (PP1). *P_{CLB2}-GLC7*, a meiosis-conditional null allele of *GLC7*, was created by replacing the endogenous promoter of *GLC7* with a mitosis-specific promoter from *CLB2*. These new results support our conclusion that Red1^{14A} is not a phosphoprotein.

REFERENCES

1. Bailis, J. M., and G. S. Roeder. 2000. Pachytene exit controlled by reversal of Mek1-dependent phosphorylation. *Cell* **101**:211–221.
2. Bailis, J. M., and G. S. Roeder. 1998. Synaptonemal complex morphogenesis and sister-chromatid cohesion require Mek1-dependent phosphorylation of a meiotic chromosomal protein. *Genes Dev.* **12**:3551–3563.
3. Benjamin, K. R., C. Zhang, K. M. Shokat, and I. Herskowitz. 2003. Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Ime2. *Genes Dev.* **17**:1524–1539.
4. Bishop, A. C., and K. M. Shokat. 1999. Acquisition of inhibitor-sensitive protein kinases through protein design. *Pharmacol. Ther.* **82**:337–346.
5. Bishop, D. K., D. Park, L. Xu, and N. Kleckner. 1992. *DMC1*: a meiosis-specific yeast homolog of *E. coli* recA required for recombination, synaptonemal complex formation, and cell cycle progression. *Cell* **69**:439–456.

6. Bishop, D. K., and D. Zickler. 2004. Early decision; meiotic crossover interference prior to stable strand exchange and synapsis. *Cell* **117**:9–15.
7. Blat, Y., R. U. Protacio, N. Hunter, and N. Kleckner. 2002. Physical and functional interactions among basic chromosome organizational features govern early steps of meiotic chiasma formation. *Cell* **111**:791–802.
8. Bolte, M., P. Steigemann, G. H. Braus, and S. Irniger. 2002. Inhibition of APC-mediated proteolysis by the meiosis-specific protein kinase Ime2. *Proc. Natl. Acad. Sci. U. S. A.* **99**:4385–4390.
9. Busygina, V., et al. 2008. Hed1 regulates Rad51-mediated recombination via a novel mechanism. *Genes Dev.* **22**:786–795.
10. Carballo, J. A., A. L. Johnson, S. G. Sedgwick, and R. S. Cha. 2008. Phosphorylation of the axial element protein Hop1 by Mec1/Tel1 ensures meiotic interhomolog recombination. *Cell* **132**:758–770.
11. Carlile, T. M., and A. Amon. 2008. Meiosis I is established through division-specific translational control of a cyclin. *Cell* **133**:280–291.
12. Cha, R. S., B. M. Weiner, S. Keeney, J. Dekker, and N. Kleckner. 2000. Progression of meiotic DNA replication is modulated by interchromosomal interaction proteins, negatively by Spo11p and positively by Rec8p. *Genes Dev.* **14**:493–503.
13. Cheng, C. H., et al. 2006. SUMO modifications control assembly of synaptonemal complex and polycomplex in meiosis of *Saccharomyces cerevisiae*. *Genes Dev.* **20**:2067–2081.
14. de los Santos, T., and N. M. Hollingsworth. 1999. Red1p, a MEK1-dependent phosphoprotein that physically interacts with Hop1p during meiosis in yeast. *J. Biol. Chem.* **274**:1783–1790.
15. Dirick, L., L. Goetsch, G. Ammerer, and B. Byers. 1998. Regulation of meiotic S phase by Ime2 and a Clb5,6-associated kinase in *Saccharomyces cerevisiae*. *Science* **281**:1854–1857.
16. Eichinger, C. S., and S. Jentsch. 2010. Synaptonemal complex formation and meiotic checkpoint signaling are linked to the lateral element protein Red1. *Proc. Natl. Acad. Sci. U. S. A.* **107**:11370–11375.
17. Foiani, M., et al. 1996. A meiosis-specific protein kinase, Ime2, is required for the correct timing of DNA replication and for spore formation in yeast meiosis. *Mol. Gen. Genet.* **253**:278–288.
18. Grushcow, J. M., et al. 1999. *Saccharomyces cerevisiae* checkpoint genes *MEC1*, *RAD17* and *RAD24* are required for normal meiotic recombination partner choice. *Genetics* **153**:607–620.
19. Haase, S. B., and S. I. Reed. 2002. Improved flow cytometric analysis of the budding yeast cell cycle. *Cell Cycle* **1**:132–136.
20. Henderson, K. A., and S. Keeney. 2005. Synaptonemal complex formation: where does it start? *Bioessays* **27**:995–998.
21. Hochwagen, A., and A. Amon. 2006. Checking your breaks: surveillance mechanisms of meiotic recombination. *Curr. Biol.* **16**:R217–R228.
22. Hollingsworth, N. M., and B. Byers. 1989. *HOP1*: a yeast meiotic pairing gene. *Genetics* **121**:445–462.
23. Hollingsworth, N. M., and L. Ponte. 1997. Genetic interactions between *HOP1*, *RED1* and *MEK1* suggest that *MEK1* regulates assembly of axial element components during meiosis in the yeast *Saccharomyces cerevisiae*. *Genetics* **147**:33–42.
24. Hong, E. J., and G. S. Roeder. 2002. A role for Ddc1 in signaling meiotic double-strand breaks at the pachytene checkpoint. *Genes Dev.* **16**:363–376.
25. Jordan, P., et al. 2009. Ipl1/Aurora B kinase coordinates synaptonemal complex disassembly with cell cycle progression and crossover formation in budding yeast meiosis. *Genes Dev.* **23**:2237–2251.
26. Keeney, S. 2001. Mechanism and control of meiotic recombination initiation. *Curr. Top. Dev. Biol.* **52**:1–53.
27. Kinoshita, E., E. Kinoshita-Kikuta, and T. Koike. 2009. Separation and detection of large phosphoproteins using Phos-tag SDS-PAGE. *Nat. Protoc.* **4**:1513–1521.
28. Kinoshita, E., E. Kinoshita-Kikuta, K. Takiyama, and T. Koike. 2006. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell Proteomics* **5**:749–757.
29. Klein, F., et al. 1999. A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis. *Cell* **98**:91–103.
30. Lin, F. M., Y. J. Lai, H. J. Shen, Y. H. Cheng, and T. F. Wang. 2010. Yeast axial-element protein, Red1, binds SUMO chains to promote meiotic interhomologue recombination and chromosome synapsis. *EMBO J.* **29**:586–596.
31. Lydall, D., Y. Nikolsky, D. K. Bishop, and T. Weinert. 1996. A meiotic recombination checkpoint controlled by mitotic checkpoint genes. *Nature* **383**:840–843.
32. Majka, J., and P. M. Burgers. 2003. Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. *Proc. Natl. Acad. Sci. U. S. A.* **100**:2249–2254.
33. Malathi, K., Y. Xiao, and A. P. Mitchell. 1997. Interaction of yeast repressor-activator protein Ume6p with glycogen synthase kinase 3 homolog Rim11p. *Mol. Cell. Biol.* **17**:7230–7236.
34. Mitchell, A. P., S. E. Driscoll, and H. E. Smith. 1990. Positive control of sporulation-specific genes by the *IME1* and *IME2* products in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **10**:2104–2110.
35. Neale, M. J., J. Pan, and S. Keeney. 2005. Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature* **436**:1053–1057.
36. Niu, H., et al. 2005. Partner choice during meiosis is regulated by Hop1-promoted dimerization of Mek1. *Mol. Biol. Cell* **16**:5804–5818.
37. Niu, H., et al. 2009. Regulation of meiotic recombination via Mek1-mediated Rad54 phosphorylation. *Mol. Cell* **36**:393–404.
38. Pringle, J. R. 1975. Methods for avoiding proteolytic artefacts in studies of enzymes and other proteins from yeasts. *Methods Cell Biol.* **12**:149–184.
39. Rockmill, B., and G. S. Roeder. 1998. Telomere-mediated chromosome pairing during meiosis in budding yeast. *Genes Dev.* **12**:2574–2586.
40. Roeder, G. S., and J. M. Bailis. 2000. The pachytene checkpoint. *Trends Genet.* **16**:395–403.
41. Sasanuma, H., et al. 2008. Cdc7-dependent phosphorylation of Mer2 facilitates initiation of yeast meiotic recombination. *Genes Dev.* **22**:398–410.
42. Schwacha, A., and N. Kleckner. 1997. Interhomolog bias during meiotic recombination: meiotic functions promote a highly differentiated interhomolog-only pathway. *Cell* **90**:1123–1135.
43. Shinohara, M., K. Sakai, T. Ogawa, and A. Shinohara. 2003. The mitotic DNA damage checkpoint proteins Rad17 and Rad24 are required for repair of double-strand breaks during meiosis in yeast. *Genetics* **164**:855–865.
44. Smith, A. V., and G. S. Roeder. 1997. The yeast Red1 protein localizes to the cores of meiotic chromosomes. *J. Cell Biol.* **136**:957–967.
45. Smith, K. N., A. Penkner, K. Ohta, F. Klein, and A. Nicolas. 2001. B-type cyclins *CLB5* and *CLB6* control the initiation of recombination and synaptonemal complex formation in yeast meiosis. *Curr. Biol.* **11**:88–97.
46. Stuart, D., and C. Wittenberg. 1998. *CLB5* and *CLB6* are required for premeiotic DNA replication and activation of the meiotic S/M checkpoint. *Genes Dev.* **12**:2698–2710.
47. Su, S. S., and A. P. Mitchell. 1993. Identification of functionally related genes that stimulate early meiotic gene expression in yeast. *Genetics* **133**:67–77.
48. Tsubouchi, H., and G. S. Roeder. 2006. Budding yeast Hed1 down-regulates the mitotic recombination machinery when meiotic recombination is impaired. *Genes Dev.* **20**:1766–1775.
49. Ubersax, J. A., et al. 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**:859–864.
50. Valentin, G., E. Schwob, and F. Della Seta. 2006. Dual role of the Cdc7-regulatory protein Dbf4 during yeast meiosis. *J. Biol. Chem.* **281**:2828–2834.
51. Vidan, S., and A. P. Mitchell. 1997. Stimulation of yeast meiotic gene expression by the glucose-repressible protein kinase Rim15p. *Mol. Cell. Biol.* **17**:2688–2697.
52. Wan, L., T. de los Santos, C. Zhang, K. Shokat, and N. M. Hollingsworth. 2004. Mek1 kinase activity functions downstream of *RED1* in the regulation of meiotic double strand break repair in budding yeast. *Mol. Biol. Cell* **15**:11–23.
53. Wan, L., et al. 2008. Cdc28-Clb5 (CDK-S) and Cdc7-Dbf4 (DDK) collaborate to initiate meiotic recombination in yeast. *Genes Dev.* **22**:386–397.
54. Wan, L., C. Zhang, K. M. Shokat, and N. M. Hollingsworth. 2006. Chemical inactivation of Cdc7 kinase in budding yeast results in a reversible arrest that allows efficient cell synchronization prior to meiotic recombination. *Genetics* **174**:1767–1774.
55. Wang, T. F., N. Kleckner, and N. Hunter. 1999. Functional specificity of MutL homologs in yeast: evidence for three Mlh1-based heterocomplexes with distinct roles during meiosis in recombination and mismatch correction. *Proc. Natl. Acad. Sci. U. S. A.* **96**:13914–13919.
56. Woltering, D., et al. 2000. Meiotic segregation, synapsis, and recombination checkpoint functions require physical interaction between the chromosomal proteins Red1p and Hop1p. *Mol. Cell. Biol.* **20**:6646–6658.
57. Xu, L., B. M. Weiner, and N. Kleckner. 1997. Meiotic cells monitor the status of the interhomolog recombination complex. *Genes Dev.* **11**:106–118.
58. Yeong, F. M., H. H. Lim, Y. Wang, and U. Surana. 2001. Early expressed Clb proteins allow accumulation of mitotic cyclin by inactivating proteolytic machinery during S phase. *Mol. Cell. Biol.* **21**:5071–5081.
59. Zickler, D., and N. Kleckner. 1999. Meiotic chromosomes: integrating structure and function. *Annu. Rev. Genet.* **33**:603–754.
60. Zubenko, G. S., and E. W. Jones. 1981. Protein degradation, meiosis and sporulation in proteinase-deficient mutants of *Saccharomyces cerevisiae*. *Genetics* **97**:45–64.