Replication Checkpoint Enforced by Kinases Cds1 and Chk1

Michael N. Boddy, Beth Furnari, Odile Mondesert, Paul Russell*

Cdc2, the kinase that induces mitosis, is regulated by checkpoints that couple mitosis to the completion of DNA replication and repair. The repair checkpoint kinase Chk1 regulates Cdc25, a phosphatase that activates Cdc2. Effectors of the replication checkpoint evoked by hydroxyurea (HU) are unknown. Treatment of fission yeast with HU stimulated the kinase Cds1, which appears to phosphorylate the kinase Wee1, an inhibitor of Cdc2. The protein kinase Cds1 was also required for a large HU-induced increase in the amount of Mik1, a second inhibitor of Cdc2. HU-induced arrest of cell division was abolished in *cds1 chk1* cells. Thus, Cds1 and Chk1 appear to jointly enforce the replication checkpoint.

Cell cycle checkpoints ensure that chromosomal DNA is replicated and repaired before nuclear division (*1*, *2*). In mammalian cells, loss of checkpoint control results in rearrangements, amplification, and loss of chromosomes, events that are causally associated with cancer. In yeasts, checkpoint controls are vital for survival when DNA is damaged or replication is inhibited.

Cdc2, the cyclin-dependent kinase that initiates mitosis, is the ultimate target of the DNA replication and repair checkpoints. In the fission yeast *Schizosaccharomyces pombe*, Cdc2 is inhibited by phosphorylation on Tyr^{15}. This phosphorylation is catalyzed by the kinases Wee1 and Mik1 and reversed by the phosphatase Cdc25. Inhibitory phosphorylation of Cdc2 is crucial for replication and repair checkpoints in fission yeast and human cells (*3*–*6*). Chk1 may enforce the DNA repair checkpoint by phosphorylating and inhibiting Cdc25 (*6*–*8*). Chk1 is only required for the repair checkpoint and not for the replication checkpoint evoked by HU (*9*). However, an HU-induced arrest is rapidly abrogated after inactivation of temperature-sensitive Wee1 protein in a *mik1* background (*10*), showing that the replication checkpoint requires inhibitory phosphorylation of Cdc2. Wee1 and Mik1 are not individually required for an HU arrest, suggesting that Wee1 and Mik1 may be coordinately regulated by the replication checkpoint. Experiments were designed to test this hypothesis.

The abundance of Wee1 was unaffected by HU (*11*). We tested whether Wee1 was phosphorylated by an HU-activated kinase, with attention focused on the NH_2-terminal regulatory domain (*12*). GST:$Wee1^{152}$, a fusion of glutathione S-transferase to amino acids 11 to 152 of Wee1, was expressed in bacteria. Protein preparations yielded full-length GST:$Wee1^{152}$ and GST:$Wee1^{70}$ (Fig. 1A), the latter being a truncation product containing ~70 amino acids of Wee1 (*13*). These proteins were bound to glutathione (GSH)-Sepharose and mixed with lysates from cells harvested at intervals during incubation with HU (*14*). The proteins bound to GSH-Sepharose were washed, incubated with [γ-^{32}P]adenosine triphosphate, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B). GST:$Wee1^{152}$ was phosphorylated in the 0-min sample, whereas GST:$Wee1^{70}$ phosphorylation was negligible. Phosphorylation of both proteins increased during incubation in HU. Unfused GST was not phosphorylated. Thus, GST:$Wee1^{70}$ associates with and is phosphorylated by an HU-regulated kinase, whereas GST:$Wee1^{152}$ is phosphorylated by this kinase and another that is active before HU treatment.

HU prevents DNA replication by inhibiting ribonucleotide reductase and thereby reducing deoxyribonucleotide pools, but it may also have other effects. However, mutational inactivation of *cdc22*, which encodes the large subunit of ribonucleotide reductase (*15*), caused phosphorylation of GST:$Wee1^{70}$ in cell lysates (Fig. 1C), indicating that the HU effect is due to inhibition of DNA replication.

GST:$Wee1^{70}$ phosphorylation was assayed in lysates from temperature-sensitive *cdc25-22* cells that were arrested in G_2 phase, incubated in HU for 45 min, and then released from G_2 arrest in medium containing HU. HU failed to induce phosphorylation of GST:$Wee1^{70}$ in lysates prepared from *cdc25-22* cells arrested in G_2 (Fig. 1D). Upon release from G_2, GST:$Wee1^{70}$ phosphorylation and cell septation increased coincidentally. Septation marks the end of mitosis (M) and the beginning of DNA replication (S); thus, GST:$Wee1^{70}$ phosphorylation increased as cells encountered the HU arrest. No increase in GST:$Wee1^{70}$ phosphorylation was detected in a mock HU-treated culture (Fig. 1D). These

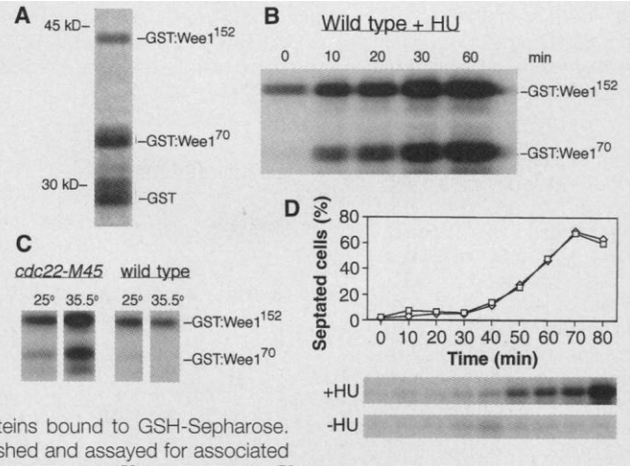

Fig. 1. Association of an HU-regulated kinase with the NH_2-terminus of Wee1. (**A**) SDS-polyacrylamide gel of GST:$Wee1^{152}$ and degradation products produced in bacteria and purified with GSH-Sepharose. The major degradation products are GST fused to ~70 amino acids of Wee1 (GST:$Wee1^{70}$) and unfused GST. Proteins were stained with Coomassie blue. (**B**) Cells were treated with 12 mM HU for a 1-hour time course at 30°C. Cell lysates were incubated with GST:Wee1 proteins bound to GSH-Sepharose. The GSH-Sepharose was washed and assayed for associated kinase activity. The positions of GST:$Wee1^{152}$ and GST:$Wee1^{70}$ after 12% SDS-PAGE are shown. (**C**) Mutational inactivation of *cdc22*, encoding the large subunit of ribonucleotide reductase, enhances phosphorylation of GST:$Wee1^{70}$. Cells carrying the temperature-sensitive *cdc22-M45* allele or wild-type cells were grown at 25°C. The cultures were split and maintained at 25°C or incubated at 35.5°C for 90 min. GST:Wee1 phosphorylation assays were performed as described above. (**D**) Activation of the kinase that phosphorylates GST:$Wee1^{70}$ in extracts from *cdc25-22* cells released into HU from a G_2 arrest. A culture of *cdc25-22* cells grown at 25°C was shifted to 35.5°C for 4 hours. The culture was split and treated with 12 mM HU (+HU, □) or mock treated (−HU, ◇) for 45 min at 35.5°C. Both cultures were returned to 25°C, and samples were taken every 10 min. The septation index marks the completion of mitosis (top). Lysates from each time point were assayed for GST:$Wee1^{70}$ kinase (bottom). GST:$Wee1^{70}$ phosphorylation occurred in the HU-treated samples after exit from M, whereas phosphorylation of GST:$Wee1^{70}$ remained low in samples from the mock-treated culture. In the mock-treated culture, activity of the kinase that phosphorylates GST:$Wee1^{152}$ increased as cells underwent mitosis.

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observations indicate that the kinase that phosphorylates GST:Wee1^{70} is activated when cells attempt DNA replication with insufficient deoxyribonucleotides.

Phosphorylation of GST:Wee1^{70} was abolished in lysates made from *rad1* and *rad3* checkpoint mutants (Fig. 2A), suggesting that the kinase that phosphorylates GST:Wee1^{70} may be involved in the replication checkpoint. Experiments were undertaken to identify this kinase. Chk1 was excluded by experiments with lysates made from *chk1* cells (Fig. 2B). The involvement of Cds1, a protein kinase that is important for survival of HU treatment (*16*), was indicated by the observation that GST:Wee1^{70} phosphorylation was abolished in a lysate prepared from HU-treated *cds1* cells (Fig. 2B). An experiment was performed to determine whether Cds1 coprecipitated with GST:Wee1^{70}. Lysates from HU-treated cells in which genomic *cds1* encoded a protein with a COOH-terminal HAHIS tag [consisting of two hemagglutinin (HA) epitopes followed by hexahistidine] were incubated with GST:Wee1 or GST proteins, washed, and analyzed by immunoblotting. Cds1^{HAHIS} precipitated with GST:Wee1 but not with unfused GST (Fig. 2C). Rad3 did not associate with GST:Wee1 (*11*). Cds1 purified from fission yeast phosphorylated GST:Wee1^{70} in vitro, producing a two-dimensional tryptic phosphopeptide map that was identical to a map made from GST:Wee1^{70} phosphorylated by its associated kinase (*11*). These data strongly suggest that Cds1 is the HU-regulated kinase that associates with and phosphorylates GST:Wee1^{70}.

Association of Cds1 and Wee1 was also examined in vivo. GST:Cds1 was expressed from the thiamine-repressible *nmt1* promoter (*17*). As a control, we used a strain that expressed GST:Chk1. The GST fusion proteins were purified with GSH-Sepharose and analyzed by immunoblotting. Cdc25 (but not Wee1) associated with GST:Chk1, whereas Wee1 (but not Cdc25) associated with GST:Cds1 (Fig. 2D).

HU may increase the abundance of Cds1, it may make Cds1 competent to associate with Wee1, or perhaps the replication checkpoint enhances the kinase activity of Cds1. Cells expressing Cds1^{HAHIS} were treated with HU or mock-treated, and lysates were made and precipitated with GST:Wee1 proteins. Association of Cds1^{HAHIS} with GST:Wee1 was detected in lysates from cells treated with HU, whereas Cds1^{HAHIS} did not associate with GST:Wee1 in mock-treated samples (Fig. 3A). Ni^{2+}–nitrilotriacetic acid (NTA) precipitation of Cds1^{HAHIS} by the hexahistidine sequence showed that Cds1^{HAHIS} was equally abundant in HU- and mock-treated cell lysates. Therefore, HU treatment enhanced association of Cds1 with GST:Wee1 (*18*). Cds1^{HAHIS} was also purified from HU- or mock-treated cells and assayed in an autophosphorylation assay. Phosphorylation of Cds1 was increased in samples from cells treated with HU (Fig. 3B). Similar results were obtained with GST:Wee1^{70} as a substrate (*11*). Immunoblot analysis confirmed that similar amounts of Cds1^{HAHIS} were purified from HU- and mock-treated cells (Fig. 3B). Thus, HU apparently both activates Cds1 as a kinase and stimulates binding to Wee1.

Phosphorylation of Wee1 by Cds1 may play a role in the DNA replication checkpoint. However, *wee1* mutants arrest division in response to HU (*3*); thus, another protein that controls Cdc2 Tyr^{15} phosphorylation must also be regulated by the replication checkpoint. The kinase Mik1 phosphorylates Cdc2 on Tyr^{15} (*4*); therefore, the abundance of Mik1 was monitored in HU-treated cells with a strain expressing

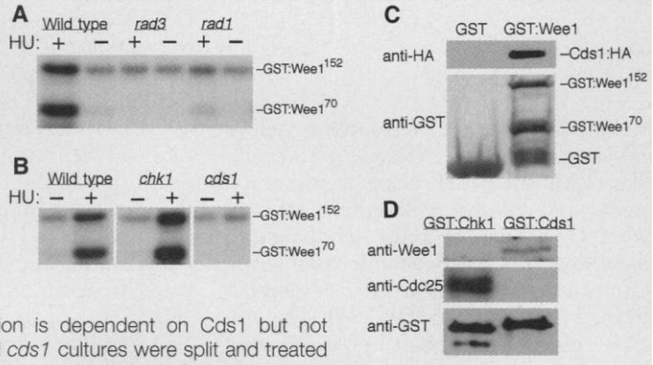

Fig. 2. Phophorylation of GST:Wee1^{70} by the kinase Cds1. (**A**) Phosphorylation of GST:Wee1^{70} is dependent on Rad1 and Rad3. Wild-type, *rad1*, and *rad3* strain cultures were split and treated for 1 hour with HU or mock-treated and then assayed for GST:Wee1^{70} phosphorylation as described in Fig. 1. (**B**) GST:Wee1^{70} phosphorylation is dependent on Cds1 but not Chk1. Wild-type, *chk1*, and *cds1* cultures were split and treated for 1 hour with HU or mock treated. Phosphorylation of GST:Wee1 was assayed. (**C**) Cds1 interacts with GST:Wee1 but not with GST. Lysates from HU-treated cells expressing epitope-tagged Cds1 [Cds1^{HAHIS} (Cds1:HA)] expressed from the *cds1* genomic locus were incubated with GST or GST:Wee1 proteins bound to GSH-Sepharose. After extensive washing, samples were resolved by 10% SDS-PAGE and subjected to immunoblotting with antibodies to HA and GST (anti-HA and anti-GST). (**D**) Wee1 interacts with GST:Cds1 but not with GST:Chk1 in vivo. Strains carrying integrated *nmt1:GST:chk1*$^+$ or *nmt1:GST:cds1*$^+$ constructs were grown in minimal medium (EMM2) without thiamine at 30°C for 16 hours to induce expression of GST fusion proteins. Cells were lysed, and GST fusion proteins were purified with GSH-Sepharose. After extensive washing, samples were resolved by 10% SDS-PAGE and subjected to immunoblotting with anti-Wee1, anti-Cdc25, and anti-GST. A ~110-kD protein corresponding to Wee1 was detected in the GST:Cds1 sample, whereas a ~80-kD protein corresponding to Cdc25 was detected in the GST:Chk1 sample. The ~110-kD protein was not detected in samples prepared from Δ*wee1* cells (*11*). GST:Cds1 and GST:Chk1 migrate at positions corresponding to ~82 and ~80 kD, respectively.

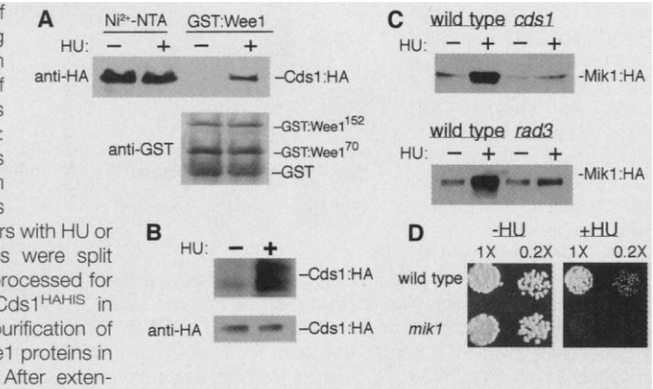

Fig. 3. Stimulation by HU of Cds1 kinase activity, binding of Cds1 to GST:Wee1^{70} in lysates, and accumulation of Mik1. (**A**) HU stimulates Cds1 binding to GST:Wee1^{70}. A culture of cells expressing Cds1^{HAHIS} from the *cds1* genomic locus was split and treated for 3.5 hours with HU or mock-treated. The cultures were split again, and samples were processed for Ni^{2+}-NTA purification of Cds1^{HAHIS} in denaturing conditions or purification of proteins that bind GST:Wee1 proteins in nondenaturing conditions. After extensive washing, samples were resolved by 10% SDS-PAGE and subjected to immunoblot analysis. (**B**) HU activates Cds1 kinase activity. In a separate experiment, Cds1^{HAHIS} was purified in native conditions and assayed for autophosphorylation activity (top). Immunoblot analysis confirmed that similar amounts of Cds1^{HAHIS} were recovered in the HU- and mock-treated samples (bottom). (**C**) Cds1- and Rad3-dependent increase in Mik1 abundance in HU-treated cells. Wild-type, *cds1*, and *rad3* cells in which genomic *mik1* encoded Mik1^{HAHIS} (Mik1:HA) protein were incubated for 4 hours in YES at 25°C with 12 mM HU or mock-treated. Mik1^{HAHIS} protein was purified by Ni^{2+}-NTA purification in denaturing conditions and detected by immunoblotting with anti-HA. (**D**) Mik1 is important for survival of HU treatment. Serial dilutions (1X, 500 cells; 0.2X, 100 cells) of wild-type and *mik1* cells were plated in YES medium supplemented with 0 or 6 mM HU and incubated for 2 days at 30°C.

Mik1^{HAHIS} from the genomic locus. Immunoblot analysis showed that the amount of Mik1^{HAHIS} increased in cells treated with HU (Fig. 3C). The abundance of *mik1* mRNA was unchanged in HU-treated cells (*11*); thus, HU must affect Mik1 synthesis or turnover. The HU-induced increase of Mik1^{HAHIS} was largely abolished in *cds1* cells and *rad3* cells (Fig. 3C), indicating that this response may be regulated by the replication checkpoint. Mik1 is a dose-dependent inhibitor of mitosis (*4*); thus, the increase of Mik1 abundance in HU-treated cells should help to enforce the checkpoint. Indeed, *mik1* mutants exhibited enhanced sensitivity to HU (Fig. 3D).

Expression of a large amount of GST:Cds1 from the *nmt1* promoter caused a cell cycle arrest, as indicated by highly elongated cells (Fig. 4). The arrest occurred in *rad3* and *chk1* mutants, indicating that Cds1 functions downstream of Rad3 and independently of Chk1. Expression of GST:Cds1 had no effect in cells that express Cdc2Y15F, a form of Cdc2 that cannot be phosphorylated by Wee1 or Mik1. These findings support the conclusion that Cds1 transmits a checkpoint signal.

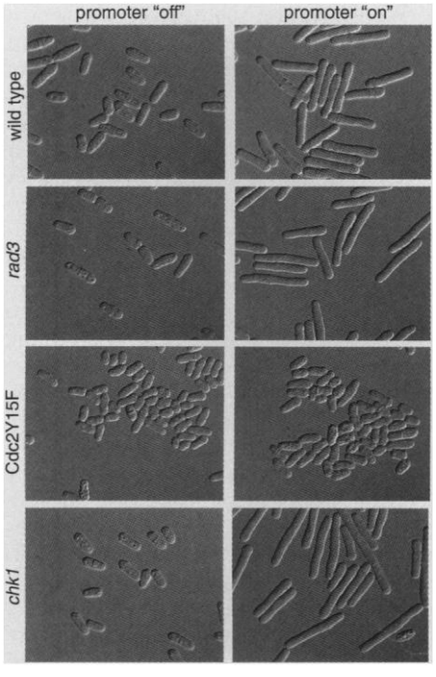

Fig. 4. Cell cycle arrest caused by expression of large amounts of GST:Cds1. Cells having an integrated *nmt1:GST:cds1*$^+$ construct were incubated in *nmt1*-repressing medium (promoter off) or *nmt1*-inducing medium (promoter on) for 19 hours at 30°C. GST:Cds1 expression induced cell cycle arrest in wild-type, *rad3*, and *chk1* strains. Flow cytometry analysis confirmed that these cells were arrested with a 2C DNA content (*11*). Cells expressing Cdc2Y15F, a form of Cdc2 in which tyrosine at position 15 is replaced with phenylalanine, were insensitive to GST:Cds1.

Cells lacking Cds1 arrest division in response to HU, indicating that the Cds1 signal is supplemented by other checkpoint proteins (*16*). We therefore examined the replication checkpoint in synchronous cultures of *cds1 chk1* double-mutant cells; *cds1*, *chk1*, and *rad3* single-mutant cells; and wild-type cells (*19*). HU arrested division in wild-type cells and in *cds1* and *chk1* single-mutant cells (Fig. 5A). In contrast, *cds1 chk1* double-mutant cells underwent division with kinetics that were similar to *rad3* cells. Thus, the checkpoint is abolished in *cds1 chk1* cells. Indeed, the *cds1 chk1* double-mutant cells were acutely sensitive to killing by HU (Fig. 5B). These findings are consistent with a recent study (*20*) and support a model in which Cds1 has a direct role in enforcing the replication checkpoint.

These findings indicate that Cds1 and Chk1 are dual effectors of the replication checkpoint. Chk1 may regulate Cdc25, whereas Cds1 appears to phosphorylate Wee1 and is required to increase the abundance of Mik1. The effect of phosphorylation of Wee1 by Cds1 is unknown and remains to be confirmed with in vivo studies, but it is striking that the replication checkpoint both increases the kinase activity of Cds1 and stimulates binding to Wee1 in cell extracts. The large HU-induced increase in the abundance of Mik1 helps to explain why *cdc25 wee1* double-mutant cells arrest division in response to HU and reaffirms the importance of Cdc2 Tyr^{15} phosphorylation in the replication checkpoint (*21*). Cds1 shares substantial sequence identity with the kinase Rad53p, a protein involved in the DNA replication checkpoint in *Saccharomyces cerevisiae* (*2*). These facts suggest that studies of Cds1 and Rad53p may help to frame investigations of replication checkpoints in human cells.

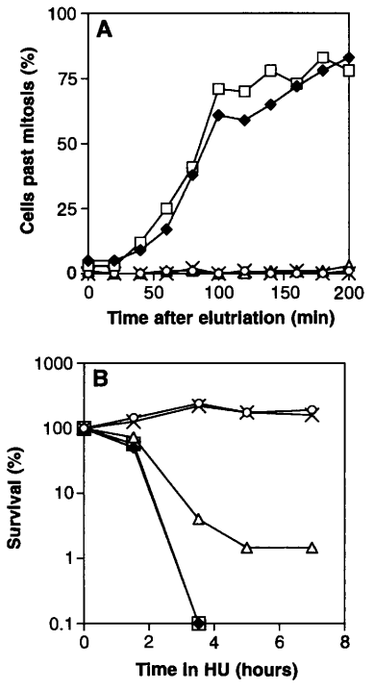

Fig. 5. Abolishment of replication checkpoint in *cds1 chk1* cells. (**A**) Synchronous cultures were obtained by centrifugal elutriation. Progression through mitosis in the presence of 12 mM HU was monitored by scoring binucleate or septated cells. The *cds1 chk1* double-mutant (□) and *rad3* (◆) cells underwent mitosis in the presence of HU, whereas division was arrested in control strains [wild-type (○) and *cds1* (△) and *chk1* (×) single-mutant cells]. (**B**) The *cds1 chk1* double-mutant and *rad3* cells were equally hypersensitive to killing by 12 mM HU. Wild-type and *chk1* cells were not sensitive to HU, whereas *cds1* cells were moderately sensitive.

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13. A polymerase chain reaction (PCR) fragment was made encoding amino acids 11 to 152 of Wee1, incorporating a 5′ Nde I site and 3′ Not I site–stop codon. This fragment was cloned into a modified pGEX vector and transformed into the *Escherichia coli* BL21 (DE3) expression host (*22*). GST:Wee1^{152} protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM) to exponentially growing cells at an absorbance at 600 nm (A_{600}) of 0.6 in LB media plus ampicillin (50 μg/ml) at 372°C. After 2 hours at 37°C, cells were collected by centrifugation and stored at −70°C in portions (200-μl pellet volume). Cells were lysed in 600 μl of lysis buffer [50 mM tris (pH 8), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% NP-40, leupeptin-aprotinin-pepstatin (5 μg/ml), and 1 mM phenylmethylsulfonyl fluoride] by sonication and centrifuged at 13,000*g* for 10 min at 4°C. GSH-Sepharose (50-μl bead volume) was added to the supernatant and incubated at 4°C for 30 min. The beads were then washed three times with 1 ml of lysis buffer. Key experiments involving the GST:Wee1^{70} truncation product were replicated with a precise fusion of GST and amino acids 1 to 72 of Wee1.
14. Strains of the following genotypes were used: PR109, wild type; NB2117, *cds1::ura4*$^+$; NB2118, *cds1:2HA6His:ura4*$^+$; NR1826, *rad3::ura4*$^+$; OM591, *cdc22-M45*; NR1593, *rad1::ura4*$^+$; NR1592, *chk1::ura4*$^+$; BF1758, *nmt1:GST:chk1:leu1*$^+$; BF1916, *nmt1:GST:cds1:leu1*$^+$; OM2173, *mik1:2HA6His:ura4*$^+$; OM2183, *mik1:2HA6His:ura4*$^+$ *cds1::ura4*$^+$; PR712, *mik1::ura4*$^+$; and BF2115, *cds1::ura4*$^+$ *chk1::ura4*$^+$. All strains were *leu1-32 ura4-D18*. All *nmt1* promoter constructs were integrated at the *leu1-32* locus. Growth media

and general methods for *S. pombe* have been described [S. Moreno, A. Klar, P. Nurse, *Methods Enzymol.* **194**, 795 (1991)]. Unless otherwise indicated, yeast cultures were grown in YES medium at 30°C. YES consists of glucose, yeast extract, and amino acid supplements. HU was used at a concentration of 12 mM. Purification of GST fusion proteins and hexahis-tagged proteins expressed in *S. pombe*, immunoblotting, and kinase assays were performed as described [K. Shiozaki and P. Russell, *Nature* **378**, 739 (1995)].
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17. To tag genomic *cds1*$^+$ with a sequence encoding two copies of the HA epitope and hexahistidine, we made a Pst I–Not I fragment having nucleotides 205 to 1465 of the *cds1*$^+$ open reading frame by PCR. This fragment was introduced into pRIP42-Spc1HA6H after digestion with Pst I and Not I enzymes (*22*). The construct was linearized with Nhe I and transformed into PR109. Integration and tagging were confirmed by Southern (DNA) hybridization and immunoblotting, with function of Cds1^{HAHIS} confirmed by lack of HU sensitivity. The *nmt1:GST:cds1*$^+$ construct was prepared as described for the *nmt1:GST:chk1*$^+$ construct (*7*).
18. The in vivo interaction between GST:Cds1 and Wee1 shown in Fig. 2D did not require HU treatment, presumably because GST:Cds1 was overexpressed.
19. Cells were grown in YES medium to an A_{600} of 1.0 and then treated with HU (12 mM) for 90 min at 30°C. Synchronous cells were then obtained by centrifugal elutriation with a Beckman JE-5.0 elutriation rotor. These cells were diluted to an A_{600} of 0.3 in YES containing 12 mM HU and grown at 30°C for a further 4 hours. Cells were scored for progression through mitosis by microscopic observation. HU sensitivity studies were carried out with cells grown to an A_{600} of 0.3 in YES medium and then diluted to 13,000 cells/ml in YES media containing HU (12 mM), followed by growth at 30°C for a further 8 hours. Samples (75 μl) were taken at regular intervals, plated onto YES plates, and grown for 4 days at 30°C to determine survival.
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23. We thank N. Rhind, J.-M. Brondello, C. McGowan, and K. Shiozaki for their advice and the Scripps Cell Cycle Groups for their support and encouragement. Funded by NIH.

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Chaos, Persistence, and Evolution of Strain Structure in Antigenically Diverse Infectious Agents

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The effects of selection by host immune responses on transmission dynamics was analyzed in a broad class of antigenically diverse pathogens. Strong selection can cause pathogen populations to stably segregate into discrete strains with nonoverlapping antigenic repertoires. However, over a wide range of intermediate levels of selection, strain structure is unstable, varying in a manner that is either cyclical or chaotic. These results have implications for the interpretation of longitudinal epidemiological data on strain or serotype abundance, design of surveillance strategies, and the assessment of multivalent vaccine trials.

New epidemics of an infectious disease can be triggered by the evolution of a novel antigenic type or strain that evades the acquired immunity within the host population created by its predecessors. The most studied case is the influenza virus, where major shifts in the structure of surface antigens can often trigger worldwide pandemics of the novel variant (*1*, *2*). The antigens that are most likely to exhibit diversity are those under strong selection by host immune responses. These polymorphic antigens may be ranked by the degree to which the associated immune response reduces the reproductive or transmission success of the pathogen. We demonstrated previously that those antigens that elicit the strongest immune response, which in turn have the strongest impact on transmission success, may be organized by immune selection acting within the host population into sets of nonoverlapping variants (*3*). For example, in the case of two antigens each encoded by a distinct locus with two alleles, namely **a** and **b** at one locus and **c** and **d** at the other, four genotypes exist (**ac**, **ad**, **bc**, and **bd**). One set of nonoverlapping variants is **ac** and **bd** (a discordant set) and the other is **bc** and **ad**. The pathogen population may exhibit a discrete strain structure where one set of nonoverlapping variants exists at much greater frequency than the other. For this pattern to emerge and be stable over time, the intensity of acquired immunity to a specific variant antigen (encoded by a given allele) within the host population must reduce considerably the transmission success or fitness of all subsequent infections by genotypes possessing that allele. Pathogen populations may therefore be categorized into discrete "strains" or serotypes according to the genetic loci that encode antigens eliciting immune responses with the greatest effect on the transmission success of the infectious agent (*3*).

Here, we show that pathogens that possess antigens that do not elicit an immune response that is strong enough to induce a discrete stable strain structure may exist as a set of strains that exhibit cyclical or chaotic fluctuations in frequency over time. They may still be organized by immune selection into discrete groups of variants, where all the members in a given group have different alleles at every locus, but the dominancy of the group, relative to that of other groups, may fluctuate widely over time, either cyclically or chaotically. Pathogen antigens that elicit immune responses that have little effect on transmission success will not be organized to express a discrete nonoverlapping strain structure. In this case, all possible allele combinations will be maintained at abundances commensurate with their individual transmission success or fitness.

We define the conditions under which each of these three outcomes arises, in terms of the biological characteristics of both the pathogen and the immune response of the host to the various antigens of the infectious agent, using a model in which a pathogen strain is defined by the m alleles that exist at each of n loci. We take no account of other important biological complications such as mutation, seasonality in transmission, time delays between the acquisition of infection and infectiousness to susceptible hosts, or genetic diversity in the host population, influencing the immune responses to particular antigenic variants. These exclusions are deliberate, because we wish to assess the impact of selection imposed by acquired immunity in the host population on temporal trends in the frequencies of different variants and the evolution of the associated strain structure in the pathogen population.

Strains that do not share any alleles (hereafter referred to as a discordant set) are assumed not to interfere with each others' transmission success or fitness as mediated by host immune responses. The degree to which infection with a given strain limits the ability of another strain that shares any alleles to infect the same host is defined by a cross-protection or cross-immunity parameter γ. If $\gamma = 0$, then the strains do not induce cross-protective responses, whereas if $\gamma = 1$, then there is complete cross-protection. It is also assumed that immunity to a given strain i does not prevent infec-

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