Identification of Ubiquitin Ligase Activity of RBCK1 and Its Inhibition by Splice Variant RBCK2 and Protein Kinase Cβ

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We previously identified a RING-IBR protein, RBCK1, as a protein kinase C (PKC) β- and ζ-interacting protein, and its splice variant, RBCK2, lacking the C-terminal half including the RING-IBR domain. RBCK1 has been shown to function as a transcriptional activator whose nuclear translocation is prevented by interaction with the cytoplasmic RBCK2. We here demonstrate that RBCK1, like many other RING proteins, also possesses a ubiquitin ligase (E3) activity and that its E3 activity is inhibited by interaction with RBCK2. Moreover, RBCK1 has been found to undergo efficient phosphorylation by PKCβ. The phosphorylated RBCK1 shows no self-ubiquitination activity in vitro. Overexpression of PKCβ leads to significant increases in the amounts of intracellular RBCK1, presumably suppressing the proteasomal degradation of RBCK1 through self-ubiquitination, whereas coexpression with PKCe and PKCe and PKCζ shows no or little effect on the intracellular amount of RBCK1. Taken together, the E3 activity of RBCK1 is controlled by two distinct manners, interaction with RBCK2 and phosphorylation by PKCβ. It is possible that other RING proteins, such as Parkin, BRCA1, and RNF8, having the E3 activity, are also down-regulated by interaction with their RING-lacking splice variants and/or phosphorylation by protein kinases.

RBCK1, a RING-IBR (RING-in between RING fingers) protein, was identified by the yeast two-hybrid screening of a rat brain cDNA library using protein kinase Cζ (PKCζ) β as bait (1) and was shown to interact with not only PKCβ but also PKCζ. RBCK1 consists of a ubiquitin-like sequence (1), a coiled-coil region, an novel zinc finger motif (2), another coiled-coil region, and a RING-IBR domain (3), arranged from the N to C terminus (see Fig. 1A). RBCK1 mRNA is ubiquitously expressed in normal rat tissues. RBCK1 possesses a transcriptional activity, and its RING-IBR domain interacts with DNA fragments containing a TGG-rich sequence, indicating that RBCK1 is a “transcriptional factor.” The RING finger motif occurring around the middle of the whole sequence (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 (MEKK1) (4). RBCK1 is localized in both the nucleus and cytoplasm (5), possessing a classical Leu-rich nuclear export signal as well as the nuclear localization signal. These intracellular localization signals, thus, allow the nucleocyttoplasmic shuttling of RBCK1 (5). Furthermore, intranuclear RBCK1 localizes with a promyelocytic leukemia protein (PML) and a CREB-binding protein (CBP) present in the nuclear bodies. The transcriptional activity of RBCK1 is up-regulated by interaction with CBP, whereas the CBP-enhanced activity is down-regulated by interaction with PML (5). RBCK2, lacking the C-terminal half of RBCK1 including the RING-IBR domain, was also identified as an alternative splice variant of RBCK1 (6). RBCK2 functions as an anchoring protein for the parental RBCK1 and represses its transcriptional activity by tethering it within the cytoplasm (7). On the other hand, another splice variant of RBCK1, named HOIL-1, was recently reported to be a ubiquitin ligase E3 (8). Furthermore, an autosomal recessive juvenile parkinsonism-related gene product, Parkin, also a RING-IBR protein, shows an E3 activity (9). These findings strongly suggest that RBCK1 functions not only as a transcriptional factor but also as a ubiquitin ligase E3.

In the ubiquitin-proteasome system, E3 plays a crucial role in the recognition of specific substrate protein and facilitates polyubiquitination of the substrate proteins by the help of ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2. The polyubiquitinated substrate proteins are sorted to the proteasome for degradation (10). Conventional regulation of E3 activity is based on the modification of specific substrates. For example, the phosphorylated forms of IkBa and β-catenin are able to interact with an FWD1 subunit of the SCF complex (an E3 enzyme), which initiates polyubiquitination of IkBa and β-catenin (11, 12). Only the oxidized form of iron regulatory protein 2 (IRP2) associates with HOIL-1 and is degraded (8). On the other hand, there are only a few E3-interacting proteins that have so far been found to modulate the E3 activity. The E3 activities of MDM2 and the SCF complex are enhanced by phosphorylation with glycogen synthase kinase-3 (13) and modification of the Cul1 subunit by Nedd8 (14), respectively.
Ubiquitin Ligase Activity of RBCK1

In this paper we demonstrate that RBCK1, reported previously as a transcriptional factor (1), also has a ubiquitin ligase E3 activity. Furthermore, we show that the E3 activity is inhibited by phosphorylation by PKCβ, similar to Parkin (15) and, more intriguingly, by interaction with its splice variant RBCK2.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pTB701-FLAG-RBCK1 and pTB701-HA-RBCK1 were described previously for mammalian expression of the N-terminal FLAG- and HA-tagged RBCK1 (FLAG-RBCK1 and HA-RBCK1), respectively (1, 16). A plasmid pTB701-FLAG-RBCK2 was described previously for mammalian expression of N-terminal FLAG-tagged RBCK2 (FLAG-RBCK2) (6). A plasmid pGEX-6T1-RBCK1 was also described previously for bacterial expression of the N-terminal glutathione S-transferase (GST)-fused RBCK1 (GST-RBCK1) (6). A plasmid pTB701-FLAG-RBCK1C293G was used for mammalian expression of N-terminal FLAG-tagged RING1-disrupted RBCK1 (FLAG-RBCK1C293G). A plasmid pTB701-FLAG-RBCK1 (ST mutant) used for mammalian expression of a multiple site-directed mutant of FLAG-RBCK1 containing Ala mutations at Ser-127, Thr-151, Thr-191, Ser-260, Thr-265, and Ser-275 was constructed from pTB701-FLAG-RBCK1 by using QuickChange® multisite-directed mutagenesis kit (Stratagene, La Jolla, CA). Similarly, FLAG-RBCK1 containing Asp mutation at Ser-127, Thr-151, or Thr-191 was expressed by using plasmid pTB701-FLAG-RBCK1S127D, pTB701-FLAG-RBCK1T151D, or pTB701-FLAG-RBCK1T191D. The expression plasmid pHT2 (Promega, Madison, WI) was used for the mammalian expression of N-terminal HaloTag protein (HT)-tagged FLAG-RBCK1 (HT-RBCK1). Plasmids pTB701-HA-PKCa, pTB701-HA-PKCb, pTB701-HA-PKCe, and pTB701-HA-PKCζ were described previously for mammalian expression of the N-terminal HA-tagged PKCs (HA-PKCa, HA-PKCb, HA-PKCe, and HA-PKCζ) (16). Plasmids pTB701-HA-PKCBN and pTB701-HA-PKCζN were used for mammalian expression of kinase negative mutants of HA-PKCb (HA-PKCBN) and HA-PKCζ (HA-PKCζN) (16, 17). Mammalian expression plasmids for N-terminal FLAG- and HA-tagged ubiquitin (FLAG-ubiquitin and HA-ubiquitin), pcDNA3.1(+)-FLAG-ubiquitin, and pcDNA3.1(+)-HA-ubiquitin, respectively, were gifts from Dr. Keiji Tanaka.

Immunoprecipitation and Western Blot Analysis—HEK293 cells (~1 x 10⁷ cells in 100-mm dish) 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. The cells were transfected with plasmids by electroporation or a FuGENE 6 reagent (Roche Diagnostics). The transfected cells were cultured for 60 h, washed twice with PBS, and suspended in 1 ml of the lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 50 mM NaF, 1 mM Na₃VO₄, and 1 tablet of Complete™ protease inhibitor mixture (Roche Diagnostics) per 50 ml). The cleared lysate was incubated with 10 μg of an anti-FLAG mouse monoclonal antibody M2 (Sigma) or an anti-HA mouse monoclonal antibody 12CA5 (Roche Diagnostics) on ice for 60 min and incubated with 50 μl of protein G-Sepharose 4 (50% (v/v) slurry) (GE Healthcare) at 4 °C for 30 min. The beads were washed 4 times with 1 ml of lysis buffer, resuspended in 30 μl of Laemmli sample buffer, subjected to SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Western blot analyses for epitope-tagged proteins were carried out with a horseradish peroxidase (HRP)-conjugated anti-HA mouse monoclonal antibody (dilution, 1:5000) (Roche Diagnostics) or an HRP-conjugated anti-FLAG mouse monoclonal antibody (dilution, 1:5000) (Sigma). Each PKC subtype was analyzed by using an anti-PKCα mouse monoclonal antibody (dilution, 1:1,000) (BD Biosciences), an anti-PKCβ mouse monoclonal antibody (dilution, 1:400) (BD Biosciences), an anti-PKCε mouse monoclonal antibody (dilution, 1:1000) (BD Biosciences), or an anti-PKCζ mouse monoclonal antibody (dilution, 1:3000) (Santa Cruz Biotechnology, Santa Cruz, CA) as a primary antibody and an HRP-conjugated anti-mouse IgG goat antibody (GE Healthcare) as a secondary antibody (dilution, 1:3000). HaloTag protein was analyzed by using anti-HaloTag rabbit polyclonal antibody (dilution, 1:1000) (Promega) as a primary antibody and an HRP-conjugated anti-rabbit IgG donkey antibody (GE Healthcare) as a secondary antibody (dilution, 1:5000). Some membranes were stripped in Western blot stripping solution (Nacalai Tesque, Kyoto, Japan) and then reprobed by using an anti-β-tubulin mouse monoclonal antibody (dilution 1:1000) (Sigma) as a primary antibody and an HRP-conjugated anti-mouse IgG goat antibody (GE Healthcare Bio-Science) as a secondary antibody (dilution, 1:3000). Immunoreactive bands were visualized by the enhanced chemiluminescence method with an ECL Plus (GE Healthcare) according to the manufacturer’s protocol.

Cycloheximide Chase Assay—HEK293 cells (~5 x 10⁵ cells in a 12-well plate) were transfected with 0.5 μg of pTB701-FLAG-RBCK1 by FuGENE 6 reagent, cultured for 40 h, and treated with cycloheximide (100 μg/ml) for 4, 8, and 24 h. MG132 (50 μM) was added to the medium to see the effect of proteasomal degradation. The cells were washed twice with PBS and suspended in 50 μl of the lysis buffer, and the lysate was subjected to Western blot analysis. The relative amount of FLAG-RBCK1 was estimated by chemiluminescence using a Quantity One one-dimensional analysis software (Bio-Rad).

Pulse-Chase Assay—HEK293 cells (~5 x 10⁵ cells in 12-well plates) were transfected by FuGENE 6 reagent with 0.5 μg of pHT2-RBCK1 and either 2 μg of pTB701-FLAG-RBCK2, 0.5 μg of pTB701-HA-PKCb, or 0.5 μg of pTB701-HA-PKCζ, cultured for 40 h, incubated with 5 μM HaloTag-tetramethylrhodamine ligand (Promega) for 15 min to allow the pulse labeling of HT-RBCK1, and washed twice with PBS. After the incubation for the indicated times, the cells were washed twice with PBS and suspended in 30 μl of lysis buffer. The cleared lysate (10 μl) was subjected to SDS-PAGE, and the HaloTag-tetramethylrhodamine ligand-labeled HT-RBCK1 was visualized with a fluoro-image analyzer FLA-3000G (FUJI FILM, Tokyo, Japan).

In Vitro Ubiquitin Ligase Assay—GST-RBCK1 was purified from Escherichia coli BL21 expressing GST-RBCK1 by using a glutathione-Sepharose 4B (GE Healthcare) column (5 x 10 mm). The ubiquitination reaction of GST-RBCK1 (1 μg) or the immunoprecipitated FLAG-tagged RBCK1 was carried out with E1 (100 ng, Boston Biochem, Cambridge, MA) and UbcH7.
Ubiquitin Ligase Activity of RBCK1

In Vivo Phosphorylation Analysis of RBCK1 with Phos-Tag Acrylamide Gel Electrophoresis—In the Mn$^{2+}$-Phos-tag-modified acrylamide gel (18), the phosphorylated proteins migrate slower than non-phosphorylated protein by the interaction of phosphate groups with Mn$^{2+}$-Phos-tag. HEK293T cells over-expressing either FLAG-RBCK1 or FLAG-RBCK1 (ST mutant) were treated with or without 1 μM okadaic acid in culture medium for 30 min. The cell lysates were subjected to the Mn$^{2+}$-Phos-tag SDS-PAGE (8% polyacrylamide gel including 50 μM MnCl$_2$ and 50 μM Phos-tag acrylamide (NARD Institute, Ltd. Hyogo, Japan)) and analyzed by Western blotting using an HRP-conjugated anti-FLAG antibody.

In Vitro Phosphorylation Assay—RBCK1 was excised from GST-RBCK1 using PreScission Protease$^{\text{TM}}$ (GE Healthcare) and purified by passing through a glutathione-Sepharose 4B column. Either RBCK1 (0.5 μg), UbcH7 (0.5 μg), or E1 (0.5 μg) was mixed with PKCβII (Sigma) in the phosphorylation reaction mixture (20 mM Tris-Cl (pH 7.4), 5 mM MgCl$_2$, phosphatase inhibitor mixture 1 (Sigma), and 25 mM [γ-$^{32}$P]ATP (2.3 × 10$^{16}$ Bq/mol)) and incubated at 37 °C for 30 min. The reaction mixtures were boiled with Laemmli sample buffer, subjected to SDS-PAGE, and then analyzed by autoradiography.

Identification of the Phosphorylation Site in RBCK1 by Mass Spectrometry—The RBCK1 phosphorylated in vitro by PKCβII was digested in-solution by endoproteinase Glu-C or in-gel by the combination of trypsin and endoproteinase Glu-C or trypsin alone. The digested products of RBCK1 were subjected to matrix-assisted laser desorption ionization (MALDI) quadrupole time-of-flight mass spectrometer.

In-solution digestion was carried out as follows. A solution containing 2 μg of RBCK1 was reduced with DTT (final concentration, 10 mM) for 1 h at 56°C and then alkylated with iodoacetamide (final concentration, 40 mM) for 45 min at room temperature in the dark. Subsequently, the solution was diluted with the same volume of 100 mM NH$_4$HCO$_3$ and added with endoproteinase Glu-C (0.1 μg/μl). Digestion was performed overnight, and the peptide solution was dried up to 10 μl under reduced pressure. The peptide mixture was purified using ZipTip pipette tips (Millipore, Billerica, MA) containing C18 reversed-phase resins according to the manufacturer’s protocol.

In-gel digestion was carried out as follows. The phosphorylated RBCK1 was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. The stained band was excised from the gel, cut into pieces, washed twice with 50 mM NH$_4$HCO$_3$ in 50% (v/v) acetonitrile, and dehydrated in 100% acetonitrile. The protein sample was reduced with 10 mM DTT in 100 mM NH$_4$HCO$_3$ for 1 h at 56°C and alkylated with 55 mM iodoacetamide in 100 mM NH$_4$HCO$_3$ for 45 min at room temperature in the dark. Subsequently, the gel pieces were washed twice for 5 min alternately with 100 mM NH$_4$HCO$_3$ and acetonitrile and then completely dried up under reduced pressure. Appropriate volumes of trypsin solution (25 ng/μl trypsin in 50 mM NH$_4$HCO$_3$) were added to the dried gel pieces. After incubation overnight at 37°C, the supernatant containing digested peptides was transferred to a new tube. The remaining peptides in gel pieces were eluted with the solution containing 50% (v/v) acetonitrile and 5% (v/v) formic acid. The recovered peptides were separated by SDS-PAGE and autoradiographed using a Cyclone Phospho Imager (PerkinElmer Life Sciences).
Ubiquitin Ligase Activity of RBCK1

A molecular organization of RBCK1. Amino acid residue numbers of the start and end of each motif are indicated. Ubi, ubiquitin-like; CC, coiled-coil; NZF, novel zinc finger; RING1, the first RING finger; IBR, in-between-RING finger; RING2, the second RING finger. Black and white pins indicate the PKCβ-phosphorylation sites identified (Ser-127, Thr-151, and Thr-191) and predicted (either one of Ser-260, Thr-265, and Ser-275) in this study, respectively. B, HEK293 cells coexpressing FLAG-RBCK1 and HA-ubiquitin were cultured for 60 h and treated with MG132 for 3 or 8 h. The anti-FLAG immunoprecipitates (IP) obtained from the cell lysates were analyzed by Western blotting with an anti-HA antibody (top panel) and an anti-FLAG antibody (middle panel). The anti-HA immunoprecipitates were analyzed by Western blotting with an anti-HA antibody (bottom panel). C, HEK293 cells coexpressing HA-ubiquitin (HA-Ub) and either FLAG-RBCK1 or FLAG-RBCK1C293G were processed as described in panel B, except that the amount of samples used was increased by 6-fold. D, HEK293 cells expressing RBCK1 were treated with cycloheximide (CHX) with or without MG132 for the indicated times. The cell lysates were analyzed by Western blotting with an anti-FLAG antibody (top panels) and an anti-ß-tubulin antibody (middle panels). Relative amounts of FLAG-RBCK1 were measured with the densitometric intensities of bands (bottom panel). The values are obtained from six sets of independent experiments. Error bars indicate a 95% confidence interval.

from the gels were combined into the same tube and were dried up to 10 μl under reduced pressure. The trypsin-digested sample was further diluted with 100 mM NH₄HCO₃ and added with endoproteinase Glu-C (0.1 μg/μl). The second digestion was also performed overnight, and the peptide solution was dried up to 10 μl under reduced pressure. The peptide mixture was purified using ZipTip pipette tips (Millipore) containing C18 reversed-phase resins according to the manufacturer’s protocol.

The eluted peptide solution was placed on a MALDI target plate, mixed with a matrix (2,5-dihydroxybenzoic acid; Sigma), and analyzed by an oMALDI-Qq-TOF MS/MS QSTAR Pulsar i (Applied Biosystems, Foster, CA) in the linear positive ion mode. The spectral data were analyzed for peptide identification by peptide mass fingerprinting using a MASCOT Version 1.9 (Matrix Science Ltd., London, UK) with NCBInr data base.

Immunocytochemical Analysis—HEK293 (~5 × 10⁶ cells in a 35-mm glass-bottom dish) cells transfected with either IRP2 (8). Moreover, the novel zinc finger motif of RBCK2 was found to interact with ubiquitinated proteins (2); the motif is shared by RBCK1 (see Fig. 1A). We, therefore, investigated whether RBCK1 interacts with a substrate protein for polyubiquitination, as reported for Parkin (9) and other E3 proteins (20–22). Both HA-ubiquitin and FLAG-RBCK1 were coexpressed in HEK293 cells in the presence or absence of the proteasome inhibitor MG132. In the anti-FLAG immunoprecipitates containing RBCK1, the amount of ubiquitinated proteins was significantly increased after 3 h of the MG132 treatment (Fig. 1B, top panel). Because self-ubiquitinated RBCK1 was hardly observed in the immunoprecipitates (middle panel), the major portion of the ubiquitinated proteins observed in the anti-FLAG immunoprecipitates was considered to be other ubiquitinated proteins bound to RBCK1 rather than the self-ubiquitinated RBCK1. Nevertheless, the amount of RBCK1 was also increased several fold by the incubation with MG132 for 8 h (middle panel), suggesting that RBCK1 itself is degraded as
In Vitro Self-ubiquitination Activity of RBCK1—Proteins tagged with the Lys-48-linked poly-ubiquitin chain are generally sorted to the 26 S proteasome and then degraded immediately (23). UIP28, a mouse orthologue of RBCK1, was shown to interact with UbcM4/Ubch7 (E2), which can catalyze the generation of the poly-ubiquitin chain through formation of an isopeptide bond between the ε-amino group of Lys-48 and the carboxyl group of the C-terminal Gly residue of ubiquitin molecules (23, 24). Because the RING-IBR domain of HOIL-1 is also contained in RBCK1 (25), RBCK1 is postulated to be an E3 enzyme cooperating with UbcH7 as an E2. To investigate this possibility, the purified GST-RBCK1 was mixed with the purified forms of ubiquitin, a ubiquitin-activating enzyme E1, and the ubiquitin-conjugating enzyme UbcH7. After 1 h of incubation, the samples were separated by SDS-PAGE and analyzed by Western blotting with an anti-ubiquitin antibody (Fig. 2A, top panel). The ubiquitinated proteins were observed as ladder bands in the mixture of E1, UbcH7, and GST-RBCK1 (third lane) but not in the samples without GST-RBCK1 or UbcH7 (first and second lanes). The molecular masses of ladder bands were 92-, 100-, and 108-kDa (from bottom to top), likely corresponding with those of mono-ubiquitinated GST-RBCK1 (calculated Mr, 92,090), di-ubiquitinated GST-RBCK1 (calculated Mr, 100,637), and tri-ubiquitinated GST-RBCK1 (calculated Mr, 109,184), respectively. More than 110-kDa proteins were considered as poly-ubiquitinated GST-RBCK1. To confirm the in vitro self-ubiquitination of RBCK1, the membrane was stripped and then reprobed with an anti-RBCK1 antibody (middle panel). Similarly, mono-ubiquitinated GST-RBCK1 was observed in the sample containing E1, UbcH7, and GST-RBCK1 (third lane). Probably due to the difference in the number of antigen (RBCK1 or ubiquitin) in the poly-ubiquitin chain, the GST-RBCK1 conjugated with more than three ubiquitin molecules, if any, could be detected with an anti-ubiquitin antibody but not with an anti-RBCK1 antibody. These results have, thus, corroborated that RBCK1 functions as an E3 enzyme and catalyzes the self-ubiquitination.

Inhibition of Self-ubiquitination Activity of RBCK1 by RBCK2—The RING finger is believed to be essential for the E3 activity of RBCK1. A, the purified GST-RBCK1 was incubated with ubiquitin (Ub), E1, and UbcH7 in the presence or absence of the purified RBCK2. Molar ratios of RBCK2 to RBCK1 are indicated in the upper margin. Self-ubiquitinated RBCK1 was analyzed by Western blotting with an anti-ubiquitin antibody (top panel). The same membrane was reprobed with an anti-RBCK1 antibody, which also reacts with RBCK2 (middle and bottom panels). B, HEK293 cells (about 1 × 10^7 cells) were cotransfected with the indicated amounts (μg) of pTB701-FLAG-RBCK1 and pTB701-FLAG-RBCK2. The cell lysates were subjected to Western blotting with an anti-FLAG antibody (top panel), and the membrane was reprobed with an anti-β-tubulin antibody (middle panel). The mRNA level of RBCK1 was validated by reverse transcription-PCR using the total RNA as a template (third panel). C, HEK293 cells (about 1 × 10^7 cells) were transfected with 2 μg of pTB701-FLAG-RBCK1 with or without 8 μg of pTB701-FLAG-RBCK2, cultured for 60 h, and treated with 50 μM MG132 for 8 h. The cell lysates were subjected to Western blotting with an anti-FLAG antibody (top panel), and the membrane was reprobed with an anti-β-tubulin antibody (bottom panel). The relative amounts of FLAG-RBCK1 were measured with densitometric intensities of bands and indicated below.
Various RING proteins (9, 26). In agreement with this, RBCK2, a splice variant of RBCK1 lacking the RING-IBR domain, showed no self-ubiquitination activity in the in vitro self-ubiquitination assay (Fig. 2A, bottom panel, seventh lane). On the other hand, RBCK2 was demonstrated to interact with the N-terminal half of RBCK1 in vitro and in vivo (7), and so the effect of RBCK2 on the E3 activity of RBCK1 was investigated by the in vitro self-ubiquitination assay. As shown in Fig. 2A (top and middle panels, lanes fourth through sixth panels), the purified RBCK2 inhibited the self-ubiquitination activity of RBCK1 in a dose-dependent manner. A 4-fold molar excess of RBCK2 is sufficient to inhibit the E3 activity of RBCK1 and the formation of not only poly- but also mono-, di-, and tri-ubiquitin (sixth lane). This result indicates that RBCK2 inhibits the ubiquitinating activity of RBCK1. Lower concentrations of RBCK2 efficiently inhibited the formation of poly-ubiquitin chains (more than tetra-ubiquitin) by RBCK1 (top panel), which may be caused by the insufficient ubiquitinating activity of RBCK1 for the elongation of poly-ubiquitin chain. When the effect of RBCK2 on the intracellular amount of RBCK1 was examined using HEK293 cells, the amount of expressed RBCK1 protein was increased markedly in parallel with the amount of the FLAG-RBCK2 plasmid DNA used (Fig. 2B, top panel). As a positive control, the MG132 treatment also increased the amount of FLAG-RBCK1 (Fig. 2C, third lane). The amount of RBCK1 mRNA was not influenced by the overexpression of RBCK2 (Fig. 2B, bottom panel), thus, most likely RBCK2 preventing the degradation of RBCK1. Furthermore, the in vivo half-life of RBCK1 was measured by the pulse-chase assay using HaloTag (HT) and HaloTag-tetramethylrhodamine ligand (27). HEK293 cells expressing HT-tagged RBCK1 (HT-RBCK1) with or without RBCK2 were treated with HaloTag-tetramethylrhodamine ligand, and the lysate was subjected to SDS-PAGE followed by Western blot analysis using a fluor-image analyzer (see the supplemental figure). It was revealed that the half-life of HT-RBCK1 was clearly extended by the overexpression of RBCK2. It is, thus, strongly suggested that the E3 activity of RBCK1 is negatively controlled by interaction with RBCK2 within the cells. Recently, homodimerization of a RING-containing E3 enzyme is proposed to be important for exhibiting its activity (28–30). RBCK2 may interfere with the putative homodimerization of RBCK1 by forming a heterodimer with RBCK1, as discussed below.

Phosphorylation of RBCK1 by PKCβ—Inhibition of Self-ubiquitination Activity—RBCK1 was originally isolated as a PKCβ-interacting protein (1). To study whether RBCK1 is phosphorylated within the cells, the HEK293 cells overexpressing FLAG-RBCK1 were cultured in the medium containing 32P-labeled phosphate, and incorporation of 32P into RBCK1 was examined by SDS-PAGE of the anti-FLAG immunoprecipitates followed by autoradiography. As an unequivocal result, RBCK1 was found to be significantly phosphorylated (Fig. 3A). We then investigated whether PKCβ phosphorylates RBCK1 and also whether this phosphorylation affects the self-ubiquitinating activity of RBCK1 in the cells. Before the in vivo experiments, the possibility of phosphorylation of RBCK1, E1, and UbcH7 (E2) by PKCβ was examined in vitro (Fig. 3B). The amount of PKCβ added was 20 mol % of each substrate (i.e. RBCK1, E1, and UbcH7). Although neither E1 nor UbcH7 was phosphorylated, RBCK1 was well phosphorylated by PKCβ in vitro. After a prolonged reaction time (about 1.5 h), by comparing the densitometric intensities of the radioactive bands of RBCK1 (see Fig. 3B, top left panel, second lane) with those of radioactive spots derived from known amounts of phosphate, 10.7 pmol of phosphate was incorporated in 8.9 pmol of RBCK1, indicating about 1.2 mol of phosphate per mol of RBCK1. These data indicate that RBCK1 is a good substrate of PKCβ, phosphorylating at least one Ser/Thr residue in RBCK1. Next, the purified PKCβ was incubated with the purified forms of E1, UbcH7, and GST-RBCK1 in the presence of ubiquitin and an ATP regenerating system (Fig. 4A). Co-incubation with PKCβ in the ubiquitination assay mixture was found to prevent almost entirely the self-ubiquitination of RBCK1 (fourth lane). Because the amount of added PKCβ was only 5 mol % of GST-RBCK1 used in the assay, it is strongly suggested that the self-ubiquitinating activity of RBCK1 is inhibited directly as a consequence of phosphorylation by PKCβ but not indirectly through the complex formation with PKCβ.
Determination of Phosphorylation Site(s) in RBCK1—The in vitro PKCβ-phosphorylated RBCK1 was used for determination of the phosphorylation site(s). As described under “Experimental Procedures,” the phosphorylated RBCK1 was digested in-solution by endoproteinase Glu-C or in-gel by the combination of trypsin and endoproteinase Glu-C or trypsin alone, and the generated peptides were analyzed by tandem mass spectrometry. In the first mass spectrum obtained with a quadrupole time-of-flight mass spectrometer, five peptides were found to contain a phosphate group as judged from the mass increase of 80 Da (Table 1). Amino acid sequences of peptides 1–4 were further analyzed by collision-induced dissociation and tandem mass spectrometry, in which a phosphorylated residue is detected by a 98-Da neutral loss of phosphoric acid (data not shown). In this way, Ser-127, Thr-151, and Thr-191 were finally determined as the phosphorylated residues (Table 1, peptides 1–4). In peptide 5, without successful collision-induced dissociation tandem mass sequence data, it could not be concluded that either one of the three residues (Ser-260, Thr-265, and Ser-275) was phosphorylated. To further examine whether these in vitro phosphorylated residues in RBCK1 include that (those) actually phosphorylated within the cells, all of the six identified Ser/Thr residues were mutated site-specifically into Ala. HEK293T cells expressing the resultant FLAG-RBCK1 (ST mutant) were incubated for 30 min with or without a phosphatase inhibitor okadaic acid, and the cell lysates were subjected to the Mn²⁺-Phos-tag SDS-PAGE (18) and then analyzed by Western blotting with an anti-FLAG antibody. Due to the interaction of a phosphate group with Mn²⁺-Phos-tag-modified polyacylamide, a phosphorylated protein should migrate slower than a protein without suffering phosphorylation. The wild-type RBCK1 obtained from the cells treated with okadaic acid migrated more slowly than the RBCK1 (ST mutant) protein, whereas both proteins obtained from the cells without okadaic acid treatment migrated in nearly the same speed (Fig. 4B). Therefore, the wild-type RBCK1 had undergone phosphorylation within the cells more significantly than RBCK1 (ST mutant). This result suggests that the major site(s) of RBCK1 intracellularly phosphorylated is included among the six residues, Ser-127, Thr-151, Thr-191, Ser-260, Thr-265, and Ser-275, identified in the in vitro phosphorylation by PKCβ, although there may be a minor phosphorylation site beside these residues (Fig. 4B, see the right-most lane).

Inhibition of in Vitro Self-ubiquitination Activity of RBCK1 by Phosphorylation—The self-ubiquitination activity of RBCK1 (ST mutant) was measured with or without PKCβ in vitro. FLAG-RBCK1 or FLAG-RBCK1 (ST mutant) overexpressed in HEK293T cells was immunoprecipitated with an anti-FLAG antibody and incubated with HA-tagged ubiquitin, UbcH7, and PKCβ (Fig. 4C). As already shown in Fig. 4A, RBCK1 was not ubiquitinated in the presence of PKCβ (third lane), whereas RBCK1 (ST mutant) was efficiently ubiquitinated even in the presence of PKCβ (sixth lane). It is noteworthy that RBCK1 (ST mutant) is more heavily ubiquitinated (fifth lane) than RBCK1 (second lane) in the absence of PKCβ. Presumably, the wild-type RBCK1 is partially phosphorylated in HEK293T cells by endogenous protein kinases (see Figs. 3A and B), by which the self-ubiquitination activity could be weaker than RBCK1 (ST mutant). Next, the FLAG-RBCK1 protein immunoprecipitated from HEK293T cells was treated with Ser/Thr-specific protein phosphatase 2A (PP2A) and subjected to in vitro ubiquitin ligase assay (Fig. 4D). The protein phosphatase 2A treatment was found to enhance the self-ubiquitination activity of RBCK1 (third lane). Furthermore, each phosphorylated site (Ser-127, Thr-151, Thr-191; see above) was substituted with Asp, and its self-ubiquitination activity was measured in vitro (Fig. 4E). The T151D and T191D mutations suppressed the self-ubiquitination activity significantly (fourth lane) and partially (fifth lane), respectively, whereas no suppression was observed by the S127D mutation (third lane). Taken together, the Thr-151 residue is critical for the regulation of self-ubiquitination activity by PKCβ, and Thr-191 is partially involved in the regulation. The phosphorylation stoichiometry (1.2 mol of phosphate per mol of RBCK1; see above) is considered sufficient for the regulation by PKCβ.

Effects of Coexpression of PKC Isoforms on Intracellular Amount of RBCK1—FLAG-RBCK1 was overexpressed with or without coexpression of a PKC isoform (α, β, ε, or ζ) in HEK293 cells, and the intracellular amount of RBCK1 was measured by Western blotting with an anti-FLAG antibody (Fig. 5A). Based on the band intensities, coexpression of PKCβ resulted in about a 3.5-fold increase of the intracellular amount of RBCK1 (Fig. 5B). In contrast, coexpression of PKCa, PKCe, or PKCζ showed no or little effect on the amount of RBCK1. Although overexpression of the kinase negative mutant of PKCβ (PKCβKN) or PKCζ (PKCζKN) was expected to reduce the amount of RBCK1 by its dominant-negative effect, there was no effect on the expression level of RBCK1, because the activities of endogenous PKCs seemed too high. These results indicated that PKCβ showed a dominant active effect, and the kinase activity of at least PKCβ positively contributes to the expression of RBCK1 in vivo. Moreover, the pulse-chase assay of RBCK1 corroborated that at least PKCβ prolongs the in vivo half-life of HT-RBCK1 presumably by its kinase activity (see the supplemental figure). Subsequently, either HA-PKCβ or HA-PKCBKN was overexpressed in HEK293 cells to see the effect on the endogenous RBCK1 by an immunocytochemical method using an anti-RBCK1 antibody (Fig. 6). As reported previously (5), endogenous RBCK1 was present in both the cytoplasm and nuclear bodies in all cells (panel B), whereas overexpressed PKCβ was localized evenly in the cytoplasm (panel A). It is intriguing to note that, as judged from the fluorescence intensities, expression of the endogenous RBCK1 was enhanced significantly in the cells overexpressing PKCβ (panel B, cf. peripheral cells without overexpressed PKCβ). The HA-PKCβKN did not affect the expression of endogenous RBCK1 (panel F). These results unequivocally show that overexpression of PKCβ, which shows dominant active effect, leads to the intracellular accumulation of both overexpressed and endogenous RBCK1. Collectively, RBCK1 interacts specifically with PKCβ within the cells and is phosphorylated by PKCβ, by which its self-ubiquitination activity is inhibited, leading to the intracellular accumulation of RBCK1 with its proteasomal degradation being prevented.
Ubiquitin Ligase Activity of RBCK1

A

GST-RBCK1

PKCβ

GST-RBCK1-Ub2

GST-RBCK1-Ub1

Blot: anti-Ub

B

Mock

FLAG-RBCK1

(St mutant)

Okadaic acid

Lysate

Blot: anti-FLAG

Phospho-RBCK1

RBCK1

C

FLAG-RBCK1

FLAG-RBCK1

(St mutant)

Ubch7

PKCβ

HA-Ub

Blot: anti-HA

Blot: anti-FLAG

D

PP2A treatment

FLAG-RBCK1

E

FLAG-RBCK1

Wild

S127D

T151D

T191D

Blot: anti-HA

Blot: anti-FLAG

FLAG-RBCK1-(HA-Ub)
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 (31). More than 2000 RING finger-containing proteins have been reported so far (3); they possess both or either one of the transcriptional and ubiquitin ligase (E3) activities. As demonstrated here, a RING protein RBCK1, previously shown to possess a transcriptional activity (1), shuttling between the cytoplasm and nucleus (5), has also been found to exhibit a significant ubiquinating activity, which is inhibited by interaction with its splice variant RBCK2 or phosphorylation by PKCβ.

It has been well established that the ubiquitin-proteasome system tags the target protein with poly-ubiquitin chains, and the poly-ubiquitinated proteins are mostly sorted to the 26 S proteasome and rapidly degraded. The ubiquitin-like motif of Parkin was reported to interact with ubiquitinated proteins (9) and the Rpn10 protein (32), a ubiquitin binding subunit of the 26 S proteasome. These interactions were supposed to facilitate the degradation of substrate proteins for Parkin. In this study we have shown that RBCK1 interacts with the ubiquitinated proteins (Fig. 1). It is possible that RBCK1 also interacts with the 26 S proteasome via its ubiquitin-like motif, and the ubiquitinated proteins may be directly transferred from RBCK1 to the 26 S proteasome. In the ubiquitin system, a RING-type E3 enzyme plays an important role for determining the substrate specificity and controlling the ubiquitinating activity of E2 enzyme. It is also known that certain RING-type E3 enzymes interact with themselves or other RING proteins that regulate the E3 activity. RBCK1 forms a homodimer (6), and its RING-IBR domain (from Cys-270 to His-498) interacts with itself (3).

Thus, the RING finger of RBCK1 likely contributes to the homodimer formation. As reported previously, BRCA1 and BARD1 can form a homodimer through their RING fingers but preferentially form a heterodimer when they co-exist (33). The E3 activity of BRCA1 is enhanced by the heterodimerization with BARD1 (28, 33). MDM2 and MDMX also can form a homodimer or heterodimer through their RING fingers (29), and the E3 activity of MDM2 for p53 is increased by the heterodimerization (30). The E3 activity of Parkin is enhanced by the RING finger-derived U-box protein CHIP through the heterodimerization (34). Similarly, the E3 activity of RBCK1 may be enhanced by dimerization with itself or a certain other RING protein.

We have shown here that RBCK2 inhibits the E3 activ-

3 K. Tatematsu, unpublished data.
activity is negatively regulated by RBCK2 and PKC activator, phorbol ester (39, 40). Based upon our results, it is shown that RBCK1 may be involved in the degradation of ferritin and transferrin (37, 38). The intracellular amount of the enzyme for IRP2 (8), a protein interacting with the mRNAs of many RING-lacking splice variants have been identified for RING proteins (e.g. Parkin, BRCA1, RNF8), whose function(s) remains to be uncovered. Like RBCK2, these splice variants may interact with their parental RING proteins and inhibit the E3 activity.

Phosphorylation has recently been revealed to be important for the modulation of E3 activity in the ubiquitin-proteasome system. The in vivo E3 activity of MDM2, a member of RING-protein, is up-regulated by phosphorylation by glycogen synthase kinase-3 (13). Itch, a HECT-type E3 enzyme, is activated and inactivated by phosphorylation by JNK and Fyn, respectively (35, 36). In this study we have demonstrated that the E3 activity of RBCK1 is inhibited by phosphorylation by PKCβ, and the phosphorylated RBCK1 thereby accumulates without undergoing proteasomal degradation through the self-ubiquitination. On the other hand, HOIL-1, a human orthologue of RBCK1 splice variant containing a RING-IBR domain, is an E3 enzyme for IRP2 (8), a protein interacting with the mRNAs of ferritin and transferrin (37, 38). The intracellular amount of the complex of IRP2 and mRNA is increased by treatment with a Cy2-labeled secondary antibody (panels A and E). The endogenous RBCK1 was stained with an anti-RBCK1 antibody and a Cy3-labeled secondary antibody (panels B and F). The merged images are panels C and G. Panels D and H, Nomarski image. Scale bar, 10 μm.

As demonstrated here, the autoimmune regulator (AIR) protein containing a RING-like zinc finger was shown to possess a ubiquitin ligase E3 activity (45), a DNA binding activity (46), and a transcriptional activity (47), which is enhanced by interaction with CBP (48). As proposed for RBCK1, AIR may play similar roles for CBP. Thus, the transcriptional and E3 activities of RBCK1 are probably associated with each other in exhibiting the intracellular function(s) of RBCK1.

The E3 activity of RBCK1 demonstrated in this paper should provide new insight into the functions of various RING proteins involved in the ubiquitin-proteasome system and the transcriptional machinery. Furthermore, this is the first report revealing that the E3 activity of a RING-finger protein is regulated by its RING-lacking splice variant.

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