

## Negative Regulation of JNK Signaling by the Tumor Suppressor CYLD\*

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William Reiley<sup>‡§</sup>, Minying Zhang<sup>‡</sup>, and Shao-Cong Sun<sup>¶</sup>

From the Department of Microbiology and Immunology, Pennsylvania State University College of Medicine,  
Hershey, Pennsylvania 17033

**CYLD is a tumor suppressor that is mutated in familial cylindromatosis, an autosomal dominant predisposition to multiple tumors of the skin appendages. Recent studies suggest that transfected CYLD has deubiquitinating enzyme activity and inhibits the activation of transcription factor NF- $\kappa$ B. However, the role of endogenous CYLD in regulating cell signaling remains poorly defined. Here we report a critical role for CYLD in negatively regulating the c-Jun NH<sub>2</sub>-terminal kinase (JNK). CYLD knockdown by RNA interference results in hyperactivation of JNK by diverse immune stimuli, including tumor necrosis factor- $\alpha$ , interleukin-1, lipopolysaccharide, and an agonistic anti-CD40 antibody. The JNK-inhibitory function of CYLD appears to be specific for immune receptors because the CYLD knockdown has no significant effect on stress-induced JNK activation. Consistently, CYLD negatively regulates the activation of MKK7, an upstream kinase known to mediate JNK activation by immune stimuli. We further demonstrate that CYLD also negatively regulates I $\kappa$ B kinase, although this function of CYLD is seen in a receptor-dependent manner. These findings identify the JNK signaling pathway as a major downstream target of CYLD and suggest a receptor-dependent role of CYLD in regulating the I $\kappa$ B kinase pathway.**

CYLD was originally identified as a tumor suppressor that is mutated in familial cylindromatosis (1), an autosomal dominant predisposition to multiple tumors of the skin appendages (2, 3). Recent studies reveal that CYLD is a new member of the deubiquitinating enzyme family (1, 4–6). Transient transfection studies suggest that CYLD inhibits the ubiquitination of certain signaling molecules, including members of the tumor necrosis factor receptor-associated factor (TRAF)<sup>1</sup> family (5–7). TRAFs are known as signaling adaptors of tumor necrosis factor receptor superfamily (8), but they are also involved in the

signal transduction by several other immune receptors, such as toll-like receptors, interleukin-1 receptors, and T-cell receptors (9–11). All TRAFs except TRAF1 contain a Ring finger domain known to mediate protein ubiquitination (12). Indeed, TRAF2 and TRAF6 have been shown to function as ubiquitin ligases that catalyze the synthesis of Lys<sup>63</sup>-linked polyubiquitin chains (13, 14). This type of ubiquitination, which occurs early during a cellular response, does not target protein degradation but is important for signal transduction (14–17). Interestingly, the self-ubiquitination of TRAF2 and TRAF6 is potentially inhibited by CYLD under overexpression conditions (4–6). Although it remains unclear whether CYLD regulates the ubiquitination of TRAFs under endogenous conditions, these findings suggest the possibility that CYLD may function as a negative regulator of TRAF ubiquitination and activation of downstream signaling events.

Among the downstream signaling cascades activated by TRAFs are those that lead to activation of I $\kappa$ B kinase (IKK) and three families of MAP kinases (MAPKs): c-Jun NH<sub>2</sub>-terminal kinase (JNK), extracellular signal responsive kinase, and p38 (8). IKK is known as a specific activator of NF- $\kappa$ B, a family of inducible transcription factors regulating genes involved in immune and inflammatory responses, cell growth/survival, and oncogenesis (18, 19). The MAPKs activate a number of transcription factors, including the ternary complex factor Elk-1 and members of the AP1 and cAMP-response element-binding protein/activation transcription factor families (20). Additionally, the MAPKs are involved in posttranscriptional regulation of gene expression (21–23). The biological functions of JNK, which include regulation of immune and inflammatory responses, cell growth, apoptosis, and tumor formation (24–26), are particularly diverse. Activation of JNK is mediated by a kinase cascade involving MAPK kinases and MAPK kinases. Two MAPK kinases, MKK4 and MKK7, serve as the direct kinases of JNK. MKK7 is required for JNK activation by inflammatory cytokines, whereas MKK4 is more important for JNK activation by stress signals (27).

Recent studies suggest that activation of IKK by TRAF6 and TRAF2 involves Lys<sup>63</sup>-linked ubiquitination (13, 14). This signaling mechanism appears to be important for IKK activation by specific immune receptors, including interleukin-1 receptors and T-cell receptors (11). The ubiquitination of TRAF2 has also been shown to mediate activation of JNK induced by the inflammatory cytokine TNF- $\alpha$  (15, 16). A role for CYLD in NF- $\kappa$ B regulation is suggested by some recent studies that reveal that CYLD inhibits the activation of an NF- $\kappa$ B reporter gene in transfected cells (1, 4–6). However, it is unclear whether CYLD functions as a negative regulator of the IKK or other signaling cascades downstream of various immune receptors.

In the present study, we have taken the RNA interference (RNAi)-mediated gene knockdown approach to investigate the function of endogenous CYLD in the regulation of cell signal-

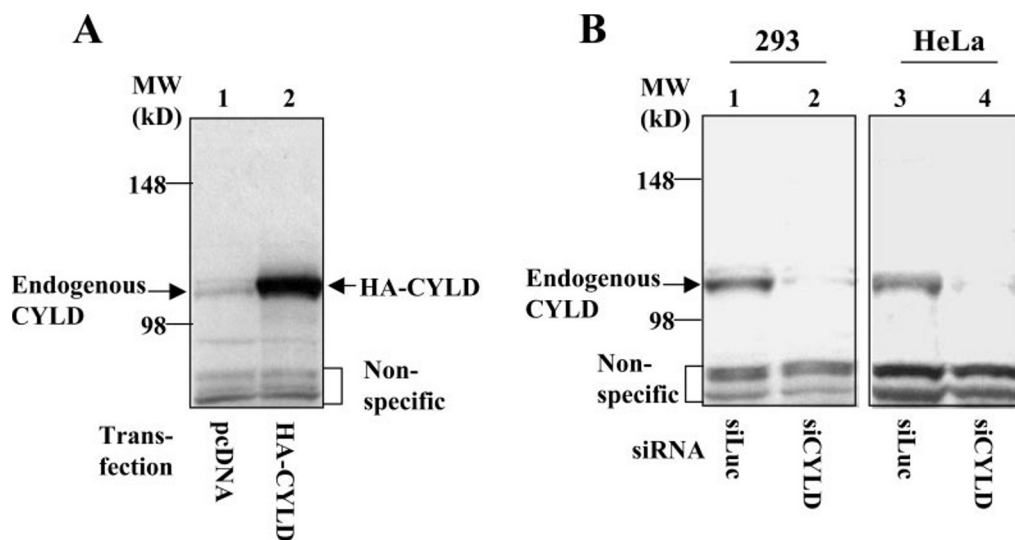
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<sup>‡</sup> These authors contributed equally to this work.

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<sup>¶</sup> To whom correspondence should be addressed. Tel.: 717-531-4164; Fax: 717-531-6522; E-mail: sxs70@psu.edu.

<sup>1</sup> The abbreviations used are: TRAF, tumor necrosis factor receptor-associated factor; IKK, I $\kappa$ B kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; TNF, tumor necrosis factor; RNAi, RNA interference; HA, hemagglutinin; siRNA, small interfering RNA; GST, glutathione S-transferase; IB, immunoblotting; EMSA, electrophoresis mobility shift assay; LPS, lipopolysaccharide.



**FIG. 1. Characterization of CYLD antibody and siRNA.** A, 293 cells were transfected with either the empty vector pcDNA or an expression vector encoding HA-tagged CYLD.  $\sim 7 \mu\text{g}$  of protein lysates were subjected to IB using anti-CYLD. The transfected HA-CYLD, endogenous CYLD, and some nonspecific protein bands are indicated. B, 293 or HeLa cells were transfected with either the control luciferase siRNA (*siLuc*) or CYLD-specific siRNA (*siCYLD*) as described under "Materials and Methods."  $\sim 20 \mu\text{g}$  of cell lysates were subjected to IB using anti-CYLD. The protein bands are indicated as in A.

ing. We demonstrate that CYLD is a key negative regulator of JNK downstream of diverse immune receptors. Further, CYLD also inhibits IKK activation, but this function of CYLD is receptor-dependent.

#### MATERIALS AND METHODS

**Plasmid Constructs**—Human CYLD was cloned by reverse transcription-PCR and inserted into the pcDNA-HA vector (28) downstream of the HA epitope tag. CYLD<sup>R</sup> is a modified form of the pcDNA-HA-CYLD in which the siRNA binding site was mutated (by site-directed mutagenesis) without altering the amino acid codons. Thus, the CYLD<sup>R</sup> retains the wild-type CYLD amino acid sequence but is resistant to siRNA-mediated suppression. GST-I $\kappa$ B $\alpha$ -(1–54) was constructed by inserting a DNA fragment encoding the first 54 amino acids of human I $\kappa$ B $\alpha$  and three copies of the HA epitope tag into the pGEX-4T-3 vector (Pharmacia Corporation). GST-c-Jun (1–79) encodes a GST-fusion protein containing the first 79 amino acids of c-Jun.

**Cell Culture and Antibodies**—Human embryonic kidney 293 cells, human cervical carcinoma HeLa cells, and human B-cell line BJAB were obtained from ATCC. 293 cells stably transfected with murine CD40 (293-CD40) were kindly provided by Dr. Steven Ley (National Institute of Medical Research, London, UK) (29). The anti-CYLD antibody was generated by injecting rabbits with a GST-fusion protein containing an N-terminal region of human CYLD (amino acid 136–301). Anti-mouse CD40 antibody was purchased from Pharmingen. The polyclonal antibodies for tubulin (TU-02), extracellular signal responsive kinase (K-23), JNK1 (C-17), JNK2 (N18), p38 (C-20), Oct1 (C-21), TRAF2 (C-20), IKK $\gamma$  (FL-419), and MKK4 (MEK-4 H-98) were purchased from Santa Cruz Biotechnology. The recombinant JNK protein, anti-MKK7, and phospho-specific antibodies recognizing activated forms of different MAPKs were purchased from Cell Signaling Technology, Inc.

**RNAi**—Small interfering RNAs (siRNAs) specific for human CYLD and luciferase were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The sense strand sequences of the oligonucleotides are as follows. CYLD siRNA, AAG UAC CGA AGG GAA GUA UAG; luciferase siRNA, AAC TTA CGC TGA GTA CTT CGA.

For siRNA delivery, 293 and HeLa cells were transfected in 6-well plates with 140 pmol of siRNA using Oligofectamine (Invitrogen). At 16–24 h following the first transfection, the cells were transfected again with the same amount of siRNA together with 300 ng of carrier DNA using Lipofectamine 2000. At about 30 h after the second transfection, the cells were used for different experiments. For stable gene knockdown using the small hairpin RNA technique, a double-stranded oligonucleotide corresponding to the CYLD siRNA was cloned into the pSUPER-retro-puro vector (Oligoengine) downstream of the U6 promoter. The generated retroviral construct, named pSUPER-shCYLD, was used to produce recombinant viruses and infect the indicated cells as described previously (30). The infected cells were enriched by

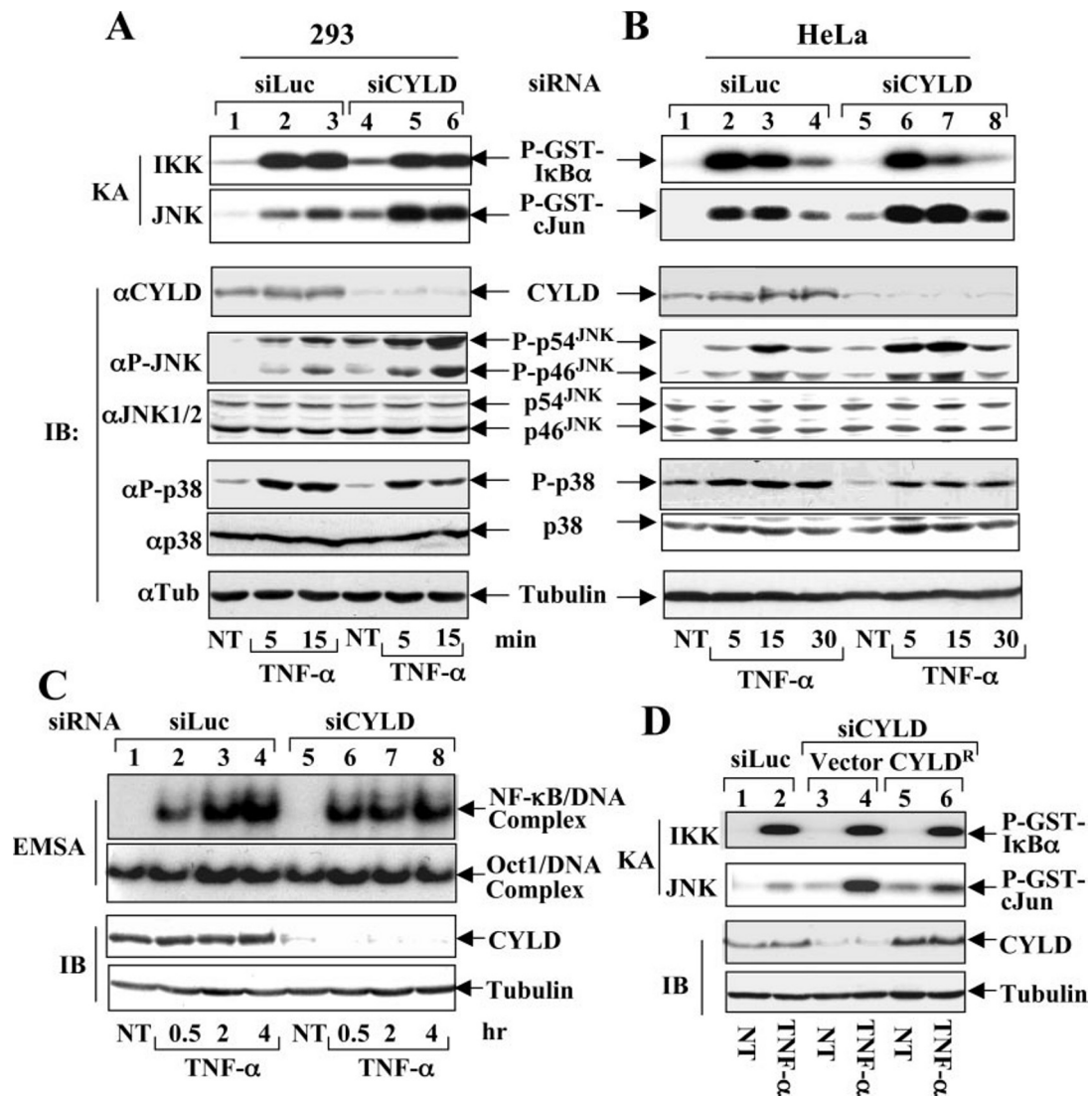
selection using puromycin. The bulk-infected cells were used in the experiments to avoid clonal variations.

**Immunoblotting (IB), *in Vitro* Kinase Assay, and Electrophoresis Mobility Shift Assays (EMSAs)**—Cell lysates were prepared by lysing the cells in a kinase lysis buffer and immediately subjecting them to IB and *in vitro* kinase assays as described previously (31). Activated MAPKs were analyzed by IB using phospho-specific antibodies. Nuclear extracts were prepared and subjected to EMSA (32) using a <sup>32</sup>P-radio-labeled high affinity  $\kappa$ B probe (5'-CAA CGG CAG GGG AAT TCC CCT CTC CTT-3') or a control probe containing the Oct-1 binding site (5'-TGT CGA ATG CAA ATC CTC TCC TT-3'); this was followed by resolving the DNA-protein complexes on native 5% polyacrylamide gels.

#### RESULTS

**CYLD Is a Negative Regulator of JNK but Not IKK in the TNF- $\alpha$  Signaling Pathway**—To systematically analyze the role of CYLD in regulation of cell signaling, we generated a CYLD-specific antibody. This antibody could readily detect the transfected CYLD (Fig. 1A, lane 2). Additionally, it also detected an endogenous protein band comigrating with the transfected CYLD (lane 1). This protein band, which was not detected by IB using a preimmune serum (data not shown), became more prominent when higher amounts of cell extracts were used in the IB (Fig. 1B, lanes 1 and 3). To confirm that this protein is endogenous CYLD, we performed RNAi assays. The expression of this endogenous protein was markedly suppressed by a CYLD-specific siRNA (*siCYLD*, Fig. 1B, lanes 2 and 4) but not by a control siRNA for luciferase (*siLuc*, lanes 1 and 3). Similar results were obtained in 293 and HeLa cells (Fig. 1B). The CYLD antibody also detected some other proteins, but this was likely because of nonspecific cross-reaction because these proteins are much smaller than the predicted mass (105 kDa) of CYLD and because their expression was not affected by the CYLD siRNA.

With the CYLD antibody and siRNA, we first examined the effect of CYLD knockdown on cell signaling stimulated by the proinflammatory cytokine TNF- $\alpha$ . In both 293 and HeLa cells, TNF- $\alpha$  stimulated the catalytic activity of IKK and JNK as demonstrated by immunocomplex kinase assays (Fig. 2, A and B, top two panels). JNK activation was also detected based on its site-specific phosphorylation *in vivo* by IB using a phospho-specific anti-JNK antibody (fourth panel). In addition to IKK and JNK, TNF- $\alpha$  stimulated the activation of the p38 MAPK (Fig. 2, A and B, sixth panel) but did not appreciably induce the activity



**FIG. 2. CYLD knockdown by RNAi results in hyperactivation of JNK but not IKK in TNF- $\alpha$ -stimulated cells.** *A* and *B*, effect of CYLD knockdown on TNF- $\alpha$ -stimulated cell signaling in 293 (*A*) and HeLa (*B*) cells. Cells were transfected with siRNA targeting CYLD or a control protein, luciferase (Luc). The cells were either not treated (NT) or stimulated with human recombinant TNF- $\alpha$  (20 ng/ml) for the indicated times. IKK and JNK were isolated by immunoprecipitation using anti-IKK $\gamma$  and anti-JNK1 plus anti-JNK2, respectively. The catalytic activity of IKK and JNK in the immune complexes was detected by *in vitro* kinase assays using GST-IkB $\alpha$  (1–54) and GST-c-Jun (1–79) substrates, respectively (*top two panels*). Phosphorylated substrates are indicated as P-GST-IkB $\alpha$  and P-GST-cJun. The cell lysates were also subjected to IB using the indicated antibodies to monitor the efficiency of CYLD knockdown (*third panel*), the *in vivo* phosphorylation of JNK (*fourth panel*) and p38 (*sixth panel*), the total protein expression of JNK1 and JNK2 (*fifth panel*) and p38 (*seventh panel*), and also tubulin loading control (*bottom panel*). *C*, EMSA to detect NF- $\kappa$ B DNA binding activity. The control and CYLD-suppressed 293 cells described in *A* were stimulated with TNF- $\alpha$ . Nuclear extracts were isolated and subjected to EMSA using a  $^{32}$ P-radiolabeled probe for NF- $\kappa$ B (*upper panel*). As a loading control, the EMSA was performed using a probe for the constitutive transcription factor Oct-1 (*second panel*). Total cell lysates were prepared from an aliquot of the cells and subjected to IB using anti-CYLD (*third panel*) and anti-tubulin (*bottom panel*). *D*, CYLD reconstitution in CYLD siRNA-transfected cells. 293 cells were transfected with either control luciferase siRNA or CYLD siRNA. In the latter case, the CYLD siRNA was cotransfected with an RNAi-resistant form of CYLD (CYLD<sup>R</sup>, lanes 5 and 6) or an empty vector (lanes 3 and 4). The cells were either not treated (NT) or stimulated with TNF- $\alpha$  for 7.5 min. Catalytic activity of IKK (*first panel*) and JNK (*second panel*) were analyzed by kinase assays as described in *A*. The cell lysates were also subjected to IB to monitor the efficiency of CYLD suppression (*third panel*) and tubulin expression (*bottom panel*).

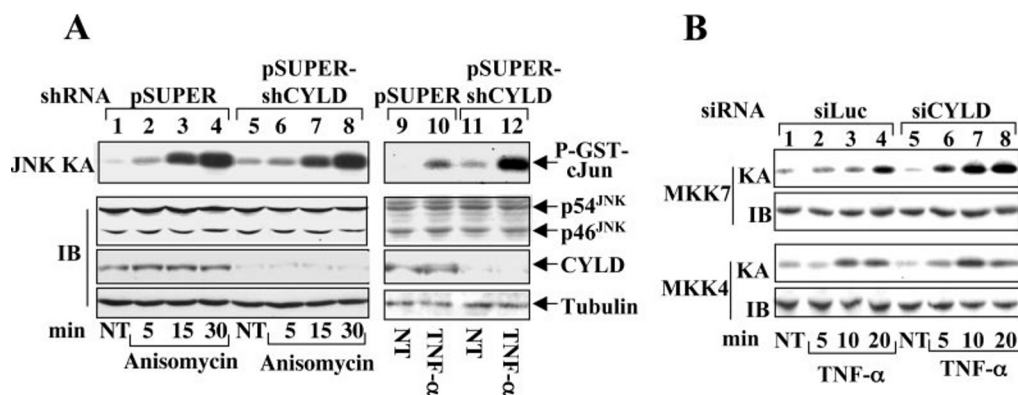
of extracellular signal responsive kinase (data not shown).

If endogenous CYLD serves as a negative regulator of TNF- $\alpha$ -stimulated cell signaling, the CYLD knockdown should result in hyperactivation of the specific kinases under the negative control of CYLD. In this regard, IKK is particularly interesting because CYLD has been shown to inhibit the induction of NF- $\kappa$ B reporter gene by various immune receptors under transient transfection conditions (4–7). To our surprise, however, the CYLD knockdown did not promote IKK activation in the TNF- $\alpha$ -stimulated 293 cells (Fig. 2*A*, *top panel*) or HeLa cells (Fig. 2*B*, *top panel*). Consistently, the TNF- $\alpha$  stimulated NF- $\kappa$ B DNA binding activity was not enhanced in the CYLD knockdown cells (Fig. 2*C*, *top panel*). Interestingly, parallel

analyses using the same cells revealed that the CYLD knockdown markedly enhanced the activation of JNK as demonstrated by both kinase assays (Fig. 2, *A* and *B*, *second panel*) and phospho-specific IB assays (*fourth panel*). The loss of CYLD also caused a low basal level of JNK activation in unstimulated cells (Fig. 2, *A* and *B*, *fourth panels*, lane 4). This result was not caused by variations in protein loading because the amounts of total JNK1 and JNK2 proteins (*fifth panel*) as well as tubulin (*bottom panel*) were comparable in the different samples. Further, the CYLD knockdown did not enhance the activation of p38 (*sixth panel*).

To further confirm that CYLD negatively regulates JNK activation in the TNF- $\alpha$  signaling pathway, we generated a





**FIG. 3. CYLD knockdown has no effect on JNK activation by a stress agent and promotes MKK7 activation by TNF- $\alpha$ .** *A*, effect of CYLD knockdown on JNK activation by a stress agent and TNF- $\alpha$ . 293 cells infected with either the empty pSUPER vector or pSUPER-shCYLD were stimulated with the stress agent anisomycin (1  $\mu$ g/ml) for the indicated time periods or TNF- $\alpha$  (20 ng/ml) for 15 min; this was followed by analyzing the JNK kinase activity by kinase assays (*top panel*) and the expression of JNK, CYLD, and tubulin by IB. *B*, hyperactivation of MKK7 in CYLD knockdown cells. 293 cells were transfected with control or CYLD siRNA and stimulated as indicated. MKK7 and MKK4 were isolated by immunoprecipitation using anti-MKK7 and anti-MKK4, respectively. The catalytic activity of isolated MKK7 (*top panel*) and MKK4 (*third panel*) were examined by kinase assays using catalytically inactive recombinant JNK as substrate. The cell lysates were also subjected to IB using anti-MKK7 (*second panel*) and anti-MKK4 (*bottom panel*) to detect the expression level of these two JNK kinases. NT, not treated.

modified form of CYLD cDNA harboring sense mutations in the siRNA-targeting site. Although such mutations do not change the amino acid sequence of CYLD, they render the expressed CYLD mRNA resistant to siRNA-mediated destruction. As expected, this modified version of CYLD (CYLD<sup>R</sup>) was efficiently expressed even in the presence of CYLD siRNA (Fig. 2*D*, *third panel*, lanes 5 and 6). More importantly, expression of CYLD<sup>R</sup> in the CYLD knockdown cells greatly reduced the level of JNK activation (*second panel*, compare lanes 4 and 6). Furthermore, the CYLD reconstitution did not affect TNF- $\alpha$ -stimulated activation of IKK (*first panel*). Together, these data demonstrate that JNK is a primary downstream target of CYLD in the TNF- $\alpha$  signaling pathway.

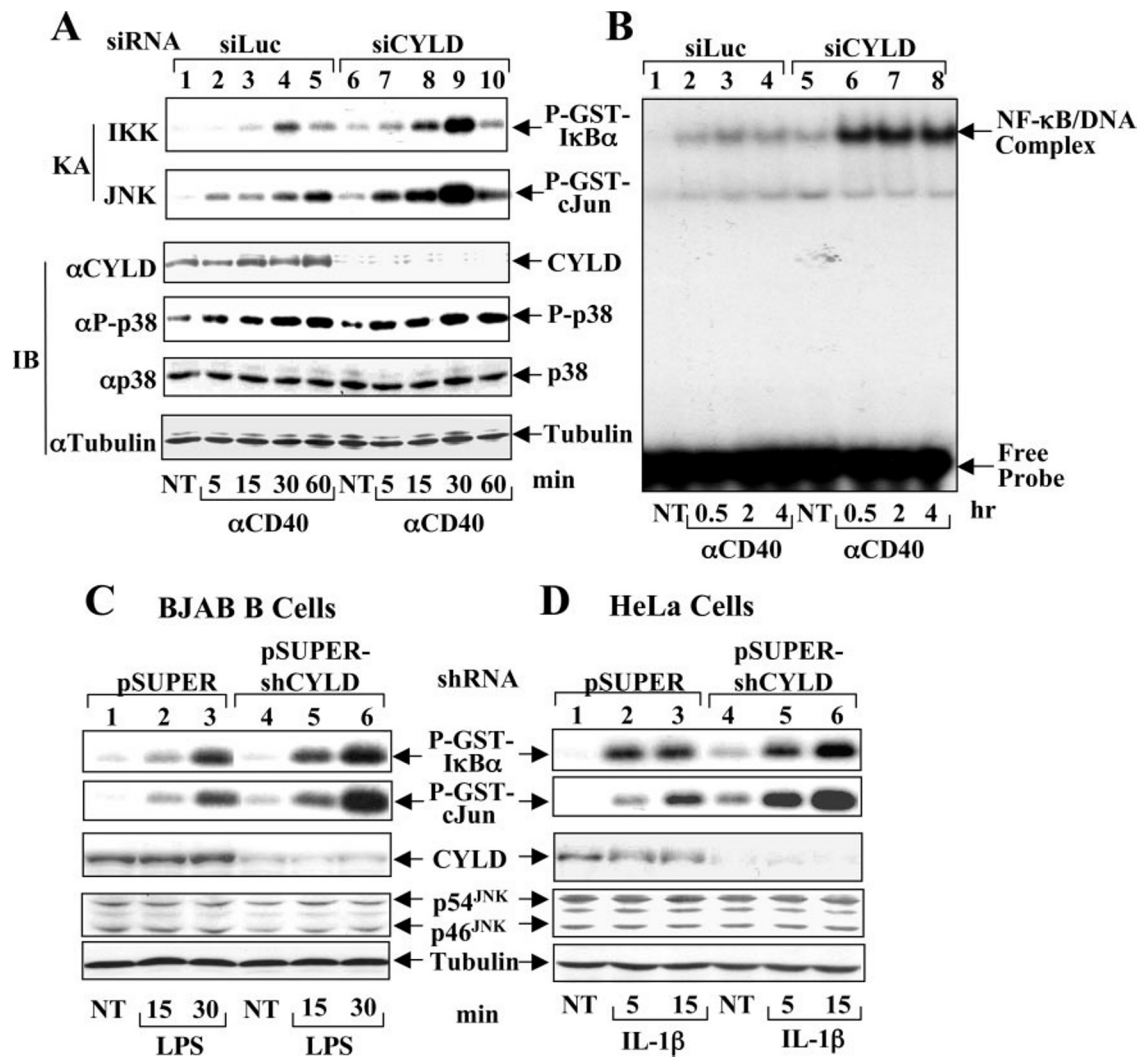
**CYLD Knockdown Has No Effect on JNK Activation by a Stress Agent**—JNK activation can be induced by both immune stimuli and stress signals, which involve different upstream signaling pathways. To assess the mechanism by which CYLD negatively regulates JNK, we examined the effect of CYLD knockdown on JNK activation by a stress stimulus, anisomycin. As expected, incubation of 293 cells with anisomycin resulted in strong activation of JNK (Fig. 3*A*, *top panel*, lanes 1–4). Interestingly, the anisomycin-induced JNK activation was not significantly affected by the CYLD knockdown (lanes 5–8). On the other hand, analysis of TNF- $\alpha$ -stimulated JNK activation using the same cells revealed a marked enhancement of this cytokine-specific JNK response by CYLD knockdown (lanes 9–12). This result indicates that CYLD does not regulate the JNK signaling pathway stimulated by stress signals. Furthermore, this finding also implies that CYLD does not directly regulate JNK but targets upstream step(s) involved in JNK activation by TNF- $\alpha$  and other immune stimuli.

**CYLD Negatively Regulates the Activation of MKK7**—Gene targeting studies suggest that JNK activation by inflammatory cytokines and stress signals involves two different upstream kinases, MKK7 and MKK4, with MKK7 being critical for cytokine-induced JNK activation (27). As a further step to investigate the mechanism underlying CYLD-mediated JNK regulation, we examined the effect of CYLD knockdown on TNF- $\alpha$ -stimulated activation of MKK7 and MKK4. These two JNK kinases were isolated from the cells by immunoprecipitation; this was followed by analyzing their catalytic activity by *in vitro* kinase assays using recombinant JNK (catalytically inactive) as substrate. As seen with JNK activation, the TNF- $\alpha$ -stimulated MKK7 activation was markedly enhanced in CYLD knockdown cells (Fig. 3*B*, *top panel*). This drastic

effect was not caused by the variation in the level of MKK7 protein expression (*second panel*). MKK4 was also weakly induced by TNF- $\alpha$  (*third panel*, lanes 1–4); however, this response was not significantly enhanced in the CYLD knockdown cells (lanes 5–8). Thus, MKK7 is an upstream target of CYLD in the JNK signaling pathway.

**CYLD Negatively Regulates JNK Activation by Diverse Stimuli**—Next we expanded our studies to investigate whether CYLD also negatively regulates JNK signaling downstream of other immune receptors. One receptor of interest is CD40, which is a member of the tumor necrosis factor receptor superfamily and mediates important immune functions via activation of IKK, JNK, as well as other MAPKs. For convenient CYLD knockdown, we used a previously characterized 293 cell line stably transfected with the murine CD40 cDNA (293-CD40, (29)). As expected from the prior studies (29), the 293-CD40 cells did not exhibit significant signaling activity under unstimulated conditions (Fig. 4*A*, *top two panels*, lane 1). However, cross-linking of CD40 with its agonistic antibody resulted in activation of both IKK (Fig. 4*A*, *top panel*) and JNK (*second panel*). Consistent with the TNF- $\alpha$ -stimulated cells, CYLD knockdown markedly enhanced the activation of JNK in the anti-CD40-treated cells (*second panel*). A detailed time course analysis revealed that the magnitude but not the kinetics of JNK activation was regulated by CYLD (*second panel*). Thus, CYLD functions as a negative regulator of JNK in both the TNF- $\alpha$  and CD40 signaling pathways. Interestingly, a parallel kinase assay revealed that the CD40-mediated IKK activation was also enhanced upon CYLD knockdown (*first panel*). Consistent with this finding, anti-CD40 stimulated hyperactivation of NF- $\kappa$ B in the CYLD knockdown cells (Fig. 4*B*, lanes 6–8). Parallel assays revealed that in contrast to the activation of IKK and JNK, the activation of p38 was not affected by CYLD knockdown (Fig. 4*A*, *fourth panel*).

To extend our studies to additional cell models, we employed a retroviral vector (pSUPER-retro-puro) to express a CYLD-specific small hairpin RNA. This approach allows gene suppression in cells with both high and low transfection efficiencies. Infection with CYLD-small hairpin RNA but not the empty pSUPER vector efficiently suppressed the expression of CYLD in BJAB B-cells (Fig. 4*C*, *third panel*). We also used the pSUPER small hairpin RNA system to knockdown CYLD in HeLa cells (Fig. 4*D*, *third panel*). As seen with the TNF- $\alpha$  and CD40 signaling pathways, CYLD knockdown greatly enhanced JNK activation by LPS and IL-1 $\beta$  (Fig. 4, *C* and *D*, *second*



**FIG. 4. CYLD knockdown promotes activation of JNK by diverse stimuli and IKK by selective stimuli.** A, CYLD knockdown results in hyperactivation of JNK and IKK by anti-CD40. 293-CD40 cells were transfected with luciferase siRNA (*siLuc*) or CYLD siRNA (*siCYLD*) and either not treated (NT) or stimulated with anti-CD40 antibody (5  $\mu$ g/ml). The catalytic activity of IKK (top panel) and JNK (second panel) was measured as described in Fig. 2A. The cell lysates were also subjected to IB assays as indicated. B, EMSA to detect NF- $\kappa$ B activation by anti-CD40. Nuclear extracts were isolated from 293-CD40 cells transfected with control (luciferase) or CYLD-specific siRNA and subjected to EMSA using the  $\kappa$ B probe. The NF- $\kappa$ B/DNA binding complex and free probe are indicated. C and D, effect of CYLD knockdown on the activation of JNK and IKK by LPS (C) and IL-1 $\beta$  (D). The indicated cells were infected with either empty pSUPER vector or pSUPER-shCYLD. The cells were stimulated with the indicated inducers; this was followed by analyzing the catalytic activity of IKK and JNK by kinase assays (top two panels) and the expression of CYLD, JNK, and tubulin by IB (bottom three panels).

panels). The IKK activation was also promoted by CYLD knockdown in LPS- and IL-1 $\beta$ -stimulated cells (Fig. 4, C and D, top panels), although it was less prominent compared with the effect on JNK activation. Thus, JNK appears to be a primary downstream target of CYLD, but IKK is also negatively regulated by CYLD downstream of certain receptors.

#### DISCUSSION

Tumor suppressor CYLD is a newly identified member of the deubiquitinating enzyme family. Although CYLD has been shown to inhibit the activation of NF- $\kappa$ B in reporter gene assays, its precise role in regulating signal transduction downstream of different immune receptors is poorly defined. In this study, we have investigated the function of endogenous CYLD using RNAi-mediated CYLD knockdown. Our data suggest that CYLD functions as a key negative regulator of the JNK signaling pathway downstream of diverse immune stimuli. We have also shown that CYLD negatively regulates IKK, although this function of CYLD is receptor dependent. Consistent with the prior NF- $\kappa$ B reporter studies (4–7), we have shown that CYLD inhibits the activation of IKK by certain cellular

stimuli, including anti-CD40, LPS, and IL-1 $\beta$  (Fig. 4). To our surprise, however, the CYLD knockdown has no appreciable effect on the TNF- $\alpha$ -stimulated activation of IKK or NF- $\kappa$ B (Fig. 2). This result was not caused by variations in CYLD knockdown or cell stimulations because parallel kinase assays reveal a remarkable elevation of JNK activation caused by the CYLD deficiency (Fig. 2).

How CYLD differentially regulates IKK and JNK is not completely understood, but one potential mechanism is attributed to the differential requirement of TRAFs in these signaling pathways. Gene knock-out studies suggest that TRAF2 gene deficiency only weakly inhibits TNF- $\alpha$ -induced NF- $\kappa$ B activation but largely abolishes the activation of JNK by TNF- $\alpha$  (33). Because TRAF2 is an upstream target of CYLD (4–6), these findings are consistent with our data that CYLD inhibits the activation of JNK but not NF- $\kappa$ B in TNF- $\alpha$ -stimulated cells (Fig. 2). Our results are also supported by two other studies that suggest an essential role for TRAF2 ubiquitination in TNF- $\alpha$ -stimulated activation of JNK (15, 16) but not that of IKK (16). The non-essential role of TRAF2 in NF- $\kappa$ B activation

by TNF- $\alpha$  is likely caused by the functional compensation by another TRAF molecule, TRAF5, because TRAF2/TRAF5 doubly deficient cells have a severe defect in NF- $\kappa$ B activation by TNF- $\alpha$  (34). Because CYLD has no effect on TNF- $\alpha$ -induced NF- $\kappa$ B activation, it is tempting to speculate that the signaling function of TRAF5 is either not regulated by ubiquitination or is controlled by a different deubiquitinating enzyme. A recent study (35) suggests that negative regulation of IKK in the TNF- $\alpha$  signaling pathway is mediated by A20, which acts by deubiquitinating the RIP kinase known to be essential for TNF- $\alpha$ -induced NF- $\kappa$ B activation (36, 37). Thus, it seems likely that downstream of the tumor necrosis factor receptor, CYLD and A20 regulate the JNK and IKK cascades by targeting deubiquitination of TRAF2 and RIP, respectively. However, the possibility that CYLD possesses additional targets cannot be excluded. In fact, the finding that CYLD negatively regulates the activation of JNK and IKK by LPS and IL-1 $\beta$  implicates a role for CYLD in negatively regulating the ubiquitination of TRAF6 because TRAF6 is an essential factor for the activation of these pathways downstream of both toll-like receptor 4 (receptor for LPS) and interleukin-1 receptor (38). At least under overexpression conditions, the ubiquitination of TRAF6 is inhibited by CYLD (5, 6).

The finding that CYLD negatively regulates JNK as well as IKK provides an insight into the tumor suppressor function of CYLD. The IKK/NF- $\kappa$ B pathway is well known for its involvement in cell survival and oncogenic transformation as well as immune responses (39). Accumulating evidence suggests that JNK is also a critical factor involved in tumorigenesis (26). The JNK signaling pathway is constitutively activated in various tumor cells (24, 40, 41) and has been shown to play an essential role in oncogenesis in a number of tumor models (42–47). Consistent with its oncogenic function, JNK has been shown to promote cell growth and survival (48–50). However, under certain conditions, JNK also functions as an inducer of apoptosis (25, 52, 53). Although the precise mechanism determining the pro- versus anti-apoptotic functions of JNK remains unclear, strong evidence suggests that prolonged activation of JNK promotes apoptosis (54–56), whereas transient activation of JNK contributes to cell survival (49). Of note, CYLD knockdown increases the magnitude of JNK transient activation but does not prolong the activation kinetics (Figs. 2B and 4). This finding supports the idea that CYLD deficiency promotes cell survival (4). In addition to its functions in oncogenesis and apoptosis regulation, JNK plays an important role in regulating immune and inflammatory responses (26). Given the important role of CYLD in regulating JNK and IKK downstream of diverse immune receptors (Figs. 2 and 4), it is tempting to speculate that this deubiquitinating enzyme may play an important role in immune regulation. Generations of CYLD knockout mice will be important for better understanding the biological function of CYLD-mediated regulation of JNK and other signaling pathways.

One interesting observation of the present study is that CYLD knockdown results in a basal level of activation of JNK as well as IKK/NF- $\kappa$ B (Fig. 2, A and B, and Fig. 4). This result indicates that the loss of CYLD is sufficient for triggering a low level of constitutive cell signaling. However, because cell lines may secrete a low level of cytokines (51), it is also possible that the constitutive kinase activity in CYLD knockdown cells is caused by the stimulatory action of endogenous cytokines. Nevertheless, these findings suggest that CYLD is a critical signaling regulator that prevents aberrant activation of JNK and IKK.

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