BUBR1 Phosphorylation Is Regulated during Mitotic Checkpoint Activation

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Abstract
Eukaryotic cells have evolved a mechanism that delays the progression of mitosis until condensed chromosomes are properly positioned on the mitotic spindle. To understand the molecular basis of such monitoring mechanism in human cells, we have been studying genes that regulate the mitotic checkpoint. Our early studies have led to the cloning of a full-length cDNA encoding MAD3-like protein (also termed BUBR1/MAD3/SSK1). Dot blot analyses show that BUBR1 mRNA is expressed in tissues with a high mitotic index but not in differentiated tissues. Western blot analyses show that in asynchronous cells, BUBR1 protein primarily exhibits a molecular mass of 120 kDa, and its expression is detected in most cell lines examined. In addition, BUBR1 is present during various stages of the cell cycle. As cells enter later S and G2, BUBR1 levels are increased significantly. Nocodazole-arrested mitotic cells obtained by mechanical shake-off contain BUBR1 antigen with a slower mobility on denaturing SDS gels. Phosphatase treatment restores the slowly migrating band to the interphase state, indicating that the slow mobility of the BUBR1 antigen is attributable to phosphorylation. Furthermore, purified recombinant His6-BUBR1 is capable of autophosphorylation. Our studies indicate that BUBR1 phosphorylation status is regulated during spindle disruption. Considering its strong homology to BUB1 protein kinase, BUBR1 may also play an important role in mitotic checkpoint control by phosphorylation of a critical cellular component(s) of the mitotic checkpoint pathway.

Introduction
Eukaryotic cells have evolved mechanisms, commonly referred to as checkpoints, that monitor their readiness to enter the next stage of the cell cycle (1, 2). Extensive research in the past has identified at least two major checkpoints (G2-M and mitotic checkpoints) that control the onset of mitosis and mitotic progression. The mitotic checkpoint is a conserved function in eukaryotic cells that delays the onset of anaphase until the microtubules are properly connected to all chromosomes. Genetic analyses have identified at least seven distinct yeast genes (MPS1, BUB1, BUB2, BUB3, MAD1, MAD2, and MAD3) that are important in regulating the mitotic checkpoint (3–5). Recently, several research groups have identified and characterized mammalian counterparts of the mitotic checkpoint components (6–9). The human MAD1 protein is hyperphosphorylated during S, G2, and mitosis, and it undergoes dramatic subcellular translocation during mitosis (6). It localizes to the centrosome during metaphase and to the spindle midzone and the midbody subsequently (6). The loss of human MAD1 function, because of sequestration by T-cell leukemia viral product Tax, appears to be responsible for the transformation of T cells by the tumor virus (6). The human MAD2 protein, structurally and functionally conserved (7), is localized at the kinetochore after chromosome condensation but not after metaphase (7). In addition, MAD2 associates with APC (or cyclosome; Ref. 10). Purified MAD2 is capable of arresting cycling Xenopus egg extracts at metaphase and blocking cyclin B degradation by preventing its ubiquitination (10). Microinjection of a MAD2 antibody into Ptk cells in mitosis induces premature anaphase (11). Both murine and human BUB1 genes have been cloned and characterized recently (8, 9, 12). Murine and human BUB1 proteins also localize to the kinetochore during early mitosis and mitotic checkpoint activation (8, 12). The human BUB1 gene has been implicated recently in the development of certain colorectal cancers (9, 13).

We and others have cloned a MAD3-like gene (alternatively termed BUBR1/MAD3/SSK1). BUBR1 encodes a protein of 120 kDa, the amino acid sequences of which resemble both MAD3 and BUB1 of the budding yeast. Structural abnormalities have been detected in the BUBR1 gene isolated from colorectal cancer samples (9), suggesting that it may also play a tumor-suppressor role. Recently, it has been shown that BUBR1 is localized to kinetochore from prophase to mid-anaphase and may have multiple functions during mitosis (14). In the present study, we report that BUBR1 is an active protein kinase capable of autophosphorylation and that its phosphorylation is regulated during mitotic checkpoint activation.
Characterization of Human BUBR1

Expression. Specific signals were quantified by densitometric scanning. Various tissues was loaded onto the blot (Fig. 1), confirming that an approximately equal amount of RNA from

BUBR1 mRNA expression levels, normalized by β-actin expression levels, and the sample identifications are shown in Table 1.

Results

Our previously cloned MAD3-like cDNA (GenBank accession number AF068760) was identical to BUBR1 (9), MAD3 (15), and SSK1 (16). To gain insights into the biological function of the putative mitotic checkpoint kinase, we first examined the pattern of BUBR1 mRNA expression in various human tissues. A survey of 50 primary human tissue blots obtained from Clontech, Inc. for BUBR1 expression (Fig. 1A) showed that thymus (Fig. 1A, blot E5), bone marrow (Fig. 1A, blot E8), and various fetal tissues (Fig. 1A, blots G1–G7) expressed a moderate-to-high level of BUBR1 transcripts. Testes contained the most abundant level of BUBR1 transcripts (Fig. 1A, blot D1). On the other hand, differentiated tissues, such as various brain tissues (Fig. 1A, blots A1–A8), heart (Fig. 1A, blot C1), and muscle (Fig. 1A, blot C3) expressed little or no detectable level of BUBR1 transcripts. The multiple tissue dot-blot was also probed with a control gene (β-actin), confirming that an approximately equal amount of RNA from various tissues was loaded onto the blot (Fig. 1B). The summarized results of BUBR1 mRNA expression, normalized by β-actin expression, are shown in Table 1.

To study the biological role of BUBR1, we raised antipeptide antiserum against BUBR1 protein using a synthetic peptide corresponding to residues 421–438 of the protein (8, 15) as an immunogen. Western blot analyses showed (Fig. 2A) that the antiserum from rabbit no. 35 (Lanes 3 and 4), but not the preimmune serum (Lanes 1 and 2), recognized an antigen ~120 kDa in both HEL (Lane 3) and Dami cell lysates (Lane 4). The antigen was barely detectable when the antiserum was preabsorbed with the BUBR1 peptide (Fig. 2A, Lanes 5 and 6), indicating that the antiserum recognized BUBR1 antigen. Immunoprecipitation followed by Western blotting confirmed the specificity of the anti-BUBR1 antiserum (Fig. 2B). Compared with the cell lysates (Fig. 2B, Lane 7), the anti-BUBR1 antiserum (Lanes 4–6), but not the preimmune serum (Lanes 1–3), immunoprecipitated BUBR1 antigen. To ascertain that the anti-BUBR1 antibody did not have a cross-reactivity with BUB1 antigen, interphase or mitotic HeLa cell lysates were immunoprecipitated with the anti-BUBR1 antibody. The immunoprecipitates were analyzed by Western blotting using the BUB1 antibody. Fig. 3A shows that the BUB1 antibody did not bring down BUB1 antigen (Lanes 3 and 4). Reciprocal experiments were also performed. Fig. 3B shows that BUB1 immunoprecipitates did not contain the BUBR1 antigen either. These combined studies strongly suggest that the anti-BUBR1 antibody does not show a cross-reactivity with BUB1, although we should point out that the anti-BUB1 antibody was not efficient in immunoprecipitation. Further studies by surveying various cell lines via Western blotting revealed that BUBR1 antigen was present, albeit at different levels, in many cell lines that were examined (Fig. 4A, Lanes 2–8). BUBR1 was barely detectable in H1Meg-1 (a myeloid progenitor; Fig. 4A, Lane 1) and PC-3 (prostatic carcinoma; Lane 9) cell lines.

To study the potential role of BUBR1 in mitotic checkpoint activation, we analyzed BUBR1 in several cell lines treated with a microtubule-disrupting agent Noc via Western blotting. Fig. 5A shows that BUBR1 was detected in asynchronous GMO637D (Lane 1), A549 (Lane 3), and HeLa (Lane 5) cells primarily as a single band of ~120 kDa. However, upon overnight Noc treatment, a new BUBR1 antigen with a slow mobility was induced in these cell lines (Lanes 2, 4, and 6). We noticed that in mitotic shake-off cells, BUB1 existed primarily as the high molecular weight form (Fig. 5A, Lane 8), whereas the interphase cells contain only the low molecular weight form (Fig. 5A, Lane 7). Many mitotic checkpoint proteins are phosphorylated during mitotic checkpoint activation. To determine whether the mobility shift of BUBR1 was attributable to phosphorylation, we immunoprecipitated BUBR1 from Noc-treated HeLa cell lysates using the anti-BUB1 antibody. The immunoprecipitates were treated with λ-phosphatase (PPase) in the presence or absence of okadaic acid, a phosphatase inhibitor. Fig. 5B shows that the slow mobility form of BUBR1 in both mitotic shake-off cell lysates (Lane 5) and asynchronous cell lysates (Lane 6) was converted to the fast mobility one after phosphatase treatment. In the presence of okadaic acid, the phosphatase failed to convert the mobility of BUBR1 (Lane 4), indicating that Noc treatment induces BUBR1 phosphorylation.

Proteins such as p55CDC involved in mediating mitotic checkpoint activation fluctuate during the cell cycle (17). In addition, there exists a potential destruction box (RSSLAE-LKS) in BUBR1 but not in BUB1 (18). To examine whether BUBR1 is also regulated at the protein level during the cell
cycle, equal amounts of A549 cell lysates from various stages of the cell cycle were analyzed for BUBR1 via Western blotting. A549 was used for synchronization because we have previously used the cell line for cell cycle study (19). Fig. 6A shows that BUBR1 was present in G1 and that its level increased (at least doubled based on densitometric scanning) during later S and G2. No phosphorylated BUBR1 was detected during G1, G1-S, S, and G2 (Fig. 5A, Lanes 2–4). In contrast, M-phase cell lysates contained the phosphorylated form of BUBR1 (Fig. 6A, Lane 5). Cell cycle status was confirmed by flow cytometric analyses of propidium iodide-stained cells (data not shown). BUBR1 does not have an obvious ATP-binding consensus sequence (9, 15). To determine whether BUBR1 is an active protein kinase, we expressed BUBR1 as a six histidine-tagged fusion protein using the baculoviral expression system. The expressed His6-BUBR1 was analyzed by SDS-PAGE, followed by Western blotting. Fig. 7A shows that His6-BUBR1 antigen was present in sf-9 cells infected with BUBR1 recombinant baculoviruses (Lanes 2) but not in the cells infected with wild-type baculoviruses (Lanes 1). Infection of sf-9 cells with a higher titer (10^3 higher in Lane 3 than that in Lane 2) of the recombinant baculoviruses resulted in a significant increase of the amount of BUBR1 antigens (Lane 3). The recombinant BUBR1 expressed in sf-9 cells (Fig. 7B, Lane 1) was purified to near homogeneity (Lanes 2 and 3) via affinity chromatography. The purified protein was immobilized onto Ni-NTA resins and assayed for autophosphorylation activity in a kinase assay as described in “Materials and Methods.” Fig. 7C shows that His6-BUBR1 (Lane 2, 10 μg of protein), but not the vehicle resins (Lane 1), is capable of autophosphorylation. We noticed that the autophosphorylation did not shift BUBR1 to the slowly migrating one.

**Discussion**

Several groups have recently cloned BUB1 (9), MAD3 (15), and SSK1 (16), which are identical to our cloned gene MAD3L (AF068760). Although the putative protein kinase has been implicated in the mitotic checkpoint control (9, 15), little was known regarding its regulation and mode of action during the mitotic checkpoint activation. In the present study, we have shown that high levels of BUBR1 mRNA expression are correlated with a high mitotic index. It is known that activation of yeast BUB1 and MAD3 halts mitotic progression when chromosomes fail to align correctly during mitosis because of disruption of microtubules (4). It is reasonable to speculate that BUBR1 protein also functions as a mitosis-safeguard protein to ensure the order of progression of mitotic events. Therefore, it is conceivable that the BUBR1 gene expression may not be required for quiescent or differentiated cells. Indeed, this notion is supported by our observations that fully differentiated primary tissues such as muscle, heart, and brain express no detectable levels of BUBR1 transcripts (Fig. 1; Table 1). Indeed, recent studies by Chan et al. (20) have convincingly shown that BUBR1 is essential for the mitotic checkpoint activation and for normal mitosis. BUBR1 proteins were present, albeit at different levels, in most cell lines examined (Fig. 4A). Dami and HEL express the most abundant BUBR1. It is interesting to note that these

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**Table 1** Summary of BUBR1 expression in 50 primary human tissues

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Sample ID</th>
<th>Relative Levels (arbitrary unit)</th>
<th>Tissue Type</th>
<th>Sample ID</th>
<th>Relative Levels (arbitrary unit)</th>
</tr>
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<td>Pancreas</td>
<td>D3</td>
<td>–*</td>
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<tr>
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<td>–</td>
<td>Pituitary gland</td>
<td>D4</td>
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<td>Adrenal gland</td>
<td>D5</td>
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<td>Thyroid gland</td>
<td>D6</td>
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<tr>
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<td>A5</td>
<td>–</td>
<td>Salivary gland</td>
<td>D7</td>
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<tr>
<td>Frontal lobe</td>
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<td>–</td>
<td>Mammary gland</td>
<td>D8</td>
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<tr>
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<td>Kidney</td>
<td>E1</td>
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<tr>
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<td>Liver</td>
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<td>Small intestine</td>
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<td>Lung</td>
<td>F2</td>
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<td>Fetal spleen</td>
<td>G5</td>
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<td>Fetal thymus</td>
<td>G6</td>
<td>60</td>
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<tr>
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<td>D2</td>
<td>10</td>
<td>Fetal lung</td>
<td>G7</td>
<td>8</td>
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* –, signals not detectable.
two cell lines are either megakaryocytic or with megakaryocyte-differentiation potentials (21), and that a significant fraction of cells are polyploid. This implies that high levels of BUBR1 may play a role in terminal differentiation of megakaryocytes by negatively affecting mitotic progression. A recent study showed that BUBR1 and BUB1 sequentially assembled onto kinetochores during prophase, and more BUBR1 and BUB1 proteins are associated with kinetochores of unaligned chromosomes (22).

We have shown that phosphorylation appears to be a primary mechanism regulating BUBR1 activity during mitotic checkpoint activation. The dramatic mobility shift observed with BUBR1, a protein of 120 kDa, after mitotic checkpoint activation suggests that the protein is phosphorylated on many sites. Our in vitro kinase assays (Fig. 7C) demonstrated that BUBR1 is capable of autophosphorylation, although its autokinase activity was rather low as compared with the immunocomplex kinase assay (data not shown). Autophosphorylated BUBR1 did not result in the mobility shift, suggesting that some other kinase activities are required for the conversion of BUBR1 to the hyperphosphorylated form. Yeast BUB1 protein appears to lie at the beginning of the mitotic checkpoint signaling pathway (1, 23), and its kinase activity is activated during the mitotic checkpoint activation (24). In addition, p38, a mitogen-activated protein kinase family member implicated in mitotic checkpoint control, is rapidly activated by Noc (25). Therefore, it would be interesting to examine whether BUB1 or p38 is the putative activity that phosphorylates BUBR1 during the mitotic checkpoint activation.

BUBR1 appears to be also regulated, to a certain extend, at protein levels (Fig. 6). Although present in G1, BUBR1 levels are significantly increased as cell progress into later S and G2 stages of the cell cycle. Recently, it has been reported that a murine BUBR1 counterpart contains a RSSLAELKS motif similar to the destruction box (18), commonly found in many

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4 Unpublished data.
precipitated with the anti-BUBR1 antibody (collected and lysed in the lysis buffer. HeLa cell lysates were immuno-
treated with Noc for 16 h. The entire population of treated cells (B,
lysates obtained by mechanical shake-off (Lane 8) were ana-
expression via Western blotting. Interphase HeLa cell lysates (Lane 7)
and mitotic cell lysates obtained by mechanical shake-off (Lane 8) were ana-
expression via Western blotting. Lane 7, Lane 8, and Lane 9 were analyzed by SDS-PAGE, followed by Western blotting using the anti-BUBR1 antibody.

proteins that are regulated in a cell cycle-dependent manner.
This motif is also present in human BUBR1 (amino acids
225–233), supporting the notion that BUBR1 protein levels
may fluctuate during the cell cycle. No phosphorylated form
of BUBR1 is detected until cells are in mitosis. This is consis-
tent with the function of mitotic checkpoint genes, i.e., to
monitor and control metaphase-anaphase transition.

Although the biochemical function of yeast MAD3 is un-
known, it has been shown recently that Saccharomyces cerevisiae MAD3, as well as MAD1 and MAD2, associate with
CDC20, a protein required for exiting from mitosis (26). The interaction between MAD3 and CDC20 appears to be cell
cycle dependent, peaking during mitosis (26). It has been suggested that CDC20 is a target of the yeast mitotic check-
point (26). Human p55CDC, a CDC20 homologue in mammals, is a phosphoprotein, and the phosphorylation peaks at
mitosis (21, 27). In addition, it has been shown that p55CDC
connects MAD2 to APC (10). Thus, it would be interesting to
examine whether BUBR1 might relay a mitotic checkpoint
signal to APC via p55CDC.

Extensive research in the past decade or so has shown
that many genes involved in cell cycle regulation or check-
point controls also play an essential role in the suppression
of tumor growth. For example, p53 regulates G1–S and G2–M
checkpoint, and the ATM gene product regulates DNA dam-
age checkpoint. Structural abnormalities that result in func-
tional defects of p53 or ATM are closely correlated with the
neoplastic transformation (28, 29). The mitotic checkpoint
regulates the initiation and segregation of chromosomes. A
failure in this checkpoint can lead to the loss or gain of
genetic materials in daughter cells, which is thought to con-
tribute to the genomic instability associated with cancer
development and progression (13). We and others have
mapped the BUBR1 gene to chromosome 15q14–15 or
14q13–15 (9), a locus shown to exhibit loss of heterozygosity in
different types of cancers (30, 31). It has been proposed that
chromosomal instability in most colorectal cancers, as well
as in many other cancers, may be a direct consequence of
mutational inactivation of mitotic checkpoint genes such as
BUB1 or BUBR1 (9). The absence of BUBR1 antigen in PC3
(Fig. 4A, Lane 9) may be attributable to abnormalities in the
BUBR1 gene because defects in this gene have been re-
ported in colorectal cancer samples (9). Obviously, further
studies will be necessary to fully understand the role of
BUBR1 in normal as well as in abnormal cell growth.

Materials and Methods
Materials. All culture media (RPMI 1640, MEM, Iscove’s modified Dul-
becco’s medium, DMEM, and McCoy’s) and antibiotics (penicillin-strep-
tomycin) were purchased from Life Technologies, Inc. (Grand Island, NY).
A multiple tissue master dot blot was purchased from Clonetech, Inc. (Palo
Alto, CA). The pT7Blue plasmid was obtained from Novagen, Inc. (Mad-
ison, WI). [α-32P]dCTP (800 Ci/mmol) was from DuPont NEN (Wilmington,
DE). Fetal bovine serum was purchased from Hyclone Laboratories (Lo-
gan, UT). Polyclonal anti-BUBR1 antisera were raised in rabbits via Re-
search Genetics (Atlanta, GA). λ phosphatase was from New England
Biolab (Beverly, MA). A baculoviral expression system was purchased from PharMingen (San Diego, CA).

Cell Lines and Treatments. Cell lines were grown in various media
supplemented with 10% FBS and antibiotics (100 μg/ml penicillin and 50
Characterization of Human BUBR1

...Cells lines included in the present study were Damí (megakaryoblastic leukemia), HEL (erythroleukemia), HEIde (myeloid progenitor), SAM-1 (megakaryocytic), LNCaP (prostatic carcinoma), DU145 (prostatic carcinoma), HeLa (cervical carcinoma), GM00637D (fibroblast), Molt4 (T-lymphocytic), A549 (transformed lung fibroblast), and PC3 (prostatic carcinoma).

A549 and HeLa cells were used for synchronization studies. G1 phase A549 cells were obtained by culture in methionine-free DMEM containing 10% FBS for 48 h. A549 cells synchronized at the G1-S boundary were achieved by sequential culture in medium containing aphidicolin (1 μM/mL) for 14 h, normal medium for 14 h, and finally, medium containing thymidine (2 mM) for 14 h. A549 cells arrested at late S and G2 were obtained through washing cells arrested at the G1-S boundary, as described above, with PBS and reculturing in fresh DMEM with 10% FBS for 6–7 h. To obtain mitotic prometaphase cells, A549 cells were treated with nocodazole (0.4 μM/mL) for 16 h. HeLa cells treated with nocodazole for 16 h were used for collection of mitotic shake-off cells. Interphase HeLa cells were the adherent fraction of HeLa cell population. The cell cycle status of the treated cells was confirmed by flow cytometric analysis of propidium iodide-stained cells.

To obtain mitotic checkpoint, A549, GM00637D, or HeLa cells were treated with nocodazole (0.4 μM/mL) for 16 h (unless otherwise specified). At the end of the treatment, cells were lysed in a lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μM/ml aprotinin, 10 μM/ml leupeptin, 10 μM/ml soy bean trypsin inhibitor, 10 mM NaF, and 300 μM okadaic acid]. Cell lysates were stored at -70°C for subsequent analyses.

**Western Blotting.** Equal amounts of cell lysates were analyzed by SDS-PAGE, followed by Western blotting using an anti-BUB1 antibody. The anti-BUB1 antiserum was raised in rabbit against BUB1 peptide (residues 421–438). The protein blots were first probed with the BUB1 antiserum, followed by a secondary antibody conjugated with horseradish peroxidase as described (19). The specific signals were quantified using an image scanner (Imager Densitometer GS-700; Bio-Rad, Richmond, CA).

**Dot Blotting.** A multiple-tissue dot blot purchased from Clontech was hybridized overnight with a 32P-labeled BUBR1 probe according to the supplier’s protocol. The blot was washed as described (12) and autoradiographed. Specific signals detected on autoradiographs were quantified using an image scanner (Imager Densitometer GS-700; Bio-Rad, Richmond, CA).

**Immunoprecipitation and BUBR1 Protein Kinase Assays.** Equal amounts of cell lysates were supplemented with an anti-BUB1 antiserum (1:500 dilution) or with the preimmune serum and incubated at room temperature for 2 h or at 4°C overnight. Protein A/G agarose beads (25 μg/mL) were then added to each immunoprecipitation mixture, and the incubation continued at room temperature for 1 h or at 4°C for 2 h. Immunoprecipitates were collected, washed three times with the lysis buffer, and analyzed by SDS-PAGE, followed by Western blotting for kinase assays. His6-BUB1 was purified using Ni-NTA resins. The purified His6-BUB1 was resuspended in a kinase buffer [10 mM HEPES (pH 7.4), 10 μM MnCl2, and 5 mM MgCl2]. Kinase assays were initiated by the addition of [γ-32P]ATP (5 μCi). The kinase mixtures were incubated at 37°C for 30 min and then analyzed by SDS-PAGE, followed by autoradiography.

**Acknowledgments**

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**References**


