Identification of Phosphorylation Sites on the Yeast Ribonucleotide Reductase Inhibitor Sml1*

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Sml1 is a small protein in Saccharomyces cerevisiae which inhibits the activity of ribonucleotide reductase (RNR). RNR catalyzes the rate-limiting step of de novo dNTP synthesis. Sml1 is a downstream effector of the Mec1/Rad53 cell cycle checkpoint pathway. The phosphorylation by Dun1 kinase during S phase or in response to DNA damage leads to diminished levels of Sml1. Removal of Sml1 increases the population of active RNR, which raises cellular dNTP levels. In this study using mass spectrometry and site-directed mutagenesis, we have identified the region of Sml1 phosphorylation to be between residues 52 and 64 containing the sequence GSSASASASSLEM. This is the first identification of a phosphorylation sequence of a Dun1 biological substrate. This sequence is quite different from the consensus Dun1 phosphorylation sequence reported previously from peptide library studies. The specific phosphoserines were identified to be Ser56, Ser58, and Ser60 by chemical modification of these residues to S-ethylcysteines followed by collision activated dissociation. To investigate further Sml1 phosphorylation, we constructed the single mutants S56A, S58A, S60A, and the triple mutant S56A/S58A/S60A and compared their degrees of phosphorylation with that of wild type Sml1. We observed a 90% decrease in the relative phosphorylation of S60A compared with that of wild type, a 25% decrease in S58A, and little or no decrease in the S56A mutant. There was no observed phosphate incorporation in the triple mutant, suggesting that Ser56, Ser58, and Ser60 in Sml1 are the sites of phosphorylation. Further mutagenesis studies reveal that Dun1 kinase requires an acidic residue at the +3 position, and there is cooperativity between the phosphorylation sites. These results show that Dun1 has a unique phosphorylation motif.

In the yeast Saccharomyces cerevisiae, Mec1 and Rad53 kinases are transducers of all known cell cycle checkpoint pathways (1). In response to DNA damage, cell cycle progression is arrested at certain phases known as checkpoints, and simultaneously cells increase their capacity to repair DNA damage. A downstream effector of the pathway modulated by Mec1 and Rad53 is ribonucleotide reductase (RNR). RNR catalyzes the conversion of nucleoside diphosphates to deoxynucleoside diphosphates, which is the rate-limiting step for dNTP synthesis. Overexpression of RNR genes rescues lethality caused by deletion of MEC1 and RAD53 genes (2). During DNA damage and at S phase, levels of dNTP pools are increased to enhance the capacity of DNA repair (2). This is achieved by transcriptional activation of RNR genes in a Mec1/Rad53-dependent manner (3, 4). The activity of RNR in yeast is also regulated by the small regulatory protein Sml1 (2). Sml1 inhibits RNR activity through an interaction with the large subunit of the RNR complex, Rnr1 (2, 5). In response to DNA damage or at S phase, the intracellular level of Sml1 is reduced significantly (6), resulting in the activation of RNR.

Zhao and Rothstein (7) demonstrated that the removal of Sml1 is triggered through its phosphorylation by Dun1 kinase. Evidence for this comes from the following observations: 1) deletion of the SML1 gene suppresses several phenotypes of the DUN1 null mutant, including its prolonged S phase; 2) in the null mutant of DUN1, both phosphorylation and degradation of Sml1 in response to DNA damage are diminished significantly; 3) Sml1 and Dun1 physically interact in vivo; and 4) Sml1 is phosphorylated by Dun1 in vitro. In S. cerevisiae, Dun1 is a particularly important serine/threonine kinase in that it acts upstream of multiple pathways such as transcriptional activation of genes required for DNA synthesis (3) and the cell cycle arrest at G2/M phase (8, 9). In addition, more than 20 different proteins physically interact with Dun1, suggesting that Dun1 may be involved in multiple pathways (10). Although the mechanism by which Dun1 transmits signals during cell cycle checkpoints to its downstream effectors is not well understood, its kinase activity is crucial for its biological function. For instance, the kinase-deficient mutants D328A and K229R, unlike wild type Dun1, cannot respond to DNA damage and do not induce the expression of the RNR3 gene (3). In addition, phosphorylation of cell cycle checkpoint proteins such as the Cdc15 repressor (11) and the DNA repair protein Rad55 (12, 13) depends on Dun1, and the pathways in which these proteins are involved are independent of Sml1. Therefore, it is likely that there are other unidentified substrates of Dun1. So far, Sml1 is the only known natural substrate of Dun1 kinase. Prior to this study, the sites of Sml1 phosphorylated by Dun1

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3 The abbreviations used are: RNR, ribonucleotide reductase; CAD, collision-activated dissociation; ESI, electrospray ionization; FTICR, Fourier transform ion cyclotron resonance; GST, glutathione S-transferase; IMAC, immobilized metal affinity chromatography; MS, mass spectrometry; SORI, sustained off-resonance irradiation; cAPK, cyclic AMP-dependent protein kinase.
Phosphorylation of Sml1

ADRI G233 Peptide—The ADRI G233 peptide (LKKLTRASFSQ) was custom synthesized at the Yale University W. M. Keck biotechnology resource center. This peptide was purified further by C18 reverse phase high performance liquid chromatography, and its purity was checked by ESI-FTICR-MS. The purified peptide was dissolved in water, and its concentration was determined by 2,4,6-trinitrobenzene sulfonic acid (Pierce) using tryptophan dissolved in water as a standard.

Expression of GST-Dun1 and Kinase Reaction—Expression of GST-Dun1 was carried out as described by Zhao and Rothstein (7). Briefly, the yeast cells (YU952-B) transformed by the GST-Dun1 expression plasmid (pWJ772-11) were grown in SC-URA raffinose, and the expression of Dun1 was induced at mid-log phase (5–6 × 10^7 cells/ml) by the addition of galactose to a final concentration of 2% (w/v). After two times of inductions, the cells were harvested by centrifugation at −80 °C. The purification of GST-Dun1 was carried out as described by Zhao and Rothstein (7) and Sanchez et al. (14). Briefly, cells were lysed in buffer C (50 mM Tris-HCl, 150 mM NaCl, 50 mM KCl, 5 mM MgCl2, 1% (v/v) Igepal 630, 10% (v/v) glycerol, 10 mM dithiothreitol, 0.1 mM Na3VO4, 30 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1× Complete™ protease inhibitor mixture, 2 μM pepstatin, pH 8.0) by glass bead disruption. The supernatant containing Dun1 was incubated with glutathione beads (glutathione-4-Sepharose Superflow: Amersham Biosciences) for 50 min and washed with buffer C. Examination of the glutathione beads by SDS-PAGE revealed pure GST-Dun1. The resin-bound GST-Dun1 was incubated with 2 bed volumes of buffer B containing 1 μM ATP, for 2 h at 30 °C. After the completion of the reaction, glutathione beads were harvested by centrifugation, and the supernatant was collected for MS analysis. For a quantitative kinase assay, 0.06 μM/μl (1 Ci = 37 GBq) [γ-32P]ATP (4,500 mCi/mmol; ICN, Costa Mesa, CA) was included in the buffer B. To compare the degree of phosphorylation in wild type Sml1 and ADRI G233 peptide, the kinase assay was conducted with substrate concentrations of 5.3 μM and 67 μM for 30 min. Immediately after the reaction, 20 μl of supernatant was spotted on a 1-cm² piece of phosphocellulose membrane. The membrane was washed in 10 mM phosphoric acid first and then with 95% (v/v) ethanol. The radioactivity on the membrane was measured by a liquid scintillation counter (LS6801; Beckman, Fullerton, CA). For autoradiography, a 15% polyacrylamide gel was prepared, a sample containing 250 pmol of Sml1 was incubated with 0.2 μg of sequencing grade trypsin (Promega, Madison, WI) at 37 °C for 10 h. To quench the reaction, acetic acid was added to a final concentration of 0.1% (v/v). For cyanogen bromide (CNBr) digestion, a sample containing 10 μg of Sml1 was dried under a speed vacuum apparatus. 10 μg of trypsin (83 kDa) in 70% (v/v) formic acid was added to the sample and incubated for 24 h. To quench the reaction, 50 μl of water was added, and the sample was dried using a speed vacuum apparatus. This step was repeated five times.

A 20-μl bed volume of GaIII-IMAC column with Pore Magic resin (PerSeptive Biosystems, Framingham, MA) was prepared as described previously (22). The CNBr-digested samples were dissolved in 10 μl of 10% (v/v) of acetic acid and manually loaded onto the column preequilibrated with 1% (v/v) acetic acid. The column was washed with 6 bed volumes of 1% (v/v) acetic acid, 6 bed volumes of a mixture consisting of water, acetonitrile, and acetic acid in a 70:30:1 respective ratio, followed by 3 bed volumes of 1% (v/v) acetic acid in water. The purified peptide was eluted with 3 bed volumes of 200 mM sodium phosphate, pH 8.5.

Derivationization of Phosphopeptides—The phosphopeptides enriched by GaIII-IMAC were desalted once with C18 reverse phase ZipTip columns (Millipore, Bedford, MA), and the sample volume was reduced to less than 5 μl using a speed vacuum apparatus. The phosphopeptides remaining in the peptides were chemically modified to S-ethylcysteine by a β-elimination reaction in the presence of ethanethiol as described previously (23, 24). Briefly, 50 μl of H2O, 4 μl of LiOH, acetonitrile, ethanol, ethanethiol mixed in a ratio of (5:14:5:5:2) was added to the sample and incubated in 37 °C for 1 h. The reaction was quenched by the addition of 25 μl of acetic acid. Because Sml1 contains only one cysteine residue (Cys18), we omitted the derivatization step, which is normally employed prior to the derivatization.

ESI-FTICR-MS—All samples were desalted with C18 reverse phase ZipTips prior to MS analysis. For positive ion analysis, samples were prepared in a 50:50 mixture of water and acetonitrile containing 0.1% HAc.

EXPERIMENTAL PROCEDURES

Yeast Strain and Plasmids—GST-Dun1 expression plasmid (pWJ772-11), a wild type Sml1 expression plasmid (pWJ-750-2) (22), and the S. cerevisiae strain U952-B (MATa, sml1ΔΔ)2 were kindly provided by Dr. Rodney Rothstein at Columbia University (New York). The procedure for constructing the C14S Sml1 mutant is described elsewhere.2 The wild type Sml1 expression plasmid was used as a template to create S56A, S58A, S60A, S61A, E63Q, and the S56A/S58A/S60A Sml1 mutants using the QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA).

Expression and Purification of Bacterially Expressed Sml1—An overnight culture of BL21 (DE3)pLysS transformed by S. cerevisiae strain U952-B-MATa, sml1ΔΔ::His1, RADS in W303 (20) were kindly provided by Dr. Rodney Rothstein at Columbia University (New York). The procedure for constructing the C14S Sml1 mutant is described elsewhere.2 The wild type Sml1 expression plasmid was used as a template to create S56A, S58A, S60A, S61A, E63Q, and the S56A/S58A/S60A Sml1 mutants using the QuikChange site-directed mutagenesis kit (Strategene, La Jolla, CA).

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Phosphorylation of Sml1

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Results from the in vitro phosphorylation of Sml1 by Dun1 where $^{32}$P is incorporated are shown in Fig. 1. Sml1 is specifically phosphorylated by Dun1 with $^{32}$P incorporation at 40–50 times greater than that of the negative control samples (Fig. 1). These results show that phosphorylation of Sml1 is solely the result of GST-Dun1 and that the kinase activity of GST-Dun1 is specific to Sml1.

The number of phosphoryl groups attached to Sml1 was determined by a combination of MS and site-directed mutagenesis. To estimate the minimum number of phosphorylation sites, intact molecules of Sml1 were first analyzed by ESI-FTICR. The attachment of a phosphate to a hydroxyl group of serine, threonine, or tyrosine results in mass increases of the proteins or peptides by 79.966 Da in monoisotopic mass, and it was possible to estimate the number of phosphates on a protein by analyzing the mass shift. We observed monophosphorylated, diphosphorylated, triphosphorylated, and unphosphorylated Sml1. These observations indicated that at least three sites of phosphorylation can be phosphorylated (Fig. 2).

To find the region of Sml1 containing the phosphorylation sites, intact molecules of Sml1 in the presence of $[\gamma^{32}\text{P}]$ATP were incubated with the GST-Dun1-bound glutathione beads. Lane 1, Escherichia coli-expressed Sml1 in the reaction buffer containing 250 μM ATP (cold) and 1 μCi/μl $[\gamma^{32}\text{P}]$ATP was incubated for 2 h with the glutathione beads preincubated with yeast lysozyme containing GST-Dun1 (see “Experimental Procedures”). 10 μl of supernatant was subjected to 15% (w/v) acrylamide SDS-PAGE. Lane 2, Sml1 was incubated with glutathione beads preincubated with the lysate of untransformed yeast cells (U962-B). Lane 3, bovine serum albumin was incubated with the GST-Dun1-bound glutathione beads. Lane 4, the GST-Dun1-bound glutathione beads alone were incubated in the reaction buffer. Lane 5, only Sml1 was incubated in the reaction buffer. Lane 6, Coomassie Blue staining of the gel presented in A. Lane 1, molecular weight markers (kDa). Lanes 2–6, the same samples in lanes 1–5 in A, C, radioactivity from 20 μl of the same samples as in A measured by a liquid scintillation counter. Subtraction of average radioactivity of all of the control samples from that of the experimental sample (Sml1 + Dun1 beads) corresponds to phosphate incorporation (mol of phosphate/mmol of protein) of 0.31. The number of replicates measured was four. The error bars represent the S.E.
sites, the protein was digested by trypsin or CNBr and then analyzed by ESI-FTICR. In the positive ion analysis of the trypsin digest, we detected peptides that covered 75% of the Sml1 sequence spanning residues 1–67 and 73–84. In the negative ion analysis, we detected peptides constituting 89% of the Sml1 sequence, which almost covered the entire Sml1 sequence with the exception of residues 16–26 (Fig. 3A). In both positive and negative ion analysis, the spectrum for the samples taken from the in vitro Dun1 phosphorylation assays showed that peptides spanning residues 33–67 (3,549.720 Da) and 34–67 (3,705.857 Da) are associated with their singly phosphorylated forms of 3,629.695 and 3,785.829 Da, respectively. In the positive ion analysis of CNBr digests, we detected four peptides that cover 34% of the Sml1 sequence spanning residues 29–39 and 81–104. However, none of these peptides was phosphorylated. In the negative ion analysis of CNBr digests, 7 or 8 peptides that cover 71% of Sml1 sequence (residues 31–106) were observed (Fig. 3B). These results showed that the peptide spanning residues 52–64 consisting of GSSASASASSLEM (1,153.539 Da) was associated with its singly phosphorylated form (Fig. 4, A and B). The phosphopeptides of Sml1 identified by both CNBr and trypsic digest are entirely consistent. The combined data generated from the CNBr and trypsic digest gave us 100% sequence coverage of Sml1.

To enrich phosphopeptides, CNBr digest samples were subjected to Ga(III) IMAC chromatography and analyzed in the negative ion mode. Singly, doubly, and triply phosphorylated forms of residues 52–64 were observed (Fig. 4C), which were absent in the negative control, which contained CNBr peptides generated from only unphosphorylated Sml1. In most of the samples, peak intensities of doubly or triply phosphorylated

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**Fig. 2. Positive ion analysis of intact phosphorylated Sml1.** A, deconvoluted mass spectrum of the negative control in which Sml1 was incubated in the presence of 250 μM ATP without GST-Dun1 for 2 h. The spectrum shows only unphosphorylated species. No peaks corresponding to the mass of phosphorylated Sml1 were observed. B, deconvoluted mass spectrum of Sml1 incubated with GST-Dun1 and 250 μM ATP for 2 h. In this particular spectrum, an internal calibration was accomplished with the known peaks of unphosphorylated Sml1. Values above each peak are the observed mass of the most abundant isotopic species. Note that the observed mass differences among different phosphorylation states corresponds closely to the calculated monoisotopic mass of HPO₃⁻, which is 79.986 Da.
species were stronger than the singly phosphorylated species. Singly phosphorylated species could only be observed prior to IMAC chromatography. To confirm that the observed mass shift was the result of phosphorylation, an ion of the doubly phosphorylated species (M-H-1 = 1,294.452 Da) was subjected to CAD. In this experiment, the species of interest was isolated in the analyzer region of the mass spectrometer, accelerated with a RF voltage and bombarded into nitrogen gas. Depending on the voltage used to accelerate the ion, we observed fragment ions of 1,196.489 and 1,098.466, which corresponded to the loss of one or two phosphoric acids (data not shown). We could not obtain a reasonable signal in the CAD experiment for singly and triply phosphorylated species, which was possibly because of the low abundance of the parent ions.

To confirm our findings further, we repeated the same experiment with the C14S mutant form of Sml1 and its proteolytic fragment (29). During purification of C14S Sm1 by gel filtration chromatography, the intact molecule was separated from a smaller degraded C14S Sm1 fragment corresponding to a mass of 8,147.073 Da. Mass spectrometric analysis of the latter revealed that the degraded peptide was an N-terminal fragment of C14S Sm1 consisting of residues 1–71 (29), which we denoted as FRAG71. We conducted the kinase reaction using both the intact and FRAG71 of the C14S mutant. Both intact C14S Sm1 and FRAG71 were singly, doubly, and triply phosphorylated (Fig. 5, A and B). Although this experiment was conducted with a mutant form of Sml1, it strongly suggests that three sites of Sml1 can be phosphorylated and that all three sites reside within the N-terminal 71 residues. In addition, triple phosphorylation of FRAG71 C14S strongly suggested that the C-terminal 33 residues, which is the minimal Rnr1 binding domain of Sml1 (30), were not required for its phosphorylation.

The data above indicated that a peptide of Sml1 consisting of residues 52–64 (GSSASASASLLE) produced by CNBr digestion includes all three phosphorylation sites. It should be noted that the above sequence does not contain any threonine residues, suggesting that only serine residues in Sml1 are phosphorylated by Dun1. However, as reported in other studies (31, 32), we too were unable to obtain sequence-specific fragmentation of the doubly phosphorylated peptide by CAD because the predominant fragmentation was the neutral loss of phosphoric acid. In addition to this, the identification of phosphorylated residues from a tandem cluster of serine residues (GSSASASASLLE) by MS is challenging. To simplify the identification of phosphoserine residues by CAD, we converted the phosphoserine residues to S-ethylcysteine by β-elimination in the presence of ethanethiol. This reaction specifically replaces phosphoryl group on serine or threonine with ethanethionyl giving a 44.008-Da mass shift from normal serine or threonine (17, 23, 24, 33). Unlike phosphoryl groups, ethanethionyl groups were more resistant to fragmentation by CAD so that fragmentation of the peptide mainly takes place at the peptide backbone (17, 23). In positive ion mode, we have observed peaks that correspond to modification of doubly and triply phosphorylated species, respectively. First we conducted CAD with a doubly modified species that was fragmented into a series of y and b type ions, from which two sites of phosphorylation were unambiguously determined (Fig. 6A). b7, b9, and b10 ions can be produced only when phosphoryl groups are attached to Ser56 and Ser60. Similarly, only two possible combinations, Ser58/Ser60 or Ser56/Ser61, can produce the observed signals for y6, y7, and y8. Overall, more than 74% (20/27) of the peaks in the tandem MS could be assigned as fragment ions of a peptide in which ethanethionyl groups (which replace phosphoryl groups) are attached to Ser56 and Ser60. The unassigned peaks do not match the possible fragments of peptides with other combinations of ethanethionyl attachment. This indicated that residues other than Ser56 and Ser60 are less likely to be the phosho-acceptors on this peptide.

CAD experiments with the triply modified species also provided a series of y and b type ions corresponding to ethanethionyl attachment at specific serine residues (Fig. 6B). y9, y8, y7, and y6 can be produced only when phosphate groups are attached to Ser56, Ser58, and either Ser60 or Ser61. Likewise, b10, b9, and b8 can be generated only when Ser56, Ser58, and either Ser56, Ser54, or Ser53 are phosphorylated. Combining these data together, it is most likely that Ser56, Ser58, and Ser60 are phosphorylated. More than 70% of the observed peaks (19/26) could be assigned as fragment ions of a peptide in which ethanethionyl groups (which replace phosphate groups) are attached to Ser56, Ser58, and Ser60.

To determine the primary phosphorylation sites of Sml1, we individually replaced Ser56, Ser58, and Ser60 with Ala and compared the degree of phosphorylation by a kinase assay based on incorporation of 32P (Fig. 7A). Compared with wild type Sml1, S60A Sml1 showed more than a 90% decrease in the amount of phosphate incorporation, whereas S58A Sml1 showed approximately a 25% decrease. No significant difference was observed between S56A and wild type Sml1. From autoradiography studies we observed that the phosphorylated form of these mutants appeared to migrate slightly faster than the wild type (in the order of S60A > S58A > S56A > wild type Sml1). These results are in agreement with the results from MS analysis. Although according to the MS results Ser56, Ser58, and Ser60 can be all phosphorylated, only the mutation at Ser60 showed a dramatic decrease in phosphate incorporation. Moreover, because of its close proximity to Ser56, Ser58, and Ser60, we constructed the S61A mutant and tested it for phosphate incorporation. The S61A mutant had a 48% phosphate incorporation (p = 0.0001) compared with wild type Sml1 (see Fig. 7B). However, Ser61 was not identified as a potential phosphorylation site by MS analysis. To determine further whether Ser61 was a potential phosphorylation site we tested the triple mutant S56A/S58A/S60A for phosphate incorporation. Our results show that the triple mutant did not show any phosphate incorporation (see Fig. 7B), strongly suggesting that it is Ser56, Ser58, and Ser60 that are the sole phosphorylation sites of Sml1. Moreover, to test for cooperativity between the phosphorylation sites, we replaced Ser56 with an aspartic acid (S60D Sml1), which mimics the negative charge resulting from phosphorylation. On average the percentage incorporation of phosphate in S60D Sml1 was 17% of wild type Sml1 (Fig. 7B). Nonetheless, this is a 2.5-fold increase in phosphate incorporation compared with S60A Sml1 (p = 0.01) (Fig. 7B). To test whether an acidic group (Glu63) located at the +3 position which is C-terminal of the three phosphorylation sites acts as a recognition site for Dun1 kinase we tested the E63Q Sml1 mutant for phosphate incorporation. Phosphate incorporation in E63Q Sml1 was 52%
FIG. 4. Negative ion analysis of Sm11 CNBr digest. A, the deconvoluted mass spectrum of the negative control of the kinase reaction subjected to CNBr digestion in which Sm11 was incubated in the presence of 250 μM ATP without GST-Dun1. As expected, no phosphorylated species were identified (see inset). In general, residues at the C terminus of peptides generated by CNBr digestion are homolactones, which can be further converted to homoserine by intramolecular hydrolysis. In this spectrum, residues at the C terminus of most of the peptides were homoserine. B, the deconvoluted mass spectrum of phosphorylated Sm11 subjected to CNBr digestion in which Sm11 was incubated with GST-Dun1 in the presence of 250 μM ATP. The peak (1,233.535) corresponds to a singly phosphorylated peptide consisting of residues 52–64 (see inset). In
that of wild type Sml1, showing a significant decrease ($p = 0.00006$). We also compared Dun1 kinase activity for wild type Sml1 with respect to a synthetic peptide (named ADR1 G233) reported previously to be phosphorylated by Dun1 (14). Using the substrate concentration of 8.3 $\mu$M no significant kinase activity above background was detected for ADR1 G233 peptide. In contrast, a significant amount of phosphorylation was detected with Sml1 as the substrate. Next, to be consistent with the experiment reported previously (14), the kinase assay was conducted at a substrate concentration of 67 $\mu$M. In this case, the average phosphate incorporation of ADR1 G233 was 11% of that in wild type Sml1 (Fig. 7B), suggesting that Sml1 is a far superior substrate for Dun1 compared with the peptide.

**DISCUSSION**

In this study, we have identified the phosphorylation sites of Sml1, the only biological substrate of Dun1 identified to date. First, we analyzed the intact phosphorylated Sml1 by ESI-FTICR-MS to estimate the number of phosphate groups attached to Sml1. We observed a mixture of singly, doubly, and triply phosphorylated Sml1 as well as unphosphorylated species. This indicated that at least three sites of Sml1 could be phosphorylated. Next, to determine regions of phosphorylation, we digested phosphorylated Sml1 with trypsin or CNBr and analyzed the proteolytic fragments. In both the trypsin and CNBr digests, we observed singly phosphorylated peptides spanning residues 34–67 or 52–64. Repeated analysis of CNBr fragments enriched by Ga(III) IMAC columns showed two to three phosphate attachments within residues 52–64. These data are consistent with the molecular mass of the intact phospho-Sml1 with three phosphate attachments. Moreover, to determine the specific residues of phosphoryl attachment, we conducted further experiments on the fragment consisting of residues 52–64. Although initial CAD experiments showed fragmentation of phosphate groups, we could not observe fragmentation at the peptide backbone. Furthermore, residues 52–64 (GSSASASASSLEM) contain a cluster of 6 serine residues...
dyes making it difficult to identify the specific phosphoserines. Therefore, the phosphoserine residues were converted into S-ethylcysteine by a β-elimination reaction in the presence of ethanethiol, which made the peptide amenable to fragment at its backbone. CAD fragmentation of the ions corresponding to the doubly phosphorylated peptide showed that Ser56, Ser58, and Ser60 were phosphorylated.

Our studies indicate that Ser 56 might be phosphorylated after Ser 58 and Ser 60 because of the following: we observed phosphorylation of all three residues in the triply phosphorylated peptide, whereas only the phosphorylation of Ser58 and Ser60 was observed in the doubly phosphorylated peptides. Some serine/threonine kinases such as casein kinase II (34) and glycogen synthase kinase 3 (35) phosphorylate multiple sites of their substrates by a “hierarchical phosphorylation mechanism” (34) in which prior phosphorylation of one site is a prerequisite for phosphorylation of other sites. We constructed several site-directed mutants to investigate whether Sml1 phosphorylation by Dun1 follows hierarchal phosphorylation and also to identify the primary phosphorylation sites. Mutation of Ser60 to Ala eliminates phosphorylation by greater than 90%. On the other hand, mutating Ser58 to Ala only had a small reduction in phosphorylation, and no significant difference between S56A and wild type Sml1 was observed. These results suggest that Ser60 is the first primary site of phosphorylation, whereas Ser 56 and Ser 58 are minor sites of phosphorylation. Moreover, although the mutation of Ser60 to Asp also showed a significantly lower level of phosphate incorporation than wild type Sml1, it was significantly higher than S60A Sml1. This shows that a negative charge at residue 60 enhances phosphate incorporation at Ser56 and Ser58, suggesting that there is positive cooperativity between the primary phosphorylation site Ser60 and the minor sites Ser56 and Ser58. Although Ser61 was not identified as a potential phosphorylation site by MS anal-

**Fig. 6.** Positive ion SORI-CAD fragmentation of species corresponding to the peptide of Sml1 consisting of residues 52–64 in which phosphoserine residues are derivatized by ethanethiol. Ser56, Ser58, and Ser60 are identified as residues with phosphoryl attachment. A, CAD fragmentation of ions corresponding to the doubly phosphorylated peptide. More than 70% of the observed peaks could be assigned when Ser56 and Ser60 were considered to be the phosphorylation sites. b* and b** ions are produced by the neutral loss of one or two H2O from b ions. Likewise, y* ions are produced by the neutral loss of one H2O from y ions. B, CAD fragmentation of ions corresponding to the triply phosphorylated peptide. More than 70% of observed peaks could be assigned when Ser56, Ser58, and Ser60 were considered to be the phosphorylation sites.
ysis, the 661A mutant showed 48% phosphate incorporation compared with wild type S111, suggesting that Ser61 could possibly be phosphorylated, or it is necessary for recognition by Dun1 kinase. However, the 666A/S68A/S60A triple mutant showed no phosphate incorporation, strongly corroborating the MS result and unambiguously identifying Ser56, Ser58, and Ser60 to be the sole S111 sites of phosphorylation by Dun1 kinase. Based on these results, we interpret the role of Ser61 to be involved in substrate recognition by Dun1. The amino acid sequence of the S111 phosphorylation sites (GSSASASASSLEM) identified in our study is completely different from that identified by the synthetic peptide substrates by Sanchez et al. (14). This study demonstrated that synthetic peptides containing a consensus cAPK recognition sequence (RRXS/TY; X, small residues; Y, residues having large hydrophobic group) can be phosphorylated by Dun1, and replacement of the −3 Arg to Ala abolishes phosphorylation. Although S111 does not contain the cAPK consensus sequence, flanking regions of a few serine residues have some similarity to it. For example, Ser75 has an Arg at the −3 position and an Ile, which is a relatively large hydrophobic group, at the +1 position (RLNSI). Similarly, Ser87 has a Lys (as a basic residue) at the −3 position and a Gly (as a small residue) at the −1 position (KFGS).

However, analysis of the wild type S111 by CNBr digest as well as the tryptic digest and FRAG71 of the C14S mutant indicated that Ser75 and Ser87 are unlikely to be phosphorylated. On the contrary, the flanking regions of the phosphorylation sites identified by our study did not show any similarity to the cAPK consensus sequence. Furthermore, we demonstrated that a synthetic peptide used by Sanchez et al. (14) can be phosphorylated by Dun1 only at a high concentration, and the relative level of its phosphorylation is only about 11% of S111. These findings show that the Dun1 recognition motif is different from the cAPK recognition motif as S111 is a better substrate of Dun1 as compared with the cAPK-based peptide.

We investigated whether the S111 phosphopeptide (GSSASASASSLEM) is similar to phosphorylation motifs found in other proteins. A Phi-BLAST search as well as simple BLAST searches in the NCBI protein sequence database showed that very few proteins have sequences similar to the S111 phospho-
Phosphorylation by Dun1, we have conducted a data base search for possible phosphorylation sites of Sm1l by several known serine/threonine kinases. Ser56, Ser58, and Ser60 were the first, second, and third most probable phosphorylation sites with scores of 0.991, 0.939, and 0.845, respectively. The recognition motifs of 10 known serine/threonine kinases were also searched, and Ser60 was predicted to be a potential phosphorylation site for only casein kinase II. The consensus specificity motif for casein kinase II is X**S/T**X/E/DX (36). If Ser60 is considered to be the site of phosphorylation then the X**S/T**X/E/DX consensus is satisfied by the ASSLEM stretch of the Sm1l phosphopeptide sequence where Glu63 is the +3 site. Our results show that mutation of Glu63 to glutamine decreases phosphate incorporation down to 52% of wild type Sm1l, and therefore it is involved in recognition by Dun1. However, the phosphorylation sites Ser56 and Ser58 of Sm1l cannot be accommodated within the casein kinase II recognition motif X**S/T**X/E/DX, suggesting that the Dun1 kinase has a unique phospho-recognition motif.

A prerequisite for phosphorylation requires that the potential sites be freely accessible to the kinase of interest. Previously, in the absence of a three-dimensional structure, we constructed a molecular model of Sm1l based on ab initio Rosetta algorithms,2 which was in close agreement with CD and NMR data (30). In this model, Ser56, Ser58, and Ser60 are located at a flexible region adjacent to a C-terminal α-helix (Fig. 8). Side chain solvent accessibilities of Ser56, Ser58, and Ser60 in our model were 30.6, 49.6, and 61.6 Å², respectively. The accessibilities tabulated for Ser56, Ser58, and Ser60 reflect 40, 64, and 80% of a side chain accessible area for serine in a random coil flanked by two Gly residues on either side (37). These results suggest that Ser56, Ser58, and Ser60 are sites relatively accessible to Dun1 kinase for phosphorylation.

The biological consequence of Sm1l phosphorylation is to reduce dramatically the cellular pools of Sm1l resulting in the elevation of RNR activity (7). At present the mechanism involving Sm1l down-regulation after its phosphorylation by Dun1 is not known. However, phosphorylation followed by degradation of proteinaceous inhibitors has been observed in the regulation of several biological processes. In S. cerevisiae, Cdc4 is a part of the SCF ubiquitin ligase complex that is responsible for recruiting several target proteins such as cyclin-dependent kinase inhibitor Sic1 (38) and p21waf1 (39), which are phosphorylated at the end of the G1 phase. Based on binding of Cdc4 to its natural targets and synthetic peptides, a consensus phosphopeptide motif for Cdc4 was identified, and it was designated as the Cdc4 phospho-degron (CPD) motif (L/I-L/P-T-P-R/C-R) (28). However, Sm1l phosphorylation sites did not show any indication of a CPD motif. Possibly degradation of Sm1l is mediated by an unidentified ubiquitin ligase that recognizes multiple phosphorylated residues. Future work will involve the identification of the pathway responsible for Sm1l degradation.

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