Nuclear-Cytoplasmic Shuttling of a RING-IBR Protein RBCK1 and Its Functional Interaction with Nuclear Body Proteins

Received for publication, November 30, 2004, and in revised form, April 14, 2005
Published, JBC Papers in Press, April 15, 2005, DOI 10.1074/jbc.M413476200

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The intracellular localization of a RING-IBR protein, RBCK1, possessing DNA binding and transcriptional activities, has been investigated. The endogenous RBCK1 was found in both the cytoplasm and nucleus. Particularly in the nucleus, it was localized in the granular structures, most likely nuclear bodies. In contrast, the over-expressed RBCK1 was detected exclusively in the cytoplasm. When the cells were treated with leptomycin B, the over-expressed RBCK1 accumulated in the nuclear bodies. These results suggest that RBCK1 possesses the signal sequences responsible for the nuclear-cytoplasmic translocation. Mutational analysis of RBCK1 has indicated that an N-terminal region containing Leu-142 and Leu-145 and a C-terminal one containing the RING-IBR domain serve as the nuclear export and localization signals, respectively. Thus, RBCK1 is a transcription factor dynamically shuttling between cytoplasm and nucleus. Furthermore, RBCK1 was found to interact with nuclear body proteins, CREB-binding protein (CBP), and promyelocytic leukemia protein (PML). Coexpression of RBCK1 with CBP significantly enhanced the transcriptional activity of RBCK1. Although PML per se showed no effect on the transcriptional activity of RBCK1, the CBP-enhanced activity was repressed by coexpression with PML, presumably through the interaction of PML and CBP. Taken together, our data demonstrate that RBCK1 is involved in transcriptional machinery in the nuclear bodies, and its transcriptional activity is regulated by nucleocytoplasmic shuttling.

The RING finger is a protein motif that binds two zinc ions in a Cys/His-rich region and mediates protein-protein or protein-DNA interactions (1). More than 2,000 RING finger-containing proteins have been reported so far (2), which possess both or either one of the transcriptional and ubiquitin ligase (E3) activities. For example, an acute promyelocytic leukemia protein (PML)1, a Ret finger protein (5), and a lung cancer-associated gene product (6) are the RING finger proteins with transcriptional activity. A RING finger LIM domain-binding protein (7), a mouse double minute-2 protein (8), and a cellular Casitas B-lineage lymphoma protein (9) are those with E3 activity. A breast cancer 1 protein (BRCA1) (10, 11) and a ubiquitin-conjugating enzyme Ubc5B-interacting protein A07 (10, 12) are the proteins with both activities. An advanced protein motif containing two RING fingers with an additional Cys/His-rich region, placed in between the RING fingers (IBR), is called a RING-IBR domain (13) and has been found in a human homologue of ariadne and an autosomal recessive juvenile parkinsonism-related gene product.

We previously identified a novel RING-IBR protein, RBCK1, by the yeast two-hybrid screening of a rat brain cDNA library using protein kinase Cβ as bait (14). RBCK1 has been found to possess transcriptional activity and consist of a ubiquitin-like sequence, two coiled-coil regions, and a RING-IBR domain, arranged from N to C terminus (see Fig. 1A). RBCK1 mRNA is ubiquitously expressed in normal rat tissues. The RING-IBR domain of RBCK1 interacts with DNA fragments containing a TGG-rich sequence. The RING finger motif occurring at the N-terminal side (RING1) is essential for the transcriptional activity of RBCK1, which is enhanced by coexpression with protein kinase A and significantly repressed by coexpression with extracellular signal-regulated kinase activator kinase 1 (MEK1) and MEK kinase 1 (15). A ubiquitin-like-truncated form of RBCK1, also known as a hepatitis B virus X-associating protein 3 (XAP3), is generated by alternative splicing of human RBCK1 gene and trans-activates Rous sarcoma virus long terminal repeat (RSV LTR) promoter (16).

RBCK1 also has been suggested to act as E3, a UbCm4 (a ubiquitin conjugating enzyme, E2)-interacting protein, UIPI2 (17), and a ubiquitin ligase-1 (HOIL-1) for a heme-oxidized iron regulatory protein-2 (IRP2) (18) are the mouse and human splice variants of RBCK1, respectively. Collectively, it is concluded that RBCK1 is a ubiquitin ligase and a transcriptional factor having a RING-IBR domain. However, RBCK1 is usually present in the cytoplasm unlike other conventional transcriptional factors.

To further elucidate cellular functions of RBCK1, we investigated its intracellular localization. In this paper, we report

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1 The abbreviations used are: PML, promyelocytic leukemia; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; RSV LTR, Rous sarcoma virus long terminal repeat; IRP2, iron regulatory protein-2; CREB, cAMP-response element-binding protein; CBP, CREB binding protein; HOIL-1, heme-oxidized iron regulatory protein-2 ubiquitin ligase-1; NES, nuclear export signal; NLS, nuclear localization signal; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; PBS, phosphate-buffered saline; LMB, leptomycin B; BRCA1, breast cancer 1; E3, ubiquitin-protein isopeptide ligase.
the identification of the nuclear export signal (NES) and the nuclear localization signal (NLS) in RBCK1, which are responsible for shuttling between the cytoplasm and nucleus. Furthermore, we demonstrate here interactions of RBCK1 with nuclear body proteins, CBP and PML, and their up- and down-regulation of the transcriptional activity of RBCK1.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—A mammalian expression plasmid pTB701-hGFPc for a C-terminally FLAG-fused protein was constructed by inserting the humanized green fluorescent protein cDNA (gGFPc) 3’-downstream of the SV40 early promoter contained in pTB701. For expression of truncated forms of RBCK1 (RBCK1(1–128) and RBCK1(270–498)), plasmid pTB701-RBCK1-hGFPc was constructed by inserting a DNA fragment encoding RBCK1 into pTB701-hGFPc. Plasmid pTB701-FLAG-RBCK1 was described previously for expression of the N-terminally FLAG-tagged RBCK1 (FLAG-RBCK1) (14). For expression of truncated forms of the N-terminally FLAG-tagged RBCK1 (FLAG-RBCK1(1–128) and FLAG-RBCK1(270–498)) plasmids pTB701-FLAG-RBCK1(1–128) and pTB701-FLAG-RBCK1(270–498) were constructed by inserting DNA fragments encoding N-terminal (from Met-1 to Glu-269) and C-terminal (from Cys-270 to His-498) halves of RBCK1 into pTB701-FLAG, respectively. For bacterial expression of an N-terminally and C-terminal (from Cys-270 to His-498) halves of RBCK1 into E. coli, plasmids pGEX-6T1-hGFPc-RBCK1 and pGEX-6T1-hGFPc-RBCK1 were constructed by inserting the PCR-amplified gGFPc gene into plasmid pGEX-6T1 (Amerham, Biosciences). An expression plasmid pGEX-6T1-hGFPc-RBCK1 (GST-fused RBCK1) was constructed by inserting the RBCK1 gene into pGEX-6T1-hGFPc. An expression plasmid pGEX-6T1-RBCK1 for the GST-fused RBCK1 (RBCK1-GST) was constructed by inserting the RBCK1 gene into pGEX-6T1-hGFPc. Plasmid pGL3-RSV-LTR used for the luciferase reporter gene assay with the RSV promoter was constructed by inserting RSV3’-LTR 5’-upstream of the luciferase gene in pGL3-Basic (Promega). A mammalian expression plasmid pRc/RSV-c-MCBP-HA/RC for the C-terminally hemagglutinin (HA) epitope-tagged CBP was a gift from S. Ishii (19). A mammalian expression plasmid pCMX-PML was a gift from A. Kakizuka (20). A mammalian expression plasmid pM-RBCK1 for GAL4 DNA-binding domain-RBCK1 was constructed previously (14). Site-directed mutagenesis (L142A/L145A) was carried out for pTB701-RBCK1-hGFPc, pTB701-FLAG-RBCK1, pGEX-6T1-hGFPc-RBCK1, and pM-RBCK1, and the resulting mutant plasmids were denoted with (L142A/L145A) after each plasmid name.

Expression of GFP-fused Proteins in Human Embryonic Kidney (HEK)293 Cells—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum at 37 °C under humidified air with 5% CO2. HEK293 cells (∼5 × 104 cells) were transfected with pTB701-RBCK1-hGFPc, pTB701-RBCK1-RK, pTB701-FLAG-RBCK1, or pTB701-FLAG-RBCK1(270–498) by FuGENE 6 with the reporter plasmid pFR-Luc (Stratagene) (1 μg) containing the firefly-derived luciferase gene 3’-downstream of the synthetic transcriptional activator consisting of the repeats of the E. coli lac operator site (17-mer), pm-RBCK1 (50 ng), and either pRC/rSV-mCBP-HA/RC (50 ng) or PCMX-PML (50 ng). After 24 h, the cells were washed once with PBS and lysed with 200 μl of passive lysis buffer (Promega). In both of the luciferase reporter gene assays, the cell lysate was assayed with a luciferase assay kit (Promega) according to the manufacturer's protocol. Transcriptional activities were shown by the relative luciferase unit divided by protein concentration, which was measured with a BCA (bicinchoninic acid) assay kit (Sigma) using bovine serum albumin as a standard.

Pull-down Assay—The cells were transfected with pRC/rSV-c-MCBP-HA/RC or pCMX-PML and lysed in 50 μl of lysis buffer containing 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 mM dithiothreitol, 1 mM NaF, 1 mM NaVO4, 10 μM L145A, and 10 μM L142A. After centrifugation, the supernatant containing the complete protease inhibitor mixture (Roche Diagnostics), and 0.5% (v/v) Nonidet P-40. Cleared lysate was incubated with 50 μl of glutathione-Sepharose 4B beads (50% slurry) immobilized with the purified RBCK1-GST (30 μg). After incubation at 4 °C for 4 h, the beads were washed twice with the lysis buffer and subjected to SDS-PAGE. Western blot analyses were carried out with an anti-human CBP rabbit polyclonal antibody (C-20) (dilution 1:1,000) of the anti-RBCK1 rabbit polyclonal antibody conjugated with Cy3 (Amersham Biosciences) as a secondary antibody (dilution 1:4,000). CBP was detected with an anti-human CBP rabbit polyclonal antibody (dilution 1:20) (Santa Cruz Biotechnology) as a primary antibody (dilution 1:1,000) and an anti-mouse IgG goat antibody conjugated with Cy3 (Amersham Biosciences) as a secondary antibody (dilution 1:1,000). CBP was detected by using an anti-CBP rabbit polyclonal antibody (dilution 1:1,000) and an anti-rabbit IgG goat antibody conjugated with Cy2 (Amersham Biosciences) as a secondary antibody (dilution 1:1,000). Endogenous CBP was detected by using an anti-CBP rabbit polyclonal antibody (dilution 1:1,000) and an anti-rabbit IgG goat antibody conjugated with Cy2 as a secondary antibody (dilution 1:1,000).

RESULTS

Effect of LMB on Intracellular Localization of RBCK1—We first investigated the intracellular localization of the over-expressed RBCK1. As shown in Fig. 1B, the GFP-fused RBCK1 was detected almost exclusively in the cytoplasm of HEK293 cells (a). When the cells were treated with leptomycin B (LMB), an inhibitor for the CRM1-dependent nuclear export (21), most RBCK1 translocated from the cytoplasm to the nucleus at 1 h after the LMB treatment (Fig. 1B, b). Although at 4 h after the treatment the protein still remained evenly in the nucleus (Fig. 1B, c), it accumulated in small granular structures inside the nucleus at 8 h after the treatment (d). Localization of the
control GFP was unaffected by the LMB treatment (Fig. 1B, e–h). These results suggest that RBCK1 has an LMB-sensitive NES sequence. However, the predominant existence of RBCK1 in the nucleus after the LMB treatment is unusual for NES-containing proteins, which are generally present in both the nucleus and cytoplasm in the presence of LMB (22), and suggests that RBCK1 possesses not only NES but also NLS sequences, like Smad1 (23) and p53 (24, 25).

**Colocalization of RBCK1 and PML in Nuclear Bodies**—PML is a major protein in the nuclear bodies (known also as NB, nuclear domain 10, or PML oncogenic domain) (20), which have granular structures of 250–500 nm in diameter and are present in the nuclei of most cells. The localization of RBCK1 in the nuclear body-like structures (Fig. 1B, d) was further examined by immunocytochemical analysis. HEK293 cells expressing RBCK1-GFP were treated with LMB, and the endogenous PML was visualized with an anti-PML antibody. In the nucleus, more than 70% of fluorescent spots derived from RBCK1-GFP overlapped with those of the nuclear bodies containing PML (Fig. 2A). Neither over-expression of RBCK1 nor LMB treatment affected the localization of PML in the nuclear bodies.

Next, the localization of endogenous RBCK1 was studied using an anti-rat RBCK1 rabbit antibody purified by affinity chromatography. Upon Western blotting of HEK293 cell lysate, the antibody could detect a 56-kDa protein (data not shown), whose size corresponds to the molecular mass (56,380) calculated from the amino acid sequence of human RBCK1 (NCB Accession number NP_112506). When the antibody was pre-treated with excess of the 24-residue oligopeptide that was used as an antigen, the 56-kDa protein band was not observed, indicating that the 56-kDa protein is an endogenous RBCK1. As shown in Fig. 2B, the endogenous RBCK1 was detected in both the cytoplasm and nucleus, unlike the over-expressed one mostly detected in the cytoplasm (cf. Fig. 1B, a). Nevertheless, RBCK1 present in the nucleus colocalized with PML at the nuclear bodies (Fig. 2B, d).

**Identification of NES and NLS**—Truncated forms of RBCK1 were used to delineate the positions of NES and NLS. The N-terminal half (from Met-1 to Glu-269) and C-terminal half (from Cys-270 to His-498) of RBCK1 were expressed as FLAG-tagged proteins in HEK293 cells, and the immunocytochemical analyses were carried out with anti-FLAG and anti-PML antibodies. The N-terminal half of RBCK1 was entirely observed in the cyto-

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**Fig. 1. Localization of over-expressed RBCK1 in cytoplasm.** A, the amino acid residue numbers of the beginning and end of RBCK1 protein and each motif are indicated below the schematic. B, HEK293 cells were transfected with pTB701-hGFPc or pTB701-RBCK1-hGFPc, treated with 20 nM LMB for 0–8 h, and fixed. The localizations of GFP and RBCK1-GFP were detected with direct fluorescence. Scale bar = 10 μm (a).

**Fig. 2. Colocalization of RBCK1 with PML in the nuclear body of HEK293 cells.** A, HEK293 cells were transfected with pTB701-RBCK1-hGFPc and treated with 20 nM LMB for 8 h. Cells were stained by using anti-PML antibody and Cy3-labeled secondary antibody. e, ~2-fold magnification of the region enclosed by a square in d. B, endogenous RBCK1 of HEK293 cells was detected with anti-RBCK1 antibody and Cy2-labeled secondary antibody. PML was detected with anti-PML antibody and Cy3-labeled secondary antibody. Scale bars = 10 μm (A, a and B, a).
plasm (Fig. 3c). In marked contrast, the C-terminal half was detected only in the dot-like structures within the nucleus (Fig. 3d), which mostly colocalized with the nuclear bodies (f) similarly to the full-length RBCK1 in the LMB-treated cells. These results show that the NES and nuclear-localizing function are contained in the N- and C-terminal halves of RBCK1, respectively. It is reasonable that the C-terminal half of RBCK1, also containing the transcriptional activation domain of RBCK1 (15), is translocated to the nuclear bodies where genes are actively transcribed (26) and activates the transcription by interacting with the transcriptional machineries.

Truncated forms of RBCK1 (from Thr-151 to Glu-269 and from Cys-270 to His-498) were also prepared as N-terminally GST-GFP-fused proteins. These proteins were microinjected into the nuclei of baby hamster kidney-21 cells together with the rhodamine B-isothiocyanate-labeled bovine serum albumin. Even 1 h after microinjection, these proteins remained in the nuclei (data not shown), whereas the GST-GFP-fused full-length RBCK1 was exported from the nucleus within 30 min (Fig. 4c, a), corroborating that the NES sequence of RBCK1 is contained in the N-terminal region (from Met-1 to Leu-150). The consensus sequence for the LMB-sensitive NES has been reported as \( \text{Lxx}_{3-5}\text{L/V/F/P/MX}_{3-5}\text{L}(L/I) \) (where \( X \) is any amino acid residue; Fig. 4b), which is recognized by an NES-receptor exportin-1/CRM1 (27). By comparing the entire RBCK1 sequence with the consensus sequence, a putative NES sequence was found at the region including Leu-142 and Leu-145 (Fig. 4a). It has been reported that the replacement of Leu by Ala in the NES sequence leads to disruption of the NES function (28). Indeed, when the GST-GFP-fused RBCK1(L142A/L145A) in which both Leu-142 and Leu-145 were substituted by Ala was microinjected into the nuclei, the protein did not translocate from the nucleus to the cytoplasm at 30 min after the microinjection (Fig. 4c, c). Also, the GFP-fused RBCK1(L142A/L145A) protein over-expressed in HEK293 cells spontaneously colocalized with PML in the nucleus as dot-like structures without the LMB treatment (Fig. 4d). These results indicate that Leu-142 and Leu-145 are essential for the NES function of RBCK1.

Consistent with the previous report that RBCK1 plays as a transcriptional activator for the RSV LTR promoter (16), RBCK1 activated the transcription of RSV LTR about 2.5-fold in the luciferase reporter assay (Fig. 4e). The NES mutant, RBCK1(L142A/L145A), localizing predominantly in the nuclear bodies, showed further enhancement of the transcriptional activity of RSV LTR promoter. A similar result has been obtained in the one-hybrid reporter assay using the GAL4 DNA-binding domain-fused RBCK1(L142A/L145A) (see Fig. 7), suggesting that the transcriptional activity of RBCK1 is down-regulated by NES.

**Interaction of RBCK1 with PML and CBP—**CBP has recently been revealed to scaffold and activate various transcription factors in the nuclear bodies (26, 29). PML, a main constituent of the nuclear bodies, was also shown to participate in the transcription mechanism involving either CBP or p53 (30, 31). In Hep-2 (26) and SK-N-SH cells (32), endogenous CBP is mostly detected in the nuclear body. However, the localization of CBP depends on cell lines; in HEK293 cells, it accumulates in nuclear body only when PML is over-expressed (32). Thus, colocalization of RBCK1 with PML in the nuclear bodies prompted us to examine the colocalization of RBCK1 with CBP. GFP-fused RBCK1 was expressed in HEK293 cells, and the cells were treated with LMB. The transfection efficiency was ~15%. In the cells, in which RBCK1-GFP was not over-expressed, the endogenous CBP was detected diffusely in the nucleus. However, in the cells, in which RBCK1-GFP was over-expressed, both RBCK1-GFP and CBP were detected in dot-like structures (Fig. 5a). Similar localization was observed with the cells over-expressing RBCK1(L142A/L145A)-GFP (Fig. 5b). These results suggest that RBCK1 interacts with not only PML but also CBP.

The interactions of RBCK1 with PML and CBP were further examined by a GST pull-down assay. The purified RBCK1-GST protein was adsorbed onto a glutathione-Sepharose 4B resin, mixed with the lysate of COS7 cells over-expressing either PML or CBP, and subjected to the pull-down assay. As shown in Fig. 6a, CBP (~265 kDa) was found to interact with RBCK1. When the lysate of COS7 cells expressing PML was analyzed by Western blotting, two bands (~58 and 62 kDa) were observed (Fig. 6b). Because the calculated molecular weight of PML is 62,006, the 58-kDa protein might be generated by proteolysis of the 62-kDa PML protein. In the GST pull-down assay, the 62-kDa PML protein was found to interact with RBCK1.

**Regulation of Transcriptional Activity of RBCK1 by CBP and PML—**CBP was shown to act as a coactivator for various transcriptional factors. PML was also shown to act as a coactivator (3, 31) or a corepressor (33, 34). It is assumed that the transcriptional activity of RBCK1 is affected by the coexpression of either CBP or PML. Thus the effects of CBP and PML on the RBCK1-dependent transcription were examined by using a luciferase reporter gene assay. The transcriptional activity of RBCK1 was enhanced by 3.6-fold by the coexpression with CBP but was unaffected by the coexpression with PML (Fig. 7). Furthermore, the CBP-enhanced transcriptional activity of RBCK1 was repressed to ~0.5 by the coexpression with PML. Similar effects of CBP and PML were observed in the transcriptional activity of the NES-disrupted RBCK1 mutant, RBCK1(L142A/L145A), which localizes exclusively in the nuclear bodies. Taken together, RBCK1 interacts functionally with PML and CBP in the nuclear bodies, and its transcriptional activity is up- or down-regulated by the interaction. It is likely that PML acts as a transcriptional repressor by interacting with the RBCK1-CBP complex, but not with the sole RBCK1.

**DISCUSSION**

A transcription factor, RBCK1, was revealed to possess NES and NLS concurrently. The NES of RBCK1 includes the leucine-rich consensus sequence, and this NES function was inhibited by the CRM1 inhibitor leptomycin B. Therefore, it was clarified that the NES of RBCK1 is involved in the CRM1 dependent nuclear-export machinery. The consensus sequences for NLS, which interacts with the importin α/β complex, are classified into two types. One type is a single cluster of basic residues, for example the simian virus 40 large T antigen-derived NLS (35). The other type consists of two basic...
regions separated by a 10-amino acid spacer (36). Although the C-terminal half of RBCK1 is preferentially localized in the nucleus, RBCK1 does not possess a typical NLS. Endogenous RBCK1 in HEK293 cells was found to localize in both cytoplasm and nucleus, whereas the over-expressed RBCK1 in the same cells localized only in the cytoplasm. We hereby propose that the cytoplasmic localization of over-expressed RBCK1 is caused by the failure of either an RBCK1 anchoring protein in the nucleus or a cytoplasmic cargo protein for the transport of RBCK1 to the nucleus. In the case of BRCA1, a RING protein- 

FIG. 4. Detection of the NES with microinjection and over-expression of mutant RBCK1 and the effect of NES disruption on the transcriptional activity of RBCK1. A, the amino acid sequence of the putative leucine-rich NES of RBCK1 is indicated below the schematic drawing of RBCK1. B, the NESs and the putative NES of RBCK1 are aligned. These NESs were reported (28, 57–61). C, GST-GFP-fused wild type RBCK1 (a) or the mutant RBCK1(L142A/L145A) (c) was mixed with rhodamine B-isothiocyanate-labeled bovine serum albumin (RITC-BSA) protein (b and d) and microinjected into the nucleus of baby hamster kidney cells. The injected cells were fixed after 30 min and observed. Scale bar = 20 μm (a). D, HEK293 cells were transfected with pTB701-RBCK1(L142A/L145A)-hGFPc, the expression plasmid of NES-disrupted RBCK1. RBCK1(L142A/L145A)-hGFPc was detected with direct fluorescence, and PML was detected with anti-PML antibody and Cy3-labeled secondary antibody. e, ~2-fold magnification of the region enclosed by a square in d. Scale bar = 10 μm (a). E, the luciferase reporter-gene assays of RSV LTR promoter were carried out with pGL3-RSV-LTR in HEK293 cells. pTB701-FLAG-RBCK1 or pTB701-FLAG-RBCK1(L142A/L145A) were transfected with pGL3-RSV-LTR. The data are expressed as a ratio to the mean value of RSV LTR promoter activity without RBCK1. Each measurement was repeated six times. Error bars indicate 95% confidence interval. RLU, relative luciferase unit.
revealed that BARD1 retains BRCA1 in the nucleus by masking its NES (39). It is therefore presumed that an unidentified RBCK1-interacting protein masks the NES of RBCK1 and fa-

**FIG. 5.** Colocalization of RBCK1 with CBP. A, HEK293 cells were transfected with pTB701-RBCK1-hGFPc and treated with 20 nm LMB for 8 h. B, HEK293 cells were transfected with pTB701-RBCK1(L142A/L145A)-hGFPc. Cells were stained by using an anti-CBP antibody and a Cy3-labeled secondary antibody (A and B). Scale bar = 10 μm (A and B, a).

**FIG. 6.** Interaction of RBCK1 with PML and CBP. A and B, COS7 cells were transfected with pRc/RSV-mCBP-HA-RK or pCMX-PML, and the cell lysates were confirmed for over-expression of CBP or PML by Western blotting with an anti-CBP antibody or an anti-PML antibody (left panels of A and B). These cell lysates were then mixed with GST or GST-RBCK1 immobilized on glutathione-Sepharose beads. After washing, beads were subjected to SDS-PAGE and immunostained with an anti-CBP antibody or an anti-PML antibody (right panels of A and B). The black and white arrowheads indicate a native (62 kDa) and partially degraded PML (58 kDa), respectively.

**FIG. 7.** Transcriptional regulation of RBCK1 with CBP and PML. The transcriptional activity of RBCK1 was measured by luciferase reporter gene assay. HEK293 cells were transfected either pM-RBCK1 or pM-RBCK1(L142A/L145A) (L/A) with pFR-Luc. These transfections were carried out with or without pRc/RSV-mCBP-HA-RK and pCMX-PML. The data are expressed as a ratio to the mean value of the GAL4 DNA-binding domain (DBD)-RBCK1 transcriptional activity without coexpression of CBP and PML. Each measurement was repeated six times. Error bars indicate 95% confidence interval. RLU, relative luciferase unit.
cilitates the nuclear translocation of RBCK1. RBCK1 was first identified as a protein kinase Cβ- and protein kinase Cε-interacting protein. Because parts of protein kinase Cβ and -ε are known to translocate to the nuclear body-like domains in phorbol ester-treated cells (40), it is hypothesized that protein kinase Cβ and -ε may be RBCK1-anchoring proteins in nuclear bodies. We demonstrated that RBCK1 colocalizes with PML in nucleus and interacts with PML and CBP. PML or CBP is also a possible candidate for a nuclear anchoring protein for RBCK1. The RBCK1-recruiting protein, which controls the localization of RBCK1, may regulate its interaction with RBCK1 via a phosphorylation signal. The forked head of the rhabdomyosarcoma protein is known as a transcription factor responsible for nucleocytoplasmic shuttling, of which the nuclear localization is mediated by phosphorylation with protein kinase B (41) and interaction with 14-3-3 protein (42). In the previous study, the transcriptional activity of RBCK1 was modulated by the expression of protein kinase A, MEK1, and MEK kinase 1 (15). Unfortunately, the subcellular localization of RBCK1 was not changed by the coexpression of these protein kinases (data not shown), but it cannot be entirely denied that other protein kinase affects the localization of RBCK1. Recently, RBCK1 has been shown to possess a ubiquitin ligase E3 activity. UbM4-interacting protein (UIP28) (17) and heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) (18) are the mouse and human homologue of RBCK1, respectively. It is postulated therefore that RBCK1 possesses a ubiquitin ligase E3 activity. Iron regulatory protein 2 (IRP2) interacts with and stabilizes a specific mRNA in the cytosol. The ubiquitination of IRP2 by HOIL-1 is supposed to be executed in the cytosol, whereas RBCK1 localizes in not only cytoplasm but also nucleus, and the nuclear RBCK1 possesses the transcriptional activating ability. These facts indicate that the role of RBCK1 may be regulated by the switch of the NLS and NES.

The nuclear body is a granular structure of 250–500 nm in diameter, and its main component protein is pML, which was shown to exert a transcriptional function in the nuclear body (31). A growing number of proteins have been demonstrated to colocalize with PML, including speckled 100-kDa protein (Sp100) (43), death domain-associated protein (44), retinoblastoma protein (45), CBP (46), p53 (4), and BRCA1 (47). These proteins are involved in the transcriptional mechanism. The chromatin-surrounding nuclear bodies are highly acetylated, and the nascent RNA is associated with the periphery of the nuclear body (26). The nuclear body is thus considered to be an active site of transcription mechanisms (48). In this report, RBCK1 interacts with PML and CBP, and was demonstrated to be a novel component protein of the nuclear body. CBP connects the DNA-binding transcriptional factors with the general transcription machinery (4), our results indicate that PML, by contrast, represses the transcriptional activity of the CBP-RBCK1 complex. It is important to elucidate how p53 and RBCK1 share the transcriptional function of the CBP-PML complex in the nuclear body at the molecular level.

The functions of autoimmune regulator are very similar to that of RBCK1. Autoimmune regulator binds to CBP (50) and the specific nucleotide sequence (51) and activates the transcription (52). The DNA-associating ability of autoimmune regulator is dependent on its homodimer or homotetramer formation. In a previous experiment, the complexes of RBCK1 and the RBCK1-specific nucleotide sequences were observed as two bands by using electrophoretic mobility shift assay (14). This result suggested that RBCK1 also binds to the specific nucleotides with a monomer and dimer. The autoimmune regulator has two plant homeodomain-type zinc-finger motif, which binds zinc in a cross-brace topology between anti-parallel β-strands reminiscent of RING finger (53), and the N terminus plant homeodomain is important domain for the ubiquitin ligase activity of the autoimmune regulator (54). Some RING proteins, such as BRCA1 (10, 11) and A07 (10, 12), were also reported to possess both transcriptional and ubiquitin ligase activity. Thus, RBCK1 and some ubiquitin ligases may regulate transcription via its ubiquitin ligase activity. In some cases, ubiquitination of the transcription factor is the important step of the transcriptional activation (55, 56). However, it is unknown how the two distinct activities of RBCK1 are involved in the function of nuclear bodies. It is important to more precisely define the function of nuclear bodies in transcription and ubiquitination.
