Box1 and 2 (box1/2) are conserved cytoplasmic motifs located in the membrane proximal region of cytokine receptors, including the human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor common βc. Deletion of box1/2 abrogated all the examined activities of GM-CSF, and this phenomenon is explained by the loss of binding by Jak2. To test if a molecule other than Jak2 interacting with the box1/2 region plays a role in GM-CSF receptor signal transduction, we screened for molecules interacting with the box1/2 region by a pull-down assay using recombinant purified protein of GST fused with the βc box1/2 region and a Ba/F3 cell lysate. The mouse homologue of Mad2 protein, which plays an important role in the M phase of the cell cycle, was revealed to associate with the box1/2 region specifically. Peptides corresponding to the box1 sequence also bound to Mad2, and mutation of the box1 decreased the Mad2 interaction. Deletion analysis indicated that interaction with box1/2 occurred through the C-terminal portion of Mad2. Mad2 is known to change affinity for binding partners cell cycle dependently. Binding affinity of Mad2 to box1/2 increased in the late M phase, suggesting the possibility that GM-CSF participates in regulation of the M phase checkpoint through interaction with Mad2.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that stimulates the proliferation and differentiation as well as survival of various hematopoietic cells (1). The receptor of human (h) GM-CSF (GM-CSFR) consists of two subunits, α and β, both of which are members of the cytokine receptor superfamily (2). The α subunit is specific to hGM-CSFR, whereas the β subunit (βc) is shared by IL-3, GM-CSF, and IL-5 receptors (2). GM-CSF induces tyrosine phosphorylation of βc and various cellular proteins and activates early response genes such as c-fos, c-jun, and c-myc, as well as stimulates cell proliferation in hematopoietic cells and fibroblasts (3). GM-CSF activates various signaling molecules, including Jak2, STAT5, mitogen-activated protein kinase cascade kinases, and phosphatidylinositol 3-kinase (2). βc contains box1 and box2 regions (box1/2), which are conserved among the cytokine receptor superfamily, and 8 tyrosine residues located in its cytoplasmic region (4, 5). To better comprehend signaling events involved in cell proliferation, we and others analyzed biological activities of various mutants of βc (6–8) and found that the box1 region was essential for all of the hGM-CSFR signals we examined. Although the box1/2 region is essential and sufficient for cell proliferation and survival, a tyrosine residue(s) is also required for mitogen-activated protein kinase and c-fos promoter activation (8, 9). Because box1 is assumed to bind Jak2, it is likely that activation of Jak2 is sufficient and essential for hGM-CSFR signals through interaction with the box1 region of βc. This notion was supported by observations that dominant negative Jak2 suppressed all the GM-CSFR signaling and activities we investigated (7). In addition, Jak2 knockout mice showed defects in GM-CSF-dependent colony formation (10, 11).

The box1 region contains the proline-X-proline sequence, which is conserved among members of the cytokine receptor superfamily (12). Mutation analysis of the motif suggested a crucial role for the motif in a variety of receptor-mediated signalings. The G-CSF receptor mutant in which the conserved proline-X-proline is substituted by Ala-X-Ala can induce neither DNA synthesis nor cell proliferation (13). The mutant growth hormone receptor failed to induce either cell proliferation or spi-1 induction (14, 15). Similar results were obtained in the case of gp130 and the prolactin receptor (12, 16, 17). Although the importance of the box1 region in various receptors has been noted, structural information about this region has been awaited. Thus, the role and mechanism of box1 function have remained unsolved.

We have now identified Mad2 (mitotic arrest-deficient 2) protein as a box1/2-binding protein. Mad2 was first discovered as a gene responsible for the mitotic checkpoint in yeast (18). The knockout of Mad2 in mice showed that Mad2 is essential in mouse cells after embryonic day 6.5, although it is dispensable for normal cell division in yeast (19). During mitosis, Mad2 localized at an unattached kinetochore monitors for correct spindle-kinetochore attachment, a prerequisite for initiation of anaphase (20). The Mad2-dependent monitoring system, maintained until the completion of spindle attachment, prevents anaphase initiation through Mad2-Cdc20 complex formation (21–23). Cdc20 activates the ubiquitin ligase activity of an-
aphase promoting complex (APC), after which the activated APC promotes the initiation of anaphase. There are a variety of Mad2 binding partners, including molecules not related to the mitotic checkpoint (24–27). Here, we found that hGM-CSFR βc acts as one of such partners of Mad2 and that their interaction is regulated in a cell cycle-dependent manner.

**EXPERIMENTAL PROCEDURES**

Reagents—Recombinant murine IL-3 (mIL-3) expressed in silk-worms, Bombyx mori, was purified as described (28). Recombinant hGM-CSF was a gift from Schering-Plough (Madison, NJ). Anti-Mad2, anti-hGM-CSFR receptor βc, and anti-GST (B-14) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Mad2 was from Transduction Laboratory (Lexington, KY), and anti-phospho-

**Plasmid Construction**—The plasmids encoding GST fusion protein were constructed by using pGEX-5X-1 vector. His-tagged recombinant proteins were constructed by using the QI-

**GST pull-down assay and immunoprecipitation**—The lysate (10° cells/ml) prepared from Ba/F3 cells was incubated overnight at 4°C with GST fusion proteins and glutathione Sepharose. GST fusion protein and associated protein(s) were eluted with 15 μl glutathione and dialyzed against 20 mM Tris-HCl, pH 7.5. The samples were lyophilized and dissolved in suitable buffer for further analyses.

**For immunoprecipitation analysis, cell lysates (2 × 10⁷ cells/sample) were incubated with an appropriate antibody and protein G-Sepharose 4 FF (Amersham Pharmacia Biotech) overnight at 4°C. The bands were visualized by using an ECL Western blotting detection system (Amersham Pharmacia Biotech).**

**Cell Culture and Cell Lysate Preparation**—The mouse IL-3-dependent, pro-B cell line Ba/F3 stably expressing both α and β subunits of hGM-CSFR (Ba/F3-wild) was maintained in RPMI 1640 containing 5% fetal calf serum, 0.25% mg/ml of Na3H, 50 units/ml penicillin, and 50 μg/ml streptomycin. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Whole cell lysates used for pull-down assay and immunoprecipitation were prepared by using lysis buffer (40 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.25% Nonidet P-40, 2 mM EDTA, 50 mM NaF, 20 mM β-glycerophosphate, 1 mM NaVO₃, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml apro-

**E. coli strain carrying a pREP4 plasmid), and purification of the protein was done as follows. Briefly, the cells were incubated in hypotonic buffer (10 mM Hapes, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 50 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) for 15 min. Nonidet P-40 was added to the cell suspension at a final concentration of 0.6%. The Nonidet P-40-insoluble fraction (enriched in nuclei) was separated by centrifugation (15,000 × g for 30 s). The pellets were washed with hypotonic buffer two times and incubated in extraction buffer (50 mM Hapes, pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.5% Nonidet P-40) and disrupted by sonication at 4°C.

**For preparation of cell cycle-synchronized cell lysates, HeLa cells were arrested at the G1/S boundary by a double-thymidine block (30). Briefly, HeLa cells were treated for 14 h with Dulbecco’s modified Eagle’s medium containing 2 mM thymidine, released for 10 h in normal medium, and then treated again with thymidine for 14 h. Thereafter, the cells were released in normal medium and harvested after incubation for the times indicated in the figure. For Ba/F3 cells, cells were depleted of mL-3 for 12 h to become arrested in the G1 phase. hGM-CSF (2 ng/ml) was then added, and the cells were harvested at the indicated time points. The phase of the cell cycle was confirmed by examining DNA contents by propidium iodide staining and flow cytometry (FACSscan, Becton Dickinson, San Jose, CA). The lysates from each time point were prepared as described above. Protein amounts of the lysates were quantified with a BCA protein assay kit (Pierce) to confirm equal extraction efficiency among the samples. The lysate from each time point was divided into two tubes, and incubated with antibody (2 μg, for Ba/F3 cells), GST-box1/2 purified protein (for HeLa cells), or anti-p55CDC antibody (2 μg). Associated proteins were precipitated either with glutathione-Sepharose beads or protein G beads and blotted with SDS-PAGE followed by Western blotting using anti-

**Electrophoresis, Blotting, Amino Acid Sequence, and Cloning of cDNA—Two-dimensional electrophoresis, a combination of isoelectric focusing and SDS-PAGE, was carried out according to O’Farrell (31). Protein spots were detected either by silver staining or Western blotting. Silver staining of the PAGE gel was done with a 2D Silver Stain II kit (Bio-Rad, Richmond, CA). Anti-phospho-p55CDC antibody (2 μg) was associated with proteins by SDS-PAGE followed by Western blotting using anti-Mad2 antibody.

For amino acid sequencing, two-dimensional separated gels were stained with 0.25% Coomassie Brilliant Blue and bands of 25 and 75 kDa were excised and treated with 0.2 μg of Achromobacter protease I (a gift from Dr. Masaki, Ibaraki University (Ref. 32)) at 37°C for 12 h in 1 μl Tris-HCl, pH 9.0, containing 0.1% SDS. The peptides generated were extracted from the gel and separated on columns of DEAE-SPW (2 × 20 mm; Tosoh, Tokyo, Japan) and Micromass RP-18 (2 × 25 mm; Kanto Chemical, Tokyo, Japan) connected in series with a model 1100 (Hewlett Packard, Palo Alto, CA) liquid chromatography system. Peptides were eluted at a flow rate of 0.1 ml/min, with a linear gradient of 0–60% solvent B, where solvents A and B were 0.09% (v/v) aqueous trifluoroacetic acid and 0.075% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile, respectively. Selected peptides were sequenced by Edman degradation in a model 477A automated protein sequencer (PerkinElmer Life Sciences) connected on-line to a model 120A PTH (PerkinElmer Life Sciences) and also examined by matrix-assisted laser desorption ionization time of flight mass spectrometry with a Reflex MALDI-TOF (Bruker-Franzen Analytik, Bremen, Germany) in linear mode, with 2-mercaptobenzothiazole used as a matrix. For cloning of full-length mouse Mad2 cDNA, PCR primers were designed according to the GenBank™ mouse Mad2 equivalent sequence of expressed sequence tag. PCR fragments were recovered by use of a TA
Mad2 Interaction with hGM-CSF Receptor βc

RESULTS

Isolation of Box1/2-interacting Proteins by Pull-down Analysis—To determine whether proteins other than Jak2 bind to the box1/2 region, we conducted pull-down assays using the recombinant box1/2 region of βc and Ba/F3 cell lysates. GST protein fused with the βc box1/2 region (GST-box1/2) or the cytoplasmic region of α subunit (GST-α) was constructed, as shown in Fig. 1A. GST-box1/2, GST-α, and GST protein were purified by using a glutathione column and then incubated with the Ba/F3 cell lysate. Proteins were precipitated by glutathione-Sepharose 4B beads and analyzed by two-dimensional electrophoresis. To identify GST protein-binding proteins derived from E. coli, purified GST-box1/2, GST-α fusion proteins, and GST protein were also subjected to two-dimensional electrophoresis, and all the gels were silver-stained. We then compared the six panels precisely and found two proteins that specifically bound to GST-box1/2. Fig. 1B shows the pattern of GST-box1/2-binding proteins, and arrows indicate specific binding proteins with approximate molecular masses of 25 and 75 kDa. The 25-kDa protein was not detected in samples prepared with GST or GST-α, and only a residual amount of the 75-kDa one co-precipitated with GST or GST-α. Because these proteins were not observed in the gel with GST-box1/2 protein alone, these were probably specific binding proteins that originated from Ba/F3 cells. We excised these spots and subjected them to microsequencing. The amino acid sequence revealed that two fragments derived from the 75-kDa protein were contained in GRP78 protein (33). Because GRP78 exclusively localizes in the endoplasmic reticulum and its structure is closely related to Hsp-70 (33), we speculate that the binding of GRP78 to GM-CSFR is an artificial event only observed in an in vitro system. A fragment derived from the 25-kDa protein corresponded to amino acids 193–199 of the human Mad2 protein. As the 25-kDa spot was recognized by an anti-Mad2 antibody in two-dimensional Western blotting analysis (data not shown), this 25-kDa protein is probably the mouse counterpart of the human Mad2. We also analyzed the immunoreactivity of the anti-Mad2 antibody toward GST protein-precipitated proteins by SDS-PAGE followed by Western blotting. The anti-Mad2 antibody recognized a band with a molecular mass of 25 kDa, and this band was observed only with the GST-box1/2 samples incubated with the Ba/F3 cell lysate (Fig. 1C, lane 7).

To determine whether the endogenous full-length βc protein binds to Mad2, we carried out co-immunoprecipitation analysis, using anti-βc antibody (specific for human βc) and Ba/F3 cells expressing hGM-CSFR. As shown in Fig. 1D, Mad2 protein co-immunoprecipitated with βc, thereby suggesting an association of Mads with the native receptor. We next asked whether this association is direct, and for this we used a binding assay of recombinant Mad2 and box1/2 proteins. Full-length mouse Mad2 coding region cDNA was isolated by PCR using synthesizing primers according to GenBank® mouse Mad2 equivalent sequences. The recently published sequence of the mouse Mad2 (19) completely matched the sequence of our PCR product. To obtain purified Mad2 for the binding assay, we constructed histidine-tagged Mad2 protein (His-Mad2) and purified it. Then, binding activity of His-Mad2 protein and GST-box1/2, GST-α, or GST proteins was examined. As shown in Fig. 2 (lanes 1–3), when the protein complex was precipitated with glutathione-Sepharose 4B beads, only the GST-box1/2 co-precipitated with His-Mad2. Likewise, when proteins were precipitated by Ni²⁺ beads, ARROW indicates Mad2 detected with anti-Mad2 antibody.

Fig. 1. Pull-down assay of Ba/F3 cell lysates and GST proteins fused with hGM-CSF receptor. A, schematic representation of GST-α and GST-box1/2, which contain GST portion fused with cytoplasmic region of hGM-CSFR α subunit and box1/2 region of βc, respectively. AH is the conserved α homology region. TM, transmembrane. B, silver staining pattern after two-dimensional gel electrophoresis of GST-box1/2-binding proteins. Pull-down assay was done by using GST-box1/2, GST-α, or GST itself, and co-precipitated proteins were separated by two-dimensional electrophoresis. Purified GST proteins themselves were also applied to two-dimensional electrophoresis. A total of six panels were carefully compared, and two proteins that bound to the GST-box1/2 specifically were identified. Panel shows the pattern of proteins binding to GST-box1/2, and specific proteins are indicated by the arrows. IEF, isoelectric focusing. C, Western blotting pattern of GST protein-binding proteins. GST-box1/2, GST-α, or GST itself was incubated with or without Ba/F3 cells lysate, and proteins precipitated with GSH beads were separated by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane, and Western blotting was done with anti-Mad2 antibody. D, co-immunoprecipitation of Mad2 and hGM-CSF βc. Immuno precipitation (IP) was done by using Ba/F3 cell lysate with anti-βc or control IgG and was followed by Western blotting. The blotted protein was detected with anti-Mad2 antibody. The arrow indicates Mad2 detected with anti-Mad2 antibody.
again only the GST-box1/2 co-precipitated with the His-Mad2 protein (lanes 10–12). In contrast, neither GST-α nor GST was precipitated together with His-Mad2. Lanes 4–6 and 7–9 indicate that nearly the same amount of proteins precipitated in each lane. These results indicate that Mad2 association with box1/2 is specific and direct.

**Amino Acid Sequence of Box1/2 Required for Mad2 Binding**—We next examined the requirement of the box1/2 sub-region for binding to Mad2 protein, using a βc-derived peptide conjugated with SulfoLink beads. Various peptides corresponding to a portion of the box1/2 region or its derivatives were synthesized and conjugated with SulfoLink beads at their N-terminal cysteine residue (Fig. 3A). The association with Mad2 was examined by incubating these peptides with Ba/F3 cell lysate, and the proteins that bound to the peptide beads were precipitated and separated by SDS-PAGE. Fig. 3B shows the Western blotting pattern obtained by using anti-Mad2 antibody for reaction with the precipitated proteins. Mad2 co-precipitated with the peptide-beads that covered the entire box1 region (lane 2). We next examined the binding potential of various box1/2-derived peptides. Neither box2 peptide nor control peptide precipitated Mad2 protein (lanes 4 and 7). Amino acids “PNP” conserved within box1 region of many cytokine receptors are thought to be critical for signal transduction as determined by mutation analysis of several receptors (12). When a peptide carrying a mutation within this conserved PNP motif (box1/ANA) was used for precipitation, the amount of co-precipitated Mad2 was significantly reduced (lane 3). It was also reported that 8 amino acid residues are critical for receptor activation (7, 34). The box1 peptide, which lacks these critical residues (ΔBox1), abrogated Mad2 precipitation (lane 6). The peptide corresponds to a joint region of box1 and box2 also could not precipitate Mad2 (lane 5). These results suggest that the conserved region of box1 plays an important role in binding to Mad2 protein. We also checked direct binding between recombinant purified His-Mad2 and these peptides, and essentially the same results (data not shown) as obtained with the Ba/F3 cell lysate emerged.

**Region of Mad2 Required for Binding to βc**—To analyze the region of Mad2 required for binding to βc, we next constructed deletion mutants of Mad2, as schematically shown in Fig. 4A. Mutants were fused at their N terminus with a histidine tag for purification. Purified histidine-tagged Mad2 mutant proteins were incubated with purified GST box1/2, and the proteins were precipitated using glutathione-Sepharose 4B beads and analyzed by Coomassie Brilliant Blue staining (Fig. 4B). The GST-box1/2 could bind wild type (lane 2) as well as mutant Mad2 lacking the N-terminal portion (ΔN, lane 4), but the amount of precipitated ΔN-Mad2 was clearly less than that of the wild-type Mad2, thus indicating that the N-terminal region of Mad2 was not essential for but influenced the binding affinity between Mad2 and box1/2. In contrast, mutant Mad2 lacking the C terminus (ΔC) did not bind to GST-box1/2 (lane 3). In view of these data, we speculate that the C-terminal portion (54 residues, amino acid positions 152–205) is sufficient and is required to interact with GST-box1/2. The anti-Mad2 antibody, which recognizes the ΔN mutant but not the ΔC mutant interfered with the association between box1/2 protein and Mad2 of Ba/F3 lysates, in a dose-dependent manner (Fig. 4C). In contrast, control antibodies, anti-γ-catenin, and anti-phosphotyrosine (4G10), did not affect Mad2 binding, thereby supporting the conclusion that the C-terminal region of Mad2 is important and sufficient for binding between box1/2 and Mad2.

**Cell Cycle-dependent Binding of Mad2 to Box1/2 of βc**—Mad2 associates with various cell cycle-related proteins in a cell cycle phase-dependent manner (21, 22, 30, 35, 36). To determine whether the association between Mad2 and βc is also affected during the cell cycle, we prepared HeLa cell lysates at various phases of the cell cycle. HeLa cells were arrested at G1 by a thymidine double block and released from arrest by removing thymidine. Cells were harvested at the time points indicated in Fig. 5A, and a portion was used to analyze the cell cycle. Cell lysates prepared from harvested cells were divided to two samples. With one being incubated with GST-box1/2 protein and precipitated with glutathione-Sepharose 4B beads. And the other immunoprecipitated with anti-p55 CDC (Cdc20) antibody. The co-precipitated Mad2 was then analyzed by Western blotting (Fig. 5A). As expected, association between Mad2 and p55CDC was not observed at the G1/S phase but became slightly visible 6 h after release from the thymidine block and was dramatically enhanced with a peak at 9 h, as reported (30). At that time, the total amount of Mad2 found in the total lysate (bottom panel) had increased between 6 and 9 h and then continued to increase gradually along with cell cycle progression. A low level of association between Mad2 and box1/2 was observed 9 h after the release from the thymidine block. In contrast to the finding that the association of p55CDC and Mad2 was transient, the extent of association between Mad2 and box1/2 increased for at least 15 h. These results suggest that p55CDC and box1/2 may associate with Mad2 through different mechanisms. In addition, when GST-box1/2-precipitated membrane was blotted with anti-p55CDC, no band was observed, which meant that a triple complex of Mad2, p55CDC, and βc may not have formed. Using fractionated cell lysates, we next examined whether or not the changes in the binding pattern were caused by a change in the subcellular localization of Mad2. The assay of GST-box1/2 and Mad2 binding was done as described in Fig. 5A except that fractionated HeLa cell lysates were used. HeLa cell lysates were divided into 0.6% Nonidet P-40 soluble fraction (cytoplasmic portion) and high salt-extracted fraction (nuclear fraction), and the binding assay using recombinant purified GST-box1/2 was done. The total amount of Mad2 in the fractions was analyzed by Western blotting. As shown in Fig. 5B, binding of Mad2 with GST-box1/2 was seen only with the cytoplasmic fraction. The time course of binding was similar to that observed for the total cell extract. In contrast, no visible binding was observed with Mad2 from the nuclear fraction, although there was a greater amount of Mad2 in this fraction. These results indicate that the change in the binding property between box1/2 and Mad2 may
be regulated by a change in affinity, not by a change in subcellular localization. Using Ba/F3 cells, we next examined the binding property of Mad2 in various phases of cell cycle progression. Ba/F3 cells were depleted of mIL-3 for 12 h and then re-stimulated with hGM-CSF. At selected time points, cell cycle progression was analyzed by using propidium iodide (Fig. 5C). The affinity of Mad2 for box1/2 region also changed during cell cycle progression (35). Interestingly, the affinity of Mad2 for the box1/2 region also changed during cell cycle progression in both Ba/F3 and HeLa cells. We found that the C terminus of Mad2 was important for binding with βc. Because one of the Mad2 M phase binding partners, p55CDC (Cdc20), binds to the C terminus of Mad2 (39), βc and p55CDC may use a similar mechanism to bind to Mad2. When we examined the time course of the Mad2/βc interaction, the binding became visible slightly later than that of Mad2/p55CDC protein in both HeLa and Ba/F3 cells, suggesting that a sequence similar to the common motif observed in the box1 region of cytokine receptors (12). Because peptide analysis showed the importance of the conserved amino acid motif of the β box1 region for Mad2 binding, a similar motif may also play a role in the binding between Mad2 and the insulin receptor. The insulin receptor activates Jak1 and Jak2 (37), but the region of this receptor responsible for binding to Jak proteins has not yet been revealed. Because Jak2 is assumed to interact with the box1/2 region of cytokine receptors, it thus can be speculated that Jak proteins bind to the insulin receptor through the same region as Mad2. Mad2 dissociates from insulin receptor by insulin stimulation (27). We examined the effects of hGM-CSF stimulation on Mad2 binding with βc, but no clear change of Mad2 and βc binding state up to 10 h after hGM-CSF stimulation was observed (data not shown). However, Mad2/βc association was increased after 12 h of hGM-CSF stimulation with the cell cycle progression. The addition of insulin induces tyrosine phosphorylation of the insulin receptor, and a C-terminal phosphorylated tyrosine residue is assumed to decrease binding affinity for Mad2 (27). In the case of βc, the absence of a tyrosine residue in the box1/2 region may be a possible reason for the lack of effect of hGM-CSF for Mad2 and βc binding in the early phase after stimulation.

The C terminus of Mad2 appeared to be important for binding with βc. Previous studies showed the importance of the Mad2 C terminus for binding with other Mad2 partners such as Cdc20 and Mad1 in mammalian and yeast systems (38, 39). The Mad2 contains the HORMA (for Hop1p, Rev7p, and Mad2) domain (40), which was found by comparative analysis of Mad2 proteins, thus indicating the possibility that the unique feature of Mad2 interaction with other proteins can be explained by the combinatorial effects of TPR and HORMA domains. The Mad2 contains the HORMA (for Hop1p, Rev7p, and Mad2) domain (40), which was found by comparative analysis of Mad2 proteins, thus indicating the possibility that the unique feature of Mad2 interaction with other proteins can be explained by the combinatorial effects of TPR and HORMA domains.

Mad2 is known to change its affinity toward interacting molecules during cell cycle progression (35). Interestingly, the affinity of Mad2 for the box1/2 region also changed during cell cycle progression in both Ba/F3 and HeLa cells. We found that the C terminus of Mad2 was important for binding with βc. Because one of the Mad2 M phase binding partners, p55CDC (Cdc20), binds to the C terminus of Mad2 (39), βc and p55CDC may use a similar mechanism to bind to Mad2. When we examined the time course of the Mad2/βc association precisely, the binding became visible slightly later than that of Mad2/p55CDC protein in both HeLa and Ba/F3 cells, suggesting that...
additional mechanisms may differently regulate the affinity of βc and p55CDC for Mad2. We found that Mad2 recovered in the 0.6% Nonidet P-40-soluble fraction bound to GST-box1/2 but that no binding occurred with the fraction extracted by the high salt condition, suggesting that the change in binding property is mainly caused by a change in affinity rather than one in subcellular localization. The trigger and mechanism of its changing of binding affinity of Mad2 to other partner proteins has not been clarified. We speculate that Mad2 may be modified in response to hGM-CSF stimulation, because GM-CSF activates cascades of kinases. Indeed, at the C terminus region of Mad2, there are several potential phosphorylation sites, but no report has appeared to suggest phosphorylation of the Mad2. To examine posttranslational modification of Mad2, we extensively examined mobility profile of Mad2 by two-dimensional gel electrophoresis, but Mad2 prepared from different phases of the cell cycle had the same mobility profile (data not shown). Further examination of tyrosine phosphorylation by Western blotting showed no detectable tyrosine phosphorylation. Taken together, our data indicate that some other mechanism(s) than phosphorylation is probable for the modification of Mad2.

Mad2 plays a major role in the nucleus as a member of the APC complex, and changing of the subcellular localization of Mad2 during cell cycle progression was reported. Mad2 appeared within the kinetochore in the G2/M phase but disappeared after cytokinesis (42). We also observed immunohistochemically a similar pattern of the subcellular localization of Mad2 overexpressed in Ba/F3 cells (data not shown). We hypothesize that the cytokine receptor anchors Mad2 in the cytoplasm until the appropriate time and that after its release, Mad2 is translocated to the nucleus where it functions as a member of the M phase checkpoint. Because the binding affinity between Mad2 and βc gradually increased after M phase, it is feasible that the cytokine receptor captures Mad2 after completion of spindle formation and keeps it until the appropriate time. As cytokine receptors are known to be internalized after ligand binding, the possibility exists that internalized GM-CSFR meets Mad2 in the cytoplasm.

The point of the cell cycle regulated by growth factors is assumed to be mainly in the G1/S phase transition. For example, lysates obtained at the indicated time points were divided to two portions, and proteins of both samples were precipitated either with anti-p55CDC antibody (upper panel) or GST-box1/2 (middle panel), and the co-precipitated Mad2 was analyzed by Western blotting. The amount of Mad2 in the total cell lysate was analyzed by Western blots using anti-Mad2 antibody (bottom panel). IP, immunoprecipitation. In B, essentially the same experiments as in A were done except that HeLa cell lysates were fractionated into cytoplasm and nuclear portions. Then, both fractions were precipitated with GST-box1/2, and a part of each was subjected to Western blotting using anti-Mad2 antibody to examine the total amount of Mad2. C and D, Ba/F3 cells were depleted of mIL-3 and then re-stimulated by hGM-CSF. Cells were collected at the indicated time points, and immunoprecipitation using anti-p55CDC (A) or -βc (C) antibodies or cell cycle analysis (C) were done. The intensity of co-immunoprecipitated Mad2 bands is also presented (D).
ple, the Ba/F3 cell line, which was used in this present study, is mIL-3-dependent, and classical experiments using transient stimulation of Ba/F3 cells indicate that the presence of cytokine only at the G1/S phase can promote completion of the whole cell cycle (data not shown). Most assays are employed to analyze signals and functions of growth factors that are early events after growth factor stimulation. Thus, although the regulation of the G1/S phase by growth factor is extensively studied, the regulation of the G2/M phase by growth factors has received less attention. How does the cytokine receptor contribute to M phase progression? Our results suggest new mechanism of regulation of the cell cycle by cytokine in the M phase.

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