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Ro52-mediated Monoubiquitination of IKKβ Down-regulates NF-κB Signalling

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Upon activation, NF-κB translocates into the nucleus and initiates biological events. This NF-κB signalling is mainly regulated by the protein kinase IKKβ. Early in this signalling pathway, IKKβ is phosphorylated for activation by several factors, such as pro-inflammatory cytokines and the Tax oncoprotein of HTLV-1. In cells infected by HTLV-1, IKKβ is persistently phosphorylated and conjugated with monoubiquitin due to Tax expression. Although this Tax-induced monoubiquitination appears to be an important regulation system for IKKβ, how the monoubiquitination occurs is unknown and its role in NF-κB signalling is still unclear. Here, we show that an E3-ubiquitin ligase Ro52 interacts weakly with wild-type IKKβ but strongly with a phosphomimetic mutant IKKβ to conjugate monoubiquitin in cooperation with an E2-ubiquitin-conjugating enzyme UbcH5B. These results suggest that the Tax-induced phosphorylation of IKKβ causes an interaction with Ro52 for the subsequent monoubiquitination. NF-κB reporter assays have shown that the IKKβ activity is suppressed by wild-type Ro52, but not by its inactive mutant. In addition, monoubiquitin fusion of IKKβ reduced its activity for NF-κB signalling. We also found that Ro52 dramatically reduces the level of Tax. These results suggest that Ro52 down-regulates Tax-induced NF-κB signalling by monoubiquitinating IKKβ and by reducing the level of Tax.

Key words: IKK, ligase, Ro52, Tax, ubiquitin.

Abbreviations: HTLV-1, human T-cell leukaemia virus type 1; iHOP, information hyperlinked over proteins; HEK, human embryonic kidney; HA, haemagglutinin; RH, RGS-poly His; protein phosphatase 2A, PP2A.

NF-κB is one of the major transcription factors and plays roles in many biological events. In resting cells, NF-κB is sequestered in the cytoplasm by the inhibitory protein IκB, which binds to the nuclear localization sequence of NF-κB (*1*). NF-κB is transiently activated by various biological inducers through cell-surface receptors such as tumour necrosis factor (TNF) receptor, Toll-like receptor, lipopolysaccharide (LPS) receptor and members of the immunoglobulin superfamilies (*2*). NF-κB is also persistently activated in cells expressing the Tax protein of human T-cell leukaemia virus type 1 (HTLV-1), which is an oncovirus (*3*). Despite differences in the initiating step, the inducers, such as TNF-α, LPS and Tax, lead to the activation of the multicomponent kinase IKK through its phosphorylation. In turn, the activated IKK phosphorylates IκBα, which is subsequently ubiquitinated and degraded by proteasomes. This degradation of IκBα allows NF-κB to translocate into the nucleus because its nuclear localization sequence is exposed. In the nucleus, NF-κB promotes the transcription of many genes involved in inflammation, immunity, tumourigenesis and apoptosis (*1*). Thus, the IKK complex is a key regulator in the NF-κB signalling pathway.

IKK is composed of three subunits: IKKα and IKKβ, which are protein kinases and IKKγ (also called NEMO), a regulatory subunit (*4*). IKKβ and IKKγ are responsible for phosphorylation of IκBα, whereas IKKα phosphorylates distinct cellular substrates in an alternative pathway for NF-κB activation (*5*). The catalytic activity of IKKβ is stimulated by signals through cell-surface receptors that trigger its phosphorylation at Ser-177 and Ser-181 (*4*). In contrast to its transient pattern of phosphorylation and activation in cells stimulated by these signals, IKKβ is also chronically phosphorylated and activated in cells expressing Tax protein of HTLV-1 as described earlier (*6*). In human cells activated by Tax, IKKβ is conjugated with a single ubiquitin molecule (monoubiquitin). Importantly, this monoubiquitination is contingent upon Tax-induced phosphorylation of IKKβ at Ser-177 and Ser-181 (*7*). Although this indicates an important mechanistic link among phosphorylation, ubiquitination and the biological action of IKKβ, how monoubiquitination of IKKβ is catalysed in cells remains unknown.

Ubiquitin, a 76-amino-acid polypeptide, is highly conserved in evolution, with a difference of only three amino acids between the human and yeast homologues (*8*). The C-terminus of ubiquitin contains a conserved Gly residue that is activated to form a thiol-ester linkage with the Cys residue of E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to E2 ubiquitin-conjugating

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enzyme to form another thiol-ester linkage. Subsequently, with the aid of E3 ubiquitin ligase, ubiquitin becomes covalently attached to Lys residues of target proteins through the formation of isopeptide bonds (*8*). In many cases, the internal Lys residue of ubiquitin further forms an isopeptide bond with the C-terminal Gly residue of another ubiquitin molecule to create a polyubiquitin chain. This chain serves as a proteasome-targeting signal (*8*). In proteasomes, polyubiquitinated proteins are degraded in an ATP-dependent manner (*8*). Thus, polyubiquitination of cellular proteins is well recognized as a targeting signal to proteasomes for degradation. However, ubiquitination has recently emerged as a regulatory signal for biological events other than proteasome-mediated degradation. Notably, monoubiquitination seems to play an intimate role in protein–protein interactions and trafficking of proteins between various cellular compartments (*9*). Although IKKβ is monoubiquitinated, whether this monoubiquitination is involved in these proteasome-unrelated biological events is unknown. In addition, the role of the monoubiquitination in NF-κB signalling is still unclear.

Ro52 is a RING-finger protein expressed in most tissues and cells (*10*). Ro52 is well known as an autoantigen that is recognized by anti-Ro52 autoantibodies found in the sera of patients with several autoimmune diseases, especially systemic lupus erythematosus and Sjögren's syndrome (*10*). Previously, we identified Ro52 as an E3 ubiquitin ligase (*11*). Ro52 has recently been shown to catalyse ubiquitination of several proteins, including Ro52 itself (*11*), UnpEL/Usp4 (*12*), IRF-8 (*13*) and TRIM5α (*14*). To identify more substrates of Ro52-mediated ubiquitination, we searched the online Information Hyperlinked over Proteins (iHOP) database (http://www.ihop-net.org) and found a description that Ro52 interacts with IKKβ. Because IKKβ is monoubiquitinated in cells expressing Tax protein, we hypothesized that Tax protein induces the interaction of IKKβ with the E3 ubiquitin ligase Ro52 for its monoubiquitination. Here, we show this to be true and also show that Ro52-mediated monoubiquitination of IKKβ plays an important role in the down-regulation of NF-κB signalling.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—Human embryonic kidney (HEK) 293, HEK293T and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics.

Antibodies—Mouse anti-HA antibody (16B12) was purchased from Covance (Richmond, CA, USA). Mouse anti-RH antibody (specific for the amino acid sequence, RGSHHHH) was purchased from QIAGEN (Santa Clara, CA, USA). Mouse anti-FLAG antibody (M2) was purchased from Sigma (St Louis, MO, USA). Mouse anti-GST antibody (GST-12) and mouse anti-HTLV-1 Tax (1A3) antibody (#sc-57872) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of cDNAs—The cDNAs of human ubiquitin (*15*), Ro52 (*11*), IKKβ, IKKγ, NUB1 (*16*) androgen receptor (AR) and E2 ubiquitin-conjugating enzymes, such as UbcH2, UbcH5B, UbcH7, UbcH10 and hCDC34 (*11*), were amplified using polymerase chain reaction (PCR) with appropriate primers from human testis, heart or brain cDNA library (Invitrogen, Carlsbad, CA, USA). The cDNAs of AIPL1 (*17*), Tax (*18*) and YopJ (*19*) were kindly provided by Dr Melanie M. Sohocki (Columbia University, New York, NY, USA), Dr Junichi Tsukada (University of Occupational and Environmental Health, Kitakyushu, Japan) and Dr Kim Orth (The University of Texas Southwestern Medical Center, Dallas, TX, USA), respectively.

Plasmid Construction and Transfection—To express proteins tagged with an epitope at the N-terminus in mammalian cells, the cDNAs were inserted into the plasmid vectors pcDNA3/HA-N (*20*), pcDNA3/FLAG-N (*21*) or pcDNA3/RH-N (*22*) as described previously (*15*). To express Ro52 tagged with the RH epitope at the C-terminus in mammalian cells, the cDNA of Ro52 was inserted into the plasmid vector pcDNA3/RH-C (*23*). The plasmids were transfected into HEK293, HEK293T and HeLa cells using FuGENE6 (Roche Applied Science, Indianapolis, IN, USA) or Lipofectamine 2000 (Invitrogen).

Site-directed Mutagenesis—To abolish the ligase activity of Ro52, a Cys-to-Ala substitution was generated in Ro52 at Cys-16 (*11*). To abolish the activity of YopJ, a Cys-to-Ser substitution was generated at Cys-172 in its Cys-box domain (*14*). To confirm the monoubiquitination site of IKKβ, a Lys-to-Arg substitution was generated in IKKβ at Lys-163. For these purposes, the cDNAs of wild-type Ro52, YopJ, and IKKβ were mutated by PCR-based site-directed mutagenesis as described previously (*24*). The mutated cDNAs of Ro52 and YopJ were subcloned into pcDNA3/RH-C and pcDNA3/FLAG-N, respectively. To replace both Ser-177 and Ser-181 of IKKβ with the phosphomimetic glutamic acid (IKKβ.SE), the cDNA of wild-type IKKβ was also mutated by PCR-based site-directed mutagenesis. The mutated cDNA was subcloned into pcDNA3/HA-N, pcDNA3/RH-N or pcDNA3/FLAG-N.

Plasmid Construction for Direct Fusion of Monoubiquitin to IKKβ—For the monoubiquitin fusion, we used a deletion mutant of ubiquitin (termed UbG), in which the last Gly residue at the 76th amino acid of wild-type ubiquitin was deleted. Because of this deletion, UbG-fused proteins are not hydrolysed by ubiquitn C-terminal hydrolases. To amplify a cDNA encoding UbG, PCR was used. The cDNA of UbG was then inserted into pcDNA3/IKKβ.SE-RH to generate plasmid pUbG-IKKβ.SE-RH for the expression of Ub-IKKβ.SE-RH.

Anti-FLAG-bead Immunoprecipitation—To immunoprecipitate FLAG-tagged proteins, such as FLAG-Ro52 and FLAG-IKKβ, HEK293T cells were transfected to express FLAG-tagged proteins and other proteins using FuGENE6 reagent. Twenty hours after transfection, cells were lysed for 30 min at room temperature in 1 ml of lysis buffer [50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10 mM NaF, 0.05% SDS] containing a protease inhibitor cocktail (Roche Applied Science). After centrifugation at 15,000 r.p.m. for 15 min, the cell extracts were subjected

to immunoprecipitation with 40 μl of anti-FLAG M2-agarose beads (Sigma) for 2 h at room temperature. The FLAG M2-agarose beads were washed three times with 750 μl of lysis buffer and treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β-mercaptoethanol. Afterward, the solubilized proteins were analysed by western blotting using rabbit anti-FLAG antibodies and other antibodies.

TALON-bead Precipitation of RH-IKKβ—To solubilize all derivatives of RH-IKKβ in 6 M guanidine–HCl and biochemically analyse them, we performed TALON-bead precipitation as described previously (*23*, *25*, *26*). Briefly, 1×10^6 HEK293T cells were co-transfected by FuGENE6 (Roche) to express RH-IKKβ, HA-ubiquitin and other proteins. Twenty hours after transfection, the cells were harvested and lysed in lysis buffer [20 mM Tris–HCl (pH 8.0), 6 M guanidine–HCl, 100 mM NaCl]. In this lysis buffer, all proteins, including de-ubiquitinating enzymes, were denatured by 6 M guanidine–HCl. Therefore, the ubiquitinated IKKβ was stable during the procedure. DNA in the lysate sample was sheared with a 22-gauge needle. The lysate was then incubated with cobalt-ion-charged TALON beads (Clontech, Palo Alto, CA, USA) for 1 h at room temperature. Because the sequence of the RH tag was RGSHHHHHH, RH-IKKβ could be purified by TALON beads. The beads were washed once with the lysis buffer and twice with washing buffer [20 mM Tris–HCl (pH 7.0), 15 mM imidazole, 8 M urea, 100 mM NaCl]. Finally, the beads were washed twice with PBS and treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β-mercaptoethanol. The solubilized proteins were analysed by western blotting using anti-HA antibody and anti-RH antibody.

Western Blotting—Protein samples were treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β-mercaptoethanol. After SDS–PAGE, western blotting was performed according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). As a secondary antibody, horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology) was used.

In Vitro Ubiquitination Assay—For this assay, we first expressed several recombinant proteins in bacteria, using the eukaryotic expression vectors pGEX-2TK (Amersham Pharmacia Biotech) and pTrcHisB (Invitrogen) as described previously (*11*, *24*). These proteins included GST (glutathione S-transferase)-fused IKKβ.SE (GST-IKKβ.SE), RH-tagged ubiquitin (RH-Ub), poly-His (His_6)-tagged Ro52 and E2 ubiquitin-conjugating enzymes. Next, bead-immobilized GST-IKKβ.SE was incubated with RH-Ub, E1 ubiquitin-activating enzyme (Boston Biochem, Cambridge, MA, USA), His_6-Ro52 and His_6-E2 enzyme in a reaction buffer [50 mM Tris–HCl (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, 2 mM DTT] for 30 min at 37°C. After reaction, the beads were washed by a washing buffer [25 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.5% NP-40] and treated for 30 min at 50°C in a sample-treating solution containing 2% SDS and 5% β-mercaptoethanol. Finally, the solubilized GST-IKKβ.SE was analysed by western blotting using anti-RH antibody to detect ubiquitinated IKKβ.SE and anti-GST antibody to detect both unubiquitinated and ubiquitinated IKKβ.SE.

Reporter Assay—To investigate the role of Ro52 in Tax-mediated activation of NF-κB signalling, Tax (FLAG-tagged) was expressed with empty vector, RH-tagged Ro52 (wild type) or Ro52 (C16A) in HeLa cells transfected with phRL-TK plasmid (Promega, Madison, WI, USA) and a 3× NF-κB luciferase reporter plasmid (NF-κB-Luc), which was a gift from Dr Jinke Cheng (The University of Texas M. D. Anderson Cancer Center). Fifteen hours after transfection, the cell extract was prepared and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) (*27*) according to the manufacturer's instructions. Luciferase activity was measured using the Lumistar Galaxy automated microplate luminescence reader (BMG Labtechnologies, Durham, NC, USA). Luciferase activity was then determined and normalized according to the *Renilla* luciferase activity induced by phRL-TK transfection. The relative luciferase activity was determined using a representative transfection experiment performed in triplicate.

To bypass the phosphorylation of IKKβ without the expression of Tax, IKKβ.SE was also expressed with or without Ro52 in HEK293 cells transfected with the phRL-TK and NFκB-Luc plasmids. Fifteen hours after transfection, the cell extract was assayed for luciferase activity as described earlier.

To investigate the effect of monoubiquitin fusion to IKKβ, RH-tagged IKKβ (wild-type), IKKβ.SE or Ub-IKKβ.SE was expressed in HEK293 cells transfected with the phRL-TK and NFκB-Luc plasmids. Fifteen hours after transfection, the cell extract was assayed for luciferase activity as described earlier.

All of the values were presented as the mean ± standard error from at least three independent experiments. The statistical significance of the data was evaluated using the Student's *t*-test. Probability values below 0.05 ($P < 0.05$) were considered significant and probability values below 0.01 ($P < 0.01$) were considered highly significant.

RESULTS

Interaction Between Ro52 and IKKβ in HEK293T Cells—We previously found that Ro52 is an E3 ubiquitin ligase (*11*). To identify its substrates, we first searched the iHOP database (http://www.ihop-net.org) for Ro52-interacting proteins. In the database, we found a report that Ro52 interacts with IKKβ, which is required for proteolytic inactivation of IκBα, a principal cytoplasmic inhibitor of NF-κB (*28*). Although the interaction between Ro52 and IKKβ was listed in the iHOP database, we could not find any published reports describing this interaction in the MEDLINE database. Therefore, we performed a co-immunoprecipitation assay to determine whether Ro52 interacts with IKKβ in human cells. Specifically, in HEK293T cells, FLAG-tagged Ro52 was co-expressed with HA-tagged IKKβ (wild-type) or IKKβ.SE, which is a constitutively active form of IKKβ due to substitutions with the phosphomimetic glutamic acid at Ser-177 and Ser-181 (*6*, *7*, *29*). We then

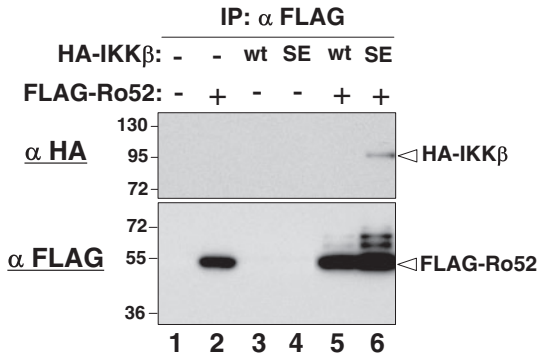

Fig. 1. **Co-immunoprecipitation of IKKβ with Ro52 in HEK293T cells.** Empty vector or FLAG-tagged Ro52 was coexpressed with HA-tagged IKKβ (wild-type or phosphomimetic mutant SE) in HEK293T cells as indicated. Cell lysates were incubated with mouse anti-FLAG antibody-conjugated agarose beads for immunoprecipitation. Co-immunoprecipitated proteins were analysed by western blotting using rabbit anti-HA antibody (upper panel). Immunoprecipitated FLAG-Ro52 was confirmed by western blotting using rabbit anti-FLAG antibody (lower panel). Molecular size markers are shown in kilodaltons.

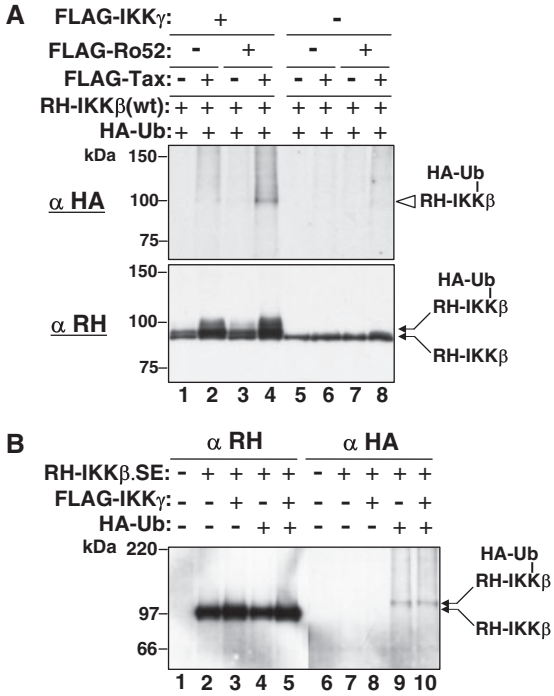

Fig. 2. **Ro52-mediated monoubiquitination of IKKβ *in vivo*.** (A) Tax-induced monoubiquitination of IKKβ. HEK293T cells were transfected with expression vectors for FLAG-tagged Tax, FLAG-tagged Ro52, RH-tagged IKKβ (wild-type), FLAG-tagged IKKγ, and HA-tagged ubiquitin as indicated. Total cell lysates were prepared in 6 M guanidine–HCl to precipitate RH-IKKβ by cobalt-coated TALON beads. Afterwards, western blotting was performed to detect ubiquitinated RH-IKKβ by anti-HA antibody (upper panel) and all derivatives of RH-IKKβ by anti-RH antibody (lower panel). Molecular size markers are shown in kilodaltons. (B) IKKγ-independent monoubiquitination of IKKβ mutant SE. HEK293T cells were transfected with expression vectors for RH-tagged IKKβ.SE, FLAG-IKKγ and HA-ubiquitin as indicated. Total cell lysates were prepared to precipitate RH-IKKβ.SE by TALON beads. Western blotting was then performed to detect all derivatives of RH-IKKβ.SE by anti-RH antibody (lanes 1–5) and ubiquitinated RH-IKKβ.SE by anti-HA antibody (lanes 6–10).

immunoprecipitated FLAG-Ro52 with anti-FLAG antibody and analysed the immunoprecipitate by western blotting using anti-FLAG antibody and anti-HA antibody. As shown in the upper panel of Fig. 1, the co-immunoprecipitated IKKβ.SE was clearly detected (lane 6). Importantly, wild-type IKKβ was also detected when the film was exposed to the western-transferred membrane for a longer time (data not shown). These results suggest that Ro52 interacts with IKKβ in overexpressed cells and that the interaction of Ro52 with the phosphomimetic mutant IKKβ.SE is much stronger than that with wild-type IKKβ. So far, we have been unable to detect the interaction between Ro52 and IKKβ in cells without overexpression, presumably because of limited expression of the relevant IKKβ species and Ro52 at the endogenous protein level.

Ro52-mediated Monoubiquitination of Wild-type IKKβ in Tax-expressing Cells—Carter *et al*. (7) recently showed that the Tax oncoprotein induces monoubiquitination of IKKβ. Since our co-immunoprecipitation assay suggested an interaction between IKKβ and the E3 ubiquitin ligase Ro52, we hypothesized that the Tax-induced monoubiquitination of IKKβ is catalysed by Ro52. To test this hypothesis, we performed an *in vivo* ubiquitination assay. We programmed HEK293T cells with expression vectors for IKKγ, Ro52, Tax, RH-tagged IKKβ and HA-tagged ubiquitin. Because the RH tag is RGSHHH HHH (RGS-polyhistidine), RH-IKKβ was then precipitated by cobalt-coated TALON beads under strong denaturing conditions that abolish ubiquitin editing by cellular isopeptidases. The precipitate was then solubilized and analysed by western blotting using anti-HA antibody to detect ubiquitinated RH-IKKβ and anti-RH antibody to detect all derivatives of RH-IKKβ. As shown in Fig. 2A, we detected monoubiquitination of IKKβ when Tax was coexpressed (lane 1 versus 2). Importantly, IKKγ was required for this monoubiquitination (lane 2 versus 6). Thus, we confirmed Tax-induced monoubiquitination of IKKβ (7). More importantly, the co-expression of Ro52 significantly increased the monoubiquitination of IKKβ (lane 2 versus 4), suggesting that IKKβ was monoubiquitinated by the E3 ubiquitin ligase Ro52.

When we performed the *in vivo* ubiquitination assay (Fig. 2A), we weakly detected the polyubiquitination of IKKβ in addition to its monoubiquitination. Strangely, the polyubiquitination was almost undetectable when IKKβ was detected by western blotting using the anti-RH antibody (the lower panel of Fig. 2A, lanes 2 and 4). On the other hand, however, the polyubiquitination was weakly detected when the anti-HA antibody was used for western blotting (the upper panel of Fig. 2A, lanes 2 and 4). Why did these two antibodies produce such discrepant results in terms of degree of polyubiquitination? We believe that a polyubiquitin chain

on RH-IKKβ consists of multiple molecules of HA-ubiquitin. Because each of these molecules reacts with an anti-HA antibody, the polyubiquitin chain is labeled with multiple molecules of anti-HA antibody, which causes the anti-HA antibody to detect the polyubiquitin chain much more strongly than is actually the case. In contrast, detection of the polyubiquitinated RH-IKKβ by anti-RH antibody reflects the actual level of the expression, because the anti-RH antibody reacts only with a single RH-epitope of the polyubiquitinated RH-IKKβ. Based on this explanation, our findings suggest that the monoubiquitinated form of IKKβ predominantly exists in the actual population of ubiquitinated IKKβ and that this modification is mediated by Ro52.

Tax- and IKKγ-independent Monoubiquitination of IKKβ.SE in HEK293T Cells—Carter *et al*. (*6*, *7*) reported that IKKβ was constitutively monoubiquitinated in Tax-deficient cells when they expressed a constitutively active mutant of IKKβ, in which Ser-177 and Ser-181 were both replaced with the phosphomimetic glutamic acid (IKKβ.SE) (*29*). Although these studies showed that Tax induction is not required for monoubiquitination of IKKβ.SE, whether IKKγ was required for monoubiquitination of IKKβ.SE was unknown. To answer this question, we conducted experiments with HEK293T cells expressing RH-IKKβ.SE and HA-ubiquitin in the presence or absence of IKKγ. Ubiquitinated and unubiquitinated forms of RH-IKKβ.SE were purified from total cell lysates by TALON beads, resolved by SDS–PAGE, and then analysed by western blotting using anti-HA antibody to detect ubiquitinated RH-IKKβ.SE and anti-RH antibody to detect all derivatives of RH-IKKβ.SE. As shown in Fig. 2B, we detected monoubiquitination of RH-IKKβ.SE in cells transfected with IKKγ (lane 10). Importantly, monoubiquitination of RH-IKKβ.SE was also detected to the same extent in cells not transfected with IKKγ (lane 9), indicating that neither Tax induction nor IKKγ overexpression is required for monoubiquitination of IKKβ.SE.

Ro52-mediated Monoubiquitination of IKKβ.SE in Tax-deficient Cells—We next examined the extent to which monoubiquitination of IKKβ.SE is increased by co-expression of Ro52 in the absence of Tax induction and IKKγ overexpression. Briefly, we conducted experiments with HEK293T cells expressing RH-IKKβ.SE and HA-ubiquitin in the presence or absence of Ro52. Ubiquitinated and unubiquitinated forms of RH-IKKβ.SE were then purified by TALON beads and analysed by western blotting using anti-HA antibody and anti-RH antibody as described earlier. As shown in Fig. 3A, Ro52 strongly increased the monoubiquitination of IKKβ.SE in cells without transfection of Tax and IKKγ (lane 9 versus 10).

In addition to the TALON-bead precipitation assay, we also performed an immunoprecipitation assay to confirm this finding. We transfected HEK293T cells to express FLAG-IKKβ.SE and HA-ubiquitin in the presence or absence of Ro52. Ubiquitinated and unubiquitinated forms of FLAG-IKKβ.SE were immunoprecipitated by anti-FLAG antibody-conjugated agarose beads and analysed by western blotting using anti-HA antibody and

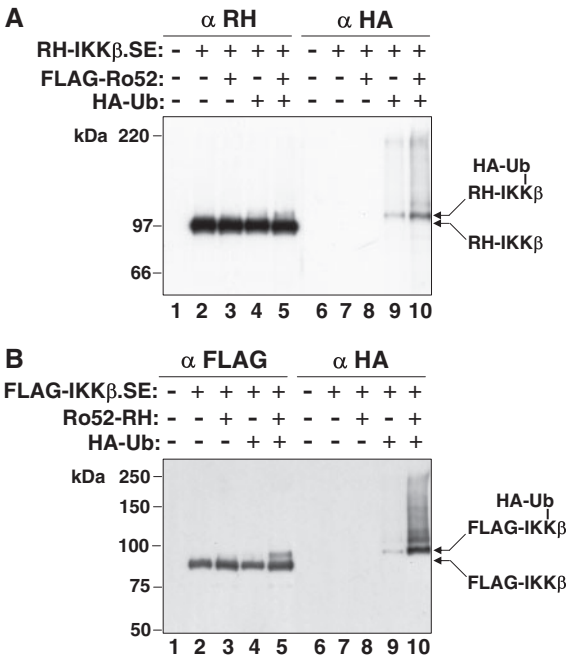

Fig. 3. **Ro52-mediated monoubiquitination of IKKβ.SE without Tax induction and IKKγ overexpression.** (A) TALON-bead precipitation of RH-tagged IKKβ.SE. HEK293T cells were transfected with expression vectors for RH-IKKβ.SE, FLAG-Ro52 and HA-ubiquitin as indicated. Total cell lysates were prepared to precipitate RH-IKKβ.SE by TALON beads. Western blotting was then performed to detect all derivatives of RH-IKKβ.SE by anti-RH antibody (lanes 1–5) and ubiquitinated RH-IKKβ.SE by anti-HA antibody (lanes 6–10). (B) Anti-FLAG-bead immunoprecipitation of FLAG-tagged IKKβ.SE. HEK293T cells were transfected with expression vectors for FLAG-IKKβ.SE, Ro52-RH and HA-ubiquitin as indicated. Total cell lysates were prepared to immunoprecipitate FLAG-IKKβ.SE by mouse anti-FLAG antibody-conjugated beads. Western blotting was then performed to detect all derivatives of FLAG-IKKβ.SE by rabbit anti-FLAG antibody (lanes 1–5) and ubiquitinated FLAG-IKKβ.SE by rabbit anti-HA antibody (lanes 6–10).

anti-FLAG antibody. As shown in Fig. 3B, we obtained results similar to those of the TALON-precipitation experiment (see above). Namely, Ro52 strongly increased the monoubiquitination of IKKβ.SE in cells that were not transfected with plasmids to express Tax and IKKγ (lane 9 versus 10).

Ro52-mediated Monoubiquitination of Mutant IKKβ (K163R)—Previously, Carter *et al*. showed that a mutant IKKβ (K163R), in which a Lys-to-Arg substitution was generated at Lys-163, is not monoubiquitinated in cells expressing Tax. Furthermore, they showed that IKKβ.SE with K163R mutation is not monoubiquitinated in cells, either. Based on these results, they concluded that Lys-163 serves as a site for monoubiquitination of IKKβ (*30*). However, because they did not express or induce E3 ubiquitin ligase for the monoubiquitination, they might not detect monoubiquitination of mutant IKKβ (K163R). To examine this possibility, we performed *in vivo* ubiquitination assay in the presence or absence of Ro52 overexpression. Specifically, we conducted

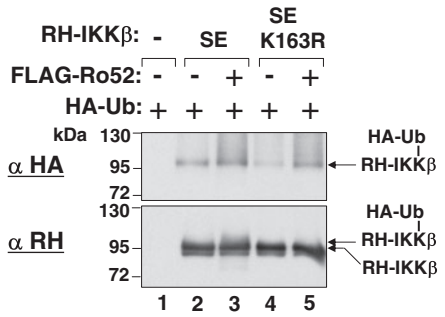

Fig. 4. **Ro52-mediated monoubiquitination of mutant IKKβ (K163R).** HEK293T cells were transfected with expression vectors for RH-IKKβ (SE or SE/K163R), FLAG-Ro52 and HA-ubiquitin as indicated. Total cell lysates were prepared to precipitate RH-IKKβ (SE or SE/K163R) by TALON beads. Western blotting was then performed to detect all derivatives of RH-IKKβ (SE or SE/K163R) by anti-RH antibody (lower panel) and ubiquitinated RH-IKKβ (SE or SE/K163R) by anti-HA antibody (upper panel).

experiments with HEK293T cells expressing HA-ubiquitin and RH-IKKβ.SE or RH-IKKβ.SE/K163R (which possesses replacement of Lys-163 with Arg in RH-IKKβ.SE) in the presence or absence of FLAG-Ro52 overexpression. As shown in Fig. 4, IKKβ.SE/K163R was weakly, but clearly monoubiquitinated inthe absence of Ro52 overexpression in HEK293T cells (lane 4). Importantly, this monoubiquitination was increased in the presence of Ro52 overexpression (lane 5). These results were confirmed by repeated experiments. Thus, our results strongly suggest that one of the Lys residues other than Lys-163 is monoubiquitinated in K163R mutant and that this monoubiquitination is catalysed by E3 ubiquitin ligase Ro52. In other words, Lys-163 does not serve as a monoubiquitination site in IKKβ.

Although we detected monoubiquitination of K163R mutant, Carter *et al*. (*30*) did not. This is probably because their experimental conditions were different from ours and because an E3 ubiquitin ligase, such as Ro52, was not expressed or induced in their experiments, as described earlier.

IKKβ-specific Monoubiquitination Mediated by RING Domain of Ro52 in HEK293T Cells—Ro52 possesses a RING-finger domain between amino acid residues 16 and 54 (*11*, *31*, *32*) (Fig. 5A). Recent results from several laboratories have indicated that the RING-finger proteins recruit E2 enzymes through their RING domain and act as E3 enzymes (*33*). We recently showed that Ro52 functions as an E3 enzyme and that its activity is abolished by a Cys-to-Ala mutation of the conserved Cys-16 residue (C16A) in the RING-finger domain (*11*) (Fig. 5A). To determine whether monoubiquitination of IKKβ is dependent on the function of the RING-finger domain of Ro52, we used this substitution mutant (C16A). Specifically, we examined the effect of this substitution on Ro52-mediated monoubiquitination of IKKβ, using an *in vivo* ubiquitination assay. In brief, FLAG-tagged IKKβ.SE and HA-ubiquitin were co-expressed with RH-tagged wild-type Ro52 or C16A in HEK293T cells. FLAG-IKKβ.SE was then immunoprecipitated from cell lysates, solubilized and analysed by western blotting using anti-HA antibody to detect ubiquitinated FLAG-IKKβ.SE and anti-FLAG antibody to detect all derivatives of FLAG-IKKβ.SE. As shown in Fig. 5B, we strongly detected monoubiquitination of FLAG-IKKβ.SE in cells when co-expressed with wild-type Ro52 (lane 3). In contrast, the monoubiquitination of FLAG-IKKβ.SE was extremely weak when co-expressed with empty vector (lane 2) or C16A mutant of Ro52 (lane 4). This faint monoubiquitination of IKKβ.SE might have been catalysed by the wild-type Ro52 or other E3 ubiquitin ligases that are endogenously expressed in HEK293T cells. These results indicate that monoubiquitination of IKKβ is dependent on the function of the RING-finger domain of Ro52.

Next, we examined whether the Ro52-mediated monoubiquitination is specific to IKKβ. We expressed FLAG-Ro52 and HA-ubiquitin with RH-tagged IKKβ.SE or other proteins such as AIPL1 (*17*), NUB1 (*16*) and AR in HEK293T cells. RH-tagged proteins were then purified from total cell lysates by TALON beads, resolved by SDS–PAGE and analysed by western blotting using anti-HA antibody to detect the ubiquitination of RH-tagged proteins and anti-RH antibody to detect all derivatives of RH-tagged proteins. As shown in Fig. 5C, we detected monoubiquitination of RH-IKKβ.SE (lane 2) but not of the other proteins (lanes 3–5), indicating that the Ro52-mediated monoubiquitination is specific to IKKβ.

In Vitro Monoubiquitination of IKKβ Mediated by the Ligase Function of Ro52—Although we hypothesized that Ro52 functions as an E3 ubiquitin ligase to monoubiquitinate IKKβ, this hypothesis then raised the question as to which E2 enzyme is utilized for the monoubiquitination of IKKβ. Previously, we found that Ro52 ubiquitinates itself (self-ubiquitination) and TRIM5α in cooperation with the E2 ubiquitin-conjugating enzyme UbcH5B (*11*, *14*), suggesting that Ro52 also ubiquitinates IKKβ in cooperation with UbcH5B. We therefore performed an *in vitro* ubiquitination assay to test this possibility. In the assay, GST-fused IKKβ.SE was expressed in bacteria and purified using glutathione-sepharose beads. GST-IKKβ.SE immobilized on the beads was then incubated with recombinant E1 enzyme and different recombinant E2 enzymes (UbcH2, UbcH5B, UbcH7, UbcH10 and hCDC34, which were produced in bacteria) in the presence of RH-tagged ubiquitin and poly-His (His_6)-tagged Ro52. In this *in vitro* system, His_6-Ro52 served as a potential E3 enzyme and GST-IKKβ.SE served as a substrate. After incubation, GST-IKKβ.SE was solubilized and analysed by western blotting using anti-RH antibody and anti-GST antibody. As shown in Fig. 6A, incubation of GST-IKKβ.SE in the reaction mixture containing UbcH2, UbcH7, UbcH10 or hCDC34 did not result in its ubiquitination (lanes 2, 4, 5 and 6), whereas incubation of GST-IKKβ.SE in the reaction mixture containing UbcH5B resulted in its monoubiquitination (lane 3). These results indicate that IKKβ is monoubiquitinated *in vitro* and that this monoubiquitination is catalysed by UbcH5B but not by the other E2 enzymes tested.

In general, ubiquitin conjugates to the substrate in the presence of E1, E2 and E3 enzymes (*8*). These proteins

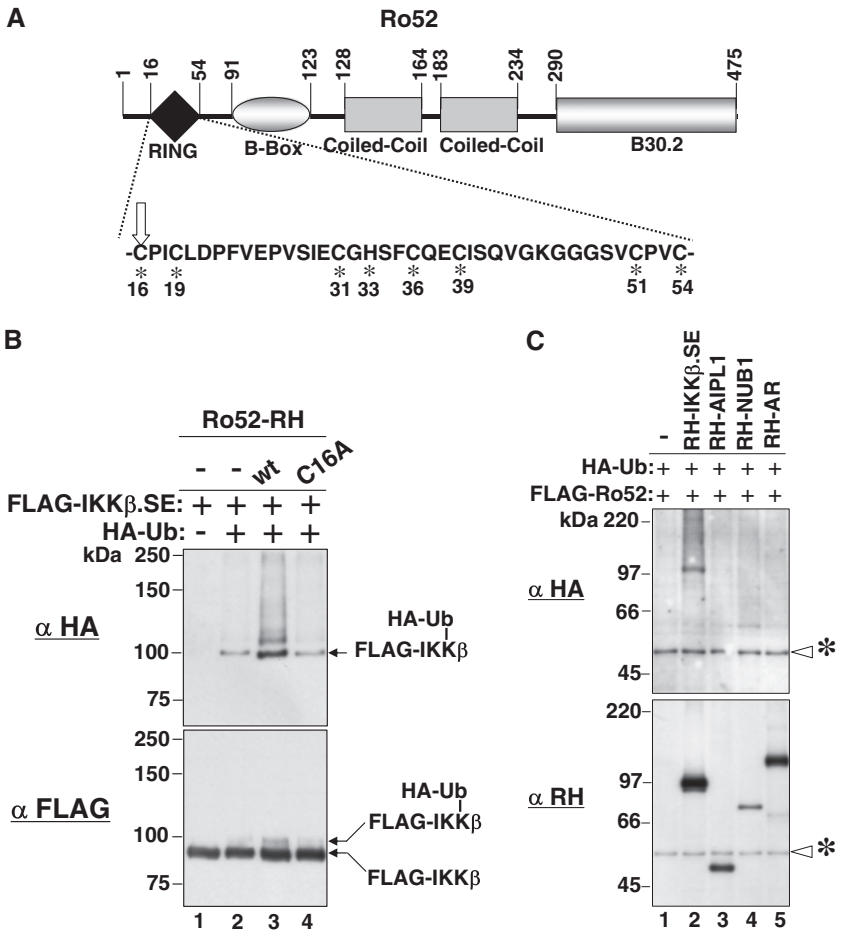

Fig. 5. **IKKβ-specific monoubiquitination mediated by RING domain of Ro52 in HEK293T cells.** (A) Schematic presentation of domain structure of Ro52. The domain structure of the entire Ro52 molecule and the amino acid sequence of its RING-finger domain are shown. Asterisks indicate conserved Cys and His residues in the RING-finger domain. Arrow indicates Cys-16, which was substituted to Ala to generate an inactive mutant of Ro52 (C16A). (B) *In vivo* monoubiquitination of IKKβ dependent on RING domain of Ro52. HEK293T cells were transfected with expression vectors for FLAG-tagged IKKβ.SE, RH-tagged Ro52 (wild-type or RING mutant C16A), and HA-tagged ubiquitin as indicated. Total cell lysates were prepared to immunoprecipitate FLAG-IKKβ.SE by mouse anti-FLAG antibody. Western blotting was then performed to detect ubiquitinated FLAG-IKKβ.SE by rabbit anti-HA antibody (upper panel) and all derivatives of FLAG-IKKβ.SE by rabbit anti-FLAG antibody (lower panel). (C) Substrate-specificity of Ro52-mediated monoubiquitination. HEK293T cells were transfected with expression vectors for FLAG-Ro52, HA-ubiquitin and RH-tagged substrates (IKKβ.SE, AIPL1, NUB1 and AR) as indicated. Total cell lysates were prepared to precipitate RH-tagged substrates by TALON beads. Western blotting was then performed to detect ubiquitinated RH-tagged proteins by anti-HA antibody (upper panel) and all derivatives of RH-tagged proteins by anti-RH antibody (lower panel). Nonspecific bands are indicated by asterisks.

are the minimum ones required for ubiquitination to occur. To confirm that these proteins are also essential for the monoubiquitination detected in lane 3 of Fig. 6A, we performed another *in vitro* ubiquitination assay (Fig. 6B). As a positive control, glutathione-sepharose beads coated with GST-IKKβ.SE were incubated in the complete reaction mixture containing RH-ubiquitin, E1 enzyme, UbcH5B (E2 enzyme) and Ro52 (E3 enzyme) (Fig. 6B, lane 5). As a negative control, glutathione-sepharose beads alone (*i.e.* not coated with GST-IKKβ.SE) were incubated in the complete reaction mixture (Fig. 6B, lane 6). In the other reactions, glutathione-sepharose beads coated with GST-IKKβ.SE were incubated in an incomplete reaction mixture lacking one of these components (Fig. 6B, lanes 1–4). After incubation, GST-IKKβ.SE was solubilized and analysed by western blotting using anti-RH antibody and anti-GST antibody. As shown in Fig. 6B, incubation of GST-IKKβ.SE in the complete reaction mixture resulted in the monoubiquitination of GST-IKKβ.SE (lane 5), whereas incubation of GST-IKKβ.SE in the incomplete reaction mixture lacking one component did not lead to the ubiquitination of GST-IKKβ.SE (lanes 1–4). These results indicate that ubiquitin, E1 enzyme, UbcH5B as an E2 enzyme, and Ro52 as an E3 enzyme are the minimum requirements for the *in vitro* monoubiquitination of IKKβ.

Disruption of Ro52-mediated Monoubiquitination of IKKβ by YopJ—YopJ is one of the *Yersinia* outer proteins encoded by pathogenic *Yersinia* species. YopJ acts

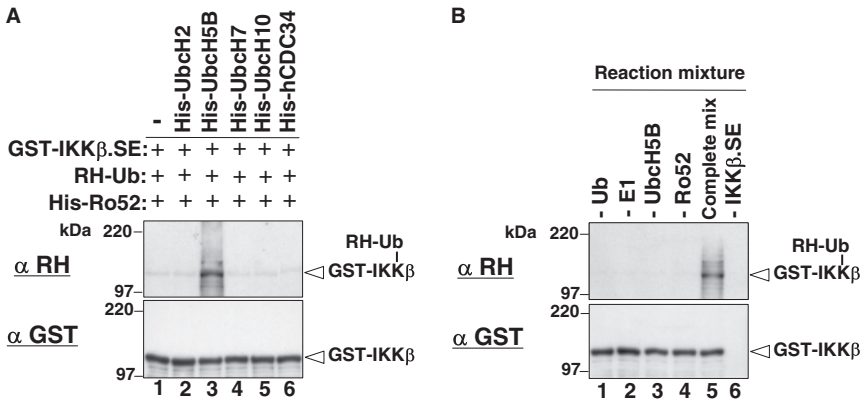

Fig. 6. ***In vitro* monoubiquitination of IKKβ.** (A) UbcH5B-dependent monoubiquitination of IKKβ. GST-fused IKKβ.SE was immobilized on beads and incubated in the reaction mixture containing RH-tagged ubiquitin, recombinant E1 enzyme and various poly-His-tagged recombinant E2 enzymes (UbcH2, UbcH5B, UbcH7, UbcH10 and hCDC34). After this reaction, GST-IKKβ.SE on the beads was washed to remove the reaction mixture and solubilized in SDS-treating solution. GST-IKKβ.SE was then analysed by western blotting using anti-RH antibody to detect ubiquitinated GST-IKKβ.SE (upper panel) and anti-GST antibody to detect both unubiquitinated and ubiquitinated GST-IKKβ.SE (lower panel). Molecular size markers are shown on the left in kilodaltons. (B) Minimum requirements for the *in vitro* monoubiquitination of IKKβ. In the *in vitro* ubiquitination assay shown above, the complete reaction mixture contained RH-ubiquitin, E1 enzyme, UbcH5B as an E2 enzyme and Ro52 as an E3 enzyme. To determine the minimum requirements for the *in vitro* monoubiquitination of IKKβ, GST-IKKβ.SE immobilized on beads was incubated in the incomplete reaction mixture lacking one of these components (lanes 1–4). As a positive control, GST-IKKβ.SE immobilized on beads was incubated in the complete reaction mixture (lane 5). As a negative control, glutathione-sepharose beads alone without immobilization of GST-IKKβ.SE were incubated in the complete reaction mixture (lane 6). After the reaction, the beads were washed and treated in SDS-containing solution to solubilize GST-IKKβ.SE. Then, GST-IKKβ.SE was analysed by western blotting using anti-RH antibody to detect ubiquitinated GST-IKKβ.SE (upper panel) and anti-GST antibody to detect both unubiquitinated and ubiquitinated GST-IKKβ.SE (lower panel). The incomplete reaction mixture shown in lanes 1, 2, 3 and 4 lacked ubiquitin, E1 enzyme, UbcH5B and Ro52, respectively.

as a de-ubiquitinating enzyme or acetyltrasferase (see DISCUSSION section) and interferes with the NF-κB signalling pathway (*19*, *34–36*). In particular, monoubiquitination of IKKβ was previously shown to be disrupted by YopJ (*7*). To examine whether YopJ disrupts monoubiquitination of IKKβ even in the presence of highly expressed Ro52, we further performed an *in vivo* ubiquitination assay. In other words, we tested whether Ro52-mediated strong monoubiquitination of IKKβ is disrupted by YopJ. Specifically, in the presence or absence of overexpressed Ro52, RH-tagged IKKβ.SE and HA-tagged ubiquitin were expressed in HEK293T cells along with empty vector, FLAG-tagged wild-type YopJ or mutant YopJ (C172S), in which Ser was substituted for the active-site Cys-172. RH-IKKβ.SE was then precipitated by TALON beads, solubilized and analysed by western blotting using anti-HA antibody to detect ubiquitinated RH-IKKβ.SE and anti-RH antibody to detect all derivatives of RH-IKKβ.SE (Fig. 7). As shown in the upper and lower panels, we detected weak monoubiquitination of IKKβ.SE when co-expressed with ubiquitin (lane 1). This monoubiquitination was disrupted by wild-type YopJ (lane 2). YopJ (C172S), however, did not affect the ubiquitination of Ro52 (lane 3), because of the substitution of Ser for the active-site Cys-172. Thus, we confirmed the activity of YopJ, which was reported previously (*7*). Next, we performed the same assay in the presence of highly expressed Ro52 (lanes 4–6). Although Ro52 strongly increased the monoubiquitination of IKKβ.SE (lane 4), it was completely disrupted by wild-type YopJ (lane 5), but not by the C172S mutant (lane 6). Taken together, we confirmed that YopJ

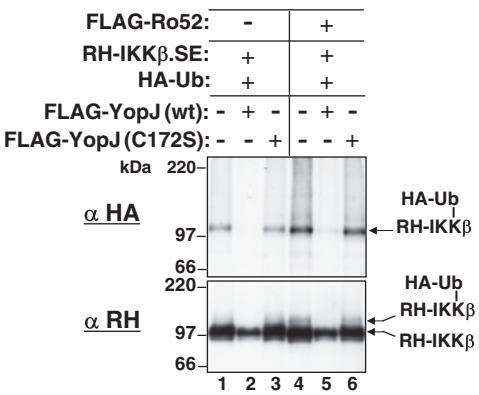

Fig. 7. **Effect of YopJ expression on Ro52-mediated monoubiquitination of IKKβ.** In the presence or absence of overexpressed FLAG-Ro52, RH-tagged IKKβ.SE and HA-tagged ubiquitin were expressed in HEK293T cells along with empty vector, FLAG-tagged wild-type YopJ or inactive mutant YopJ (C172S) as indicated. RH-IKKβ.SE was then precipitated by TALON beads, solubilized and analysed by western blotting using anti-HA antibody to detect ubiquitinated RH-IKKβ.SE (upper panel) and anti-RH antibody to detect all derivatives of RH-IKKβ.SE (lower panel).

effectively disrupts the monoubiquitination of IKKβ even when the monoubiquitination is promoted by overexpressed Ro52.

Role of Ro52 in Tax-induced Activation of NF-κB Signalling—To determine whether Ro52 regulates NF-κB signalling through monoubiquitination of IKKβ

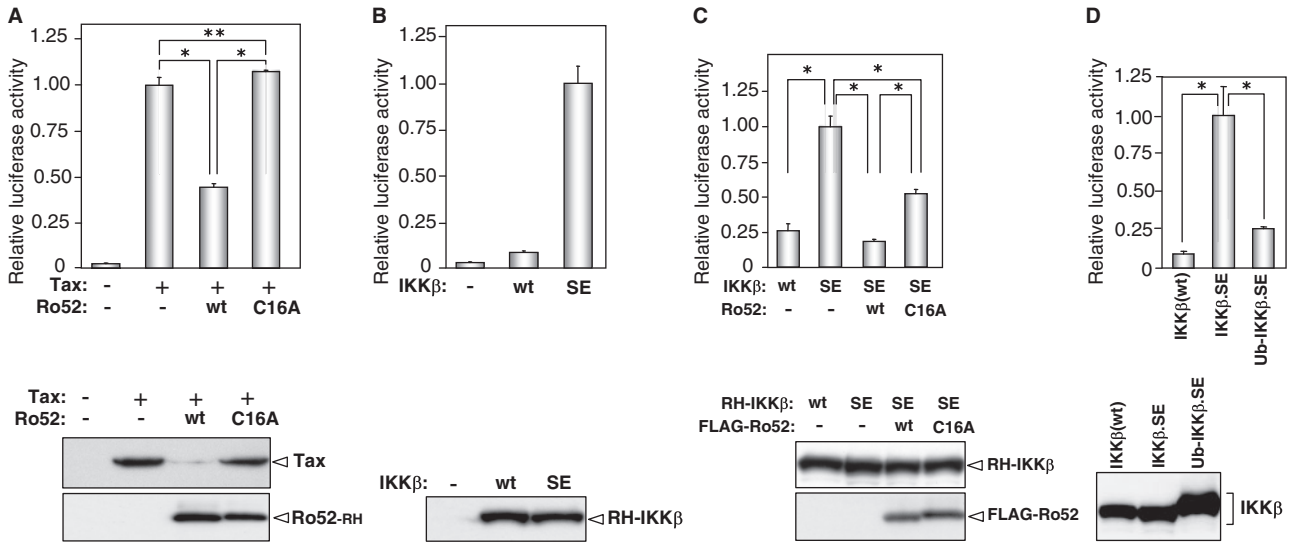

Fig. 8. **NF-κB reporter assays.** (A) Role of Ro52 in Tax-induced NF-κB signalling. Tax (FLAG-tagged) was co-expressed with wild-type Ro52 or its C16A mutant (RH-tagged) in HeLa cells transfected with the NF-κB reporter plasmid. Fifteen hours after transfection, the cell extract was prepared and assayed for luciferase activity. Each bar represents the mean ± standard error ($^{*}P < 0.01$, $^{**}P > 0.05$) (upper panel). Exogenously expressed proteins for the reporter assay were analysed by western blotting using anti-Tax antibody to detect Tax and anti-RH antibody to detect Ro52-RH (lower panel). (B) Enhanced activity of IKKβ mutant (SE) to promote NF-κB signalling. Empty vector or RH-tagged IKKβ (wild-type or SE mutant) was expressed in HEK293 cells transfected with the NF-κB reporter plasmid. Fifteen hours after transfection, the cell extract was prepared and assayed for luciferase activity. Each bar represents the mean ± standard error (upper panel). Exogenously expressed RH-IKKβ (wild-type and SE mutant) for the reporter assay were analysed by western blotting using anti-RH antibody (lower panel). (C) Role of Ro52 in IKKβ-mediated activation of NF-κB signalling. RH-tagged IKKβ (wild-type or SE mutant) was co-expressed with empty vector or FLAG-tagged Ro52 (wild-type or C16A mutant) in HEK293 cells transfected with the NF-κB reporter plasmid. Fifteen hours after transfection, cell extracts were prepared and assayed for luciferase activity. Each bar represents the mean ± standard error ($^{*}P < 0.01$) (upper panel). Exogenously expressed proteins for the reporter assay were analysed by western blotting using anti-RH antibody to detect RH-IKKβ and anti-FLAG antibody to detect FLAG-Ro52 (lower panel). (D) Effect of monoubiquitin fusion to IKKβ on IKKβ-mediated activation of NF-κB signalling. To determine the effect of monoubiquitin fusion, wild-type IKKβ (control), IKKβ.SE or monoubiquitin-fused IKKβ.SE was expressed with empty vector, Ro52 (wild type) or Ro52 (C16A) in HEK293 cells transfected with the NF-κB reporter plasmid. Fifteen hours after transfection, cell extracts were prepared and assayed for luciferase activity. Each bar represents the mean ± standard error ($^{*}P < 0.01$) (upper panel). Exogenously expressed RH-tagged IKKβ (wild-type, SE mutant and monoubiquitin-fused SE mutant) for the reporter assay were analysed by western blotting (lower panel).

in cells, we performed reporter assays using an NF-κB reporter construct. First, we investigated the effect of Ro52 overexpression on Tax-induced activation of NF-κB signalling (Fig. 8A). Specifically, Tax was co-expressed with Ro52 (wild-type) or its mutant (C16A) in HeLa cells transfected with the NF-κB reporter plasmid. As shown in the upper panel of Fig. 8A, NF-κB reporter activity was suppressed when co-expressed with wild-type Ro52. Importantly, the suppression was not observed when co-expressed with mutant Ro52 (C16A), suggesting that NF-κB-dependent gene expression induced by Tax is suppressed by the E3 ligase activity of Ro52 (Fig. 8A). In this experiment, we also examined expression levels of proteins (Tax and Ro52-RH) by western blotting (lower panel, Fig. 8A). Unexpectedly, the expression level of Tax was dramatically reduced when co-expressed with wild-type Ro52, but not with its inactive mutant.

Role of Ro52 in IKKβ-induced Activation of NF-κB Signalling—As described earlier, we found that Ro52 down-regulates Tax-induced NF-κB signalling by reducing the expression of Tax. However, down-regulation of NF-κB signalling might not result only from this Ro52-mediated reduction of Tax. Because Ro52 monoubiquitinates IKKβ, we hypothesized that this monoubiquitination is also involved in the down-regulation of NF-κB signalling. To test this hypothesis, we used IKKβ.SE, the constitutively active form of IKKβ (*6*, *7*, *29*), because it allows us to bypass the phosphorylation of IKKβ without the expression of Tax and IKKγ as described earlier. To confirm the activity of IKKβ.SE, we first performed a reporter assay using an NF-κB reporter construct (Fig. 8B). Briefly, empty vector, IKKβ (wild-type) or IKKβ.SE was expressed in HEK293 cells transfected with the NF-κB reporter plasmid. Then, NF-κB-dependent gene expression was determined. As expected, the expression of IKKβ (wild-type) weakly promoted the NF-κB reporter activity, whereas the expression of IKKβ.SE strongly promoted it (upper panel, Fig. 8B). Importantly, although the expression levels of wild-type IKKβ and IKKβ.SE were the same (lower panel, Fig. 8B), we detected a great difference in their activities.

After we confirmed the activity of IKKβ.SE, we further examined the effect of Ro52 expression on the activity of IKKβ (Fig. 8C). Specifically, IKKβ.SE was co-expressed with empty vector, Ro52 (wild-type) or its mutant (C16A)

in HEK293 cells transfected with the NF-κB reporter plasmid. Then, the level of luciferase activity was determined. As shown in the upper panel of Fig. 8C, IKKβ.SE strongly promoted the NF-κB reporter activity when co-expressed with empty vector. In contrast, the reporter activity was suppressed to 23% when co-expressed with wild-type Ro52. However, the suppression was mild (~50%) when co-expressed with mutant Ro52 (C16A).

Effect of Monoubiquitin Fusion to IKKβ on NF-κB Signalling—As described earlier, our biochemical studies revealed that IKKβ is monoubiquitinated by Ro52 when co-expressed with Ro52 in HEK293T cells. Furthermore, the reporter assay shown in Fig. 8C revealed that the activity of the RING finger domain of Ro52 down-regulates IKKβ-induced NF-κB signalling. These observations raise the question of whether NF-κB-dependent gene expression is suppressed by Ro52-mediated monoubiquitination of IKKβ. To address this, we used monoubiquitin-fused IKKβ. Specifically, we examined whether the direct fusion of monoubiquitin to IKKβ modulates IKKβ-induced NF-κB signalling. In the molecule of monoubiquitin-fused protein, however, the monoubiquitin links to the N-terminal Met residue of the protein via an α-peptide bond. This linkage is artificial, not natural, in cells. In the naturally monoubiquitinated protein in cells, monoubiquitin links to the target Lys residue of the protein via an isopeptide bond. Thus, the monoubiquitin fusion is different from natural monoubiquitination, exactly, but monoubiquitin-fused proteins are structurally similar to monoubiquitinated proteins. Therefore, using the direct fusion of a monoubiquitin to target proteins, we are able to investigate the biological relevance of protein monoubiquitination (*37*).

By using monoubiquitin-fused IKKβ (Ub-IKKβ) in NF-κB reporter assay, we examined whether the monoubiquitin fusion modulates IKKβ-induced NF-κB signalling (Fig. 8D). In brief, wild-type IKKβ (control), IKKβ.SE or monoubiquitin-fused IKKβ.SE was expressed in HEK293 cells transfected with the NF-κB reporter plasmid. Afterwards, the cell extract was assayed for luciferase activity (Fig. 8D). As shown in the upper panel of Fig. 8D, monoubiquitin fusion of IKKβ.SE led to a significant reduction in luciferase activity compared to unubiquitinated IKKβ.SE. Thus, NF-κB-dependent gene expression is suppressed by monoubiquitin fusion of IKKβ. This result supports our hypothesis that NF-κB-dependent gene expression is down-regulated by Ro52-mediated monoubiquitination of IKKβ.

DISCUSSION

IKKβ is phosphorylated for activation of NF-κB signalling by various inducers, such as cytokines, including TNF and other pro-inflammatory cytokines, and the oncoprotein Tax derived from HTLV-1. Importantly, cytokines transiently phosphorylate IKKβ by binding to cell-surface receptors (*2*), whereas Tax persistently phosphorylates IKKβ and thereby continuously transduces NF-κB signalling (*7*), which is responsible for chronic inflammatory diseases, T-cell lymphoma and T-cell leukaemia (*38*, *39*). Mechanisms of transient phosphorylation of IKKβ by cytokines have been well investigated. Specifically, the cytokine TNF leads to rapid phosphorylation and activation of IKKβ, followed by dephosphorylation and inactivation within 30–60 min (*4*). The mechanism of persistent phosphorylation of IKKβ by Tax has also been reported. Namely, Tax binds directly to IKKγ to induce persistent phosphorylation and subsequent activation of IKKβ (*7*). Recent data have further indicated that Tax forms a stable ternary complex with IKKγ and protein phosphatase 2A (PP2A). In this context, PP2A is inhibited or diminished by Tax (*3*). Although PP2A is a negative regulator of phosphorylated IKKβ (because PP2A dephosphorylates IKKβ), IKKγ-bound Tax inhibits PP2A to maintain IKKβ in a phosphorylated, active state (*3*).

Recently, researchers showed that the TNF-induced transient response does not enable the stable formation and/or detection of monoubiquitination of IKKβ, whereas the Tax-induced persistent response does enable the detection of monoubiquitination of IKKβ (*30*). To confirm this, we also performed *in vivo* ubiquitination assays. As expected, monoubiquitination of IKKβ could not be detected at all in cells treated with TNF even when an E3 ubiquitin ligase Ro52 was overexpressed (data not shown). This is probably because IKKβ was rapidly dephosphorylated and was not able to interact stably with Ro52 (see below). In contrast, we easily detected monoubiquitination of IKKβ in cells expressing Tax as described in Results. Thus, as Carter *et al*. proposed, persistent and stable phosphorylation of IKKβ seems to be required for monoubiquitination of IKKβ.

At the first step of ubiquitination, substrates are recognized by E3 ubiquitin ligases. In some cases, the substrates interact with E3 ligases only after their post-translational modification, such as phosphorylation or hydroxylation. For example, in response to appropriate signals, IκBα is phosphorylated on Ser-32 and Ser-36 and then recognized by β-TRCP, which is a subcomponent of an SCF-type E3 ligase (*40*). Hypoxia-inducible factor (HIF)-1α is another example. In response to hypoxia, HIF-1α is hydroxylated on Pro-564 and then recognized by pVHL, which is a subcomponent of an E3 ligase (*41*, *42*). Likewise, IKKβ is persistently and stably phosphorylated in cells expressing Tax (*3*) and then seems to be recognized by an E3 ligase Ro52 through interaction. Indeed, our immunoprecipitation assay showed that Ro52 interacts with IKKβ, but the interaction with the phosphomimetic form of IKKβ is much stronger than that with the wild-type of IKKβ. Therefore, we believe that the phosphorylation of IKKβ is required for the tight interaction between IKKβ and Ro52. As described in RESULTS section, however, we have thus far been unable to detect the interaction between Ro52 and IKKβ in cells without overexpression, presumably because of limited expression of the relevant IKKβ species and Ro52 at the endogenous protein level.

The interaction of IKKβ with the E3 ubiquitin ligase Ro52 then leads to monoubiquitination of IKKβ in the presence of an E2 ubiquitin-conjugating enzyme, such as UbcH5B. In general, monoubiquitination plays roles in proteasome-unrelated biological events (*43–46*). Therefore, we hypothesized that Ro52-mediated monoubiquitination of IKKβ plays a role in Tax-induced NF-κB signalling. In other words, Ro52 modulates the NF-κB

signalling through monoubiquitination of IKKβ. To test this hypothesis, we performed NF-κB reporter assays. Our studies revealed three things. First, Ro52 inhibits Tax-induced NF-κB signalling by its E3 ligase activity. Second, Ro52 inhibits IKKβ-induced NF-κB signalling by its E3 ligase activity. Third, monoubiquitin fusion to IKKβ inhibits IKKβ-induced NF-κB signalling. These findings strongly suggest that Ro52-mediated monoubiquitination of IKKβ leads to inhibition of Tax-induced NF-κB signalling. In these studies, we unexpectedly found another important explanation for Ro52-mediated inhibition of Tax-induced NF-κB signalling. Namely, the E3 ligase activity of Ro52 dramatically reduces the expression of Tax. Although the detailed mechanism is currently under investigation, we believe that this reduction of Tax expression also plays a crucial role in the Ro52-mediated inhibition of Tax-induced NF-κB signalling.

As described earlier, Ro52 monoubiquitinates IKKβ to down-regulate NF-κB signalling. Interestingly, this monoubiquitination can be disrupted by the *Y. pestis* virulence factor YopJ. How does YopJ disrupt monoubiquitination? YopJ has homology to de-ubiquitinating enzymes (*47*). Recently, two groups demonstrated that recombinant YopJ has de-ubiquitinating activity, and they concluded that YopJ functions as a de-ubiquitinating enzyme (*34*, *35*). On the other hand, other groups concluded that YopJ is an acetyltransferase that inhibits the activation of host MAP kinase kinases (MKKs) by modifying serine and/or threonine residue(s) in the activation loop, thereby preventing their modification by phosphorylation (*19*, *36*). These apparently contradictory findings have not yet been resolved, and it remains possible that YopJ has both protease and acetyltransferase activities (*48*). Despite these controversial functions of YopJ, our results show that YopJ strongly disrupts Ro52-mediated monoubiquitination of IKKβ, indicating that YopJ is a potent inhibitor against Ro52 in monoubiquitination of IKKβ. As an overall effect, however, YopJ inhibits NF-κB signalling, probably because YopJ affects functions of other substrates as well as Ro52.

Tax-induced monoubiquitination of IKKβ was first reported by Carter *et al*. (*7*). In the study presented here, we further investigated this using NF-κB reporter assays, which supported our hypothesis that monoubiquitination of IKKβ down-regulates NF-κB signalling. Thus, previous studies and our own suggest that Tax not only induces phosphorylation of IKKβ for activation of NF-κB signalling, but also induces the subsequent monoubiquitination of IKKβ for inactivation of NF-κB signalling. It is of interest that Tax expression sequentially leads to activation and inactivation of IKKβ in the presence of Ro52. Tax, however, usually induces persistent activation of NF-κB signalling. This is probably because monoubiquitinated species of IKKβ are very limited in cells. To increase the level of monoubiquitinated IKKβ and efficiently down-regulate NF-κB signalling, the E3 ligase(s), such as Ro52, may need to be induced in cells.

Persistent activation of IKKβ is induced by Tax and importantly, this type of activation is responsible for chronic inflammatory diseases and cancers (*38*, *39*). If the persistent activation of IKKβ can be down-regulated or terminated, then patients with these diseases might be treated. Since the findings presented here suggest that the E3 ubiquitin ligase Ro52 plays roles in this down-regulation by monoubiquitinating IKKβ and by reducing levels of Tax, further research involving Ro52 may lead to the development of new therapeutic targets for these diseases.

FUNDING

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CONFLICT OF INTEREST

None declared.

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