Members of the interferon regulatory factor (IRF) family of transcription factors have been implicated in host defense, cell growth, and immune regulation (1). Ten members of this family have been identified thus far: IRF-1, IRF-2, IRF-3, IRF-4/Ip, ISG56, ISG54, ISG55, ISG58, ISG59, and IRF-10. In addition, IRF-8 and IRF-1 synergistically activate the expression of IFN-α/β receptors, which are critical for the induction of type I interferon (IFN-α/β) responses, leading to the production of IFN-α/β and the activation of downstream immune effectors (2).

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level of a transcription factor reflects the balance between synthesis and degradation. Although much is known about the mechanism regulating the synthesis of IRF-8 and IRF-1, little is known about the mechanism of degradation of IRF-8 and IRF-1 and its role in regulating IL-12 expression. In the present study we report that ubiquitination of IRF-8 by the E3 ligase Cbl targets IRF-8 for degradation, which results in down-regulation of IL-12 expression.

MATERIALS AND METHODS

Reagents and Antibodies—N-Carboxbenzoxyl-L-leucyl-L-norleucinal (MG132), leupeptin, trans-epoxysuccinyl-L-leucylamido-4-guanidino butane, N-α-tosyl-L-lysine chloromethyl ketone, and LPS were from Sigma. Lactacystin was obtained from Calbiochem-Novabiochem. IFN-γ was obtained from R&D Systems (Minneapolis, MN). Antibodies against IRF-8, IRF-1, IRF-4, β-actin, and ubiquitin were obtained from Santa Cruz. HA mAb 12CA5 was from Roche Applied Science, and FLAG mAb M2 was obtained from Sigma.

Plasmid Constructs—Full-length IRF-8 cDNA and IRF-8 carboxy-terminal deletion mutants were inserted into the mammalian expression vector pcDNA3.1. The IRF-1 expression plasmid was a gift from T. Taniguchi (University of Tokyo, Tokyo, Japan). The HA-ubiquitin plasmid and HA-ubiquitin mutant plasmids K48R, K29R, and K63R were kindly provided by Dr. Ze’ev Ronai (Mount Sinai School of Medicine). The Cbl dominant negative plasmid was a gift from Dr. Reuben Siraganian (National Institutes of Health).

Peritoneal Macrophages—Peritoneal macrophages were isolated from C57BL/6 and Cbl−/− mice 3 days after injection (intraperitoneal) of 2 ml of thioglycollate medium. Cells were cultured in DMEM containing 10% fetal bovine serum and antibiotics.

Cell Lines—The RAW 264.7 murine macrophage cell line (American Type Culture Collection) was maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. Cbl−/− murine fibroblast cells were obtained from Dr. H. Band (Harvard Medical School) and maintained in DMEM with 10% fetal bovine serum and antibiotics.

Transfection—293T cells were transiently transfected using DNA-calcium phosphate precipitate as described previously (28). For each transfection, 10–20 μg of plasmid was used.

IL-12 p40 Promoter Assay—RAW264.7 cells were transiently transfected using Superfect (Qiagen). For each transfection, 2.5 μg of plasmid was mixed with 100 μl of DMEM (without serum and antibiotics) and 10 μl of Superfect reagent. After 10 min at room temperature, 600 μl of DMEM complete medium was added, and the mixture was added to cells in 6-well plates. Luciferase activity was measured 16–24 h later. When indicated, murine IFN-γ (10 ng/ml) or LPS (1 μg/ml) was added to the culture for 6–12 h before harvest. The cells were harvested with receptor lysis buffer (Promega) and 1 ml of cell extract was assayed for luciferase as described previously (11). Cells were co-transfected with a constitutively active cytomegalovirus promoter-β-galactosidase reporter plasmid to normalize experiments for transfection efficiency.

IL-12 p40 Enzyme-linked Immunosorbent Assay—RAW264.7 cells transduced with retrovirus-encoded IRF-8 and dominant negative Cbl or green fluorescent protein were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 24 h. In addition, peritoneal macrophages from wild type and Cbl−/− mice were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 24 h. The supernatants were collected for quantification of IL-12 p40 by enzyme-linked immunosorbent assay (R&D Systems).

Retroviral Transduction—For retroviral transduction we used a derivative of the Moloney murine leukemia virus vector pMP412 (29). To prepare pseudotyped virus, human 293 EbnaT cells were seeded at a density of 4 × 10^4 in 10-cm dishes. The next day, cells were transfected using DNA-calcium phosphate precipitate containing 2.5 μg of plasmid pMD.G encoding vesicular stomatitis virus G protein, 7.5 μg of plasmid pMD.OGP encoding gag-pol, and 10 μg of the retroviral expression construct. 48 h after transfection, the viral supernatant was collected, centrifuged at 800 × g, and used to infect cells. RAW264.7 cells (5 × 10^4) were suspended in 12 ml of viral supernatant in the presence of Polybrene (4 μg/ml), aliquoted into 6-well plates, and spun at 800 × g in a microtiter rotor at room temperature for 1 h.

Immunoblotting—Cell lysates and pre-stained molecular mass markers were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBST; incubated with anti-IRF-8, anti-ubiquitin, anti-IRF-1, anti-IRF-4, anti-β-actin, or anti-glycereraldehyde-3-phosphate dehydrogenase antibodies (1:2000) for 1–2 h; washed with TBST; and stained with peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:5000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL kit; Santa Cruz).

RESULTS

IRF-8 in Peritoneal Macrophages Is Degraded by the Proteasome—We have shown previously that IFN-γ and LPS synergistically induce IRF-8 protein expression in macrophages (11), and we now examine the stability of IRF-8. Thioglycollate-elicited macrophages from C3H/OUJ mice activated for 6 h with IFN-γ (10 ng/ml) and LPS (1 μg/ml) were incubated with fresh medium in the presence or absence of the proteasome inhibitor MG132 (10 μM), and at various time intervals cell lysates were assayed by immunoblotting for IRF-8. The expression of IRF-8 decreased with a half-life between 2 and 5 h, but in the presence of proteasome inhibitor MG132, IRF-8 became stable (Fig. 1A). Equivalent results were obtained with cells pretreated with cycloheximide (10 μM) for 30 min (Fig. 1B). We have also examined two other members of the IRF family, IRF-1 and IRF-4. IRF-1 was degraded in a pattern similar to that of IRF-8, and the degradation was again blocked by MG132 (Fig. 1B). However, IRF-4, which is constitutively expressed in macrophages, was stable (Fig. 1B). Inhibitors of calpains and lysosomal enzymes had no effect on IRF-8 degradation (Fig. 2). Notably, lactacystin, a more specific proteasome inhibitor than MG132, blocked degradation of IRF-8 to the same extent as MG132 (Fig. 2). These results indicate that the proteasome is involved in the degradation of IRF-8 and IRF-1.

Ubiquitination of IRF-8 in RAW264.7 Murine Macrophages—We next addressed the pathway leading to proteasomal degradation. Ubiquitin has diverse cellular functions, including marking proteins for degradation by the proteasome. As a model for peritoneal macrophages, we used the macrophage cell line RAW264.7, in which IRF-8 is also degraded rapidly (Fig. 3A). Cells lysates from IFN-γ- and LPS-activated RAW264.7 cells were immunoprecipitated with IRF-8 antibody and immunoblotted with anti-ubiquitin antibody. A high molecular mass smear typical of multi-ubiquitinated proteins was detected, which was increased following incubation with MG132 (Fig. 3B). In contrast, IRF-4, which is stable, was not ubiquitinated. To confirm the ubiquitination of IRF-8 in RAW264.7 cells, 48 h after co-transduction with retroviruses encoding IRF-8 and HA-ubiquitin, cell lysates were immunoprecipitated with anti-IRF-8 antibody and immunoblotted with anti-HA antibody. Ubiquitinated protein was present only in samples in which IRF-8 was expressed (Fig. 3C). These data indicate that both endogenously and exogenously expressed IRF-8 in RAW264.7 cells are subject to ubiquitination.

The Carboxyl-terminal Region of IRF-8 Controls Degradation—The stability of IRF-1 is controlled by carboxyl-terminal domain (30). The homology between IRF-1 and IRF-8 carboxyl-terminal domains suggests that the stability of IRF-8 might also be controlled by the carboxyl terminus. To examine this, we first had to show that IRF-8 degraded in 293T cells comparably to macrophages, which was found to be the case (Fig. 4A), and IRF-4 was stable in both cell types (Fig. 4A). Ubiquitina-
Fig. 1. IRF-8 is subject to proteasomal degradation in peritoneal macrophages. A, thioglycollate-elicited peritoneal macrophages were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 6 h, washed three times, and incubated with fresh medium in the presence or absence of MG132 (10 μM). Cell lysates were subjected to 10% SDS-PAGE, and after transfer to nitrocellulose, the blots were immunostained for IRF-8 and β-actin. B, thioglycollate-elicited peritoneal macrophages were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 6 h, cycloheximide (10 μg/ml) was added throughout the incubation, and the cells were cultured in the presence or absence of MG132 (10 μM). Cell lysates were analyzed as described before for IRF-8, IRF-1, IRF-4, and β-actin.

Fig. 2. IRF-8 degradation is blocked by proteasome inhibitors. Thioglycollate-elicited peritoneal macrophages were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 6 h, cycloheximide (10 μg/ml) was added, and the cells were incubated with fresh medium in the presence of calpastatin (5 μM), Nα-p-tosyl-l-lysine chloromethyl ketone (TPCK; 50 μM), trans-epoxysuccinyl-l-leucylamido-4-guanidinobutane (E64; 50 μM), leupeptin (50 μM), MG132 (10 μM), and lactacystin (10 μM) for 5 h. Cell lysates were analyzed for IRF-8 and β-actin.

Full-length IRF-8 or the two carboxyl-terminal truncated expression plasmids and HA-ubiquitin. Co-immunoprecipitation experiments of 293T cells co-transfected with IRF-8 constructs and HA-ubiquitin showed that the IRF-8 truncation mutants were not ubiquitinated (Fig. 5C). The stability and lack of ubiquitination of IRF-8 carboxyl-terminal truncation mutants indicate that elements in this domain are required for E3 recognition and targeting for proteasome.

Dominant Negative Ubiquitin Mutants Block IRF-8 Degradation—Although ubiquitin Lys-48 is the primary site of isopeptide polyubiquitination required to target protein for degradation (31), polyubiquitin chains linked through Lys-29 have also been identified both in cells and in vitro and also target proteins for degradation (32). In contrast, ubiquitin Lys-63 is involved in endosomal trafficking and in activation of TLR pathways. K48R and K29R ubiquitin mutants act as ubiquitin dominant negatives, resulting in premature termination of ubiquitin chains and inhibition of degradation by the proteasome. We have shown that degradation of IRF-8 is proteasome-dependent, whereas IRF-4 is not ubiquitinated proteasome-independent. To confirm that ubiquitination is required for degradation, we co-transfected into 293T cells IRF-8 and IRF-4 together with ubiquitin mutants K48R, K29R, and K63R. Co-transfection of K48R or K29R ubiquitin mutants resulted in higher expression of IRF-8 but had no effect on IRF-4 (Fig. 6). There was no change of glyceraldehyde-3-phosphate dehydrogenase, which is degraded by the lysosomal pathway. These data confirm that ubiquitination of IRF-8 is required for degradation.

Cbl Is an E3 Ligase That Targets IRF-8 Degradation—Members of the Cbl family are ubiquitin ligases that function as negative regulators of activated tyrosine kinase-coupled receptors. Cbl proteins contain, in addition to a typical E3 ring domain, a conserved amino-terminal phosphotyrosine-binding PTB domain, which targets the ubiquitination of activated protein tyrosine kinases and other phosphotyrosine-containing proteins (33, 34). Because it has been reported that IRF-8 is constitutively tyrosine-phosphorylated in vivo (17), we speculated that Cbl may also be involved in IRF-8 degradation. We first confirmed that Cbl was expressed in macrophages and 293T cells by immunoblotting (Fig. 7A). Co-transfection of IRF-8 and Cbl dominant
negative plasmids into 293T cells resulted in increased expression of IRF-8 as predicted if Cbl were the E3 ligase mediating ubiquitination of IRF-8 (Fig. 7B). If Cbl is involved in the degradation of IRF-8, there should be direct physical interaction between IRF-8 and Cbl. To test this, we co-transfected FLAG-tagged IRF-8 or IRF-8 carboxyl-terminal deletion mutants (IRF-8-(1–390) and IRF-8-(1–356)) with Cbl into 293T cells. Immunoprecipitation of FLAG-IRF-8 from cell lysates resulted in co-precipitation of Cbl, demonstrating that a complex was formed between the two proteins. However, neither IRF-8 carboxyl-terminal truncation mutant interacted with Cbl (Fig. 7C).

To demonstrate the importance of Cbl in IRF-8 degradation, we co-transduced retrovirus-encoded IRF-8 and HA-ubiquitin into Cbl+/+ and Cbl−/− fibroblasts. After 2 days, the cells were treated with or without MG132 (10 μM) for 4 h, and whole cell lysates were then immunoprecipitated with an anti-IRF-8 antibody and immunoblotted with anti-HA antibody (B). RAW264.7 cells were incubated for 48 h after co-transduction with retrovirus encoding IRF-8 and HA-ubiquitin, and cell lysates (500 μg) were immunoprecipitated with anti-IRF-8 antibody and immunoblotted with anti-HA mAb (C).

Fig. 3. Ubiquitination of IRF-8 in RAW264.7 murine macrophages. RAW264.7 cells were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 6 h, cycloheximide (10 μg/ml) was added for 30 min, and the cells were incubated with fresh medium in the presence or absence of MG132 (10 μM). Cell lysates were analyzed for IRF-8 and β-actin (A). Cell lysates (500 μg) were immunoprecipitated with anti-IRF-8 antibody and immunoblotted with anti-ubiquitin antibody (B). RAW264.7 cells were incubated for 48 h after co-transduction with retrovirus encoding IRF-8 and HA-ubiquitin, and cell lysates (500 μg) were immunoprecipitated with anti-IRF-8 antibody and immunoblotted with anti-HA mAb (C).

Fig. 4. Ubiquitination of IRF-8 in 293T cells. 293T cells were transfected for 36 h with IRF-8 or IRF-4 plasmids. Cells were then pretreated with cycloheximide (10 μg/ml) for 30 min and incubated in the presence or absence of MG132 (10 μM) as indicated. Cell lysates were analyzed by immunoblotting for IRF-8 and IRF-4 (A). 293T cells were co-transfected for 48 h with IRF-8 and HA-ubiquitin. Cell lysate (500 μg) was immunoprecipitated with anti-IRF-8 antibody and immunoblotted with anti-HA mAb (B).
hypothesis that the degradation of IRF-8 affects IL-12 expression. To approach this question, RAW 264.7 cells were co-transfected for 30 h with an IL-12 p40 promoter reporter and IRF-8 or IRF-8 truncation mutants with anti-FLAG mAb (B). RAW 264.7 cells were co-transfected with IL-12 p40 promoter reporter and IRF-8 plasmids for 30 h. IL-12 p40 promoter activity was significantly up-regulated in Cbl−/− cells compared with Cbl+/+ cells (Fig. 9B). In addition, we co-transduced into RAW264.7 cells retrovirus-encoded IRF-8 and dominant negative Cbl or green fluorescent protein as a control and found IL-12 p40 protein production was significantly enhanced in cells co-transduced with IRF-8 and dominant negative Cbl after activation with IFN-γ and LPS for 24 h (Fig. 9C). To confirm these experiments, we stimulated peritoneal macrophages from both wild type and Cbl−/− mice, and the data showed that IL-12 p40 production was significantly higher in Cbl−/− cells (Fig. 9D). Taken together, the results suggest IRF-8 degradation mediated by Cbl down-regulates IL-12 expression.

DISCUSSION

This study describes the relationship between the degradation of IRF-8 and IL-12 gene expression. We have shown that the proteasome inhibitor MG132 blocks the rapid degradation of IRF-8 in primary macrophages, macrophage cell lines, and 293T cells. Ubiquitination of IRF-8 was demonstrated by retroviral co-transduction of RAW 264.7 cells with IRF-8 and HA-ubiquitin. Furthermore, dominant negative ubiquitin mutants K48R and K29R clearly increase the expression of IRF-8, showing a physical interaction. In contrast to IRF-8, carboxyl-terminal truncations of IRF-8 are more stable and are not ubiquitinated, and they fail to interact with Cbl, indicating the importance of carboxyl-terminal domain. Cbl is an E3 ligase that interacts with IRF-8, and IRF-8 ubiquitination is significantly attenuated in Cbl−/− cells. The stable carboxyl-terminal truncation mutant more strongly induces IL-12 p40 promoter activation than IRF-8, and similarly, IRF-8-induced IL-12 p40 promoter activity is stronger in Cbl−/− cells than in Cbl+/+ cells. Co-transduction of dominant negative Cbl significantly increased IL-12 p40 protein production induced by IFN-γ and LPS. Furthermore, macrophages from Cbl−/− mice have
enhanced IL-12 p40 production compared with wild-type mice. These results indicate that the degradation of IRF-8 mediated by Cbl down-regulates IL-12 expression, suggesting a novel mechanism for the regulation of cytokine gene expression by the ubiquitin-proteasome pathway.

IRF-8 is a transcription factor that plays an important role in the immune system (5). The IRF-8 promoter has both a GAS sequence, induced by IFN-γ, and a nuclear factor-κB site, induced by LPS (10). Interaction of these two sites accounts for the synergistic induction of IRF-8 by IFN-γ and LPS in macrophages. In vivo experiments showed that IRF-8−/− mice are deficient in synthesis of IL-12 and IFN-γ and, as a consequence, are unable to mount a Th1-mediated immune response (20, 35). Additional studies revealed that IRF-8 binds to the IFN-γ-stimulated response element site of the IL-12 promoter and is directly involved in the activation of IL-12 p40 gene expression (18, 37). In addition, IRF-8 controls other proteins important in inflammation, such as inducible nitric-oxide synthase, IL-18, and IL-1 (11, 38, 39). Although the Th1 immune response is important for host defense against microbial pathogens, an unregulated Th1 immune response with enhanced production of Th1 cytokines is found in many autoimmune or inflammatory diseases. Therefore, the balance between the synthesis and degradation of IRF-8 is important to the host.

Like IRF-8, we also found that IRF-1 was degraded rapidly, consistent with a recent report that rapid degradation of IRF-1 protein in COS7 cells was inhibited by MG132 (30). IRF-3, another IRF family member, which regulates the expression of the interferon-β gene, is also degraded by the proteasome pathway, but only when phosphorylation on serine and threonine residues has been induced by virus infection (40). Therefore, it appears that the proteasome pathway is vital for the degradation of several IRF family members. Interestingly, IRF-4, another member of IRF family that is homologous to IRF-8, was quite stable in macrophages and 293T cells. The reasons for the differences in protein stability among IRF family members are not clear.

Targeting of cellular proteins for proteolysis by the proteasome is a highly complex and tightly regulated process (31). Ubiquitin, an evolutionarily conserved protein of 76 residues, has diverse cellular functions, including marking proteins for degradation by the proteasome or trafficking to the lysosomal compartment and activation of signaling pathways. However, because some non-ubiquitinated proteins are degraded by the proteasome, and in other cases ubiquitination serves other functions than as a degradation signal, ubiquitination alone does not allow one to conclude that a protein is degraded by the proteasome. In the present study, we show that the dominant negative ubiquitin mutants K48R and K29R significantly increase the stability of IRF-8, confirming a link between ubiquitination and degradation of IRF-8.

Several degradation signals in protein substrates have been implicated to control the ubiquitin-proteasome pathway (43), including PEST sequences, degradation signals of a hydrophobic nature, phosphorylation-dependent signals, and the carboxyl-terminal sequence (44–47). The IRF-1 carboxyl-terminal 39 residues are thought to control the stability of IRF-1 protein, and the hydrophobic nature of the carboxyl-terminal of IRF-1 was proposed as a degradation signal (30). Phosphorylation of IRF-3 carboxyl-terminal region is a signal for its subsequent degradation, implying that this carboxyl-terminal region may function as the phosphorylation-dependent degradation signal recognized by an E3 ligase complex (40). Our experiments using the IRF-8 carboxyl-terminal truncated mutants indicate that the carboxyl-terminal 390–424 residues of IRF-8 are required for ubiquitination and degradation. The carboxyl-terminal region does not have typical PEST sequences but is hydrophobic, in comparison with other regions of IRF-8.

Our results show that Cbl is an E3 ligase for IRF-8. The RING finger domain and phosphotyrosine-binding domain (PTB) of Cbl are required for ubiquitination in vitro and in vivo (48). Biochem-
ical mutational analysis and crystal structure data demonstrate that the RING finger mediates binding to E2 enzymes. We find that the residues 390–424 at the carboxyl terminus of IRF-8 are required both for physical interaction with Cbl and for degradation, but this sequence does not contain any tyrosine residues. However, there is a tyrosine residue that is very close to the carboxyl terminus that might serve as a PTB docking site in conjunction with more carboxyl-terminal residues.

Although our data showed that Cbl is involved in IRF-8 degradation, the fact that IRF-8 is still degraded in cCbl-deficient macrophages suggests that some other E3 ligases may also contribute to the control of IRF-8 degradation. Our future study will address two issues: 1) identification of other E3 ligases responsible for IRF-8 stability, and 2) detailed dissection of Cbl in the control of protein stability of other IRF family members including IRF-1.

The IL-12 p40 gene, reflecting its importance in the generation of a Th1 versus a Th2 immune response, is subject to complex transcriptional regulation by multiple transcription factors. Nuclear factor-κB, CAAT/enhancer-binding protein, AP-1, IRF-1, and IRF-8 have all been reported as important activators of IL-12 p40 transcription (49–53). Although these studies suggest that these transcription factors are activators of IL-12 p40 gene, how the degradation of transcription factors affects IL-12 p40 promoter activation is incompletely understood. In the present study, we found that an IRF-8 carboxyl-terminal truncation mutant much more strongly activates IL-12 p40 promoter and that a dominant negative Cbl mutant significantly enhanced IRF-8-induced IL-12 p40 promoter activation. Furthermore, IRF-8-induced IL-12 p40 promoter activation was up-regulated in Cbl−/− cells in contrast to Cbl+/+ cells, and co-transduction of dominant negative Cbl significantly enhanced IRF-8-induced IL-12 p40 production. These results indicate that the ubiquitin-proteasome pathway is involved in the regulation of IL-12 gene expression, suggesting that balanced control of transcription factor expression level is an important mechanism for the modulation of target gene expression.

Regulated protein degradation is critical for many cellular processes, including control of the cell cycle and inflammation. Deeper understanding of the mechanism for IRF-8 degradation may facilitate the modulation of this transcription factor, which is central to a competent Th1 immune response. Recent studies revealed that IRF-1 and IRF-3 are subject to proteasomal degradation, implying conservation of the signals controlling degradation among various members of the IRF family. A future study will address identification and characterization of the specific ubiquitin-conjugating enzymes involved in the degradation of IRF family members, which will help understand the consequence of degradation of transcription factors in immune responses.
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REFERENCES

1. Nguyen, H., Hiscott, J., and Pitha, P. M. (1997) Cytokine Growth Factor Rev. 8, 293–312
2. Barnes, B., Lubyova, B., and Pitha, P. M. (2002) J. Interferon Cytokine Res. 22, 59–71
3. Nehyba, J., Hrdlickova, R., Burnside, J., and Bose, H. R., Jr. (2002) Mol. Cell. Biol. 22, 3942–3957
4. Veals, S. A., Schindler, C., Leonard, D., Fu, X. Y., Aebersold, R., Darnell, J. E., Jr., and Levy, D. E. (1992) Mol. Cell. Biol. 12, 3315–3324
5. Tamura, T., and Ozato, K. (2002) J. Interferon Cytokine Res. 22, 145–152
6. Weisz, A., Marx, P., Sharf, R., Appella, E., Driggers, P. H., Ozato, K., and Levi, B. Z. (1992) J. Biol. Chem. 267, 25589–25596
7. Politis, A. D., Sivo, J., Driggers, P. H., Ozato, K., and Vogel, S. N. (1992) J. Immunol. 148, 801–807
8. Nelson, N., Kanno, Y., Hong, C., Contursi, C., Fujita, T., Fowkes, B. J., O’Connell, E., Hu-Li, J., Paul, W. E., Jankovic, D., Sher, A. F., Coligan, J. E., Thornton, A., Appella, E., Yang, Y., and Ozato, K. (1996) J. Immunol. 156, 3711–3720
9. Kantakamalakul, W., Politis, A. D., Marecki, S., Sullivan, T., Ozato, K., Fenton, M. J., and Vogel, S. N. (1999) J. Immunol. 162, 7417–7425
10. Kanno, Y., Kosak, C. A., Schindler, C., Driggers, P. H., Ennist, D. L., Gleason, S. L., Darnell, J. E., Jr., and Ozato, K. (1993) Mol. Cell. Biol. 13, 3951–3963
11. Xiong, T., Zhu, C., Li, H., Chen, F., Mayer, L., Ozato, K., Ukeleis, J. C., and Plevy, S. E. (2003) J. Biol. Chem. 278, 2271–2277
12. Egwuagu, C. E., Sztein, J., Chan, C. C., Mahdi, R., Nussenblatt, R. B., and Chepelnisky, A. B. (1994) Dev. Biol. 166, 557–568
13. Li, W., Nagineni, C. N., Ge, H., Effros, B., Chepelninsky, A. B., and Egwuagu, C. E. (2002) J. Biol. Chem. 274, 9686–9691
14. Li, W., Nagineni, C. N., Effros, B., Chepelninsky, A. B., and Egwuagu, C. E. (1999) Dev. Biol. 210, 44–55
15. Nelson, N., Marks, M. S., Driggers, P. H., and Ozato, K. (1993) Mol. Cell. Biol. 13, 588–599
16. Weisz, A., Kirchhoff, S., and Levi, B. Z. (1994) Int. Immunol. 6, 1125–1131
17. Sharf, R., Meraro, D., Arzil, A., Thornton, A. M., Ozato, K., Petricoin, E. F., Larner, A. C., Schaper, F., Hauser, H., and Levi, B. Z. (1997) J. Biol. Chem. 272, 9785–9792
18. Masumi, A., Tamaoki, S., Wang, I. M., Ozato, K., and Komuro, K. (2002) PLoS Med. 13, 348–353
19. Trinchieri, G., and Scott, P. (1995) Res. Immunol. 146, 423–431
20. Sharrot, Kensten, T., Contursi, C., Masumi, A., Sher, A., and Ozato, K. (1997) J. Exp. Med. 186, 1523–1534
21. Lahohf, M., Perrick, D., Mittrucker, H. W., Duncan, G. S., Bischof, S., Röhlinghoff, M., and Mak, T. W. (1997) Immunity 6, 681–689
22. Taniguchi, T., Ogasawara, K., Takaoka, A., and Tanaka, N. (2001) Annu. Rev. Immunol. 19, 623–653
23. Neurath, M. P., Pinotto, S., and Glimcher, L. H. (2002) Nat. Med. 8, 567–573

FIG. 9. Degradation of IRF-8 regulates IL-12 expression. A. IL-12 p40 reporter plasmid was co-transfected into RAW264.7 cells with IRF-8 or IRF-8 carboxyl-terminal truncation mutant IRF-8-(1–390), and luciferase activity was determined 30 h after transfection. B. IL-12 p40 promoter reporter was co-transfected into Cbl+/+ and Cbl−/− fibroblasts with IRF-8 or vector plasmids for 30 h. Cell lysates were collected for the determination of luciferase activity. C. RAW264.7 cells were co-transduced with retrovirus-encoded IRF-8, dominant negative Cbl or green fluorescent protein for 2 days. Cells were then activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 24 h. The supernatants were collected, and IL-12 p40 production was determined by enzyme-linked immunosorbent assay. D. peritoneal macrophages from wild type and Cbl−/− mice were activated with IFN-γ (10 ng/ml) and LPS (1 μg/ml) for 24 h. The supernatants were collected for determination of IL-12 p40 protein production.
24. Rosloniec, E. F., Latham, K., and Guedez, Y. B. (2002) *Arthritis Res.* 4, 333–336
25. Abbas, A. K., Murphy, K. M., and Sher, A. (1996) *Nature* 383, 787–793
26. Sher, A., and Coffman, R. L. (1992) *Annu. Rev. Immunol.* 10, 385–409
27. Kuchroo, V. K., Das, M. P., Brown, J. A., Ranger, A. M., Zamvil, S. S., Sobel, R. A., Weiner, H. L., Nabavi, N., and Glimcher, L. H. (1995) *Cell* 80, 707–718
28. Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) *Cell* 14, 725–731
29. Pickl, W. F., Pimentel-Muinos, F. X., and Seed, B. (2001) *J. Virol.* 75, 7175–7183
30. Nakagawa, K., and Yokosawa, H. (2000) *Eur. J. Biochem.* 267, 1680–1686
31. Ciechanover, A., and Schwartz, A. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 2727–2730
32. Koege, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999) *Cell* 96, 635–644
33. Joazeiro, C. A., Wing, S. S., Huang, H., Leveron, J. D., Hunter, T., and Liu, Y. C. (1999) *Science* 286, 309–312
34. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) *Cell* 102, 533–539
35. Hofschke, T., Lohler, J., Kanno, Y., Fehr, T., Giese, N., Rosenbauer, F., Lou, J., Knobeloch, K. P., Gabriele, L., Waring, J. F., Bachmann, M. F., Zinkernagel, R. M., Morse, H. C., III, Ozato, K., and Horak, I. (1996) *Cell* 87, 307–317
36. Giese, N. A., Gabriele, L., Doherty, T. M., Tadesse-Heath, L., Contursi, C., Epstein, S. L., and Morse, H. C., III. (1997) *J. Exp. Med.* 186, 1535–1546
37. Wang, I. M., Contursi, C., Masumi, A., Ma, X., Trinchieri, G., and Ozato, K. (2000) *J. Immunol.* 165, 271–279
38. Kim, Y. M., Im, J. Y., Han, S. H., Kang, H. S., and Choi, I. (2000) *J. Immunol.* 165, 3198–3205
39. Marecki, S., Riedeau, C. J., Liang, M. D., and Fenton, M. J. (2001) *J. Immunol.* 166, 6829–6838
40. Lin, R., Heybroeck, C., Pitha, P. M., and Hiscott, J. (1998) *Mol. Cell. Biol.* 18, 2986–2996
41. Verma, R., and Deshaies, R. J. (2000) *Cell* 101, 341–344
42. Pickart, C. M. (2001) *Mol. Cell* 8, 499–504
43. Laney, J. D., and Hochstrasser, M. (1999) *Cell* 97, 427–430
44. Rogers, S., Wells, R., and Rechsteiner, M. (1996) *Science* 234, 364–368
45. Johnson, P. R., Swanson, R., Rakshitina, L., and Hochstrasser, M. (1998) *Cell* 94, 217–227
46. Lawson, T. G., Gronros, D. L., Evans, P. E., Bastien, M. C., Michalewich, K. M., Clark, J. K., Edmonds, J. H., Graber, L. H., Werner, J. A., Lurvey, B. A., and Cate, J. M. (1999) *J. Biol. Chem.* 274, 9994–9980
47. Mitsui, A., and Sharp, P. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6054–6059
48. Waterman, H., and Yarden, Y. (2001) *FEBS Lett.* 490, 142–152
49. Murphy, T. L., Cleveland, M. G., Kolessa, P., Magram, J., and Murphy, K. M. (1990) *Mol. Cell. Biol.* 10, 5258–5267
50. Plevy, S. E., Gemberling, J. H., Hsu, S., Dorner, A. J., and Smale, S. T. (1997) *Mol. Cell. Biol.* 17, 4572–4588
51. Zhu, C., Gagnidze, K., Gemberling, J. H., and Plevy, S. E. (2001) *J. Biol. Chem.* 276, 18519–18528
52. Maruyama, S., Sumita, K., Shen, H., Kanoh, M., Xu, X., Sato, M., Matsumoto, M., Shinomura, H., and Asano, Y. (2003) *J. Immunol.* 170, 997–1001
53. Zhu, C., Rao, K., Xieng, H., Gagnidze, K., Li, F., Horvath, C., and Plevy, S. (2003) *J. Biol. Chem.* 278, 39372–39382