Human BUBR1 Is a Mitotic Checkpoint Kinase that Monitors CENP-E Functions at Kinetochores and Binds the Cyclosome/APC

G.K.T. Chan, S.A. Jablonski, V. Sudakin, J.C. Hittle, and T.J. Yen
Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

Abstract. Human cells express two kinases that are related to the yeast mitotic checkpoint kinase BUB1. hBUB1 and hBUBR1 bind to kinetochores where they are postulated to be components of the mitotic checkpoint that monitors kinetochore activities to determine if chromosomes have achieved alignment at the spindle equator (Jablonski, S.A., G.K.T. Chan, C.A. Cooke, W.C. Earnshaw, and T.J. Yen. 1998. Chromosoma. 107: 386–396). In support of this, hBUB1 and the homologous mouse BUB1 have been shown to be important for the mitotic checkpoint (Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggins, J.K. Willson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Nature 392:300–303; Taylor, S.S., and F. McKeon. 1997. Cell. 89:727–735). We now demonstrate that hBUBR1 is also an essential component of the mitotic checkpoint. hBUBR1 is required by cells that are exposed to microtubule inhibitors to arrest in mitosis. Additionally, hBUBR1 is essential for normal mitotic progression as it prevents cells from prematurely entering anaphase. We establish that one of hBUBR1’s checkpoint functions is to monitor kinetochore activities that depend on the kinetochore motor CENP-E. hBUBR1 is expressed throughout the cell cycle, but its kinase activity is detected after cells have entered mitosis. hBUBR1 kinase activity was rapidly stimulated when the spindle was disrupted in mitotic cells. Finally, hBUBR1 was associated with the cyclosome/anaphase-promoting complex (APC) in mitotically arrested cells but not in interphase cells. The combined data indicate that hBUBR1 can potentially provide two checkpoint functions by monitoring CENP-E-dependent activities at the kinetochore and regulating cyclosome/APC activity.

Key words: kinetochore • hBUBR1 • mitotic checkpoint • anaphase-promoting complex • CENP-E

Accurate chromosome segregation relies on the interactions between the kinetochore and microtubules of the spindle. The process by which kinetochores capture a microtubule is stochastic in nature, a chance encounter, which is prone to error (Nicklas, 1997). Thus, it is critical that cells are able to delay exit from mitosis until all chromosomes have aligned at the spindle equator. The mitotic checkpoint is able to determine whether chromosomes have aligned by monitoring the kinetochore, a structure that mediates attachment of the chromosome to microtubules and is directly responsible for chromosome motility (Inoue and Salmon, 1995; Yen and Schaar, 1996; Rieder and Salmon, 1998). The checkpoint is sensitive to the presence of even a single unattached kinetochore, as this is sufficient to delay cells from exiting mitosis (Rieder et al., 1995). The precise biochemical mechanism by which an unattached kinetochore blocks entry into anaphase remains undefined, but it appears to be sensitive to kinetochore microtubule attachments (Rieder et al., 1994) and to the level of tension that is exerted on the kinetochore (Li and Nicklas, 1995).

Molecular insights into the mitotic checkpoint have come from the identification of seven genes in budding yeast that are essential for the mitotic checkpoint. In the presence of spindle or kinetochore defects, the MPS1 kinase (Weiss and Winey, 1996) and the BUB1/BUB3 kinase complex (Hoyt et al., 1991; Roberts et al., 1994), along with MAD1, MAD2, MAD3, and BUB2, are required to block cells in mitosis (Li and Murray, 1991; Hardwick and Murray, 1995). The mitotic checkpoint appears to be highly conserved throughout evolution, as homologues of many of the yeast checkpoint proteins have also been identified in Drosophila and vertebrates. A nalysis of MAD1 (Chen et al., 1998; Jin et al., 1998), MAD2 (Chen et al., 1996; Li and Benezra, 1996), BUB1, BUBR1, and BUB3 (Taylor and McKeon, 1997; Basu et al., 1998; Cahill et al., 1998; Chan et al., 1998; Jablonski et al., 1998; Taylor et al., 1998) in higher eukaryotes show that these proteins accumulate at kinetochores where they are postulated to monitor kinetochore functions and participate in generating the anaphase signal. Consistent with this possibil-
ity, unattached kinetochores exhibited a higher level of some of these checkpoint proteins than kinetochores that were aligned at the spindle equator. Functional studies have shown that MAD1 and MAD2 are essential components of the mitotic checkpoint in vertebrate cells and in cycling Xenopus egg extracts (Chen et al., 1996; Li and Benezra, 1996; Waters et al., 1998). Similarly, mouse BUB1 (mBUB1) has also been shown to be essential for the mitotic checkpoint (Taylor and McKeon, 1997). The target of the mitotic checkpoint in both yeast and vertebrates is the cyclosome/anaphase-promoting complex (APC),1 a multisubunit E3 ubiquitin-ligase that specifies the proteolytic destruction of specific proteins to initiate the onset of anaphase (Sudakin et al., 1995; King et al., 1996; Hershko and Ciechanover, 1998). MAD2 was found to interact with the cyclosome/APC in mitotically arrested cells and inhibit its ubiquitination activity in vitro and in vivo (Li et al., 1997; Chen et al., 1998; Fang et al., 1998; Gorbsky et al., 1998). Genetic and biochemical studies have shown that the association between MAD2 and the cyclosome/APC is mediated by p55CDC/cdc20 (Fang et al., 1998; Huang et al., 1998; Kallio et al., 1998; Kim et al., 1998), an evolutionarily conserved protein that is essential for the metaphase–anaphase transition (Dawson et al., 1995; Visintin et al., 1997; Kallio, 1998). The mechanism by which unaligned chromosomes specify the inhibition of the cyclosome/APC by MAD2 is unclear, but a tentative model suggests that unattached kinetochores serve to convert MAD2 into an inhibitor of the cyclosome/APC (Chen et al., 1998; Gorbsky et al., 1998). This possibility is partly supported by the finding that recombiant human MAD2 can form a homotetramer and this complex is more efficient at inhibiting the cyclosome/APC than monomeric forms of MAD2 (Fang et al., 1998).

In spite of the significant advances in our understanding of MAD2 function, the picture remains incomplete due to the lack of understanding of the functions of the other checkpoint proteins. The situation in mammalian cells may be even more complex than in budding yeast as the function and structure of mammalian kinetochores is vastly more complicated and may require a more elaborate checkpoint monitoring system. This possibility is consistent with the recent finding that mammalian cells express two BUB1-related kinases that appear to have evolved from a common ancestral BUB1 kinase. hBUB1 (the homologue of mBUB1) and hBUBR1 are human BUB1-related kinases that were found to be mutated in 2 out of 20 colorectal carcinomas that exhibited a chromosome instability phenotype (Cahill et al., 1998). The mutations identified in hBUB1 were confirmed to interfere with the mitotic checkpoint as the mutant protein disrupted the activity of the wild-type hBUB1 in a dominant negative fashion (Cahill et al., 1998). Although colorectal carcinomas that were heterozygous for hBUB1 mutations were also identified in the study (Cahill et al., 1998), the role of hBUBR1 in the mitotic checkpoint was not tested.

hBUBR1 was also independently isolated based on its similarities with a portion of the yeast checkpoint protein MAD3 (Taylor et al., 1998). The significance of this similarity is unknown but it is noteworthy that other members of the BUB1 kinase family also share the same MAD3 homology domain (Roberts et al., 1994; Taylor and McKeon, 1997; Chan et al., 1998). Clues to hBUB1 function came when it was found to associate with the kinetochore motor CENP-E (Chan et al., 1998). Although this interaction was initially identified in a yeast two-hybrid screen for proteins that associate with CENP-E, a stable complex of hBUB1 and CENP-E was detected in HeLa cells. CENP-E is a kinetochore-associated kinesin motor protein whose function is in microtubule attachment to kinetochores and in the alignment of chromosomes to the metaphase plate (Schaar et al., 1997; Wood et al., 1997). The function of CENP-E is likely to be monitored by the mitotic checkpoint as cells invariably were arrested in mitosis when their kinetochores lacked CENP-E functions (Schaar et al., 1997). Taken together, we postulated that the CENP-E–hBUB1 complex might be integral parts of a mechano sensor that link kinetochore motility with checkpoint control (Chan et al., 1998).

In this paper, we establish the importance of the hBUBR1 kinase in the mitotic checkpoint and provide mechanistic insights into its checkpoint functions. We show that one function of hBUBR1 is to monitor kinetochore activities that require CENP-E. We also demonstrate that hBUBR1 is a mitotic-specific kinase that associates with a large fraction of the cyclosome/APC during mitosis. We propose a working model that integrates these two activities to account for how local defects at the kinetochore are detected, relayed, and amplified at a global level to block premature entry into anaphase. The similarities shared between hBUBR1 and the MAD2–p55CDC complex suggests that they can function as an integrated complex or act along parallel pathways in the mitotic checkpoint.

Materials and Methods

Cell Culture

HeLa cells were grown in DME supplemented with 10% FBS. Hela cells adapted for growth in suspension were cultured in SME supplemented with 10% FBS and 1% pluronic acid. K562 suspension cultures were grown in RPMI 1640 supplemented with 10% FBS. ALL cells were grown in a humidified 37°C chamber at 5% CO2. Centrifugal elutriation was performed using a Beckman centrifuge (J2-MC) with the JE5.0 rotor as described (Gately et al., 1998). Samples were taken from each fraction and analyzed for cell cycle distribution by FACS. HeLa cells arrested in mitosis were obtained by growing them in the presence of nocodazole (0.06 μg/ml) for 12–16 h. Both normal and nocodazole-blocked mitotic cells were harvested from the culture dishes by mechanical shakeoff.

Construction and Expression of Mutant hBUBR1

Full-length gfp-hBUBR1 expression construct was described previously (Chan et al., 1998). The mutant gfp-hBUBR1 kinase construct was made by deleting nine amino acids (amino acids 795–803, KVSSQPVPW) from the most conserved kinase subdomain II (Hunter, 1987; Hancock and Quinn, 1991). Deletion was accomplished by PCR-based mutagenesis. Transient transfections were performed by lipofection using LT2 (PanVera) as described (Chan et al., 1998).

Microinjection

HeLa cells were plated onto gridded coverslips and were synchronized at

1 Abbreviations used in this paper: ACA, ant centromere autoimmune antibodies; APC, anaphase-promoting complex; CENP-E, centromere protein E; DAPI, 4',6-diamidino-2-phenylindole.
cells were injected 1–2 h after they were released from the G1/S boundary. Microinjection was performed with a semi-automatic injector (model 5412; Eppendorf Scientific, Inc.) and the Ependorf automated micromanipulator (model 5402). For each coverslip, 200–300 cells were injected with each antibody over the course of ~30 min. Multiple coverslips were injected to accommodate time course experiments where samples had to be taken at different times. The affinity-purified hUBR1 and CENP-E antibodies used for the injection experiments have been previously described (Schaar et al., 1997; Chan et al., 1998). Nonimmune IgG was isolated from sera, which was originally used to affinity purify hUBR1 antibodies, and verified by immunofluorescence and Western blots to no longer contain detectable levels of hUBR1 antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Stained cells were examined with either a TEC-1 CCD camera (Dage-MTI) that was controlled with IP LabSpec software (Phase 3 Imaging Systems). Cells were then fixed with antibody incubations. To minimize loss of loosely attached mitotic or apoptotic cells, the coverslips were centrifuged at 200 × g for 3 min in a clinical centrifuge (GPK R; Beckman). For time-lapse microscopy, HeLa cells were attached on 35-mm glass bottom microwell dishes (TeK Corp.), and cells were monitored with a 60× Planapo objective mounted on a Nikon Eclipse TE300 inverted microscope. Cells were kept at 37°C with a 35-mm tissue culture dish stage warmer regulated by a temperature controller (TC-102; Medical Systems Corp.). Time-lapse images were captured using a Sensys CCD camera (Photometric) that was controlled by Image-Pro Plus software (Phase 3 Imaging Systems).

**Immunofluorescence Microscopy**

Cells on coverslips were fixed, permeabilized, and stained as described (Chan et al., 1998). Immunoprecipitations were performed using a rat anti-hUBR1 antibody that was generated and affinity-purified the same way as the previously described rabbit hUBR1 antibodies (Chan et al., 1998; Jablonski et al., 1998). Mitotic cells were visualized using an appropriate primary antibody that was coupled to Cy2 or Texas red (Jackson ImmunoResearch Laboratories). DNA was stained with 0.1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). Stained cells were examined with either a 40 or 100× PlanNeofluor objective mounted on a Nikon Microphot SA microscope that was equipped with epifluorescence optics. Images were captured with a TEC-1 CCD camera (Dage-MTI) that was controlled with IPLabSpec v2.0.1 (Scanalytics Inc.) and contrast enhanced with A dobe Photoshop 5.0 (A dobe Systems Inc.).

**Immunoprecipitation and Western Blots**

Cells were harvested, washed, and lysed in NP-40 lysis buffer (1% NP-40, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM DTT) supplemented with protease and phosphatase inhibitors as described previously (Gately et al., 1998). Cell lysates were centrifuged at 10,000 × g for 15 min. The protein concentrations of the lysates were determined (BCA protein assay; Pierce Chemical Co.) and adjusted to the same protein concentration. Immunoprecipitation was performed with 300 or 500 μg of lysate and the final concentrations of antibodies were 2 μg/ml. For FPLC separations, 500 μl was loaded onto a Superose 6 column (A mershac Pharma Biotech Inc.), and proteins were eluted in NP-40 lysis buffer with protease and phosphatase inhibitors. Fractions containing cyclosome/APC were combined, concentrated with a Centri 100 (A micon Inc.), and incubated with the hUBR1 antibodies. To solubilize hUBR1 that is tumble in PBS with the kinetochore, chromosomes were boiled in 2% SDS for 2 min, dialyzed with 20 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 10 mM β-glycerophosphate. Beads were incubated in kinase buffer that contained 50 μM ATP and 20 μM CTP-γ[32P]ATP at 25°C for 15 min. Cold ATP was added to a final concentration of 0.5 mM and the reaction was allowed to proceed for another 15 min before SDS sample buffer was added. The immunoprecipitations—kinase samples were boiled and separated through a 4–15% gradient gel, fixed, dried, and then subjected to autoradiography. Quantitation was done using a Bio Imaging analyzer (Fuji BA S1000; Fuji PhotoFilm Co., Ltd.).

**Results**

**hUBR1 Is Required for the Mitotic Checkpoint**

Two complementary approaches were used to examine if hUBR1 function is required for the mitotic checkpoint in HeLa cells. Our first strategy to inhibit hUBR1 activity was to microinject hUBR1 antibodies into cells and test the response of the injected cells to the microtubule inhibitor nocodazole. The hUBR1 antibodies used for the injection experiments have been shown not to co-react with hUBR1 as determined by Western blots and immunoprecipitations (Chan et al., 1998; Jablonski et al., 1998). HeLa cells synchronized at the G1/S boundary were micro-injected with hUBR1 or nonimmune antibodies shortly after they were released from the G1/S boundary. Approximately 2 h before the synchronized cells were expected to enter mitosis, nocodazole was added to the medium and the injected cells were sampled at various times during the ensuing 8 h (Fig. 1, A and B). Mitotic cells were scored by the presence of condensed chromosomes which aggregated near the center of the cell when the spindle is abolished by nocodazole (Fig. 1 A, rows 1 and 2, left). Approximately 12 h after release from the G1/S boundary, most cells have entered mitosis. At this time, there was no significant difference between the mitotic indices of the cells that were injected with hUBR1 or the nonimmune antibodies. Thus, injection of antibodies did not interfere with mitotic entry. Nonimmune antibodies were diffusely distributed throughout the mitotic cell (Fig. 1 A, row 1, middle) and did not interfere with the localization of hUBR1 at kinetochores of the unaligned chromosomes (Fig. 1 A, row 1, right). In contrast, the injected hUBR1 antibodies were concentrated at the kinetochores of the unaligned chromosomes (Fig. 1 A, row 2, middle). The presence of hUBR1 at kinetochores of these cells was independently confirmed by staining with hUBR1 antibodies (Fig. 1 A, row 2, right).

When cells were examined at later times after nocodazole treatment, the mitotic index of cells injected with nonimmune IgG remained high, whereas the mitotic index of cells injected with hUBR1 antibodies was about three-fold lower (Fig. 1 B). The decrease in the mitotic index of...
cells that were injected with the hUBR1 antibodies was accompanied by a fivefold rise in the number of interphase cells with aberrantly shaped nuclei relative to the negative control (Fig. 1A, row 3, and B). The flattened morphology as well as the presence of nuclei indicated that these cells exited mitosis (Fig. 1A, row 3). The presence of paired centromeres, as revealed by staining with an anticentromere autoimmune (ACA) serum, suggested that either the chromosomes did not separate or had separated but re-replicated (Fig. 1A, row 3, right, inset).

To directly confirm that inhibition of hUBR1 abrogated the mitotic checkpoint and caused cells to exit mitosis, time-lapse videomicroscopy was used to follow the fate of nocodazole-treated cells that were microinjected with hUBR1 antibodies. Fig. 1C shows that within 30 min after the cell entered mitosis, the cell began to flatten out. 2 h later, the presence of a nucleus demonstrated that it had clearly exited mitosis (Fig. 1C, 150'). At this level of resolution, we could not determine how rapidly cells injected with hUBR1 antibodies exited mitosis. Uninjected cells on the same coverslip as well as cells injected with nonimmune antibodies remained blocked in mitosis for up to 16 h (data not shown).

To obtain independent verification of the microinjection results, we attempted to disrupt endogenous hUBR1 function by targeting an hUBR1 mutant to kinetochores. We created a mutant by deleting nine amino acids (residues 795–803) from subdomain II of the conserved catalytic core of the hUBR1 kinase. Western blots confirmed that both mutant and wild-type hUBR1 were expressed at equivalent levels (data not shown). Both mutant and wild-type gfp:hUBR1 were transfected into synchronized HeLa cells shortly after they were released from the G1/S boundary (Fig. 2). Nocodazole was added ~2 h before the cells were expected to enter mitosis. By 12 h after release from the G1/S boundary, mitotic cells expressing both mutant and wild-type gfp:hUBR1 at their kinetochores were identified (Fig. 2A, rows 1 and 2). After overnight incubation in the presence of nocodazole, the mitotic index of cells expressing the mutant gfp: hUBR1 was ~2 times lower than for wild-type gfp: hUBR1-transfected cells (Fig. 2B). In concordance with the microinjection results, many of the cells expressing the mutant hUBR1 exited mitosis and formed aberrant shaped nuclei (Fig. 2A, row 3, and B). The accumulation of the gfp:hUBR1Δkinase in the cytoplasm of these aberrant interphase cells is consistent with the distribution of endogenous hUBR1 in interphase cells as previously reported (Chan et al., 1998). The microinjection and transfection experiments demonstrate that hUBR1 kinase is likely to be an essential component of the mitotic checkpoint.

hUBR1 Is Essential for a Normal Mitosis

We have previously shown that hUBR1 can form a complex with the kinetochore motor CENP-E (Chan et al., 1998). If hUBR1 can monitor kinetochore microtubule interactions mediated by CENP-E as part of its normal checkpoint function, disruption of hUBR1 might cause cells to exit mitosis prematurely. Alternatively, hUBR1 might regulate CENP-E functions that are important for chromosome alignment. In this case, loss of hUBR1 should interfere with CENP-E functions at the kinetochore and block cells in mitosis with unaligned chromosomes (Schaar et al., 1997). To test the importance of hUBR1 during normal mitosis, the fates of synchronized cells that were either injected with hUBR1 antibodies or transfected with the gfp:hUBR1Δkinase were determined. Approximately 12 h after release from the G1/S boundary, cells that were injected with nonimmune IgG were found in prometaphase and metaphase with the majority being at metaphase (Fig. 3A, row 1). Immunofluorescence staining revealed that the metaphase cells exhibited detectable levels of hUBR1 at their kinetochores despite the presence of nonimmune antibodies throughout the cell (Fig. 3A, row 1, middle and right). For cells injected with the hUBR1 antibody, most of the mitotic cells were in prometaphase. The injected hUBR1 antibodies were found throughout the cell including kinetochores (Fig. 3A, row 2, middle). The presence of hUBR1 antibodies at the kinetochores did not interfere with CENP-E localization (Fig. 3A, row 2, right). Although a few metaphase cells injected with hUBR1 were seen, we consistently identified anaphase cells that contained lagging chromosomes (Fig. 3A, row 3, left). These cells appeared to be in late anaphase as they contained a mature cleavage furrow that was concentrated with CENP-E (Fig. 3A, row 3, right). The presence of lagging chromosomes suggested that these cells exited mitosis with unaligned chromosomes and gave rise to the high frequency of aberrantly divided cells that accumulated at later time points (Fig. 3A, row 4, and B). The nuclei of the cells injected with hUBR1 antibodies were abnormally shaped and DNA was invariably found in between the two divided cells (Fig. 3A, row 4, left). The DNA in between the cells was derived from chromosomes because they contained centromeres as determined by ACA staining (Fig. 3A, row 5, right, inset). However, we cannot distinguish whether the foci of ACA staining represent separated centromeres or fragmented centromeres. This cut phenotype was specific for hUBR1 antibodies as this was not observed in uninjected cells or cells injected with nonimmune antibodies. In fact, hUBR1 antibody injection did not produce any normal telophase cells (Fig. 3B).

Similar results were obtained when synchronized HeLa cells were transfected with the mutant hUBR1Δkinase. In transfected cells that had progressed into mitosis, the mutant hUBR1 was concentrated at kinetochores (Fig. 3C, top right). At later times, there was a large increase in aberrantly divided cells with the cut phenotype (Fig. 3C, bottom left). Both mock and wild-type gfp:hUBR1-transfected cells divided normally and did not exhibit the cut phenotype (data not shown). The combined data suggest that disruption of hUBR1 function caused the cells to exit mitosis in the presence of unaligned chromosomes.

hUBR1 Monitors CENP-E Kinetochore Functions

We have previously shown that disruption of CENP-E functions at kinetochores prevented chromosome alignment and arrested cells in mitosis for >12 h (Schaar et al., 1997). To test whether hUBR1 is required for this checkpoint,
we coinjected hBUBR1 and CENP-E antibodies into synchronized HeLa cells, and then compared the fates of these cells to those that were injected with CENP-E antibodies alone. Injection of CENP-E antibodies blocked the assembly of CENP-E onto kinetochores (Fig. 1 A, left and middle) but did not interfere with kinetochore localization of hBUBR1 (Fig. 4 A, left gallery, row 1) or the distribution of other centromere components that were recognized by ACA (Fig. 4 A, left gallery, row 2). As shown previously, the mitotic block induced by injection of CENP-E antibodies is the direct result of disruption of kinetochore function and not bipolar spindle formation (Fig. 4 A, left gallery, row 3).

Cells coinjected with CENP-E and hBUBR1 antibodies

Figure 1. hBUBR1 is essential for cells to arrest in mitosis in the presence of nocodazole. (A) Immunofluorescence staining of HeLa cells injected with antibodies and exposed to nocodazole. (row 1 and 2) 12 h after release from the G1/S boundary, nonimmune- and hBUBR1 antibody-injected cells have entered mitosis in the presence of nocodazole with unaligned chromosomes (left). Injected antibodies were visualized with Cy5 anti-rabbit secondary antibodies (middle) and endogenous hBUBR1 was visualized with a rat anti-hBUBR1 antibody and Cy2 anti-rat secondary antibodies (right). (row 3) Cells injected with hBUBR1 (middle) exited mitosis in the presence of nocodazole and formed aberrantly shaped nuclei (left) that contained paired centromeres as determined by ACA and Cy2 anti-human secondary antibody staining (right, arrowheads in the inset). Chromosomes and nuclei were visualized by DAPI staining (left). Images in rows 1 and 2 were captured with a 100× objective, whereas row 3 was visualized with a 40× objective. (B) Histogram comparing the fates of HeLa cells injected with nonimmune or hBUBR1 antibodies after exposure to nocodazole. Synchronized HeLa cells were injected within 2 h after their release from the G1/S boundary, nocodazole was added 8 h after release from the G1/S boundary and sampled 4, 6, and 8 h later. HeLa cells normally enter mitosis between 10–12 h after release from the G1/S boundary. The histogram compares the mitotic (open bars), multi-nucleated (striped bars), and apoptotic (dotted bars) indices of cells injected with hBUBR1 and nonimmune antibodies at 16 h after release from the G1/S boundary. Approximately 80–120 injected cells were counted for each experiment. The plotted values are the means plus SD from at least three experiments. (C) A single cell microinjected with hBUBR1 antibodies and incubated in the presence of nocodazole was followed by time-lapse videomicroscopy shortly after it entered mitosis (time 0). Frames were selected from a 4.5-h long time-lapse experiment. Actual time in minutes is labeled in the bottom right corners of each frame. Bars, 10 μm.
entered mitosis. Immunofluorescence staining confirmed that hBUBR1 remained at kinetochores (Fig. 4 A, right gallery, row 1, right) of the injected cells. 16 h after release from the G1/S boundary, we counted cells that were either in mitosis, telophase, aberrantly divided cut, or apoptosis. Cells injected with just CENP-E antibodies were arrested in mitosis with unaligned chromosomes (Fig. 4 B). No cells were found to be apoptotic, in telophase, or cut. Examination of the cells that were coinjected with hBUBR1 and CENP-E antibodies showed an ∼5-fold reduction in the number of mitotic cells relative to cells injected with CENP-E alone (Fig. 4 B). The reduction of mitotic cells was accompanied by a large increase in the number of aberrantly divided cells that exhibited the cut phenotype (Fig. 4 A, right gallery, rows 2 and 3) along with a slight increase in apoptotic cells. Normal telophase cells that were

---

**Figure 2.** Overexpression of a hBUBR1 kinase prevents cells from arresting in mitosis in the presence of nocodazole. (A) 12 h after release from the G1/S boundary, cells expressing gfp:hBUBR1 (WT) and the gfp:hBUBR1 kinase mutant enter mitosis in the presence of nocodazole. Both the gfp:hBUBR1 (WT) and the gfp:hBUBR1 kinase mutant were concentrated at kinetochores (right). After overnight incubation in nocodazole, cells that expressed the gfp:hBUBR1 kinase mutant had exited mitosis and formed aberrantly shaped nuclei (left). Gfp:fusion constructs were directly visualized by autofluorescence. Chromosomes and nuclei were visualized with DAPI. (B) Fates of HeLa cells that expressed wild-type gfp: hBUBR1 or gfp: hBUBR1 kinase and exposed to nocodazole. Synchronized HeLa cells were transfected 2 h after release from the G1/S boundary with wild-type gfp: hBUBR1 or gfp: hBUBR1 kinase; after 5 h, the transfection mix was removed and nocodazole was added. The mitotic (open bars), multinucleated (striped bars), and apoptotic (dotted bars) indices of wild-type gfp: hBUBR1 and gfp: hBUBR1 kinase after overnight incubation (∼20 h after release from the G1/S boundary) in nocodazole was compared. Approximately 120-150 transfected cells were counted and scored for each experiment. The plotted values are the means plus SD from at least three experiments. Bars, 10 μm.
coinjected with the hBUBR1 and CENP-E antibodies were not found. These data showed that hBUBR1 is an essential component of the mitotic checkpoint that is sensitive to kinetochore functions that were specified by CENP-E.

**Characterization of hBUBR1 Protein Expression and Kinase Activity**

Having established that hBUBR1 is an essential component of the mitotic checkpoint, we wanted to investigate its
mechanism of action by characterizing its expression and kinase activity as a function of the cell cycle. hBUBR1 was immunoprecipitated from K562 erythroleukemic cells and HeLa cells that were separated into different phases of the cell cycles by centrifugal elutriation. Portions of the immunocomplexes were used to determine steady-state levels of protein and kinase activity. We assayed the immunoprecipitated hBUBR1 for autokinase activity as a recent study showed that a transfected hBUBR1 exhibited autokinase activity (Taylor et al., 1998). However, hBUBR1 isolated from K562 and HeLa (data not shown) exhibited no detectable autokinase activity (Fig. 5A, top) or kinase activities towards exogenous substrates (data not shown) even though it was expressed throughout the cell cycle of the cell. 

Figure 4. hBUBR1 is required for mitotic arrest resulting from loss of CENP-E functions at kinetochores. Synchronized HeLa cells were injected with rabbit anti-CENP-E and hBUBR1 antibodies and sampled at 12 or 16 h after release from the G1/S boundary. (A) Immunofluorescence staining of injected cells at these time points. (left gallery) Cells injected with CENP-E antibodies arrest in mitosis with unaligned chromosomes. Cells were stained for the injected CENP-E antibodies (middle), localization of endogenous hBUBR1, ACA, and tubulin (rows 1-3, respectively, right). (right gallery) Cells coinjected with CENP-E and hBUBR1 antibodies (middle) enter mitosis with hBUBR1 at kinetochores (right, row 1). At the 16-h time points (rows 2 and 3), the cells coinjected with CENP-E and hBUBR1 antibodies exit mitosis with unaligned chromosomes to produce divided cells with lagging chromosomes (inset in left) and multiple nuclei that reform around unsegregated or incompletely separated chromosomes. Phase-contrast image show cells were tethered at the stem-body (row 2, right). Injected antibodies were stained with Cy5 anti-rabbit secondary antibodies (middle columns of both galleries). Endogenous hBUBR1 was stained with anti-rat hBUBR1 and visualized with Cy2 anti-rat secondary antibody. ACA was visualized with Cy2 anti-human secondary antibodies. Microtubules were detected with antitubulin mAbs and Cy2 anti-human secondary antibodies. Chromosomes and nuclei were stained with DAPI (left column). (B) Comparison of the fates of cells injected with CENP-E antibodies, hBUBR1+CENP-E antibodies, and nonimmune IgG. After cells were released from the G1/S boundary for 16 h, the percentage of cells injected with the various antibodies that were in mitosis (open bars), normal telophase (white dotted bar), cut (checkered bar), and apoptosis (black dotted bar) were quantitated and compared. The plotted values are the means plus SDs from at least three experiments. Bars, 10 μm.
both cell types (Fig. 5 A, middle and bottom). hBUBR1 levels remained fairly constant throughout the cell cycle of K 562 cells, whereas hBUBR1 levels in Hela cells fluctuated with the cell cycle. hBUBR1 level was lowest in G1 and steadily increased as cells progressed towards mitosis. If hBUBR1 is a mitosis-specific kinase, its activity would have escaped detection because the vast majority of the cells that were elutriated into the late fractions were in G2 and only a very minor fraction was in mitosis. Therefore, we compared the hBUBR1 kinase activity between interphase and mitotically arrested Hela cells. hBUBR1 autokinase activity remained undetectable in interphase cells, but was highly active in mitotically arrested cells (Fig. 5 B). The same profile of hBUBR1 kinase activity was obtained with exogenous substrates such as myelin basic protein and a carboxyl-terminal fragment of CENP-E (data not shown). The increase in hBUBR1 kinase activity in mitotic cells was not due to increased expression of the protein or its association with the hBUB3 subunit relative to interphase cells (Fig. 5 B, bottom). However, hBUBR1 mobility was reduced in mitotically blocked cells relative to the interphase cells (Fig. 5 B, middle right). The upshift was due to phosphorylation as the electrophoretic mobility of hBUBR1 was no longer retarded after phosphatase treatment (Fig. 5 C, compare lanes 1 and 2). In vivo labeling experiments showed that hBUBR1 was also phosphorylated during interphase but became hyperphosphorylated in mitosis (data not shown). To further test if the hyperphosphorylation of hBUBR1 was related to the mitotic checkpoint, we compared hBUBR1 in mitotically blocked cells to those that were released from the block and had exited mitosis (Fig. 5 C, right). The majority of hBUBR1 was hyperphosphorylated in mitotically blocked cells relative to normal mitotic cells. 1 h after their release from the mitotic block, hBUBR1 was no longer hyperphosphorylated (Fig. 5 C, compare lanes 4 and 5).

In the previous experiments, hBUBR1 kinase activity was determined in cells that had already been blocked in mitosis for many hours. As a checkpoint kinase, hBUBR1 should respond to spindle defects even after cells have reached metaphase. To test this, we isolated mitotic Hela cells by mechanical shakeoff, and then exposed them to nocodazole for various times. Microscopic analysis revealed that hBUBR1 levels increased slightly after 30 and 60 min in nocodazole, but returned to the initial level after 2 and 4 h of nocodazole treatment. The most dramatic change was that hBUBR1 was quantitatively hyperphosphorylated within 15 min of spindle disruption and this high level of phosphorylation was maintained throughout the block.

**hBUBR1 Associates with the Cyclosome/APC**

Next, we examined the native size of hBUBR1 between interphase and mitotically blocked cells. Gel filtration chromatography revealed that hBUBR1 exists primarily in an ~500-kD complex in interphase cells (Fig. 7 A, top). In mitotically arrested cells, hBUBR1 was associated with the ~500-kD complex, but was also found in a larger complex that overlapped with the cyclosome/APC (Fig. 7 A, bottom). The possibility that hBUBR1 was associated with the cyclosome/APC was tested by immunoprecipitating hBUBR1 from the fractions containing the cyclosome/APC and probing for the presence of the APC subunits hscdc27, hscdc16 (Tugendreich et al., 1995), and APC7 (Yu et al., 1998). The results showed that all three of the APC subunits, coimmunoprecipitated with hBUBR1 in the mitotically blocked lysates (Fig. 7 B, middle). Although some hBUBR1 was detected in the cyclosome/APC containing fractions in interphase cells (Fig. 7 A, top), this was not associated with the cyclosome/APC (Fig. 7 B, left), and most likely resulted from smearing of the 500-kD complex in the gel filtration column. This finding along with the absence of cyclosome/APC subunits in immunoprecipitates from nonimmune IgG demonstrated that the cyclosome/APC specifically associates with hBUBR1 in mitosis.

We estimated the proportion of the cellular pool of the cyclosome/APC that was associated with hBUBR1 in mitotically arrested cells by comparing the relative levels of various APC subunits in hBUBR1 immunoprecipitates and the remaining supernatant. When ~30% of the total pool of hBUBR1 was immunoprecipitated from mitotic lysates, roughly 16–20% of the total pool of hscdc16, hscdc27, and APC7 was also found in the precipitate. Assuming that the remaining pool of hBUBR1 that was not immunoprecipitated can also associate with the APC, we
can extrapolate that ~50% of the total pool of A PC can be associated with hBUBR1. Since the gel filtration data show that ~50% of the total pool of hBUBR1 comigrates with the A PC, we estimate that about 25% of the total pool of A PC is associated with hBUBR1 in mitosis.

**Discussion**

**hBUBR1 Is Essential for the Mitotic Checkpoint**

The results from this study provide details about the function and biochemical properties of hBUBR1 kinase. We demonstrate that hBUBR1 is essential for the mitotic checkpoint as disruption of hBUBR1 function prevented cells from arresting in mitosis in the presence of nocodazole. Instead, the cells exited mitosis without dividing and formed highly aberrant nuclei that contained unsegregated chromosomes. The behavior of hBUBR1-defective cells is very similar to that exhibited by cells with defective MAD1, MAD2, or BUB1 functions. Depletion of MAD2 and MAD1 from Xenopus egg extracts resulted in the inability to block mitosis in the presence of nocodazole (Chen et al., 1996, 1998). Introduction of anti-MAD2 antibodies into cells abrogated their ability to arrest in mitosis in the presence of nocodazole (Li and Benezra, 1996) or taxol (Waters et al., 1998). Overexpression of a mutant form of mBUB1 (Taylor and McKeon, 1997) and hBUB1 (Cahill et al., 1998) prevented cells from arresting in mitosis in the presence of nocodazole.

Our studies also showed that hBUBR1 provided an essential function during normal mitosis by preventing cells from prematurely exiting mitosis. It was unlikely that disrupting hBUBR1 functions directly interfered with chromosome alignment since we were able to identify metaphase cells that were injected with hBUBR1 antibody or expressed the hBUBR1 kinase-defective mutant. As the frequency of such metaphase cells was rare, the most likely fate of many of the prometaphase cells that we saw was premature exit from mitosis. This interpretation is supported by the identification of aberrant anaphase cells with lagging chromosomes. The lagging chromosomes most likely were chromosomes that had not achieved...
alignment at the metaphase plate when cells decided to exit mitosis. This defect would explain the high frequency of abnormally divided cells that contained chromatin trapped by the cleavage furrow. At present, we have not directly determined precisely how rapidly cells progress through mitosis when hBUBR1 function is inhibited. However, studies have shown that disruption of MAD2 in human keratinocyte cells accelerated mitotic progression by 6.5 min. Despite the similarity in the outcomes of all these studies, it remains to be seen how MAD2, BUB1, and BUBR1 relate to each other at a mechanistic level. All of these proteins are localized at the kinetochore, they may act in parallel by monitoring different activities of the kinetochore or work as an integrated complex to generate the wait anaphase signal from the kinetochore. These two possibilities are supported by the fact that the two BUB1-related kinases do not share overlapping functions as neither one is able to maintain the mitotic checkpoint alone.

**hBUBR1 Is Sensitive to CENP-E Functions at Kinetochores**

Our data show that one checkpoint function of hBUBR1 is to monitor kinetochore functions that are specified by CENP-E. When kinetochores are depleted of CENP-E, cells arrest in mitosis with unaligned chromosomes but a normal spindle. Our data suggest that hBUBR1 activity at the kinetochore is important for generating the wait anaphase signal. This possibility is supported by the finding that hBUBR1 was still present on kinetochores that lacked CENP-E. Furthermore, microinjection of hBUBR1 antibodies abrogated the mitotic arrest and caused cells to exit mitosis. If hBUBR1 was contributing to the wait anaphase signal from the CENP-E–depleted kinetochores, it must be able to do so when it is not in direct contact with CENP-E. Under normal circumstances, we speculate that interactions between CENP-E and microtubules can directly regulate hBUBR1 activity by altering its interactions with CENP-E.

Although such a feedback mechanism can link kinetochore attachments to the checkpoint, the threshold level of CENP-E activity that is required to activate or inactivate hBUBR1 activity remains to be determined. We have shown that when kinetochore–microtubule attachments of metaphase cells are dissolved by nocodazole, the most dramatic change was the quantitative hyperphosphorylation of the kinetochore-bound hBUBR1. Perhaps the phosphorylation state of hBUBR1 at the kinetochore is related to the number of kinetochore microtubules that are attached to CENP-E and the level of tension that results from these interactions. At metaphase, the levels of both CENP-E and hBUBR1 have been shown to be reduced (Chan et al., 1998; Jablonski et al., 1998) along with other checkpoint proteins. Under these circumstances, there may be insufficient hBUBR1 kinase activity that is generated from the kinetochore to sustain the checkpoint components that lie downstream of the kinetochore. Studies have shown that it takes ~30 min from the time the last chromosome becomes aligned at the metaphase plate until the onset of anaphase (Rieder et al., 1995). This may be the time that is required for the checkpoint signal to fall and to allow cyclosome/APC activity to reach a critical level that is necessary to drive cells into anaphase. Before this critical level of cyclosome/APC activity is reached, the checkpoint can be reactivated by unattached kinetochores to once again inhibit cyclosome/APC. Although we proposed that hBUBR1 kinase activity is sensitive to the mechanical ac-

---

Figure 6. hBUBR1 kinase in metaphase Hela cells are stimulated by disruption of the spindle. Mitotic shakeoff cells that were nearly uniformly at metaphase were exposed to nocodazole for the times indicated and hBUBR1 complexes were immunoprecipitated from cell lysates and probed for autokinase activity. (A) hBUBR1 autokinase activity from each time point was quantitated with a PhosphorImager and normalized to metaphase cells (time 0). This is a valid comparison as equal amounts of hBUBR1 were used for the kinase assay. (B) Western blots of hBUBR1 immunoprecipitates probed with hBUBR1 (top) and hBUB3 (middle). Lysates obtained from the same experiment were separately probed for cyclin B expression (bottom). (C) Western blot of the insoluble chromosome fractions obtained from the experiment described above was probed for hBUBR1.

High resolution videomicroscopy showed that disruption of MAD2 in human keratinocyte cells accelerated mitotic progression by 6.5 min. Despite the similarity in the outcomes of all these studies, it remains to be seen how MAD2, BUB1, and BUBR1 relate to each other at a mechanistic level. All of these proteins are localized at the kinetochore, they may act in parallel by monitoring different activities of the kinetochore or work as an integrated complex to generate the wait anaphase signal from the kinetochore. These two possibilities are supported by the fact that the two BUB1-related kinases do not share overlapping functions as neither one is able to maintain the mitotic checkpoint alone.
tivities of the kinetochore, activation of its kinase may require additional biochemical changes such as mitosis-specific phosphorylations. As kinetochores are now known to also contain cdc2 (Rattner et al., 1990), MAP kinase (Shapiro et al., 1998; Zecevic et al., 1998), and hBUB1 (mBUB1) kinases (Taylor and McKeon, 1997; Jablonski et al., 1998), any one of these may be important for regulating hBUBR1 kinase at the kinetochore.

hBUBR1 Is Associated with the Cyclosome/APC in Mitosis

We infer from the interaction between hBUBR1 and cyclosome/APC that hBUBR1 might provide global checkpoint functions by directly inhibiting cyclosome/APC. This is supported by the finding that partially purified hBUBR1 from HeLa cells inhibited ubiquitination activity of the APC in vitro (Sudakin, V., and T. Yen, unpublished results). By comparing the amount of APC that communoprecipitated with hBUBR1 to that in the remaining supernatant, we estimated that 25% of the total pool of APC was bound to hBUBR1. As MAD2–p55cdc was also found to be associated with only a fraction of the total pool of APC, it is possible that the hBUBR1–APC complex represents a different subpopulation of the APC. As hBUBR1 kinase activity is stimulated when the spindle is disrupted, hBUBR1 may directly phosphorylate and inhibit cyclosome/APC activity or phosphorylate specific substrates so that they are no longer recognized by the cyclosome/APC.

As proposed for MAD2–p55cdc (Gorbsky et al., 1998), the interactions between hBUBR1 and the cyclosome/APC should be inherently unstable so that cells can proceed into anaphase when the checkpoint is extinguished. As hBUBR1 appears to form a fairly stable complex with the cyclosome/APC in cells arrested in mitosis, its kinase activity may be labile. This is supported by the observation that the in vitro hBUBR1 kinase activity had decayed when it was isolated from cells whose spindle had been disrupted by nocodazole for an hour as compared with the mitotic cells that were examined 30 min earlier. As the phosphorylation status of the soluble pool of hBUBR1 did...
not strongly correlate with its kinase activity, additional factors may influence hBUBR1 kinase activity. Despite the delay in the soluble pool of hBUBR1 kinase activity, sufficient kinase activity was probably maintained in the mitotically arrested cells by the continued presence of unattached kinetochores.

**Proposed Mechanism of Action of hBUBR1**

The cumulative results obtained for hBUBR1 suggest that it can provide checkpoint functions at the local and global levels. In the simplest model, unattached kinetochores contain active hBUBR1 kinase that can rapidly activate the soluble pool of hBUBR1 through autophosphorylation. This then leads to inhibition of the cyclosome/APC and exit from mitosis. This model of hBUBR1 function shares features proposed for MAD2-p55cdc and MAD2/ MAD1 (Chen et al., 1998; Gorbsky et al., 1998; Kallio et al., 1998). The overall view is that a soluble pool of checkpoint proteins is used to block cyclosome/APC activity, but the inhibitory status of these checkpoint proteins must be maintained through interactions with the unattached kinetochore. We propose that hBUBR1 can accomplish this through an autokinase loop that may also include MAD2-p55cdc complex. As MAD2 is not known to be hyperphosphorylated in mitotically blocked cells, hBUBR1 might phosphorylate one of its associated subunits such as p55cdc (Weinstein, 1997) or MAD1 (Chen et al., 1998; Jin et al., 1998). Biochemical analysis of yeast MAD1 showed that it is hyperphosphorylated in mitotically arrested cells and that this is dependent on the M PS1 and BUB1 kinases (Harwood and Murray, 1995). A throug vertebrate MAD1 does not undergo phosphorylation-dependent shifts in its migration in S D S-P A G E (Chen et al., 1998; Jin et al., 1998), it does not eliminate the possibility that MAD1 is hyperphosphorylated in mitosis. It is possible that in vertebrate systems, MAD1, MAD2, p55CDC, and hBUB1 act in parallel with hBUBR1 or through more complex interactions. Clearly, it will be important to clarify the biochemical relationship amongst these proteins to resolve whether they act together or along separate pathways in vertebrate cells.

The authors would like to thank B. Conner for excellent technical support, and Dr. M. Campbell (both from Fox Chase Cancer Center) for suggestions and comments.

This work was supported by U nited States Public Health Services grant GM 44762: A merican Cancer Society, core grant CA 06927, and an appropriation from the Commonwealth of Pennsylvania. T. Y en is supported by a Leukemia Society Scholar’s A ward. V. Sudakin is supported by a Human F rontier postdoctoral fellowship.

Submitted: 6 A pril 1999
Revised: 30 J uly 1999
A ccepted: 4Au gus t 1999

References

Basu, J., E. Logarinho, S. Herrmann, H. Boubda, Z. Li, G.K. Chan, T. Yen, C.E. Sunkel, and M.L. Goldberg. 1998. Localization of the Drosophila checkpoint control protein Bub3 to the kinetochore requires Bub1 but not Cdc15 or Rad50. Chromosoma. 107:376–385.

Cahill, D.P., C. Lengauer, J. Yu, G.J. Riggs, J.K. Wilson, S.D. Markowitz, K.W. Kinzler, and B. Vogelstein. 1998. Mutations of mitochrond checkpoint genes in human cancers. Nature. 392:300–303.

Chan, G.K.T., B.T. Schaar, and T. Yen. 1998. Characterization of the kineto-
derman, and A. Hershko. 1995. The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol. Biol. Cell. 6:185-197.

Taylor, S.S., and F. McKeon. 1997. Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell. 89:727-735.

Taylor, S.S., E. Ha, and F. McKeon. 1998. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. J. Cell Biol. 142:1-11.

Tugendreich, S., J. Tomkiel, W. Earnshaw, and P. Hieter. 1995. CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell. 81:261-268.

Visintin, R., S. Prinz, and A. Amon. 1997. CD C20 and CDH1: a family of substrate-specific activators of A PC-dependent protolysis. Science. 278:460-463.

Waters, J.C., R.H. Chen, A.W. Murray, and E.D. Salmon. 1998. Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. J. Cell Biol. 141:1181-1191.

Weinstein, J. 1997. Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc, a mammalian homolog of CD C20/Flzzy/Slp1. J. Biol. Chem. 272:28501-28511.

Weiss, E., and M. Winey. 1996. The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. J. Cell Biol. 132:111-123.

Wood, K.W., R. Sakowicz, L.S. Goldstein, and D.W. Cleveland. 1997. CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. Cell. 91:357-366.

Yen, T.J., and B.T. Schaar. 1996. Kinetochore function: molecular motors, switches and gates. Curr. Opin. Cell Biol. 8:381-388.

Yu, H., J.M. Peters, R.W. King, A.M. Page, P. Hieter, and M.W. Kirschner. 1998. Identification of a cullin homology region in a subunit of the anaphase-promoting complex. Science. 279:1219-1222.

Zecevic, M., A.D. Catling, S.T. Eblen, L. Renzi, J.C. Hittle, T.J., Yen, G.J. Gorbsky, and M.J. Weber. 1998. Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. J. Cell Biol. 142:1547-1558.