MBLR, a new RING finger protein resembling mammalian Polycomb gene products, is regulated by cell cycle-dependent phosphorylation

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Abstract

Background: The RING finger proteins function in a variety of fundamental cellular processes. The products of some members of the Polycomb group (PcG) bear ring finger domains and are defined as a subclass of RING finger proteins. Among them are Drosophila posterior sex combs and suppressor 2 of zeste, whose RING fingers are conserved in vertebrate PcG proteins Mel18 and Bmi1.

Results: We have identified a new mammalian RING finger protein, termed MBLR due to its structural similarity to Mel18 and Bmi1 (Mel18 and Bmi1-like RING finger protein). MBLR interacts with some PcG proteins: in vitro biochemical data support the idea of a direct interaction of MBLR’s RING finger domain with Ring1B, which is highly homologous to one of the mammalian PcG genes, Ring1A. We also show that MBLR acts as a transcriptional repressor in transiently transfected cells, as is the case for other PcG proteins. Immunocytochemical analysis reveals that MBLR protein is localized in a fine-grained distribution throughout the nucleoplasm in interphase cultured cells and in a fainter diffuse cytoplasmic distribution in mitotic cells. In addition, we find that serine 32 of MBLR is specifically phosphorylated during mitosis, most likely by CDK7, a component of the basal transcriptional machinery.

Conclusion: Similarities to previously defined PcG proteins suggest that MBLR should be included in the same subclass of RING finger proteins as Mel18 and Bmi1. Although the biological relevance of the cell cycle-related phosphorylation remains to be demonstrated, serine 32 phosphorylation could nevertheless be functionally important.

Introduction

The RING finger motif is characterized by a cysteine-rich Zn2+-binding domain and defines a superfamily of diverse proteins (Borden & Freemont 1996; Freemont 1993). The RING finger domains are involved in protein–DNA and protein–protein interactions and E2-dependent ubiquitinations and, consequently, function in a variety of fundamental cellular processes including the regulation of gene expression, signal transduction and proteolysis (Fremont 2000; Jackson et al. 2000; Joazeiro & Weissman 2000). Although cysteine-cysteine/histidine pairs are conserved among RING finger proteins, amino acid sequences between these cysteine-cysteine/histidine pairs and at the linking region between the two finger-like domains have diverged, thereby providing a molecular basis for the functional heterogeneity of the RING finger domains.

Functional insights into the RING finger domains are in part obtained from genetic and molecular analyses of Drosophila and mammalian Polycomb group (PcG) gene products. The Drosophila PcG proteins posterior sex combs (Psc), and its neighbouring and related gene
product, the suppressor two of zeste [Su(z)2], share sequence homology with the mammalian PcG proteins, Mel18 and Bmi1 in their RING finger domains. This homology defines a subclass of RING finger proteins which are conserved between invertebrates and vertebrates (Brunk et al. 1991; Ishida et al. 1993; Tagawa et al. 1990; van Lohuizen et al. 1991). Interestingly, all of the RING finger proteins listed above contain the helix-turn-helix (HTH) domain.

The PcG genes were first identified in Drosophila as a group of genes required for the maintenance of stable repression of Hex cluster genes during development (Bienz & Muller 1995; Kennison 1995; Paro 1995; Pirrotta 1997b; 1998; Simon 1995). Biochemical and immunohistochemical analyses indicate that Drosophila PcG gene products function as large multimeric protein complexes which are thought to act by changing the local chromatin structure (Orlando & Paro 1995; Paro 1995; Pirrotta 1997b). Synergistically genetic interactions between mutant alleles of different Drosophila PcG genes indicate their capability to affect the expression of Hex genes in a gene dosage-dependent manner and this is in accordance with the action of PcG gene products in multimeric protein complex (Franke et al. 1992; Shao et al. 1999).

Psc and Su(z)2 encode large proteins exhibiting a sequence similarity in their RING finger and HTH domains. Su(z)2, although not strictly a PcG protein, functions as a modifier of zeste loci like Psc, enhancer of zeste [E(z)] and sex comb on midleg (Scm) and seems to interact genetically with Psc and Scm mutations (Platero et al. 1996; Walrath & Elgin 1995; Wu & Howe 1995). The Psc and Su(z)2 products have been shown to co-localize on more than 80 sites in salivary gland polytene chromosomes, and their association with chromosomes is dependent on the active E(z) protein (DeCamillis et al. 1992; Franke et al. 1995; Rastelli et al. 1993; Zink & Paro 1989). The RING finger domain of the Psc protein is involved in the interaction with polycomb (Pc) protein, which is thought to be essential for its repression of gene expression (Kyba & Brock 1998). The Drosophila lethal(3)73Ah gene has been shown to encode a RING finger protein homologous to the Psc and Su(z)2 proteins, but its specific function has not yet been determined (Irminger-Finger & Nothiger 1995).

An increasing number of mammalian genes structurally and functionally related to Drosophila PcG proteins are being identified. Mammalian Bmi1 and Mel18 share highly homologous domains with Psc and Su(z)2 but are much smaller in weight (Tagawa et al. 1990; van Lohuizen et al. 1991). Mice bearing null mutations in either of these genes show rather subtle posterior transformations of their axial skeletons (Akasaka et al. 1996; von der Luit et al. 1994), accompanied by extended domains of Hox gene expression in the paraxial mesoderm to the neighboring, anterior preverbata (Akasaka et al. 1997; van der Luit et al. 1994, 1996). Recently, we have shown that Mel18/Bmi1 double mutant mice exhibit a strong synergistic effect in their ectopic expression of Hex cluster genes (Akasaka et al. 2001). For example, Hoxb3, Hoxb6 and Hox6 expression domains are all shifted up to the forebrain at day 9.5, whereas the Hoxd4 expression domain is less affected in these mutant mice. One possible explanation for the different extent among the Hex gene de-repression is the existence of another Psc resembling gene product, which has a function redundant with Mel18/Bmi1. Quite recently, NSPc1 was isolated, a gene sharing sequence similarity to Mel18 and Bmi1 (Nunes et al. 2001). However, it is not clear whether NSPc1 plays a compensatory role in Hex gene de-repression.

Moreover, it has been shown that Bmi1 and Mel18 regulate cell proliferation and senescence via repression of the Ink4a/Arf locus (Jacobs et al. 1999a,b). It has also been reported that chromatin association of Bmi1 inversely correlates with its phosphorylation status in a cell cycle-dependent fashion (Yonksen et al. 1999). This is the first report on the modification and cell cycle-dependent regulation of a PcG protein. Elucidation of the mechanism of PcG modifications should provide a better understanding of how and when PcG proteins form large protein complexes at specific genomic targets.

In this study, we have attempted to identify another Mel18/Bmi1 related gene based on structural similarities to their RING finger domains and identified human and mouse MBLRs (Mel18- and Bmi1-like RING finger proteins). Our experiments demonstrate the physical association of MBLR with Ring1A, Ring1B and Bmi1, transcriptional repression, and the subcellular localization of the MBLR protein. These observations suggest that the functions of MBLR protein are similar to those of Mel18 and Bmi1. We also show that the phosphorylation of MBLR occurs during mitosis and that CDK7, a component of basal transcriptional machinery (Bregman et al. 2000; Kaldis 1999; Nigg 1996), can phosphorylate MBLR. Furthermore, we identified the phosphorylation site of MBLR as serine 32. The implications of our observations are that MBLR is included in same subclass of RING finger proteins such as Mel18 and Bmi1 and that phosphorylation is a key modification step for the cell cycle-dependent regulation of MBLR.
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Results

Isolation of cDNAs coding for proteins that have high sequence homology with the RING finger domain of Mel18 and Bmi1

In an effort to structurally isolate genes encoding RING fingers related to those of Mel18 and Bmi1, we searched the Human Novel Transcripts (Hunt) Human Full Length cDNA Database <http://helix-www.hri.co.jp/443/hrfldb/> using the BLAST algorithm (Yudate et al. 2001). A BLAST search of the Hunt database using the peptide sequences of the RING finger domain of Mel18 and Bmi1 revealed a Helix clone with a high sequence similarity. However, translation of this cDNA clone was interrupted by an insertion. We designed several primers and obtained a cDNA, which contains a single large open reading frame (ORF) encoding a polypeptide of 352 amino acids (aa) (Fig. 1A). The predicted first methionine is defined by a good fit with the Kozak’s consensus sequence A/GXXATGG (the reliability of ATGpr program <http://www.hri.co.jp/atgpr/> is 0.78). Because of its similarity to the RING finger domain of Mel18 and Bmi1, we termed this protein MBLR (Mel18- and Bmi1-like RING finger protein).

A comparison of the amino acid sequence of MBLR with Mel18 and Bmi1 revealed that MBLR consists of at least three distinct domains (Fig. 1B). Highly homologous domain (HD) to Mel18 and Bmi1 is located in the central region of MBLR protein (amino acids 125–216).
The RING finger domain (amino acids 136–174) is seen in the N-terminal half of the HD exhibiting 62% and 64% identities to the analogous domain of Mel18 and Bmi1, respectively (Fig. 1C). The C-terminal half of the HD also shows extensive similarity to not only Mel18 and Bmi1 but also Drosophila Psc and Su(z)2. The overall identities of the HD are 58% (Mel18), 55% (Bmi1), 49% (Psc) and 36% (Su(z)2). A HTH domain is predicted in the C-terminal region (amino acids 240–324) of MBLR by using the method of Chou & Fasman (1978), which is also characteristic of Mel18 and Bmi1, while the amino acid sequence is rather diverged. However, amino acid identities in this region are 31% (Mel18) and 28% (Bmi1) and the amino acid similarities are 54% (Mel18) and 49% (Bmi1). Thus, the tertiary structure of MBLR in this domain could be similar to the analogous domain of Mel18 and Bmi1. The N-terminal region (amino acids 1–101) containing proline- and glutamic acid-rich domains is a unique structure for MBLR, while the C-terminal PEST region seen in Mel18 and Bmi1 is absent in MBLR. The amino acid sequence between the RING finger and HTH domains have a 38% identity with that of Mel18 and Bmi1. The MÔTE search program (GODMÔTE, MEMSAT and PSORT) could not predict any known domain except for the RING finger.

Based on this sequence, we isolated the murine counterpart of MBLR gene. In order to isolate mouse MBLR (mMBLR), we performed a TBLASTN search of the GenBank database using the human MBLR sequence and obtained overlapping expressed-sequence tag (EST) cDNA clones (AA414523, AA764577, AL034737, AA242737, AA920315, AA121824, AA153848, AA43956, AA139711, AA860491 and AA675640). We then performed 5’ RACE on a mouse embryo cDNA library and finally obtained an 1828 bp cDNA, which contains an ORF encoding a mouse MBLR (353 amino acids) (Fig. 1A). A comparison between the DNA sequences of the human and mouse MBLR cDNAs showed good conservation in the coding regions, which encode proteins with almost identical amino acid sequences (Fig. 1A, identity; 87%).

The expression of MBLR gene in adult and embryonic tissues

To determine the expression pattern of the hMBLR gene we analysed polyA mRNA transcripts from various adult human tissues by Northern blot analysis. High levels of a 2.5 kb human MBLR transcript were detected in testis and lower levels in heart (Fig. 1D, left upper panel). After a longer exposure of the membrane, the transcript was also detected in other tissues (Fig. 1D, right upper panel). RT-PCR analysis confirmed this ubiquitous expression pattern (Fig. 1D, Lower panel). A longer hMBLR transcript (about 4.5 kb) was also detected, although at lower levels than that of the predominant 2.5 kb transcript. Analysis of mMBLR transcripts in the developing embryo by whole mount in situ hybridization of 10.5 days post-coitus (dpc) mouse embryo revealed almost ubiquitous expression. The strongest MBLR hybridization signals were detected in the facial mesenchyme, branchial arches and limb buds (Fig. 1E).

Expression and subcellular localization of endogenous MBLR protein

To examine the expression and subcellular localization of hMBLR, we obtained the polyclonal anti-hMBLR antibody, pABR0, raised against the recombinant hMBLR protein. To evaluate the specificity of this antibody, we performed a Western blot analysis of extracts from HEK293 cells transiently transfected with a Myc-hMBLR construct and with extracts from U2OS osteosarcoma cells, which are known to express high levels of many PcG proteins. Using pABR0, we detected bands in both cell extracts with mobilities corresponding to polypeptides of approximately 60 and 65 kDa. This coincides with the mobility of the transfected hMBLR detected with the anti-Myc antibody (Fig. 2A, left and right panels, respectively). Neither over-expressed nor endogenous hMBLR protein was detected with preimmune serum (Fig. 2A, central panel). The pABR0 antibody cross-reacts to the murine MBLR in a specific manner (data not shown). Therefore, this antibody appears to have the expected specificity for hMBLR and was used in subsequent experiments. The presence of additional bands in these blots may be an indication that the MBLR protein may undergo post-translational modification.

Using the anti-hMBLR antibody, pABR0, we confirmed the expression of endogenous MBLR in various adult tissues by Western blotting (Fig. 2B). Consistent with Northern blotting, the highest expression level was detected in testis, and lower levels are seen in ovary, heart, stomach, liver, thymus and kidney. However, there still remains the discrepancy of the expression level between adult and embryonic heart. One possibility is that the amount of MBLR protein might be controlled during developing stages.

We next analysed the subcellular localization of the endogenous MBLR protein in tissue culture cell lines (U2OS, L cells and CHP134 cells) and in primary cultures (Emfis). In all cases, we found that hMBLR is
MBLR is regulated by cell cycle dependent phosphorylation exclusively seen in the nucleus of the interphase cells, excluded from the nucleolus (Fig. 2C). The staining of these cell lines using pre-immune serum did not show any specific staining (data not shown).

**MBLR is a transcriptional repressor**

Mel18 and Bmi1, like all other PcG proteins, act as transcriptional repressors when tethered on the episomal reporter plasmid (Bunker & Kingston 1994; Cohen et al. 1996; Schoorlemmer et al. 1997). The structural similarity of MBLR to Mel18 and Bmi1 prompted us to examine its transcriptional activity in transiently transfected mammalian cells. The coding sequences of hMBLR, Mel18 and Bmi1 were fused to the GAL4DBD of pM plasmid. The transcriptional activity of these fusion proteins were assessed on a reporter plasmid in which luciferase expression is driven by several GAL4-binding sites (Fig. 2D, right). Co-transfection of pM-hMBLR plasmid and the reporter plasmid into L cells resulted in up to a 50% repression of luciferase activity in a dose-dependent manner (Fig. 2D, left). A GAL4DBD-hMBLR fusion protein did not show any significant repression on a reporter plasmid lacking GAL4-binding sites (data not shown). In the same assay, both GAL4DBD-Mel18 and GAL4DBD-Bmi1-fusion proteins showed a higher repressor activity than that of GAL4DBD-hMBLR (Fig. 2D, left). The same results were obtained in U2OS cells (data not shown).

**MBLR interacts with Ring1B via its highly homologous domain (HD)**

Because of the structural and functional resemblance of mammalian MBLR to Mel18 and Bmi1, we asked whether MBLR is part of the PcG complex. To address this, we investigated the interaction of hMBLR with a number of components of the PcG complex (Mel18, Bmi1, Ring1A, Ring1B and Mph2) using a yeast two-hybrid assay. We found that hMBLR interacted strongly with Ring1A and Ring1B, moderately with Bmi1, and weakly with Mph2, but not at all with Mel18 or the negative control (lamin) (Fig. 3A, top panel). In addition, the screening of about $7 \times 10^6$ clones of a mouse 11 dpc embryo cDNA library using LexA-hMBLR as bait resulted in half of the positive clones encoding Ring1B (the other hMBLR-interacting proteins identified in this screening will be described elsewhere). Because of this, we decided to study the interaction between hMBLR and Ring1B in more detail by using an in vitro GST pull-down assay. We showed that $^{35}$S-hMBLR was bound to GST-Ring1B, and that $^{35}$S-Ring1B interacted with...
Figure 3  MBLR interacts with Ring1B via its highly homologous domain (HD). (A) Interaction of MBLR, with PcG proteins detected by yeast two-hybrid assay. MBLR was expressed as a GAL4AD fusion protein and Mel18, Bmi1, Ring1A, Ring1B and Mph2 were expressed as LexAIDBD fusion proteins in yeast. Transformants were lysed and incubated on media containing X-gal. The relative strengths of the interaction were determined by a visual inspection of the β-galactosidase assay. LexADBD-Lamin fusion protein was used as a negative control. A schematic representation of the Ring1B deletion constructs is shown on the lower right. (B) In vitro transcription translation products used for GST pull-down assay. The full-length and the indicated truncated proteins were synthesized in vitro, separated by SDS-PAGE and visualized by a BAS-2000II Bio-Imaging Analyser. Sizes of molecular weight markers are indicated in the left. A schematic representation of the constructs used in the in vitro transcription translation is shown on the right, and amino acids are numbered in the various truncated proteins. (C) GST-fusion proteins used for GST pull-down assay. The full-length and the indicated truncated proteins were bacterially produced, and then immobilized on GSH-sepharose beads. After being washed, the bound proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. Sizes of the molecular weight markers are indicated in the left. A schematic representation of the GST-fusion proteins is shown on the right, and amino acids are numbered in the various truncated proteins. (D) The full-length and indicated truncated Ring1B proteins were incubated with GST, GST-MBLR-full, GST-MBLR-ΔC, or GST-MBLR-HD immobilized on GSH-sepharose beads. After washing, the bound proteins were separated by SDS-PAGE and visualized by a BAS-2000II Bio-Imaging Analyser. Sizes of molecular weight markers are indicated on the left. A summary of the binding data is shown on the right. The interaction between each Ring1B protein and GST-fusion protein is designated by +, while +/− indicates a weak interaction, and − indicates no interaction. (E) The full-length and indicated truncated MBLR proteins were incubated with GST, GST-Ring1B-full, GST- Ring1B-ΔN, or GST- Ring1B-ΔC immobilized on GSH-sepharose beads. After washing, the bound proteins were separated by SDS-PAGE and visualized by a BAS-2000II Bio-Imaging Analyser. Sizes of molecular weight markers are indicated on the left. A summary of the binding data is shown on the right. The interaction between each MBLR protein and GST-fusion protein is designated by +, while +/− indicates a weak interaction, and − indicates no interaction. (F) The full-length Ring1A and Bmi1 proteins were incubated with GST, GST- MBLR-full, GST- MBLR-ΔC, or GST-MBLR-HD immobilized on GSH-sepharose beads. After washing, the bound proteins were separated by SDS-PAGE and visualized by a BAS-2000II Bio-Imaging Analyser. Sizes of molecular weight markers are indicated on the left. (G) Immunoprecipitation of HEK293 cells transiently co-transfected with MBLR and Ring1B expression plasmids. At 48 h after transfection of EGFP-MBLR and HA-tagged-Ring1B expression plasmids, cells were lysed and immunoprecipitated in the presence of anti-EGFP antibody. After elution by boiling, samples were analysed by 10% SDS-PAGE and Western blot with anti-HA antibody. Aliquots of the lysates were analysed by Western blot with anti-EGFP and anti-HA antibodies (input).
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Next we determined the precise binding domain for MBLR and Ring1B by using several deletion mutants of respective genes. The deletion constructs of Ring1B containing amino acids 189–338 and 1–188 fused to the GAL4DBD (Ring1B-ΔN and ΔC, respectively) interacted with MBLR similarly to full-length Ring1B in a yeast two hybrid assay (Fig. 3A, bottom panel). Further analyses were performed using the in vitro GST pull-down assay. Several deletion mutants of MBLR and Ring1B were synthesized in vitro or expressed as GST fusion proteins (Fig. 3B,C). We found that a truncated Ring1B derivative lacking amino acids 189–338 (Ring1B-ΔC) was able to bind efficiently not only to an intact MBLR protein fused to GST, but also to deleted variants lacking amino acids 239–352 and 1–102 plus 239–352 (GST-MBLR-ΔC and GST-MBLR-HD). A Ring1B derivative lacking amino acids 1–188 (Ring1B-ΔN) interacted only with the intact MBLR (Fig. 3D). An amino-terminal-truncated MBLR derivative containing amino acids 103–352 was able to bind to both GST-Ring1B-ΔC and GST-Ring1B-ΔN. However, truncated variants, MBLR proteins lacking amino acids 103–352 (MBLR-ΔC) or amino acids 1–102 plus 239–352 (MBLR-HD), were unable to interact with the C-terminal half of Ring1B fused to GST (GST-Ring1B-ΔN) (Fig. 3E). These results indicate that the direct interaction between MBLR and Ring1B occurs through two regions. One lies in the central homologous region of MBLR and the N-terminal portion of Ring1B containing the RING finger domain, whereas the other spans residues in the C-terminal portion of MBLR and Ring1B. Since the C-terminal region of MBLR encompassing residues 103–352 (MBLR-ΔN) showed weaker interaction with GST-Ring1B-ΔN compared with MBLR-full, the N-terminal region of MBLR may also assist the interaction to some extent.

Finally, we also investigated the interaction between MBLR and other PcG proteins, such as Ring1A and Bmi1. Intact 35S-labelled Ring1A and Bmi1 interact with MBLR and with either of its truncated derivatives MBLR-ΔC or MBLR-HD (Fig. 3F). Taken together, these results show that MBLR interacts directly with at least a subset of PcG proteins, and that its smallest interacting domain contains a RING finger domain, whereas the other spans residues in the C-terminal portion of MBLR and Ring1B. Since the C-terminal region of MBLR encompassing residues 103–352 (MBLR-ΔN) showed weaker interaction with GST-Ring1B-ΔN compared with MBLR-full, the N-terminal region of MBLR may also assist the interaction to some extent.

In the interphasic nucleus of some tissue culture cell lines, like the human U2OS cells, the PcG proteins such as Ring1A, Ring1B, M33, Mph1, Mph2 and Bmi1 co-localize in specific subnuclear domains, termed PcG bodies (Alkema et al. 1997a,b; Gunster et al. 1997). To investigate the distribution of hMBLR in U2OS cells, we performed a double-staining with anti-hMBLR antibody, pAbR0, and the monoclonal antibody against Ring1B. We found that, in contrast to the nuclear speckled distribution of Ring1B, hMBLR showed a fine-grained pattern distributed uniformly throughout the nucleus (Fig. 4 MBLR). Ring1B protein was excluded from the nucleolus and showed a speckled distribution throughout the nucleoplasm, as shown previously (Fig. 4, Ring1B). The different subnuclear distribution of endogenous hMBLR and Ring1B may suggest a functional difference of hMBLR with regard to the mammalian PcG proteins.

Cell cycle-dependent phosphorylation of MBLR

The intracellular localization of MBLR corresponded to that of interphase U2OS cells. However, in mitotic cells we noted the hMBLR staining outside of the condensed...
chromatin (Fig. 5A, MBLR and Merge). We asked whether these changes in cell localization could be due to a post-translational modification of hMBLR at different stages of the cell cycle. U2OS cells were synchronized at G2/M and G1/S by Nocodazole and Aphidicoline, respectively, and analysed by Western blotting with anti-MBLR, pAbR0, antibody. In extracts from cells accumulated at the G2/M boundary we detected an anti-MBLR reactive band that migrated more slowly than the band seen in extracts of non-treated cells. In contrast, extracts from cells accumulated at the G1/S boundary showed almost no slow migrating MBLR band (Fig. 5B). This suggests that MBLR undergoes a cell cycle-regulated post-translational modification.

To investigate whether the alteration of the electro-phoretic mobilities of MBLR, are related to phosphorylation, we treated immunoprecipitated MBLR with Lambda Protein phosphatase. The treatment eliminated the band shift of MBLR seen on Aphidicoline synchronized cells (Fig. 5D, lane IP+) and indicates that MBLR is post-translationally modified by phosphorylation.

Next, we wished to identify which residues were phosphorylated. The observation that MBLR–ΔN protein showed only a single migrating band on Western blots suggested that these residues should be contained within the 102 amino acid residues at the NH2-terminal end of MBLR. In this domain there are four serine residues at positions 32, 59, 61 and 71. All of them were mutated individually to alanine to examine whether these mutations had any effect on the electro-phoretic mobility of MBLR. We found that the substitution of serine 32 abolished the slowly migrating band of MBLR (Fig. 5D, lane S32A), while the mutations of serines 59, 61 and 71 had no effect on the migration of MBLR (Fig. 5D, lanes S59, 61A and S71A). These results indicate that the phosphorylation of serine 32 of MBLR is responsible for the alteration of the electro-phoretic mobility of MBLR during mitosis.

To confirm the specific phosphorylation of MBLR during mitosis, we raised an antibody against
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Phospho-MBLR peptide. Consistent with the previous characterization, this antibody recognized a single band of 65 kDa, which is the molecular mass of phosphorylated MBLR as determined by Western blotting (Fig. 6A). Indirect immunofluorescent studies revealed that the antibody specifically detected phosphorylated MBLR as a diffuse cytoplasmic distribution in the mitotic U2OS cells (Fig. 6B). This diffuse cytoplasmic distribution of phosphorylated MBLR was observed from metaphase to telophase of the mitotic cells. This specific staining pattern was not detected with pre-immune serum (data not shown).

In vitro phosphorylation of MBLR by CDK7

To confirm the phosphorylation of MBLR, we performed an in vitro kinase assay of GST-MBLR fusion protein using cell nuclear extract. For this assay we used the C-terminal deleted MBLR (amino acids 1–238) because of its better protein stability. GST-MBLR is readily phosphorylated when incubated with the cell nuclear extract (Fig. 7A, lane GST-MBLR), but GST alone is not (Fig. 7A, lane GST). To assess whether the serine 32 residue of MBLR is actually the likely substrate of phosphorylation in this reaction, we analysed the phosphorylated MBLR and S32A mutant MBLR by immunoblot analysis using anti-phospho-MBLR antibody. Only MBLR showed the phosphorylated serine 32 (Fig. 7B, lane 5) when assayed with both nuclear cell extract and ATP. This shows that the serine 32 of MBLR protein is one of the sites phosphorylated by a kinase contained with the nuclear extract. Taken together with the in vivo data (Fig. 4), the serine 32 may be functionally the most important phosphorylation site of MBLR. There are several proline-directed kinases (Pdks) including CDK7, which is known as a component of CAK (cyclin-dependent kinase (CDK)-activating kinase). CAK has been strongly implicated in cell cycle regulation and the initiation of transcription (Bregman et al. 2000; Kildis 1999; Nigg 1996). We focused on CDK7, because the phosphorylation site of MBLR had a high similarity to the known substrates for CDK7 (Fig. 7C, S118 of oestrogen receptor α and S77 of retinotic acid receptor α1) (Chen et al. 2000; Rochette-Egly et al. 1997). To examine the role of CDK7 in the phosphorylation of MBLR, we performed an in vitro kinase assay using Hela cell nuclear extract from which CDK7 was immunodepleted by anti-CDK7 antibody. We found that in this CDK7-depleted extract phosphorylation of MBLR was significantly reduced to that seen with intact extracts (Fig. 7D, right panel). We also confirmed that there was about a 70% reduction in the CDK7 content of this nuclear extract by Western blot analysis (Fig. 7D, left panel). These results indicate that, at least in vitro, MBLR is a substrate for CDK7 phosphorylation.

Discussion

In Drosophila, the presence of over 30 PcG gene loci has been suggested by genetic analyses (Pirrotta 1997a,b; Simon 1995). However, less than half of them have been molecularly or functionally characterized. To understand the function of PcG complexes, it is necessary to identify other PcG and PcG-related genes. Mel18 and Bmi1 share sequence similarity to Drosophila posterior sex combs (Psc) and its relative Su(z)2, and are the most extensively
analysed mammalian PcG proteins. Screening based on the sequence similarities and molecular interactions with known mammalian PcG proteins allow us to discover new candidates for mammalian PcG proteins. In this study, we have searched the sequence database and identified a potential candidate PcG-related gene which has a high similarity to \( \text{Mel}18 \) and \( \text{Bmi}1 \), and designated it \( \text{MBLR} \) (\( \text{Mel}18 \)- and \( \text{Bmi}1 \)-like RING finger protein).

Structural comparison of MBLR with \( \text{Mel}18 \), \( \text{Bmi}1 \) and \( \text{NSPc}1 \) and binding properties to Ring1B suggest that MBLR might be subdivided into at least three distinct sub-regions. The central region (amino acids 125–216) containing a RING finger domain (amino acids 136–174) and adjacent regions are highly homologous to the N-terminal regions of Mel18, Bmi1 and \( \text{NSPc}1 \) and are essential for binding to Ring1B as well as Mel18 and Bmi1 (Hemenway et al. 1998; Satijn & Otte 1999). The C-terminal region of MBLR is predicted to form HTH structures similar to the central region of Mel18 and Bmi1. However, Mel18 and Bmi1 strongly interact with Mph1 and Mph2 via their HTH regions (Alkema et al. 1997a; Gunster et al. 1997), and MBLR only weakly interacted with Mph2 on a yeast two-hybrid assay. This weak interaction of MBLR with Mph2 might be explained by the structural difference of the putative HTH domain. The most characteristic region for the MBLR protein is the N-terminal region, which contains proline- and glutamic acid-rich domains. A serine 32 residue in a proline-rich domain is demonstrated to be a site of cell cycle-dependent phosphorylation of MBLR. Neither \( \text{Mel}18 \), \( \text{Bmi}1 \), nor \( \text{NSPc}1 \) possesses a homologous region to the N-terminal region of MBLR, while \( \text{Mel}18 \) and \( \text{Bmi}1 \) have PEST regions in their C-terminal regions. \( \text{Bmi}1 \) is also phosphorylated in a cell cycle-dependent manner although its phosphorylation site is not yet determined (Voncken et al. 1999). Since the PEST region of \( \text{Bmi}1 \) contains several possible phosphorylation sites, part of the function of the N-terminal region of MBLR, while \( \text{Mel}18 \) and \( \text{Bmi}1 \) have PEST regions in their C-terminal regions. \( \text{Bmi}1 \) is also phosphorylated in a cell cycle-dependent manner although its phosphorylation site is not yet determined (Voncken et al. 1999). Since the PEST region of \( \text{Bmi}1 \) contains several possible phosphorylation sites, part of the function of the N-terminal region of MBLR, might be similar to those of PEST region of \( \text{Bmi}1 \) and \( \text{Mel}18 \). It has previously been demonstrated that the accumulation of \( \text{Bmi}1 \) protein to the subnuclear PcG bodies were mediated by the RING finger and its adjacent domains (Alkema et al. 1997a). Although these domains are extensively conserved between MBLR and \( \text{Bmi}1 \) and are functionally equivalent as indicated by Ring1B binding, MBLR is not involved in the PcG bodies. Therefore, the N-terminal or C-terminal domains of MBLR might affect the accumulation of MBLR to the PcG bodies.

One explanation is that unknown proteins interacting with the N- or C-terminal region of MBLR would prevent its accumulation to the PcG bodies.

**Figure 7** In vitro kinase assay of recombinant GST-MBLR (A) Recombinant GST-MBLR and control GST were incubated with \( [\gamma^{32}P] \) ATP in the presence of Hela cell nuclear extract, then precipitated with glutathione- sepharose beads and resolved by 10% SDS-PAGE. The dried gel was autoradiographed. (B) The serine 32 residue of MBLR is phosphorylated in vitro. Recombinant GST-MBLR and GST-S32A MBLR were incubated separately in the presence of nuclear extract and ATP, and subjected to SDS-PAGE. Analysis of serine 32-phosphorylated MBLR was determined by Western blot with anti-phospho MBLR antibody. (C) Comparison of amino acid sequences between the phosphorylation site of hMBLR and other substrates for CDK7. The phosphorylated serine residues are indicated by shading. (D) Effect of CDK7 depletion on in vitro phosphorylation. Recombinant GST-MBLR was incubated with ATP in the presence of CDK7-depleted Hela cell nuclear extract or control extract, immunoprecipitated with glutathione- sepharose beads and resolved by 10% SDS-PAGE. Analysis of serine 32-phosphorylated MBLR was determined as described in (B).
Although the involvement of MBLR in the mammalian PRC-1 [Polycomb repressive complex 1 (Shao et al. 1999)]-like complex remains unclear, we suggest that MBLR belongs to the subclass of RING finger proteins represented by mammalian PcG proteins, Mel18 and Bmi1, based on the following observations. First, MBLR functions as a transcriptional repressor like Mel18 and Bmi1. Second, MBLR is able to bind to mammalian PcG proteins (Ring1B, Ring1A and Bmi1) via its central domain, and over-expressed MBLR and Ring1B are co-immunoprecipitated with the lysate of transiently transfected mammalian cells. Third, although MBLR is co-immunoprecipitated from the lysate of transiently transfected mammalian cells, detectable throughout the nucleoplasm in wild-type U2OS cells, over-expression of exogenous MBLR leads to the recruitment of MBLR to PcG bodies in U2OS cells (data not shown). RYBP, a recently identified member of the mammalian PcG complex, is constitutively associated with MBLR and Ring1B in mouse embryonic extracts, but distributed throughout the nucleoplasm as seen in MBLR (Garcia et al. 1999). Furthermore, a mutant Bmi1 protein lacking the Zn finger domain induced anterior transformations of the axial skeleton in transgenic mice and displayed a diffuse nuclear localization when expressed in U2OS cells (Alkema et al. 1997a). Thus the formation of PcG bodies should be considered separately from functional aspects of PcG proteins. Mice lacking the PcG gene show a posterior transformation of the axial skeleton which correlates with an anterior shift of several Hox gene expression boundaries (Akasaka et al. 1996; Core et al. 1997; Takihara et al. 1997; van der Lught et al. 1994). To ascertain that MBLR is a PcG-related protein, it is necessary to analyse it genetically. We are currently generating MBLR-deficient mice.

In this paper, we clarified that MBLR is a phosphoprotein whose phosphorylation is regulated in a cell cycle-dependent manner. Quite recently, it was reported that Bmi1 is phosphorylated at G2/M and that hyperphosphorylated Bmi1 does not bind to chromatin (Voncken et al. 1999). We did not show that MBLR interacts directly with chromatin. However, MBLR is also phosphorylated during mitosis and dispersed in the cytoplasm. Likewise, some of the trithorax group (trxG) proteins, a protein family which has an opposing effect to PcG repression, are also phosphorylated in a cell cycle-dependent manner and excluded from the condensed chromosomes (Muchardt et al. 1996). These lines of evidence suggest that molecules which have a transcriptional memory function may be generally regulated by cell cycle-dependent phosphorylation.

We identified the presence of phosphorylated MBLR outside the condensed chromosome. Since Ring1B dissociates from the condensed chromosome during the mitotic phase (unpublished data), MBLR and Ring1B may interact mainly in the cytoplasm of mitotic cells. Although it is tempting to speculate that such an interaction is the molecular basis for the ‘cellular memory’ of transcriptional states, neither the exact role of MBLR nor the effect of the interaction of MBLR with Ring1B is understood. Nevertheless, the identification of MBLR-phosphorylation in vivo provides insight for further functional analyses.

Cyclin-dependent kinase 7 (CDK7) was first identified as the catalytic subunit of a complex able to phosphorylate and thereby activate several cell cycle-regulatory CDKs (cyclin-dependent kinases) (Fesquet et al. 1993; Poon et al. 1993; Solomon et al. 1993). Later it was also identified as a component of TFIIH, required for basal transcription by RNA polymerase II (Roy et al. 1994). In addition to the T loop of CDKs and the carboxyl-terminal domain (CTD) of the large subunit of RNA polymerase II, several other phosphorylated targets including p53, hERα and mRARα have been reported (Chen et al. 2000; Ko et al. 1997; Rochette-Egly et al. 1997). The similarity in phosphorylation sites between MBLR and hERα and mRARα prompted us to investigate whether CDK7 is involved in MBLR phosphorylation. We performed an in vitro kinase assay and obtained results that support CDK7-mediated MBLR phosphorylation. We did not demonstrate a direct interaction between MBLR and CDK7, but we hypothesize that CDK7 modulates the transcriptional repressor activity of the PcG complex following the dissociation of a part of the PcG complex through the interaction of phosphorylated MBLR and Ring1B (and perhaps other components of PcG complex).

We have shown that MBLR, which interacts with Ring1B, is phosphorylated during mitosis. In addition, we showed here that MBLR is a potential substrate for CDK7, one of the components of the basal transcriptional machinery. Although our findings indicate a potential link between the basal transcriptional machinery and the PcG complex, to determine any further involvement of CDK7 in the regulation of PcG complex it is necessary to clarify a functional role of the MBLR-phosphorylation.

**Experimental procedures**

Cloning of human and mouse MBLR genes and construction of expression plasmids

The sequence information of Hunt (Human Novel Transcript) Human Full Length cDNA Database <http://helix-www.hri.co.jp:443/HRIDB/> allowed us to synthesize specific
primes for ring-finger-S (5’-TTGTGCTCTCCGAGC- CATGGA-3’) and ring-finger-AS1 (5’-GAGTCTTCAAGTTATCTTCAGAG-3’). Then, a cDNA clone of human mMBLR (mMBLR) was obtained from an adult testis Marathon-Ready cDNA library (Clontech). For amplification of the cDNA containing the full-length open reading frame (ORF), PCR was performed using hMBLR-S (5’-CGGCCATGGGAG- GGTTGCGCGGT-3’) and ring-finger-AS2 (5’-TTGTGCTCCTG- GAGTCTCTCAAGTTATCCAGG-3’) primers. The sequence information for the mouse EST clones allowed the synthesis of two specific primers for 5’-RACE; mouse 5’-R1 (5’- CAGGAGCTTGGGCGACTTCTGCAGCCT-3’) and mouse 5’-R2 (5’-TGTGAGCAACTATGTTGACACGGTCGCC-3’). Then, a 5’-cDNA fragment of mouse mBLR (mMBLR) was obtained by using a mouse brain Marathon-Ready cDNA library (Clontech) according to the manufacturer’s instructions. For amplification of the cDNA containing the full-length ORF, PCR was performed using mouse-full-S (5’-CTCCTACTCGCCA- GATTTCGT-3’) and mouse-full-AS (5’-GGTGGACAACTATGTTGCATTTTGGGC-3’). The PCR fragments were ligated into a pCR2.1-TOPO cloning vector (Invitrogen) and sequenced. The nucleotide sequence was confirmed by sequencing several clones to avoid errors introduced during the PCR reaction. EGFP-tagged hMBLR expression plasmid (pEGFP-hMBLR-full) and Myc-tagged hMBLR expression plasmid (pDNA3-Myc- hMBLR) were constructed by ligation into the Xho I site of the pEGFP-C3 plasmid (Clontech) and pcDNA3 (Invitrogen), respectively. Several mutant constructs of pDNA3-Myc-hMBLR were generated using the GeneEditor in vitro site-directed mutagenesis system (Promega). All mutations were verified by DNA sequencing. The HA-tagged Ring1B expression plasmid (pCEP4-mRing1B) was constructed by ligation into the HindIII/ XhoI sites of the pCEP4 plasmid (Invitrogen).

**Northern blot analysis**

Northern blots derived from multiple human tissues containing 2 µg of poly(A)+ RNA per lane were obtained from Clontech. The membranes were probed with a 1.8 kb hMBLR cDNA fragment labelled with [32P]-dCTP by the random-primed labelling method and hybridized at 68 °C for 1 h. Subsequently, the membranes were washed once at room temperature (RT) in 2×SSC, 0.1% SDS, twice for 30 min each in 0.1×SSC, 0.1% SDS at 50 °C.

RT-PCR analysis cDNA from various tissue samples was obtained from Clontech. RT-PCR to evaluate the expression profile of hMBLR transcript was carried out using the following set of primers; sense, 5’-GCTAATGAAGGCACGGGACA-3’ and anti-sense, 5’-GCTAATGAAGGCACGGGACA-3’ and anti-sense, 5’-GCTAATGAAGGCACGGGACA-3’ and anti-sense, 5’-GCTAATGAAGGCACGGGACA-3’. The PCR was performed for 33 cycles which consisted of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. The amplified products were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining and UV trans-illumination. To ensure the successful completion of cDNA synthesis for each sample, G3PDH cDNA was amplified with the following primers; sense, 5’-TCAAGGTCGGAGTCAACG- GATTTCGT-3’ and anti-sense, 5’-CATGTGGGGGCGCAGG- GTCCACAC-3’, for 25 cycles.

**Antibody preparation**

The full-length ORF of hMBLR cDNA was subcloned into the XhoI site of the pcDNA-1 bacterial expression vector (Pharmacia). The GST-hMBLR fusion protein was expressed and purified, then polyclonal antibody against hMBLR was generated by subcutaneous immunization of a rabbit with the recombinant protein. Anti-hMBLR antibody was enriched by Protein A-ephaphe. Monoclonal anti-Ring1B antibodies were prepared as described (Atsuta et al. 2001). Monoclonal anti-HA antibody 12CA5 and monoclonal anti-Myc antibody 9E10 were obtained from Boehringer Mannheim and Santa Cruz Biotechnology, respectively. Monoclonal anti-CDK7, actin, and myosin antibodies were purchased from Sigma and Clontech, respectively.

**Synthesis of phosphorylated MBLR peptide and production of anti-phospho-MBLR antibody**

A hMBLR peptide, derived from MBLR amino acid residues 26–32 (Ile to T7), [CPPVPPVSP(PO3) (PPIAT)], was chemically synthesized by a previously described method (Kitagawa et al. 1996) and used as...
the antigen. Rabbits were immunized with these phosphopeptides after conjugation with keyhole limpet haemocyanin through the cysteine residues. Polyclonal antibodies were affinity-purified from these antisera by chromatography on Sepharose CL-4b beads coupled with the same phosphopeptide, followed by passage through the beads linked with a corresponding unphosphorylated peptide. The antibodies were screened for activity by using an ELISA test (Kitagawa et al. 1996).

**Immunoprecipitation and Western blot analysis**

Cells were lysed on ice for 30 min with a 1% NP-40 lysis buffer consisting of 1% NP-40, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 10 µg/ml leupeptin, 2 µg/ml pepstatin A, 10 µg/ml aprotinin, and 200 µM PMSE. Lysates were centrifuged at 15 000 r.p.m. for 10 min, then aliquots of the supernatant were incubated for 3 h at 4°C with antibodies conjugated protein A/G-agarose beads (Oncogene). After five washes with the 1% NP-40 lysis buffer, bound proteins were eluted by boiling for 5 min in 2× Laemmli sample buffer [125 mM Tris–HCl (pH 6.8), 1 mM EDTA, 4% SDS, 20% glycerol, and 0.2% bromophenol blue]. Whole-tissue extracts were prepared in PBS containing 1× complete protease inhibitor cocktail (Roche) from 3-month-old C57Bl/6 mice using PowerGen Homogenizer (Fisher Scientific). The amount of protein was assayed by Protein assay kit (Bio-Rad). All samples were resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto an Immuno-Blot PVDF membrane (Bio-Rad) for 1 h at 140 mA. Blots were blocked with TBS (phosphate buffered saline, 0.05%Tween 20) containing 5% skim milk for 1 h at room temperature and then incubated with an appropriate dilution of each antibody (1: 10 000 for anti-MBLR, 1: 500 for anti-phospho-MBLR, 1: 4 000 for anti-GFP, 1: 2000 for anti-HA, and 1:2000 for anti-Myc antibodies) in TBS for 1 h. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse Igg or anti-rabbit IgG (Amersham) for 1 h, and specific proteins were detected using an enhanced chemiluminescence system (NEM Life Science Products).

**Immunofluorescence microscopy**

Cells growing on glass cover slips were washed twice in PBS or mitotic cells were collected on to the slide glass using CytoSpin (Shandon). Then they were fixed in freshly prepared 2% paraformaldehyde for 15 min at RT. The cells were washed twice for 3 min in PBS and permeabilized with PBS containing 0.5% Triton X-100 for 5 min at RT. After washing, the cells were blocked with PBS containing 10% FBS and 0.02% Triton X-100 for 1 h at RT, and then incubated with anti-hMBLR, or anti-phospho-MBLR antibody for 30 min in PBS containing 0.02% Triton-X-100 diluted 1: 500 or 1: 50, respectively. The cells were reacted with donkey anti-rabbit IgG coupled with FITC (Jackson Immunoresearch Laboratories) or donkey anti-rabbit IgG coupled with Cy3 (Jackson Immunoresearch Laboratories) diluted 1: 300 in blocking solution for 1 h at RT. For nuclear counterstaining, the cells were incubated with TO-PRO-3 iodide (642/661) (Molecular Probes) after staining with the secondary antibody. After being washed five times for 3 min each in PBS containing 0.02% TritonX-100, the cells were embedded in Immunon Perma Floor Aqueous Mounting Medium (Shandon/Lipshaw) and analysed by confocal microscopy (LSM510 Version2.02/Carl Zeiss).

**Repression assays**

Full-length hMBLR, mouse Mel18 and Bmi1 were subcloned into the pM (fusion vector of GAL4 DNA-binding domain, Clontech). The luciferase reporter construct, pFR–Luc, contains five GAL4 binding elements upstream of the SV40 promoter (Promega). Expression values were standardized against Renilla reniformis luciferase expression from pRL–TK reference plasmid (Promega). L cells were plated the day before transfection at 1×105 cell per 24-well dish. Transfection was performed using Lipofectamine (Gibco BRL), according to the manufacturer’s instructions, and cells were harvested 40 h after transfection. Luciferase activity was measured by the Dual-luciferase reporter assay system (Promega). Luciferase expression values obtained with the reporter plasmid in the presence of empty pM effector vector were set to 100 arbitrary units, and luciferase activities in cells transfected with hMBLR, Mel18 or Bmi1 were expressed relative to those obtained with the empty GAL4 vector. The reported results represent the mean values of five independent experiments. Reporter plasmid lacking GAL4 binding sites did not give any significant repression upon hMBLR, Mel18 or Bmi1 expression plasmids (unpublished results).

**Yeast two-hybrid assay**

The full-length cDNA of hMBLR was cloned in-frame into the pGAD10 (GAL4 activation domain fusion vector, Clontech). The L40 yeast strain (MATa) containing lexA-His3 and lexA-Lac Z reporter was first transformed with pGAD10-hMBLR using the lithium acetate method (Ito et al. 1983). Murine Mel18, Bmi1, Ring1A, Ring1B, Mph2, truncated Ring1B and lamin, as a negative control, were cloned into pBTM116 (LexA DNA-binding domain fusion vector, Paul Bartel and Stan Fields), and transformed into AMR70 (MATa0). The transformed clones were mated to each other in all possible combinations on YPAD plates to test for physical interactions among the expressed polypeptides. Then Leu + and Trp + diploids were selected on Leu-Trp- plate and assayed for β-galactosidase activity. The relative strength of the interaction was determined by visual inspection of β-galactosidase filter assay (Vogel et al. 1993).

**In vitro transcription/translation and GST pull-down assay**

The full-length and deleted cDNAs were subcloned in the pcDNA3 vector (Invitrogen). RNA was synthesized with 500 ng of supercoiled plasmids and translated in the presence of 10 µCi of [35S] methionine (Amersham Pharmacia) using a rabbit reticulocyte lysate (Promega). For the in vitro GST pull-down
Manley et al. (1993), and the protein concentration was determined as 3 µg/µL. GST-MBLR (amino acid 1–238) and GST-MBLR S32A (amino acid 1–238) were expressed and purified from E. coli DH5α using Glutathione Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) and used as substrate (10 µg) in a reaction of 2 µg of the nuclear extract with 20 µM HEPES (pH 7.6), 10 mM MgCl₂, and 10 µg of [γ-32P] ATP. Each reaction mixture was incubated for 30 min at 30 °C. The beads were extensively washed three times with 1 ml of the same buffer, then bound proteins were eluted by boiling for 5 min in 15 µL of 2× Laemmli sample buffer. Samples were resolved by 10% or 15% SDS-PAGE and dried gels were analysed using a BAS-2000II Bio-Imaging Analyser (FUJIX).

**In vitro kinase assay**

Hela cell nuclear extract was prepared as described by Manley et al. (Manley et al. 1993), and the protein concentration was determined as 3 µg/µL. GST-MBLR (amino acid 1–238) and GST-MBLR S32A (amino acid 1–238) were expressed and purified from E. coli DH5α using Glutathione Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) and used as substrate (10 µg) in a reaction of 2 µg of Hela cell nuclear extract, 20 mM HEPES (pH 7.6), 10 mM MgCl₂, and 10 µg of [γ-32P] ATP. The reaction was allowed to proceed for 30 min at 30 °C, then the beads were washed three times with washing buffer (25 mM HEPES, 0.1 mM EDTA, 50 mM KCl, 10% glycerol, and 0.1% Triton X-100). The proteins were separated by SDS-PAGE. For the immunodepletion assay, 6 µg of MO-1.1 (Sigma) or 6 µg of control antibody (anti-CD31; Pharmingen) were pre-bound with 20 µL (50% slurry) of Protein G Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) for 1 h at 4 °C. The beads were extensively washed in PBS containing 0.1% Triton X-100 and incubated with 20 µL of nuclear extract for 1 h at 4 °C. After centrifugation, 5 µL of the supernatant was used for kinase assay, the same as above, except that 100 nM ATP was used instead of [γ-32P] ATP. Each reaction mixture was immediately chilled on ice and extensively washed. The glutathione-Sepharose beads were boiled in 15 µL of 2× Laemmli sample buffer to elute the GST fusion proteins.

**Accession numbers**

The human MBLR (accession no. AB47006) and the mouse MBLR (accession no. AB47007) cDNA sequences have been deposited in the DDBJ/EMBL/GenBank database.

**Acknowledgements**

We are grateful to Dr. Y. Y. Taya (National Cancer Research Institute in Japan), Dr. M. Vidal (Developmental and Cell Biology, Centro de Investigaciones Biológicas, Dr. T. Nakayama (Department of Molecular Immunology, Chiba University) Dr. R. J. Wessels and Krista Golden (Department of Cellular, Molecular and Developmental Biology, University of Michigan) for helpful discussion and critical reading of the manuscript. We thank Dr. M. Vidal for providing the Ring1B expression plasmids. We also thank to Dr. S. Matsumoto for assistance with the Northern blot and for helpful suggestions and Dr. H. T. Yudate for editing the manuscript. This project was in part supported by grants from the Ministry of Education, Culture, Sports, Science and Culture of Japan.

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Received: 24 April 2002
Accepted: 17 May 2002