WD repeat-containing mitotic checkpoint proteins act as transcriptional repressors during interphase

Young-Mee Yoon[a,b], Kwan-Hyuck Baek[a], Sook-Jung Jeong[a], Hyun-Jin Shin[a], Geun-Hyoung Ha[a], Ae-Hwa Jeon[c], Sang-Gu Hwang[b], Jang-Soo Chun[b], Chang-Woo Lee[a,*]

[a] *Research Institute, National Cancer Center, Goyang, Gyeonggi, Republic of Korea*
[b] *Department of Life Science, Gwangju Institute of Science and Technology, Gwangju, Republic of Korea*
[c] *Division of Molecular and Life Science, Pohang University of Science and Technology, Republic of Korea*

Received 23 June 2004; revised 20 July 2004; accepted 22 July 2004

Available online 26 August 2004

Edited by Gianni Cesareni

Abstract WD repeats are implicated in protein–protein interactions and regulate a wide variety of cellular functions, including chromatin remodeling and transcription. The WD repeats of the Bub3 and Cdc20 kinetochore proteins are important for the physical interactions of these proteins with Mad2 and BubR1 to yield a kinetochore protein complex capable of delaying anaphase by inhibiting ubiquitin ligation via the anaphase-promoting complex/cyclosome. Here, we show that Bub3 and Cdc20 form a complex with histone deacetylases; this interaction appears to confer transcriptional repressor activity in a heterologous DNA-binding context. In addition, inhibition of Bub3 and Cdc20 expression significantly impairs interphase cell cycle. These results indicate that Bub3 and Cdc20 play additional roles in the integration of cell cycle arrest as transcriptional repressors.

Keywords: Mitotic checkpoint protein; WD repeat; Transcriptional repression; Histone deacetylase; Cell cycle arrest

1. Introduction

The WD repeats are a conserved sequence motif usually ending with Trp-Asp (WD), and WD repeat proteins are found in all eukaryotes and have been implicated in a wide variety of crucial functions, including gene expression, RNA processing, signal transduction and cell division [1,2]. All WD repeat proteins are thought to fold into propeller-shaped structures in which the internal β-strands form a rigid skeleton that is fleshed out on the surface by specialized loops responsible for binding other proteins. A number of studies have revealed that many WD repeat-containing proteins interact with histone deacetylases (HDACs) and function as transcriptional repressors to regulate the expression of genes involved in the cell cycle and chromatin assembly [2,3]. For instance, chromatin assembly factor-1 (CAF-1), which is essential for chromatin assembly, interacts with HDAC2 through its WD repeat [2]. In addition, overexpression of the WD repeat-containing TATA-binding protein-associated factors, TAF72 or TAF73, suppresses mitotic cell cycle arrest through regulation of anaphase-promoting complex/cyclosome (APC/C)-mediated ubiquitination [3].

The mitotic checkpoint ensures accurate chromosome segregation by sensing unaligned chromosomes and delaying anaphase until proper alignment is achieved; defects in this checkpoint mechanism result in the chromosomal instability implicated in tumorigenesis. Components of the mitotic checkpoint machinery include Bub1, Bub3, BubR1 (a homolog of yeast Mad3), Mad1, Mad2, Mad3, Mps1, and CENP-E (a microtubule-dependent motor protein) [4–7]. When the spindle checkpoint is activated at unattached kinetochores, the mitotic checkpoint proteins (MCPs) BubR1, Bub3 and Mad2 bind to and inhibit Cdc20, an activator of the APC/C, and then produce a diffusible "wait anaphase" signal that inhibits the APC and delays the onset of anaphase [4–7]. Fractionation experiments have suggested that BubR1, Bub3 and Mad2 may be concomitantly present in a protein complex with Cdc20; formation of the Bub3–Cdc20 complex is mediated via WD repeats [8]. Bub3 protein levels are constant throughout the cell cycle, whereas Cdc20 protein accumulates during mitosis and is degraded to almost undetectable levels prior to cytokinesis. Although the levels of the Bub3–Cdc20 complex vary during the cell cycle, these variations do not precisely reflect the periodicity of the Cdc20 protein levels. In addition, it has been suggested that a pool of free Cdc20 exists separate from the Bub3 complex even during checkpoint activation [9]. It is interesting to note that Cdc20 associates with different proteins at different subcellular locations during the cell cycle; a dramatic change occurs in the patterns of protein association during the transitions from G2 to M and M to G1 [8–10]. Taken together, these observations suggest that the mitotic checkpoint proteins may play additional, unexplored roles in interphase cells. Of these, Bub3 and Cdc20 are good candidates to mediate protein complex assembly during the cell cycle, because these proteins contain WD repeats, which have been strongly implicated in protein–protein interactions.

Here, we investigated the role of these MCPs in interphase cells. Cell cycle analysis of siRNA-transfected cells revealed that inhibition of endogenous Bub3 or Cdc20 significantly reduced the proportion of S and G2/M cells, respectively, suggesting that Bub3 and Cdc20 may be involved in mediating the G2 and G1 phases, respectively. Furthermore, we found that WD repeat-containing Bub3 and Cdc20 are present in a

* Corresponding author. Fax: +82-31-920-1520.
E-mail address: cwlee@ncc.re.kr (C.-W. Lee).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.089

complex with HDAC1 and HDAC2, and function as transcriptional repressors in the DNA-binding context. This is the first report of a role for Bub3 and Cdc20 in the integration of cell cycle arrest during interphase.

2. Materials and methods

2.1. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Hyclone) and antibiotics. For cell cycle analysis, MRC-5 primary cells were cultured in minimum essential medium (MEM) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10% FBS and antibiotics.

2.2. siRNA synthesis

The following gene-specific sequences were used to generate siRNAs (Dharmacon): siBub3 5′-AGCGACUGUGCCAAUUCCA-3′; siMad2 5′-GGAAGAGUCGGACCACAG-3′; siCdc20 5′-CGGCAGGACUCCGGGCCGA-3′ [11]; siCdh1 5′-UGAGAAGUCUCCCAGUCAG-3′ [11]; and siLuciferase (as a control) 5′-CGT ACG CGG AAT ACT TCG A-3′ [12].

2.3. Plasmids and transfection

cDNAs of Bub1, Bub3, Mad1, Mad2, BubR1, Cdc20 and Cdh1 were amplified by PCR using templates generated by reverse transcription from HeLa cell total mRNA, with oligo-dT as a primer. PCR products were then cloned into the pGEX-KG to generate GST-Bub1, -Bub3, -Mad1, -Mad2, -BubR1, -Cdc20 and -Cdh1, respectively. The plasmids expressing Gal4 DNA-binding domain and the MCP fusion proteins, Bub1, Bub3, Mad1, Mad2, BubR1, Cdc20 or Cdh1, were constructed by insertion of each MCP open reading frame into the pSG424 vector [13]. To create the Gal4-fused Bub3 or Cdc20 deletion mutants, each DNA fragments of Bub3 or Cdc20 as indicated in Fig. 4A and B were amplified by PCR and subsequently inserted into the pSG424 vector. Gal4-Rb was described previously [14].

MRC-5 primary cells were electroporated with siRNA. Briefly, subconfluent monolayers of MRC-5 primary cells were detached from the culture dishes by trypsin treatment, washed with phosphate-buffered saline, and adjusted to a concentration of 2×10^6 cells per ml in complete medium. Then, 300 μl of the cell suspension (6×10^5 cells) was mixed with 20 μg siRNA by gentle pipetting, transferred to a 0.4-cm width electroporation cuvette (Bio-Rad), and subjected to an electric pulse at 900 μF and 220 V using a Gene Pulser (Bio-Rad). Cells were immediately transferred to 8 ml of complete media and 4 ml samples were seeded into 6-cm-diameter cell culture dishes.

2.4. Flow cytometry and immunoblotting

MRC-5 cells were harvested 24 and 48 h after siRNA electroporation for flow cytometry and immunoblotting. Cells were resuspended in 400 μl of cold PBS, fixed by dropwise addition of 360 μl cold 100% ethanol with gentle vortexing, and then placed at −20 °C. Fixed cells were washed in PBS, stained with propidium iodide (40 μg/ml) and treated with RNase A (50 μg/ml) for 30 min at room temperature. Samples of 10 000 cells were analyzed for DNA content. Data were generated with the CellQuest software (Becton–Dickinson).

Immunoblotting was performed to confirm the siRNA knock-down effect. Whole cell lysates of MRC-5 cells were prepared in lysis buffer (20 mM HEPES, pH 7.6, 250 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 20% Glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol (DTT), protease inhibitor cocktail (PIC, Roche) and 200 μM PMSF). Equal amounts of protein quantitated by Bradford assay (Bio-Rad) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. For immunoblot analysis, blots were incubated with anti-Cdh1, anti-Cdc20 or anti-Bub3 antibodies, with anti-Actin antibody used as an internal control.

2.5. Pull-down assay and immunoprecipitation

GST-Bub1, -Bub3, -Mad1, -Mad2, -BubR1, -Cdc20 and -Cdh1 encode a complete Bub1, Bub3, Mad1, Mad2, BubR1, Cdc20 and Cdh1 proteins, respectively, fused to GST in pGEX-KG and were induced and purified by conventional procedures. Fusion proteins bound to glutathione–agarose beads were incubated with 600 μg/ml extracts prepared from untreated and nocodazole treated HeLa cells for 4 h at 4 °C.

For immunoprecipitation, cell pellets from HeLa cells were first lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), protease inhibitor cocktail (PIC, Roche) and 200 μM PMSF) for 5 min on ice and centrifuged at 2000 rpm for 3 min. Then, the pellets were lysed in IPH buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA and protease inhibitor cocktail (Roche)). The protein concentration of the lysates was determined by Bradford assay (Bio-Rad). Lysates were incubated for 2 h at 4 °C with Protein-G or -A–agarose (Amersham) with immunoglobulin (IgG, Santa Cruz), to clear the lysates. The cleared lysates were then incubated overnight at 4 °C with anti-HDAC1 (Santa Cruz), anti-HDAC2 (Santa Cruz), anti-Cdc20 (Santa Cruz), anti-Cdh1 (Oncogene), anti-Bub3 (Pharmingen), anti-Mad2 (Pharmingen) or anti-pRb (Santa Cruz). The beads were then incubated for 2 h at 4 °C, and the bound proteins were eluted with 2× SDS loading buffer and analyzed by immunoblotting with the appropriate antibody.

2.6. Gene reporter assays

For luciferase assay, cell extracts were prepared in reporter lysis buffer (Promega). After removing cell debris, extracts were assayed for luciferase activity by mixing 300 μl luciferase reagent (Promega) with 60 μl cell extracts and measuring the activity with luminometer. β-Galactosidase assays were performed as described previously [15].

3. Results and discussion

3.1. WD repeat-containing Bub3 and Cdc20 are potentially involved in the regulation of cell cycle arrest during interphase

It has been well documented that mitotic checkpoint proteins such as Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3/Bub1-related kinase) and Cdc20 are localized at kinetochores. In addition, these proteins are essential for cellular progression through mitosis, as they are necessary for APC/C-mediated activation of the mitotic checkpoint [16–18]. However, these MCPs are found in a multiprotein complex even in interphase cells, suggesting that the MCP complex might additionally function at other points during the cell cycle. Interestingly, WD repeats are important in the formation of the Bub3–Mad2–Mad3–Cdc20 complex and have been strongly implicated in protein–protein interactions (Fig. 1A) [8]. In an effort to begin investigating the role of mitotic checkpoint proteins during interphase, we transfected various siRNAs designed to cause posttranscriptional gene silencing of Mad2, Bub3, BubR1, Cdc20 and Cdh1. The specificity of these siRNAs was confirmed by our observation that endogenous Mad2, Bub3, BubR1, Cdc20 and Cdh1 protein levels were reduced in siRNA-transfected cells, whereas the control luciferase siRNA had no effect on these protein levels (Fig. 1B). To examine the effect of siRNA-induced Mad2, Bub3, BubR1, Cdc20 and Cdh1 knock-down on cell cycle progression, MRC-5 cells transfected with each siRNA were harvested for flow cytometry analysis, and analyzed for the proportion of transfected cells in the G1, S and G2/M phases (Figs. 1C and D). Cells transfected with the Mad2 or BubR1 siRNA showed little difference from control cells transfected with luciferase siRNA in terms of cell cycle profile. Cells transfected with Cdh1 showed slightly increased proportions of cells in S phase, an observation that is consistent with a previous report demonstrating that Cdh1 was required for G1 cell cycle arrest [17,19]. In contrast, cells transfected with Bub3 or Cdc20 siRNA showed significant increases in the G1 and G2/M or S phase

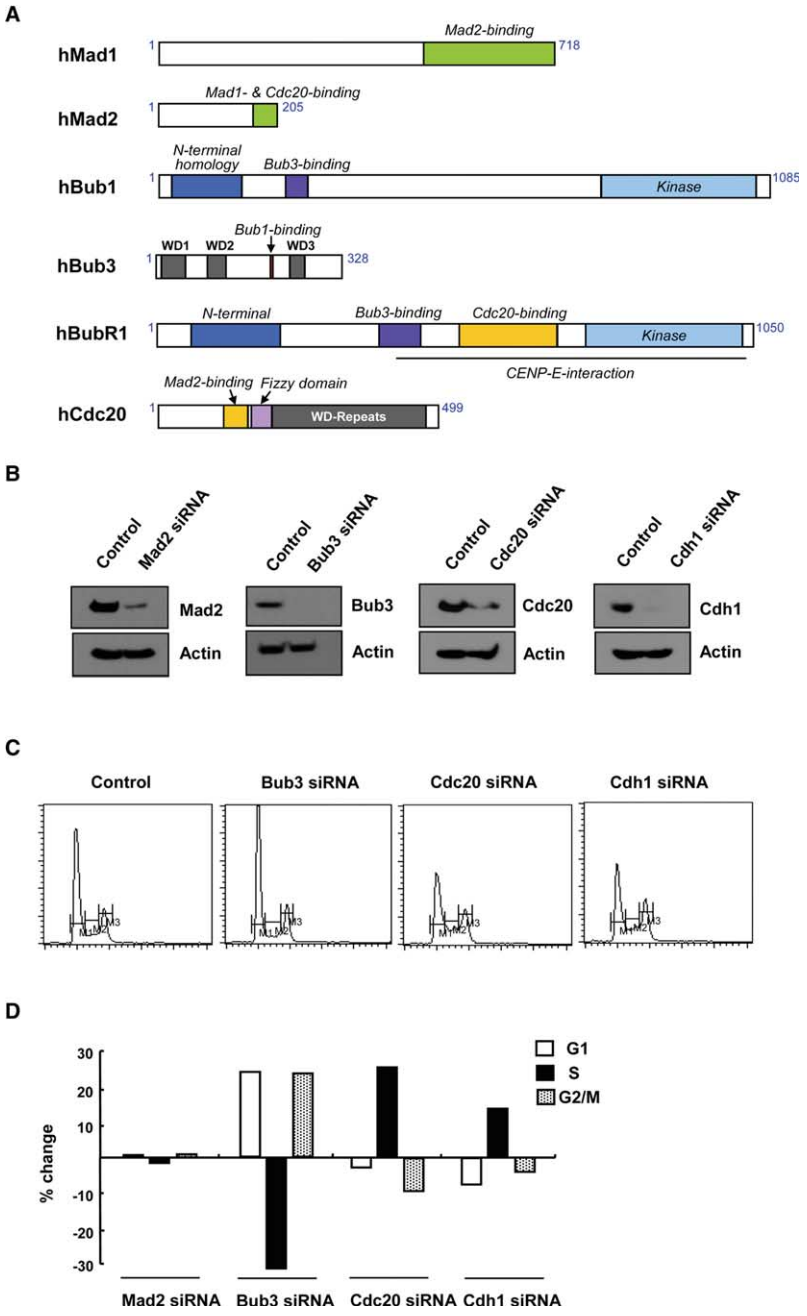

Fig. 1. Inhibition of Bub3 and Cdc20 expression significantly affects cell cycle arrest in G2 and G1 phases, respectively. (A) Schematic summary of MCP domains: The functional domains found in the various MCPs include the Bub, Mad or Cdc20-interaction domain, the kinase domain, the N-terminal homology domain, and the WD repeat region. (B) MRC-5 cells were transiently transfected with one of the following: control luciferase siRNA, Mad2 siRNA, Bub3 siRNA, Cdc20 siRNA or Cdh1 siRNA. Transfected cells were harvested and analyzed by immunoblotting with anti-Mad2, anti-Bub3, anti-Cdc20, anti-Cdh1 or anti-Actin antibodies. (C and D) Quantitative comparison of cell cycle arrest in MRC-5 cells treated with siRNA as described in Section 2. siRNA-transfected cells were harvested, stained with propidium iodide and analyzed by flow cytometry. Relative proportions of G1, S and G2/M phase cells by the different siRNA treatments were derived from the flow cytometry. M1, M2 and M3 indicate G1, S and G2/M populations of cells, respectively.

populations, respectively, with corresponding reductions in the S or G2/M phase populations. Together, these results suggest that the WD repeat-containing proteins Bub3 and Cdc20 play roles in cell cycle arrest during interphase.

3.2. MCP/Gal4 DNA-binding domain fusion proteins show transcriptional repression activity

Recent studies have demonstrated that some WD repeat proteins function as transcriptional repressors to regulate genes involved in the cell cycle and chromatin assembly [1–3]. To test the role of WD repeat-containing MCPs in transcriptional repression, we fused these proteins with the Gal4 DNA-binding domain (Gal4) to generate Gal4-Bub1, Gal4-Bub3, Gal4-Mad1, Gal4-Mad2, Gal4-BubR1, Gal4-Cdc20 and Gal4-Rb fusion proteins (Fig. 2A). Expression of the chimeric proteins in transfected cells was verified by immunoblotting analysis using an anti-Gal4 antibody (data not shown). HepG2 (lanes 1–6), HeLa S3 (lanes 7–12) and

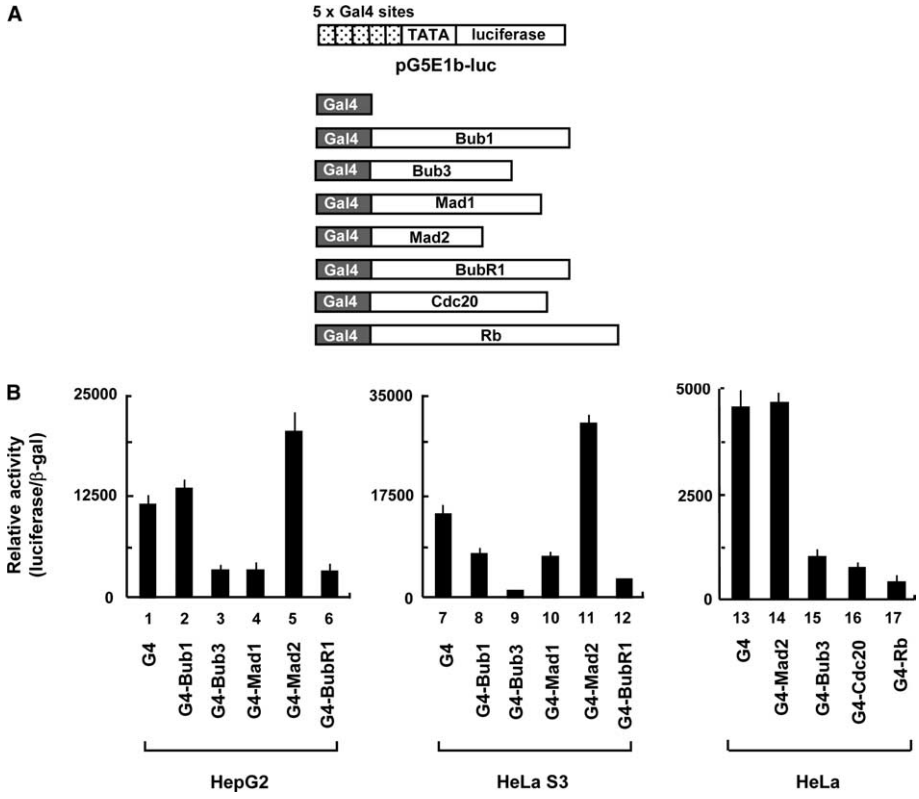

Fig. 2. Transcriptional repression activity of mitotic checkpoint proteins artificially recruited to DNA by means of the Gal4 DNA-binding domain. (A) Schematic of Gal4 (G4)-fused MCPs. (B) The Gal4 reporter, G5E1b-luciferase (1 μg), was co-transfected into HepG2 (lanes 1–6), HeLaS3 (lanes 7–12) or HeLa (lanes 13–17) cells together with G4 expression vectors (5 μg) encoding the Gal4 DNA-binding domain or the MCP fusion proteins Bub1, Bub3, Mad1, Mad2, BubR1 or Cdc20, or Rb (as a control). pCMV-βgal (1 μg) was included in each transfection as an internal control. Cells were assayed for luciferase and β-galactosidase activity 40 h post-transfection. The relative activity of luciferase to β-galactosidase is presented. The results represent the averages of at least three independent experiments.

HeLa (lanes 13–17) cells were transfected with a luciferase reporter plasmid controlled by five copies of the Gal4-binding site and the HSV tk promoter (pG5E1b-luc), together with Gal4-Bub1, Gal4-Bub3, Gal4-Mad1, Gal4-Mad2, Gal4-BubR1, Gal4-Cdc20 or Gal4-Rb (as a control). Gal4-Bub3 and Gal4-Cdc20 efficiently repressed transcriptional activity in all three tested cell lines (Fig. 2B). Unexpectedly, expression of Gal4-BubR1 and Gal4-Mad1 (to a lesser degree) also led to significant reduction of reporter gene activity (Fig. 2B). Furthermore, MCPs including BubR1 and Mad1 did not repress transcription in the absence of a heterologous DNA-binding context (data not shown), and thereby circumvent potential artifacts arising from non-specificity for the repression. In addition, we obtained similar results when these experiments were repeated in different DNA-binding contexts, such as the use of the E2F-1 DNA-binding domain instead of the Gal4 DNA-binding domain (data not shown). Taken together, these data suggest that MCPs, but not all of them, might be involved in transcriptional repression.

3.3. *Bub3 and Cdc20 may interact directly with HDAC1 and HDAC2*

The above results demonstrated that some MCPs may act as transcriptional repressors in a heterologous DNA-binding context. To examine this further, we next tested whether there is a direct interaction between HDACs and MCPs by in vitro co-immunoprecipitation and pull-down assays. *Escherichia coli*-expressed GST-Bub1, -Bub3, -Mad1, -Mad2, -BubR1, -Cdc20 and -Cdh1 fusion proteins were purified and incubated with lysates from HeLa cells cultured in the absence or presence of nocodazole (Fig. 3A). GST-Bub3, -BubR1, -Cdc20 and -Cdh1 exhibited a clear binding activity toward the HDAC1 protein, as tested by immunoblotting with an anti-HDAC1 antibody. GST-Bub1 and -Mad1 showed very weak interactions with HDAC1, whereas GST and GST-Mad2 showed no binding under the same conditions. These interactions were not influenced by nocodazole treatment. Anti-HDAC2 immunoblotting showed that HDAC2 interacted with GST-Bub3, -Mad1, -Cdc20 and -Cdh1 but not with GST-Bub1, -Mad2 or -BubR1 (Fig. 3A). Interestingly, these results appeared to parallel the transcriptional repression activity of these constructs, as presented in Fig. 2B. To further verify the data of our pull-down assays, we performed a co-immunoprecipitation experiment. Unlikely our pull-down results, HDAC1 and HDAC2 appeared to only interact with Bub3, whereas under the same experiment conditions, we could not detect binding of Mad2, BubR1 or Mad1 to HDAC1 or HDAC2, even when positive control immunoprecipitations showed clear recognition of Mad2, BubR1 and Mad1 proteins by the employed antibodies (Fig. 3B). Our failure to detect HDAC complexes containing BubR1 or Mad1 in the co-immunoprecipitations may indicate that these protein complexes could be formed by weak indirect protein–protein interactions,

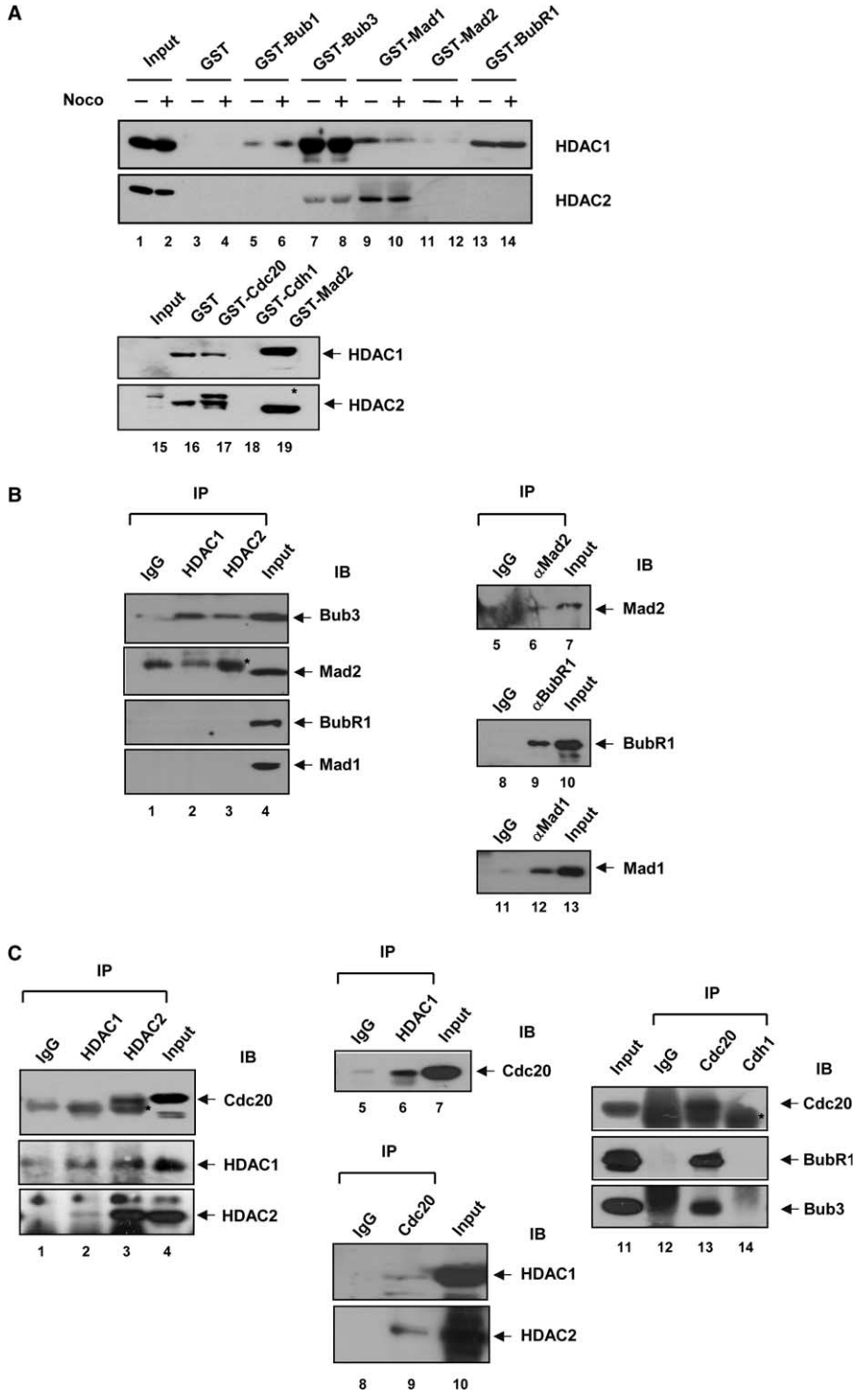

Fig. 3. WD repeat-containing Bub3 and Cdc20 are present in a complex with HDAC1 and HDAC2. (A) In vitro binding assay between HDAC1 or HDAC2 and GST fusion proteins encoding Bub1, Bub3, Mad1, Mad2, BubR1, Cdc20 and Cdh1. The indicated GST fusion proteins were incubated with extracts prepared from untreated (−) and nocodazole (Noco)-treated (+) HeLa cells. After incubation, immunoblotting was performed with either anti-HDAC1 (upper) or anti-HDAC2 (lower) antibodies. Lanes 1, 2 and 15 show the input extract (5%). (B) Co-immunoprecipitation of endogenous HDACs and mitotic checkpoint proteins from HeLa cell extracts was performed using anti-IgG (negative control), anti-HDAC1 or anti-HDAC2 followed by immunoblotting with anti-Bub3, anti-Mad2, anti-BubR1 or anti-Mad1 antibodies (left panels). As a control, immunoprecipitation was further performed with anti-IgG, anti-Mad2, anti-BubR1 or anti-Mad1 followed by immunoblotting with anti-Mad2 (lanes 5–7), anti-BubR1 (lanes 8–10) or anti-Mad1 (lanes 11–13) as indicated (right panels). (C) Co-immunoprecipitation of endogenous HDACs and Cdc20 from HeLa cell extracts was performed using anti-IgG (negative control), anti-HDAC1, anti-HDAC2 (lanes 1–4, upper and lanes 5–7) or anti-Cdc20 (lanes 8–10) followed by immunoblotting with antibodies as indicated. To normalize these immunoprecipitation conditions, cell extracts were taken at the same time as used above, and anti-Cdc20 (positive control) or anti-Cdh1 (negative control) immunoprecipitations were confirmed by immunoblotting with anti-Cdc20, anti-BubR1 and anti-Bub3 antibodies, respectively (lanes 11–14).

may compete with other proteins for HDAC, or may involve other HDAC family members. However, these points remain to be further addressed. Next, immunoprecipitation with anti-HDAC1 or anti-HDAC2 antibodies followed by immunoblotting with anti-Cdc20 antibody revealed that Cdc20 significantly interacted with HDAC1 and HDAC2 (Fig. 3C, lanes 1–7). We observed the similar result when the immunoprecipitation was performed with anti-Cdc20 and subsequent immunoblotting with anti-HDAC1 or anti-HDAC2 antibody, confirming that Cdc20 makes a complex with HDAC protiens (Fig. 3C, lanes 8–10). In a positive control experiment, we confirmed that under these experimental conditions, Cdc20 can complex with its well-defined interacting proteins, BubR1 and Bub3. The negative control confirmed that Cdh1 was not co-immunoprecipitated with these proteins. Thus, our data indicate that Bub3 and Cdc20 interact with HDACs and appear to be involved in the regulation of transcriptional repression. One interesting question remaining to be addressed is whether the interactions of Bub3 and Cdc20 with HDACs are dependent on the damage checkpoint for induction of G1 and/or G2 arrest. However, the observation that the Bub3–HDAC and Cdc20–HDAC complexes are constitutively present during interphase seems to suggest that these complexes are involved in normal cell cycle control throughout G1 and G2.

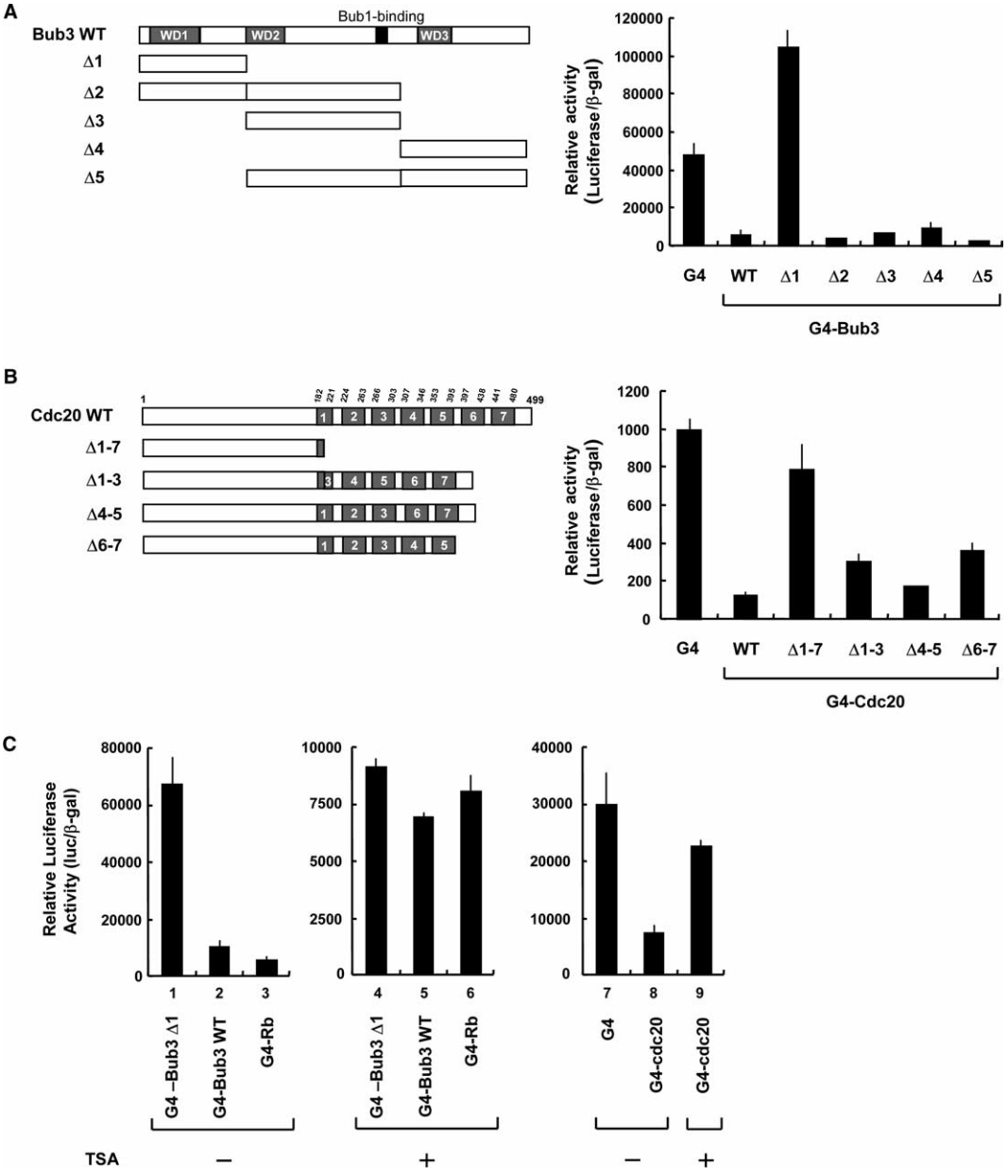

Fig. 4. The WD repeats of Bub3 and Cdc20 are responsible for transcriptional repression activity. (A and B) Schematic representations of Bub3 (A) and Cdc20 (B), with the WD repeats indicated as a shaded box. Gal4-fused Bub3 or Cdc20 deletion mutants (5 μg) were transfected together with the Gal4 reporter G5E1b-luciferase (1 μg) into HeLa cells. pCMV-βgal (1 μg) was included in each transfection as an internal control. Cells were assayed for luciferase and β-galactosidase activity 40 h post-transfection. The results shown represent the averages from at least three independent experiments. (C) Histone deacetylases participate in transcriptional repression of Bub3 and Cdc20 proteins. The Gal4 reporter G5E1b-luciferase (1 μg) was transfected into HeLa cells with G4-Bub3Δ1, G4-Bub3WT, G4-Rb or G4-Cdc20. Sixteen hours post-transfection, cells were incubated in the absence (−) or presence (+) of the histone deacetylase inhibitor, trichostatin A (TSA), for a further 24 h. The relative activity of luciferase to β-galactosidase is presented.

3.4. The WD repeats of Bub3 and Cdc20 are responsible for their transcriptional repression activity

WD repeats have been widely implicated in substrate-specific protein–protein interactions. Bub3 and Cdc20 contain three and seven WD repeats, respectively, based on their amino acid sequence similarities to the known WD repeat proteins. To examine the dependence of the observed transcriptional repression activity on these repeats, we constructed a series of Bub3 (Fig. 4A) and Cdc20 (Fig. 4B) WD repeat deletion mutants and studied their transcriptional repression activities using a luciferase reporter assay. Interestingly, Bub3 Δ2, Δ3, Δ4 and Δ5 mutants, which all contain repeats number WD2 and/or WD3, efficiently repressed the transcriptional activity to a similar degree as Bub3 WT (wild type), whereas Bub3 Δ1, which possessed only WD1, showed in the complete abrogation of repression activity (Fig. 4A). These results indicated that repeats WD2 and WD3 are involved in the transcriptional repression activity of the Bub3 protein. Using the same approach, we investigated the WD region in Cdc20 and its effects on transcriptional repression. Interestingly, the complete deletion of Cdc20 WD repeats Δ1–7 showed the almost loss of repression activity. However, although Cdc20 deletion mutants Δ1–3, Δ4–5 and Δ6–7 showed the repression activity, the activity was noticeably lower than that of Cdc20 WT, suggesting that a broad region of Cdc20 WD repeats is involved in transcriptional repression.

Next, we examined whether the transcriptional repression of WD repeat-containing Bub3 and Cdc20 is mediated by their interaction with HDAC, by assessing the ability of a histone deacetylase inhibitor, trichostatin A (TSA), to inhibit the observed transcriptional repression. HeLa cells were transfected with G4-Bub3 Δ1, G4-Bub3 WT or G4-Rb (as a control) and cultured in the absence or presence of TSA (Fig. 4C, lanes 1–6). The transcriptional repression activity of G4-Bub3 WT and G4-Rb was significantly abrogated by TSA treatment. In contrast, the activity of transcriptional repression-incompetent G4-Bub3 Δ1 was not influenced by TSA. Similarly, in the presence of TSA, the repression of G4-Cdc20 was almost completely abolished (Fig. 4C, lanes 7–9). Together, these results indicate that Bub3 and Cdc20 require histone deacetylase activity for their involvement in the process of transcriptional repression during interphase.

A series of studies have reported that the direct stochiometric binding of sets of MCPs containing BubR1, Bub3, Mad2, Cdc20 or subsets of these proteins is responsible for the inhibition of APC/C-Cdc20 [16–18]. These proteins are dissociated from intact kinetochores when cells exit from mitotic arrest; during interphase, they may be abundantly present as high levels of the complex, as basal levels of the complex, or in pools of free MCPs. Recent studies have suggested that Cdc20 (and possibly other MCPs) associates with different proteins at different subcellular locations during the cell cycle [8,9,20,21]. Recent important data by Sanchez and colleagues suggested that Cdc20 is phosphorylated on PKA consensus sites after DNA damage, and the phosphorylation regulates Cdc20 interaction with its substrates such as Clb2. In addition, they reported that the levels of Cdc20 were high in mitosis and declined rapidly as the cells completed mitosis. However, in cells with a DNA damage signal, Cdc20 levels remained high throughout the pre-anaphase arrest [21]. These observations strongly raise the possibility that MCPs may be also involved in the harmonized interphase cell cycle. Interestingly, we have recently verified that BubR1 induces apoptotic death of cells arrested at G1 and G2 after exposure to DNA damaging agents and irradiation, respectively, indicating that BubR1 is not only a sensor for monitoring the mitotic checkpoint, but it also plays an additional role during interphase [22]. Moreover, it has been well documented that another WD repeat protein, Cdh1, activates APC/C to target mitotic cyclins from the end of mitosis to the G1 phase. Recently, Saya and colleagues [19] reported that Cdh1 plays multiple roles during interphase, including mediation of Cdk inhibitor-dependent G1 arrest and DNA damage-induced G2 arrest.

Although it is not yet clear whether the WD repeat-containing mitotic checkpoint proteins Bub3 and Cdc20 directly bind DNA for regulation of transcriptional repression, this study shows for the first time that Bub3 and Cdc20 play important roles in the control of interphase cell cycle, and this function appears to be mediated by their interaction with HDAC.

Acknowledgements: We thank Dr. Frank McKeon for providing materials. This work was supported by research grant from National Cancer Center (0210100-3).

References

[1] Smith, T.F., Gaitatzes, C., Saxena, K. and Neer, E.J. (1999) Trends Biochem. Sci. 24, 181–185.
[2] Ahmad, A., Takami, Y. and Nakayama, T. (1999) J. Biol. Chem. 274, 16646–16653.
[3] Mitsuzawa, H., Seino, H., Yamao, F. and Ishihama, A. (2001) J. Biol. Chem. 276, 17117–17124.
[4] Taylor, S.S. and McKeon, F. (1997) Cell 89, 727–735.
[5] Waters, J.C., Chen, R.-H., Murray, A.W. and Salmon, E.D. (1998) J. Cell Biol. 141, 1181–1191.
[6] Chan, G.K.T., Jablonski, S.A., Sudakin, V., Hittle, J.C. and Yen, T.J. (1999) J. Cell Biol. 146, 941–954.
[7] Abrieu, A., Kahana, J.A., Wood, K.W. and Cleveland, D.W. (2000) Cell 102, 817–826.
[8] Fraschini, R., Beretta, A., Sironi, L., Musacchio, A., Lucchini, G. and Piatti, S. (2001) EMBO J 20, 6648–6659.
[9] Harper, J.W., Burton, J.L. and Solomon, M.J. (2002) Genes Dev. 16, 2179–2206.
[10] Weinstein, J. (1997) J. Biol. Chem. 272, 28501–28511.
[11] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296, 550–553.
[12] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[13] Lee, C.W., Sorensen, T.S., Shikama, N. and La Thangue, N.B. (1998) Oncogene 16, 2695–2710.
[14] Luo, R.X., Postigo, A.A. and Dean, D.C. (1998) Cell 92, 463–473.
[15] Jooss, K., Lam, E.W., Bybee, A., Girling, R., Muller, R. and La Thangue, N.B. (1995) Oncogene 10, 1529–1536.
[16] Musacchio, A. and Hardwick, K.G. (2002) Nat. Rev. Mol. Cell Biol. 3, 731–741.
[17] Peters, J.M. (2002) Mol. Cell 9, 931–943.
[18] Yu, H. (2002) Curr. Opin. Cell Biol. 14, 706–714.
[19] Sudo, T., Ota, Y., Kotani, S., Nakao, M., Takami, Y., Takeda, S. and Saya, H. (2001) EMBO J. 20, 6499–6508.
[20] Camasses, A., Bogdanova, A., Shevchenko, A. and Zachariae, W. (2003) Mol. Cell 12, 87–100.
[21] Searle, J.S., Schollaert, K.L., Wilkins, B.J. and Sanchez, Y. (2004) Nat. Cell Biol. 6, 138–145.
[22] Shin, H.J., Baek, K.H., Jeon, A.H., Park, M.T., Lee, S.J., Kang, C.M., Lee, H.S., Yoo, S.H., Chung, D.H., Sung, Y.C., McKeon, F. and Lee, C.W. (2003) Cancer Cell 4, 483–497.