

Mad1 Function in Cell Proliferation and Transcriptional Repression Is Antagonized by Cyclin E/CDK2*

Received for publication, December 23, 2004,
and in revised form, February 15, 2005
Published, JBC Papers in Press, February 18, 2005,
DOI 10.1074/jbc.C400611200

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The transcription factors of the Myc/Max/Mad network play essential roles in the regulation of cellular behavior. Mad1 inhibits cell proliferation by recruiting an mSin3-corepressor complex that contains histone deacetylase activity. Here we demonstrate that Mad1 is a potent inhibitor of the G₁ to S phase transition, a function that requires Mad1 to heterodimerize with Max and to bind to the corepressor complex. Cyclin E/CDK2, but not cyclin D and cyclin A complexes, fully restored S phase progression. In addition inhibition of colony formation and gene repression by Mad1 were also efficiently antagonized by cyclin E/CDK2. This was the result of cyclin E/CDK2 interfering with the interaction of Mad1 with HDAC1 and reducing HDAC activity. Our findings define a novel interplay between the cell cycle regulator cyclin E/CDK2 and Mad1 and its associated repressor complex and suggests an additional mechanism how cyclin E/CDK2 affects the G₁ to S phase transition.

The oncoprotein c-Myc promotes proliferation and enhances the G₁ to S phase transition at least in part by activating cyclin E/cyclin-dependent kinase 2 (CDK2),¹ which possesses essen-

tial functions in promoting S phase progression (1–3). Myc, a basic region-helix-loop-helix-leucine zipper (bHLHZip) protein, functions as a transcriptional regulator. Transcriptional activation is antagonized by Mad bHLHZip proteins that include Mad1, Mxi1, Mad3, and Mad4. These proteins, in contrast to Myc, inhibit proliferation and repress transformation (4). Myeloid precursor cells of *mad1*-deficient mice show ectopic cell divisions prior to differentiation and hyperplastic alterations in several tissues were observed in *mxi1*-deficient mice (5, 6). Inhibition of proliferation requires that Mad proteins interact with Max and with a mSin3-histone deacetylase (HDAC) repressor complex, which is recruited through the N-terminal mSin3-interaction domain (SID) (4). Together these findings suggest that Mad proteins affect proliferation by modulating chromatin structure of target genes.

Several HDAC-dependent corepressor complexes have been described in mammalian cells (7). The mSin3-HDAC repressor complex that is recruited by Mad proteins contains more than 10 subunits, including the two histone deacetylases HDAC1 and HDAC2 (4). These two enzymes show a high degree of sequence similarity and are substrates for kinases implying signal-dependent regulation (8). However, little additional information is available about the regulation of the mSin3-HDAC repressor complex. Defining its regulation will be important not only to understand Mad function in more detail but to obtain insight into the function of the complex and the associated HDACs in cell behavior control.

To obtain novel insight into the regulation of Mad1 function in the control of cell proliferation we further characterized the role of this protein in the G₁ to S phase transition. Mad1 blocks this transition dependent on its ability to recruit the mSin3-repressor complex and to interact with its heterodimeric partner Max. We observed that cyclin E/CDK2, but not other cyclin/CDKs, overcame Mad1-dependent inhibition of cell cycle progression. This was due to a direct effect on the Mad1-repressor complex. Cyclin E/CDK2 interfered with Mad1-dependent transcriptional repression and with the HDAC activity of the corepressor complex, at least in part as a consequence of resolving the complex. Together our findings provide a novel link between HDAC-dependent gene repression and cell cycle control.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1, NIH3T3, SAOS2, and COS7 cells were grown in Dulbecco's modified Eagle's medium and RK-13 cells in minimal essential medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. Quiescent 3T3-L1 cells were obtained by starvation for 36 h in medium containing 0.1% FCS. After injection quiescent cells were stimulated by the addition of FCS (final concentration of 10%). To monitor DNA synthesis cells were incubated in the presence of 100 μ M bromodeoxyuridine (BrdUrd, Roche Diagnostics, Mannheim, Germany). SAOS2 colony formation assays were performed as described previously (9).

Plasmids—Plasmids encoding Mad1 and mutants and a myc-tagged version of HDAC1 have been described previously (9, 10). For Gal-Sin3 the full-length coding regions of mSin3A was fused to the DNA binding domain of Gal4. Plasmids encoding cyclin E, cyclin D1, cyclin D2, cyclin A, CDK2, CDK4, and p27^{Kip1} were kindly provided by R. Bernards. Plasmids coding for Gal-MadN35 and Gal-Mad35(Pro) were generously provided by R. N. Eisenman. pEGFP-C1 was purchased from Clontech.

Microinjection, Staining Procedures, and Antibodies—Microinjections and stainings were performed as described previously (9). Antibodies recognizing Mad1 (C-19), mSin3B (AK-12), cyclin E (M-20), and cyclin A (C-19) were from Santa Cruz. Antibodies recognizing BrdUrd were from Fitzgerald.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (LU 466/6, SFB542 TP B8) (to B. L.) and FOG "Chromatin-mediated Biological Decisions" (to M. E.). The microinjection setup was a gift of the MUCK e.V., Hannover. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: CDK, cyclin-dependent kinase; bHLHZip, basic region/helix-loop-helix/leucine zipper; HDAC, histone deacetylase; SID, mSin3 interaction domain; FCS, fetal calf serum; mAb, monoclonal antibody; GFP, green fluorescent protein.

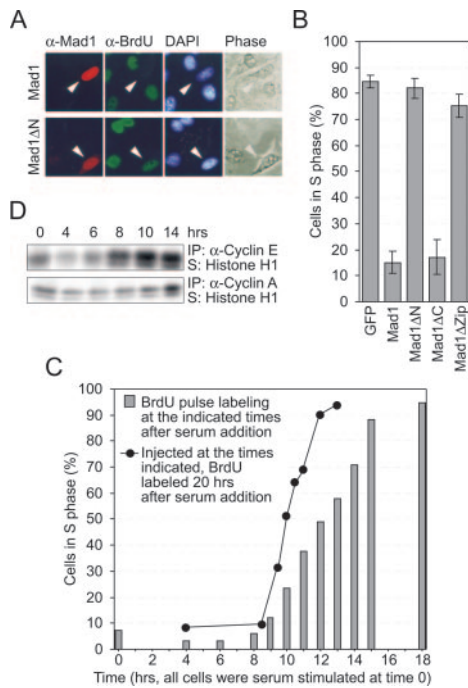


FIG. 1. Mad1 inhibits serum-stimulated S phase entry until late in G_1 . A, serum-starved 3T3-L1 cells were injected with 50 ng/ μ l pCMVmad1 or pCMVmad1ΔN that express Mad1 or Mad1ΔN, respectively, as indicated. The cells were then stimulated with FCS (10% final concentration) in the presence of BrdUrd, fixed, and stained 20 h later. Injected cells were identified using Mad1-specific antibodies (C-19). Progression into S phase was visualized by staining with anti-BrdUrd antibodies. For control the DNA was stained with DAPI and phase contrast pictures are shown. B, summary of between 4 and 10 micro-injection experiments with 50–70 stainable cells per experiment performed essentially as described in A. Injected cells were identified using Mad1-specific antibodies (Mad1, Mad1ΔN, and Mad1ΔZip with C-19 and Mad1ΔC with mAb 5C9) or by analyzing GFP that was expressed from co-injected pCMV-EGFP plasmids. C, Mad1 inhibits S phase until late in G_1 . Quiescent 3T3-L1 fibroblasts were stimulated with FCS in the presence of BrdUrd and injected with 50 ng/ μ l pCMVmad1 at the times indicated. 20 h after addition of serum the cells were fixed and stained for Mad1 and BrdUrd. The number of double positive cells is displayed as percentage of the total number of Mad1 positive cells (filled circles). Uninjected cells on parallel coverslips were pulse-labeled for 1 h with BrdUrd at the times indicated after serum stimulation. The cells were then fixed and stained for BrdUrd and the number of positive cells determined (bars). D, serum-starved 3T3-L1 fibroblasts were serum-stimulated and F-buffer lysates prepared at the times indicated. The different cyclin/CDK complexes were immunoprecipitated as indicated (IP) and the associated kinase activities determined using histone H1 as substrate (S).

Transient Transfections—Transient transfections of NIH3T3, RK-13, and COS7 cells were performed using calcium phosphate coprecipitation as described previously (22, 23). The p(Gal4)₄-mintk-luc reporter and the plasmid expressing β -galactosidase used for standardization have been described previously (23).

HDAC Assays—Complexes containing HDAC1 were immunoprecipitated from transiently transfected COS7 cells lysates prepared in Frackelton buffer (9) containing 10% glycerol using Mad1-specific antibodies (C-19) or mAb 9E10 recognizing the myc epitope tag. The HDAC assays were performed with [³H]acetate-labeled chicken reticulocyte core histones as described previously (9).

Preparation of Active Cyclin E/CDK2 Complexes and Kinase Assays—Expression and purification of human cyclin E/CDK2 complexes from insect cells infected with recombinant baculoviruses were carried out as described previously (24). Cyclin E/CDK2 activity was measured using histone H1 as substrate. 1 unit of cyclin E/CDK2 activity transferred 1 pmol of phosphate onto 5 μ g of histone H1 in kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 0.01% Tween 20, 10 mM NaF, 10 mM β -glycerophosphate, 1 mM orthovanadate, 0.01% bovine serum albumin) containing 25 μ M ATP in 30 min. Kinase reactions with immunopurified HDAC complexes were performed with 5 unit of cyclin E/CDK2 as above for 15 min.

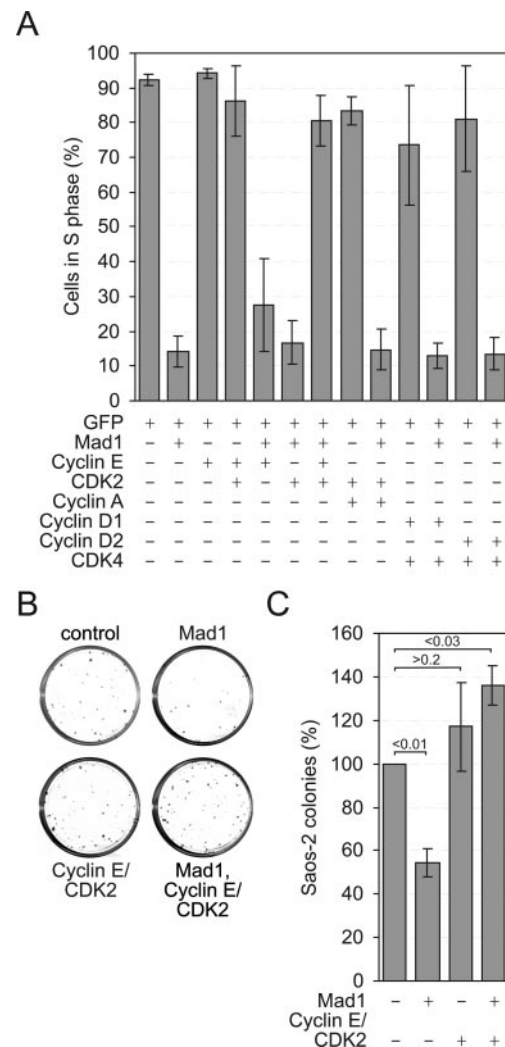


FIG. 2. Cyclin E/CDK2 inhibits Mad1 function. A, quiescent 3T3-L1 cells were co-injected with pEGFP-C1 (5 ng/ μ l) and with plasmids expressing Mad1 (50 ng/ μ l), cyclin E (40 ng/ μ l), cyclin A (40 ng/ μ l), cyclin D1 (40 ng/ μ l), cyclin D2 (40 ng/ μ l), CDK2 (30 ng/ μ l), and CDK4 (30 ng/ μ l) as indicated. The cells were stimulated with FCS in the presence of BrdUrd and fixed 20 h later. Injected cells identified by GFP expression were analyzed for BrdUrd incorporation. B, expression plasmids encoding the indicated proteins together with a neomycin resistance plasmid were transfected into SAOS2 cells, and neomycin-resistant colonies were selected and stained 21 days later. C, summary of colony formation experiments shown in B. The mean values and standard deviations of four experiments performed in triplicates are displayed. Statistical values using double sided student's *t* test analysis are given.

FCS-synchronized NIH3T3-L1 cells were harvested in F-buffer at the times indicated. Protein concentrations of the different samples were determined using the Bradford protein assay. CDK complexes were immunopurified from aliquots with equal protein concentration using antibodies specific for cyclin E or A. Kinase reactions were performed in kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β -glycerophosphate) containing 1 μ g of histone H1 and 10 μ M of [³²P]ATP at 30 °C for 15 min.

Coimmunoprecipitation Experiments—Relevant proteins were expressed in COS7 or HEK293 cells and the complexes immunoprecipitated from F-buffer whole cell lysates. The components of the complexes were analyzed by Western blotting.

RESULTS AND DISCUSSION

Mad1 Inhibits Entry into S Phase until Late in G_1 —We have shown previously that Mad1 inhibits serum-induced S phase entry in fibroblasts (9). To determine domains in Mad1 important for this function, plasmids expressing different mutants were injected into quiescent 3T3-L1 fibroblasts and progression

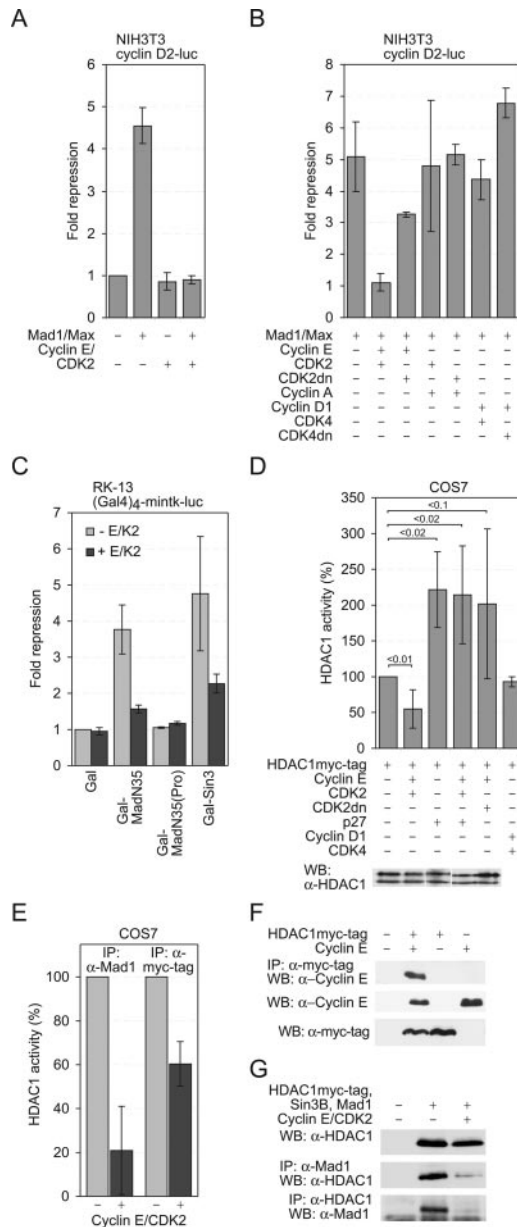


FIG. 3. Cyclin E/CDK2 inhibits Mad1-dependent gene repression and interferes with the Mad1-repressor complex. **A**, NIH3T3 cells were transiently transfected with a *cyclin D2* promoter-luciferase reporter gene construct and expression plasmids encoding Mad1 (1 μ g), Max (0.1 μ g), cyclin E (0.2 μ g), and CDK2 (0.2 μ g) as indicated. A representative experiment performed in triplicate is shown. **B**, experiments were performed as in **A** with additional expression plasmids for CDK2dn, cyclin A, cyclin D1, CDK4, and CDK4dn (all 0.2 μ g). The mean values and standard deviations of three independent experiments performed in triplicates are displayed. **C**, RK-13 cells were transiently transfected with a Gal4-responsive luciferase reporter gene construct ((Gal4)₄-mintk-luc) and with plasmids expressing the indicated Gal4-DNA binding domain (GalDBD) fusion proteins. Plasmids encoding cyclin E and CDK2 (both 0.2 μ g) were cotransfected as indicated. Mean values and standard deviations of four independent experiments performed in triplicates are shown. **D**, COS7 cells were transiently transfected with plasmids expressing the indicated proteins. HDAC1myc-tagged complexes were immunoprecipitated using mAb 9E10 and the deacetylase activity determined. The activities of control immunoprecipitates were subtracted. Mean values and standard deviations of five independent experiments (three with cyclin D1/CDK4) are shown. Statistical values using double sided Student's *t* test analysis are given. Aliquots of the lysates were analyzed for HDAC1myc tag expression (lower panel). **E**, Mad1, mSin3A, and HDAC1myc-tag were transiently expressed in COS7 cells and immunoprecipitated using mAb 9E10 (anti-myc tag) or antibodies specific for Mad1 (C19). The immunoprecipitates were incubated with or without insect cell expressed recombinant cyclin E/CDK2. The mean values and

into S phase was measured by BrdUrd incorporation. Mad1 efficiently blocked progression into S phase (Fig. 1, **A** and **B**). This was dose-dependent, and similar effects were measured in Swiss3T3 and primary rat embryo fibroblasts (data not shown). Mutants lacking either the N terminus including the SID (Mad1 Δ N) or the leucine zipper (Mad1 Δ Zip) were unable to inhibit S phase progression, while the C-terminal domain (Mad1 Δ C) was dispensable (Fig. 1, **A** and **B**). This suggests that heterodimerization with Max and recruitment of an mSin3-HDAC corepressor complex are required to inhibit entry into S phase. This is comparable with the requirements to inhibit cell proliferation and transformation (10, 11).

To determine until when in the G₁ phase Mad1 was able to inhibit S phase progression, quiescent fibroblasts were serum stimulated and then microinjected with Mad1-expressing plasmids at different times. BrdUrd-positive cells were determined 20 h after serum addition (Fig. 1C, closed circles). Mad1 efficiently inhibited progression into S phase up to 8.5 h after serum stimulation. At later time points an increasing proportion of Mad1-expressing cells entered S phase, reaching control levels by 12 h. The time requirement of control cells to enter S phase after serum stimulation was determined by BrdUrd pulse labeling of cells on parallel coverslips (Fig. 1C, bars). By 12 h 50% of control cells had entered S phase. Taking into account a delay of 30 min to allow for efficient protein synthesis following microinjection these data suggest that Mad1 was capable of inhibiting entry into S phase until late in G₁. This coincided with the activation of cyclin E-associated kinase activity whereas cyclin A-associated kinase activity appeared later (Fig. 1D). These findings indicate that Mad1 can no longer inhibit S phase once cyclin E/CDK2 is activated.

Cyclin E/CDK2 Antagonizes Mad1-dependent Inhibition of Proliferation—Next we addressed whether cyclin E/CDK2 can interfere with the ability of Mad1 to inhibit S phase progression. Coexpression of cyclin E/CDK2, but not other cyclin/CDK complexes, was able to overcome the Mad1-dependent block of S phase progression (Fig. 2A). The inability of cyclin D/CDK complexes to antagonize Mad1 function was corroborated by the finding that coexpression of E1A was not sufficient to overcome the Mad1 block (data not shown). This indicates that the retinoblastoma protein, which controls the restriction point, is not involved in this regulation (12). The cyclin E/CDK2 effect was dependent on kinase activity, since cyclin E/dominant negative CDK2 could not rescue (data not shown). To further evaluate the functional antagonism of Mad1 and cyclin E/CDK2 we performed colony formation assays. As for S phase progression cyclin E/CDK2 could overcome the Mad1-dependent inhibition of colony formation in SAOS2, U2OS, and NIH3T3 cells (Fig. 2, **B** and **C**, and data not shown). Thus in these different experimental settings cyclin E/CDK2 abolished Mad1-dependent inhibition of cell proliferation.

Cyclin E/CDK2 Interferes with Mad1-dependent Gene Repression—Two fundamentally different possibilities can be envisaged about how cyclin E/CDK2 antagonizes Mad1. First, cyclin E/CDK2 might directly affect the function of Mad1 and its associated repressor complex. Second, cyclin E/CDK2 might function downstream of Mad1. While the second possibility is

standard deviations of three independent experiments are displayed. **F**, HDAC1myc-tag and cyclin E were expressed in COS7 cells. Aliquots of F-buffer lysates were analyzed for expression of the two proteins. HDAC1myc tag was immunoprecipitated using mAb 9E10 and analyzed for associated cyclin E. **G**, HDAC1myc-tag, mSin3A, Mad1, cyclin E/CDK2 were expressed in COS7 cells as indicated. The complexes were immunoprecipitated with antibodies specific for Mad1 (C19) or for the myc-tag (9E10) and analyzed for coimmunoprecipitated proteins by Western blotting.

difficult to address due to the lack of known Mad1-regulated effectors (4), the first implies that cyclin E/CDK2 may interfere with Mad1-dependent repression of gene transcription. Indeed repression of a *cyclin D2*-reporter gene construct by Mad1 was abolished by cyclin E/CDK2 (Fig. 3A). This required CDK2 kinase activity, and similar to S phase progression, cyclin D and A complexes were inactive (Fig. 3B). One possible explanation for these findings is that cyclin E/CDK2 negatively regulates Mad1/Max DNA binding. However, neither of the two proteins was substrate of nor was DNA binding affected by this kinase (data not shown). Consistently repression by Gal-MadN35, a fusion protein of the Mad1 SID and the Gal4 DNA binding domain (13), and by Gal-Sin3 was also relieved by cyclin E/CDK2 (Fig. 3C). In contrast Gal-MadN35(Pro), which contains an inactivating mutation in the SID, did not repress and was not affected by cyclin E/CDK2 (Fig. 3C). Thus this kinase inhibits Mad1-dependent gene repression without directly phosphorylating Mad1 or Max.

Cyclin E/CDK2 Inhibits HDAC Activity—To address whether cyclin E/CDK2 affects the Mad-HDAC-repressor complex, a tagged version of HDAC1 was coexpressed with this kinase, and deacetylase activity was measured. Cyclin E/CDK2, but not cyclin D1/CDK4, resulted in a reduction of HDAC activity. In contrast coexpression of p27^{Kip1}, either with or without cyclin E/CDK2, or CDK2dn stimulated HDAC activity (Fig. 3D). Next we evaluated the effect of cyclin E/CDK2 on deacetylase activity *in vitro*. Immunoprecipitated, overexpressed HDAC activity, recruited through Mad1 or by using antibodies specific for the HDAC-associated tag, was inhibited upon phosphorylation by recombinant cyclin E/CDK2 (Fig. 3E). Together these findings suggest that cyclin E/CDK2 negatively regulates HDAC activity through phosphorylation of one or several components of the repressor complex. This is further supported by the observation that cyclin E interacted with the HDAC complex in coimmunoprecipitations (Fig. 3F) and that cyclin E bound directly to HDAC1 in glutathione *S*-transferase pull-down assays (data not shown).

In an effort to identify cyclin E/CDK2 substrates associated with the HDAC complex we performed kinase assays with recombinant cyclin E/CDK2 with immunoprecipitated repressor complex as substrate. We detected HDAC1 as substrate and identified Ser³⁴⁶ as the main *in vitro* cyclin E/CDK2 phosphorylation site. However, cell derived repressor complexes, with HDAC1 mutated at Ser³⁴⁶ to Ala, were still sensitive to cyclin E/CDK2 (data not shown). This indicated that HDAC1 is either not the critical substrate or that other proteins need to be phosphorylated within the repressor complex in addition to HDAC1 for the observed phenotype. Further studies are designed to address this issue.

The Mad1-Repressor Complex Is Sensitive to Cyclin E/CDK2—In addition to the reduction of HDAC activity in response to cyclin E/CDK2, we also observed that this kinase altered the interaction of the repressor complex with Mad1. HDAC1 was coimmunoprecipitated with Mad1 and vice versa. This interaction was substantially reduced when cyclin E/CDK2 was coexpressed (Fig. 3G). Since Mad1 is not phosphorylated by cyclin E/CDK2, mSin3 might serve as substrate that could explain the above described effect. Indeed this protein has several potential CDK phosphorylation sites. However, *in vitro* kinase assays identified mSin3 as a poor cyclin E/CDK2 substrate that was not further analyzed. Together these findings suggest that the activity of the Mad1-repressor complex is inhibited in response to cyclin E/CDK2 due to at least two distinct events. This includes loss of binding of the repressor complex to Mad1 and inhibition of HDAC activity.

Conclusions—Our findings have uncovered a functional inter-

action between cyclin E/CDK2, a component of the machinery that regulates the transition from the G₁ into the S phase of the cell cycle, with the Mad1-mSin3-HDAC repressor complex. We demonstrate that cyclin E/CDK2 overcomes the Mad1-induced block to S phase progression by interfering with the ability of Mad1 to repress genes. This is the result of a cyclin E/CDK2-dependent dismantling of the Mad1-mSin3A-HDAC1 repressor complex and of inhibition of the HDAC activity associated with the repressor complex. Other cyclin-dependent kinases did not influence the activities of Mad1 suggesting that cyclin E/CDK2 has a unique function in repressing the Mad1 repressor complex.

Cyclin E/CDK2 is essential for S phase progression in both RB-positive and RB-negative cells. This implies that this kinase has some critical substrates, in addition to RB, that control the transition from G₁ into S phase (14). In support cyclin E/CDK2 has been shown to bypass the requirement for E2F activity (15, 16). This suggests strongly that cyclin E/CDK2 regulates substrates that are rate-limiting for S phase entry, which are not targeted by cyclin D complexes. Some of these substrates, including the CDK inhibitor p27^{KIP1}, nucleophosmin/B23, Mps1, and CP110, have been identified in recent years and play important roles for various processes at the G₁ to S phase transition (17–20). Furthermore the cyclin E/CDK2 substrate NPAT is associated with the activation of histone gene transcription at the beginning of S phase (21). Our findings expand these observations by defining that the mSin3-HDAC repressor complex is targeted by cyclin E/CDK2 suggesting that additional as yet undefined substrates are involved in the regulation of gene transcription at the G₁ to S phase transition.

Acknowledgments—We thank R. Bernards, R. N. Eisenman, and C. Seiser for plasmids and E. Buerova and H. Burkhardt for excellent technical assistance. We are indebted to S. Gehring, M. Henriksson, and A. Sommer for many helpful discussions during the initial phase of this work.

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J. Biol. Chem. 2005, 280:15489-15492.

doi: 10.1074/jbc.C400611200 originally published online February 18, 2005

Access the most updated version of this article at doi: [10.1074/jbc.C400611200](https://doi.org/10.1074/jbc.C400611200)

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