The Tyrosine Kinase c-Abl Protects c-Jun from Ubiquitination-mediated Degradation in T Cells

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The cross-talk of ubiquitination with other types of posttranscriptional modifications, such as phosphorylation, regulates the stability of many proteins. We have previously demonstrated that c-Jun is a substrate of Itch, a HECT-type E3 ubiquitin ligase. c-Jun is also a substrate of the tyrosine kinase c-Abl. Here we report that genetic ablation of c-Abl accelerated c-Jun degradation. Phosphorylation of the tyrosine within the PPX motif by c-Abl inhibited c-Jun ubiquitination and its binding by Itch. The nuclear localization of c-Abl, triggered by T-cell activation signals, was essential for its activity in regulating c-Jun transcription activity. These findings define a potential molecular mechanism for the immunodeficiency in mice lacking the c-abl gene.

Many proteins can be modified by several different posttranslational modifications (1). The effects of different modifications on each other provide potential mechanisms for integration of stimuli from different signal transduction pathways. c-Jun is a substrate for ubiquitination by the HECT (homologous of E6 C terminus)-type E3 ubiquitin ligase, Itch, which regulates the stability and therefore the steady-state level of c-Jun in the cell (2, 3). Protein ubiquitination requires a cascade of at least three different enzymatic reactions. First, ubiquitin is activated by E1, the ubiquitin-activating enzyme. Next, ubiquitin is transiently transferred to E2, the ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase transfers the activated ubiquitin molecule from the E2 to a lysine residue on the substrate (4). Several different E3 ubiquitin ligases have been shown to contribute to c-Jun ubiquitination (3, 5, 6). In neurons the stability of c-Jun is regulated by the E3 ligase SCF^{Itch}, which ubiquinates c-Jun and facilitates c-Jun degradation. SCF^{Itch}-induced c-Jun ubiquitination is regulated by JNK-mediated serine/threonine phosphorylation of c-Jun (7). We previously found that c-Jun is ubiquitinated by Itch (3, 5). c-Jun is also a substrate for phosphorylation by the tyrosine kinase c-Abl (8). Tyrosine phosphorylation of c-Jun up-regulates the activations of both JNK and c-Abl in the nucleus. The site of c-Jun phosphorylation on tyrosine 170 occurs within the PPXY recognition motif for Itch. This suggested the possibility that phosphorylation by c-Abl may regulate c-Jun ubiquitination by Itch.

c-Abl is a ubiquitously expressed nonreceptor tyrosine kinase. Numerous studies have demonstrated that c-Abl is responsive both to extracellular signals, such as growth factors, cell adhesion, and cytokines, and internal signals, such as DNA damage and oxidative stress, in regulating cell proliferation, differentiation, and apoptosis (9, 10). The c-Abl protein is comprised of multiple domains including Src homology (SH) 3, SH2, and SH1 (catalytic) domains, proline-rich sequences that bind SH3-containing molecules, nuclear localization and export signals, and DNA and actin binding domains (10–12). The c-Abl kinase localizes to the cytosol, membrane, cytoskeleton, and nucleus. Both the localization and kinase activity of c-Abl are regulated within the cell (10, 13). Nuclear localization of c-Abl plays important roles in the regulation of growth, apoptosis, and DNA repair. These functions of nuclear c-Abl require its cross-talk with many nuclear proteins, such as the ataxia telangiectasia gene product, the RB, and p53 (14, 15).

It has been implicated that c-Abl might play a role in T-cell receptor (TCR) signaling because targeted disruption of the Abl1 gene in mice resulted in animals that had splenic and thymic atrophy and cell-autonomous lymphopenia (16, 17). Zipfel et al. (18) recently reported that c-Abl may play a role in T-cell activation, possibly by targeting Zap70 and the transmembrane adaptor linker for activation of T cells, providing the first evidence that cytoplasmic c-Abl is involved in TCR signaling. However, the roles of nuclear c-Abl in the modulation of immune functions remain to be defined.

Here we report that phosphorylation of c-Jun at tyrosine 170 by c-Abl kinase inhibited Itch binding to c-Jun and protected c-Jun from Itch-induced ubiquitination and degradation. Conversely, c-Abl deficiency accelerated c-Jun degradation and suppressed T-cell activation. Ablation of the Itch gene and inhibition of Itch activity abrogated the effects of c-Abl on c-Jun ubiquitination and T-cell activation. Expression of an Itch mutant lacking E3 ubiquitin ligase activity stabilized c-Jun in c-Abl-deficient T cells and reversed T-cell activation.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Plasmids, and Mice**—Anti-Xpress was purchased from Roche (Nutley, NJ), anti-HA and anti-Myc were from Sigma (St. Louis, MO), Anti-GFP was purchased from Invitrogen (Carlsbad, CA), Anti-FLAG was purchased from Sigma. Anti-Xpress was purchased from Roche (Nutley, NJ), anti-HA and anti-Myc were from Sigma. Anti-GFP was purchased from Invitrogen (Carlsbad, CA), Anti-FLAG was purchased from Sigma.

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**References**

1. The abbreviations used are: JNK, c-Jun N-terminal kinase; HECT, homologous of E6 C terminus; TCR, T-cell receptor; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; SH, Src homology; MEK, mouse embryonic fibroblast; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.

2. The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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4. The abbreviations used are: JNK, c-Jun N-terminal kinase; HECT, homologous of E6 C terminus; TCR, T-cell receptor; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; IL-2, interleukin 2; GST, glutathione S-transferase; GFP, green fluorescent protein; HA, hemagglutinin; SH, Src homology; MEK, mouse embryonic fibroblast; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.
Santa Cruz Biotechnology) (Santa Cruz, CA), anti-GST, anti-Itch, anti-phospho-tyrosine (4G10), anti-CD3, and anti-CD28 were from BD Biosciences. pEF-Jun, pEF-Itch, and pMIG-Itch/CA plasmids were constructed as reported earlier (2). c-Abl and its mutant expression plasmids were kind gifts from Giulio Superti-Furga (European Molecular Biology Laboratory, Heidelberg, Germany) (8). Mice heterozygous for c-Abl were a generous gift from Steve Goff (Columbia University, New York, NY) (16), and Itch−/− MEF cells (19) were generated in Yun-Cai Liu’s laboratory (La Jolla Institute for Allergy and Immunology, San Diego, CA).

**Transfection, Immunoprecipitation, and Western Blotting**—Transient transfections were performed by using Lipofectamine (Invitrogen) according to the manufacturer’s instructions, with 2–5 µg of total DNA/transfection. Transfected cells were pelleted and resuspended in 1× Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM NaPiP, 2 mM Na3VO4, and 10 µg/ml each of apro tinin and leupeptin). Cells were lysed for 10 min at 4 °C, and insoluble materials were removed by centrifugation at 15,000 × g (4 °C, 10 min). For immunoprecipitation, lysates (−1 × 107 cells) were mixed with antibodies (1 µg) for 2 h, followed by the addition of 30 µl of protein G-Sepharose beads (Santa Cruz Biotechnology) for an additional 2 h at 4 °C. Immunoprecipitates were washed four times with 1× Nonidet P-40 lysis buffer and boiled in 20 µl of 2X Laemmli’s buffer. Samples were subjected to 8 or 10% SDS-polyacrylamide gel electrophoresis analysis and electrotransferred onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with the indicated primary antibodies (usually 1 µg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with an enhanced chemiluminescence detection system (ECL; Amersham Biosciences). When necessary, membranes were stripped by incubation in stripping buffer (62.5 mM Tris-HCl, pH 5.7, 100 mM 2-mercaptoethanol, and 2% SDS) for 1 h at 70 °C with constant agitation, washed, and then reprobed with other antibodies as indicated.

**GST Fusion Protein Pulldown Assays**—GST pulldown assay was performed as described previously (2). GST-ItchWW was bound to glutathione-agarose beads (Sigma) and incubated for 2 h with lysates of transfected HEK293 cells. Beads were washed three times in GST wash buffer (250 mM NaCl, 10 mM Tris, pH 7.5, 1 mM dithiothreitol) and analyzed by immunoblotting.

**Primary T-cell Proliferation and IL-2 Production**—Primary T cells were isolated from the lymph nodes of 4–5-week-old Abl+/+ and Abl−/− mice. T cells were left unstimulated or stimulated with anti-CD3, anti-CD28, or both for various time periods. To determine cell proliferation, primary T cells (1 × 106 cells/well) were stimulated with anti-CD3, anti-CD28, or both for 24 h, pulsed with 1 µCi/ml of [3H]thymidine, and incubated for an additional 12 h. [3H]Thymidine incorporation was measured with a scintillation counter. For measurement of cytokine production, culture supernatants from the stimulated cells described above were collected and the concentrations of IL-2 were analyzed by ELISA. For the gene delivery to mouse primary T cells by retroviral infection, virus was produced by transfection of pMIG-Itch/CA plasmids into a packaging cell line, Plat-E (20); the supernatant was used to incubate with mouse primary T cells in the presence of anti-CD3 plus anti-CD28.

**Statistical Analysis**—All values are presented as the mean ± S.D. Statistical analysis was performed with Student’s t test using SPSS software. p < 0.05 was considered as significant.

**RESULTS**

**c-Jun Degradation Is Affected by c-Abl Expression**—c-Jun contains a PXFY consensus recognition sequence for the E3 ligase Itch. The tyrosine residue in this sequence is essential for Itch binding and ubiquitination of c-Jun (2, 3). The same tyrosine residue is a site for phosphorylation by c-Abl (8). We therefore tested the hypothesis that phosphorylation of this tyrosine by c-Abl affected c-Jun ubiquitination by Itch and the rate of c-Jun degradation. To investigate the potential influence of c-Abl on c-Jun stability, we compared the rates of c-Jun degradation in the primary T cells from wild-type mice and from mice lacking the gene encoding c-Abl (Fig. 1A). The cells were activated by treatment with anti-CD3 and anti-CD28, and the rate of c-Jun turnover was measured by addition of cycloheximide following stimulation. In wild-type primary T cells, c-Jun was degraded with a half-life of 1.6 ± 0.3 h, consistent with previous results (2). In cells lacking c-Abl, c-Jun degradation was accelerated almost 2-fold, resulting in a half-life of 0.9 ± 0.2 h (p < 0.05). As a control, the degradation of the transcription factor NF-κB was comparable between the wild-type and c-Abl−/− T cells (p > 0.1). These results indicate that the loss of c-Abl destabilizes c-Jun in primary T cells. We also found that c-Abl was degraded during T-cell activation with a half-life of 1.1 h, indicating that c-Abl may also be degraded during T-cell activation. In support of our data, previous study has demonstrated that c-Abl is a substrate for ubiquitination-mediated degradation (21).

To determine whether the difference in c-Jun degradation in these cells was due to the change in c-Abl activity, we examined the effects of c-Abl-PP, a double proline mutation (P242E,P249E) that confers constitutive activity to c-Abl, and the kinase-dead mutation of c-Abl (c-Abl/KM) on the degradation of co-expressed c-Jun in HEK293T cells (Fig. 1B). The half-life of c-Jun was extended from 1.1 ± 0.3 h to 3.2 ± 0.7 h by overexpression of constitutively active c-Abl (p < 0.05 against control). In contrast, expression of dominant negative c-Abl reduced the half-life of c-Jun 2-fold to 0.6 ± 0.1 h (p < 0.01 against control). Thus, manipulations of c-Abl activity in HEK293 cells changed the half-life of c-Jun in a manner consistent with the change observed in primary T cells lacking the c-Abl gene.

There are numerous pathways whereby changes in c-Abl activity might affect c-Jun degradation. To investigate whether the changes in the rate of c-Jun degradation caused by manipulations of c-Abl activity required the tyrosine residue in c-Jun that can be phosphorylated by c-Abl and that is required for recognition by Itch, we examined the effects of the same manipulations on the stability of c-Jun in which this tyrosine was substituted by a phenylalanine (c-JunY170F). The mutated c-Jun protein exhibited almost 3-fold slower degradation than
expression of constitutively active c-Abl partially inhibited c-Jun ubiquitination. In contrast, co-expression of dominant negative c-Abl dramatically increased the amount of high molecular weight forms of Jun (Fig. 2A). The effects of c-Abl activity on the rate of c-Jun degradation were therefore likely caused by inhibition of c-Jun ubiquitination.

Several different E3 ligases have been identified that can mediate c-Jun ubiquitination (3, 5, 6). The E3 ligase Itch recognizes a PPXY motif overlapping the site of c-Jun phosphorylation by c-Abl. We tested whether the changes in c-Jun ubiquitination caused by manipulations of c-Abl activity were mediated by Itch by comparing the effects of co-expression of constitutively active and dominant negative c-Abl in MEFS from wild-type mice and from mice lacking the gene encoding Itch (Fig. 2B). In wild-type MEFS, the expression of constitutively active c-Abl inhibited c-Jun ubiquitination, whereas expression of dominant negative c-Abl enhanced c-Jun ubiquitination, similar to the results obtained in HEK293 cells. In contrast, in MEFS lacking the gene encoding Itch, there was no detectable effect of the expression of either constitutively active or dominant negative c-Abl on the efficiency of c-Jun ubiquitination. Itch was therefore required for the changes in c-Jun ubiquitination induced by manipulations of c-Abl activity.

Recently, it has been reported that another member of Src kinases, Fyn, negatively regulates the E3 ligase activity of Itch via a direct interaction and phosphorylation of Itch (22). To test whether c-Abl inhibits Itch-mediated c-Jun ubiquitination via a similar mechanism, we examined whether c-Abl can also induce the tyrosine phosphorylation of Itch. Itch tyrosine phosphorylation was detected in HEK293 cells; overexpression of c-Abl in HEK293 cells dramatically increased the amount of high molecular weight forms of Jun (Fig. 2A). The effects of c-Abl activity on the rate of c-Jun degradation were therefore likely caused by inhibition of c-Jun ubiquitination.

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FIGURE 1. Analysis of the effect of Abl on c-Jun protein stability. A, primary T cells were isolated from Abl+/+ or Abl−/− mice, cells were stimulated with anti-CD3 plus anti-CD28, and the activated T cells were cultured in the presence of 50 μg/ml cycloheximide for different amounts of time. The protein stability of c-Jun, NF-κB, and c-Jun were analyzed by Western blotting. The protein expression level of Actin was blotted as a control (bottom lane). B, the density of each band was measured by phosphorimaging software, and the half-life of c-Jun was calculated as reported previously (18). The data represent the mean ± S.D. from three independent experiments. C, Xpress-tagged c-Jun (left panel) or Jun/Y170F (right panel) expression plasmids were transfected without or with Abl-PP or Abl/KM. Transfected cells were treated with cycloheximide (50 μg/ml) for different amounts of time as indicated. The protein levels of c-Jun were detected with anti-Xpress antibody that reflects c-Jun ubiquitination. An antibody. The half-lives of c-Jun and its Y170F mutant were calculated, and the data represent the mean ± S.D. from three independent experiments. D, the density of each band was measured using phosphorimaging software, and the half-life of c-Jun and its Y170F mutant was calculated as reported previously (18). The data represent the mean ± S.D. from three independent experiments.

wild-type c-Jun (p < 0.05). Moreover, the expression of either the constitutively active or dominant negative c-Abl caused little change in the half-life of the c-Jun/Y170F protein (p > 0.1) (Fig. 1B). The effects of the manipulations of c-Abl activity on c-Jun degradation therefore required the tyrosine residue that is the site of c-Jun phosphorylation by c-Abl and a critical determinant of Itch recognition of c-Jun.

c-Abl Inhibits c-Jun Ubiquitination by Itch—We have previously demonstrated that ubiquitination plays a central role in c-Jun degradation (2, 3); c-Abl may therefore stabilize c-Jun by inhibiting c-Jun ubiquitination. We investigated whether the manipulations of c-Abl activity in HEK293 cells altered c-Jun ubiquitination. An in vivo ubiquitination assay was performed as described previously (2, 3). When Xpress-Jun expression plasmid was co-transfected with HA-tagged ubiquitin, higher molecular weight ladders were detected in the immunoprecipitates with anti-Xpress antibody that reflects c-Jun ubiquitination. Overexpression of Itch further enhanced c-Jun ubiquitination (supplemental Fig. S1). Those data further confirm our previous findings that Itch is an E3 ubiquitin ligase of c-Jun (2, 3). We then used this in a ubiquitination assay to determine the effect of c-Abl on Itch-mediated c-Jun ubiquitination. The co-expression of constitutively active c-Abl partially inhibited c-Jun ubiquitination. In contrast, co-expression of dominant negative c-Abl dramatically increased the amount of high molecular weight forms of Jun (Fig. 2A). The effects of c-Abl activity on the rate of c-Jun degradation were therefore likely caused by inhibition of c-Jun ubiquitination.

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c-Abl Inhibits c-Jun-Itch Interaction—To investigate the molecular mechanisms of c-Abl in protecting c-Jun from ubiquitination-induced degradation, we further tested the effects of c-Abl on the interaction of c-Jun with Itch. c-Jun was immunoprecipitated from cell lysates obtained from HEK293 cells that had been transfected with kinase-competent or kinase-inactive forms of c-Abl together with Itch and c-Jun. The interaction of Itch with c-Jun was determined by co-immunoprecipitation and Western blotting. As we demonstrated previously (2, 3),

expression of constitutively active c-Abl partially inhibited c-Jun ubiquitination. In contrast, co-expression of dominant negative c-Abl dramatically increased the amount of high molecular weight forms of Jun (Fig. 2A). The effects of c-Abl activity on the rate of c-Jun degradation were therefore likely caused by inhibition of c-Jun ubiquitination.

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Itch was detected in the anti-c-Jun immunoprecipitate (Fig. 3, lane 1), indicating that Itch interacts with c-Jun. Co-expression of c-Abl/KM further enhanced the interaction of Itch and c-Jun (lane 2), whereas c-Abl-PP inhibited their interaction (lane 3). Therefore, c-Abl inhibited c-Jun ubiquitination by suppressing the interaction of c-Jun with its E3 ubiquitin ligase Itch, and the inhibitory effect of c-Abl required its kinase activity.

c-Jun has been identified as a direct substrate of c-Abl tyrosine kinase (8), suggesting that this phosphorylation event may be required for c-Abl in the inhibition of c-Jun ubiquitination and c-Jun-Itch interaction. To test this, we first confirmed c-Abl-mediated tyrosine phosphorylation of c-Jun (8). c-Jun is phosphorylated at a very low level in HEK293 cells (Fig. 3B, lane 1), whereas overexpression of c-Abl slightly enhanced c-Jun phosphorylation (lane 2). In the presence of c-Abl/KM, the phosphorylation level of c-Jun is not detectable (lane 3), which is possibly due to the dominant negative effects of c-Abl/KM in competition with the endogenous c-Abl in c-Jun phosphorylation. Expression of the constitutively active form of c-Abl, c-Abl-PP, dramatically increased c-Jun phosphorylation (lane 4). Even in the presence of c-Abl-PP, mutation of the tyrosine 170 to phenylalanine of c-Jun, Jun/YF, completely abolished c-Jun tyrosine phosphorylation (lane 5), which indicated that tyrosine 170 is the phosphorylation site for c-Abl (8). These results further confirmed that c-Abl is a kinase for c-Jun phosphorylation, which suggests that suppression of c-Jun ubiquitination and its interaction with Itch by c-Abl requires phosphorylation of the tyrosine 170 of c-Jun.

We next employed a GST fusion protein pulldown assay to test whether these c-Jun proteins that interacted with Itch are phosphorylated. The GST pulldown was analyzed by SDS-PAGE and Western blotting using anti-c-Jun (Fig. 3C, top panel) and anti-p-Y antibodies (middle panel). c-Jun was pulled down by GST-ItchWW fusion protein but not GST-only controls (top panel). However, the tyrosine-phosphorylated c-Jun was detected in the anti-c-Jun immunoprecipitates but not in the precipitates of GST-ItchWW fusion (Fig. 3C, middle panel, lanes 1 and 3). These results indicate conclusively that the E3 ubiquitin ligase can only recognize non-phosphorylated c-Jun, which suggests c-Abl-mediated tyrosine phosphorylation inhibited the interaction of c-Jun with Itch.
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TCR Signaling Mediates the Nucleus Translocation of c-Abl in T Cells—Previous study demonstrated that the nuclear translocation of c-Abl is required for c-Jun phosphorylation (8). To determine the signals that regulate the nuclear translocation of c-Abl in T cells, we examined the effects of activation signals on the nuclear translocation of c-Abl in mouse primary T cells. Purified T cells were stimulated without or with anti-CD3 or anti-CD3 plus anti-CD28 for 2 h. Subcellular fractionation was then performed using these stimulated T cells. cAMP-response element-binding protein, a protein that localizes in the cytoplasm (24), was only detected in the cytoplasmic fraction (Fig. 4A). The protein levels of c-Abl in either cytoplasm or nucleus were analyzed by Western blotting. Very low level of c-Abl was detected in the nucleus from naïve T cells (Fig. 4A, lane 1). Stimulation of mouse primary T cells with anti-CD3 antibody for 2 h significantly accumulated c-Abl into nucleus (lane 2). The nucleus translocation of c-Abl was not further enhanced by anti-CD28 stimulation (lane 3). Thus, the nuclear translocation of c-Abl is triggered by TCR signaling in T cells.

To test whether the nuclear translocation of c-Abl induces the tyrosine phosphorylation of c-Jun, a protein that predomi-
nantly localizes in the nuclei (3), we immunoprecipitated c-Jun from mouse primary T cells after stimulation and detected its tyrosine-phosphorylated form with anti-phospho-tyrosine. As shown in Fig. 4B, the tyrosine phosphorylation of c-Jun was at very low level in naïve T cells from c-Abl+/− mice, and anti-CD3 stimulation significantly enhanced c-Jun phosphorylation. Consistent with the c-Abl nuclear translocation data, anti-CD28 did not further enhance c-Jun tyrosine phosphorylation. The tyrosine-phosphorylation of c-Jun in c-Abl−/− T cells was still detectable but significantly reduced. These results indicate that c-Abl is a kinase that mediates c-Jun tyrosine phosphorylation and TCR signaling is required for c-Abl-induced c-Jun tyrosine phosphorylation.

We next examined whether the T-cell activation signals affect the interaction of c-Jun with Itch. To test this hypothesis, we analyzed the interaction of c-Jun with Itch in wild-type and in c-Abl−/− T cells during T-cell activation. As shown in Fig. 4B, the interaction of c-Jun with Itch was comparable in naïve T cells between wild type and c-Abl−/−. Interestingly, when these T cells were stimulated with anti-CD3 or anti-CD3 plus anti-CD28, the interaction of c-Jun with Itch was reduced in c-Abl+/+ T cells, whereas it increased in c-Abl−/− T cells. Thus, c-Abl inhibited c-Jun ubiquitination by blocking Jun-Itch interaction during T-cell activation.

Furthermore, we found that c-Abl enhanced c-Jun transcription activity using luciferase assay. As shown in Fig. 4C, expression of the constitutively active form of c-Abl dramatically increased AP-1 reporter. The kinase mutant of c-Abl inhibited c-Jun transcription activity, probably because c-Abl/IK competes with endogenous c-Abl in activating c-Jun. Therefore, c-Abl is a positive regulator for c-Jun transcription activity. Importantly, sequestration of c-Abl-PP by mutation of its nuclear translocation sequence, Abl-PP-Cyt, failed in activating c-Jun. The kinase mutant of c-Abl inhibited c-Jun ubiquitination by blocking Jun-Itch interaction during T-cell activation.

c-Abl Regulates T-cell Activation through Itch—Previous studies have delineated that c-Abl is required for T-cell activation (25). To investigate the molecular mechanisms of c-Abl in regulating the immune functions, we first confirmed the effects of c-Abl deficiency on the activation of T cells. c-Abl-deficient
T cells showed a decreased cell proliferation and IL-2 production in response to either anti-CD3 or anti-CD3 plus anti-CD28 stimulation when compared with wild-type T cells (Fig. 5, A and B). The differences of IL-2 production from c-Abl–/– and wild-type T cells were greater when these T cells were cultured for longer times (supplemental Fig. S2). These results further confirmed that c-Abl is required for T-cell activation. Interestingly, c-Abl-deficient T cells showed slightly decreased IL-2 production in response to PMA and ionomycin stimulation, particularly when these T cells were stimulated with PMA plus ionomycin at low concentration (Fig. 5C). Because PMA directly activates protein kinase (27) and ionomycin forms a lipid-soluble calcium complex to convey Ca²⁺ across the hydrocarbon region of the cell membrane (28), PMA plus ionomycin stimulation activates T cells bypassing the upstream signal molecule. These results suggest that downstream signal molecules or transcription factors are involved in c-Abl-mediated T-cell activation, which further supports our finding here that c-Abl regulates T-cell activation by stabilizing c-Jun transcription factor.

Because c-Abl-mediated tyrosine phosphorylation of c-Jun inhibits Itch-induced c-Jun ubiquitination, we then asked whether inhibition of Itch E3 activity rescues T-cell activation of c-Abl–/– T cells. To this end, we infected both wild-type and c-Abl–/– T cells with retrovirus that carries a dominant negative mutant of Itch, Itch/CA (2). The retrovirus that carries GFP only was used as a control. GFP-positive T cells were sorted 48 h after infection. IL-2 production from these sorted T cells was analyzed upon stimulation with anti-CD3 or anti-CD3 plus anti-CD28. As shown in Fig. 5D, expression of Itch/CA in Abl+/+T cells slightly up-regulated IL-2 production, which further confirmed our previous findings that Itch is a negative regulator for T-cell activation (2). Expression of c-Jun/Y170F mutant also rescued IL-2 production from c-Abl–/– T cells. These results suggest that c-Abl regulates T-cell activation through Itch.

The tyrosine 170 of c-Jun is the receptor for c-Abl-mediated phosphorylation, which also locates within the recognition site, PPXY, of Itch. Therefore, we further asked whether expression of the Y170F mutant of c-Jun rescues the activation of c-Abl–/– T cells. As shown in Fig. 5E, expression of c-Jun/Y170F in Abl–/– T cells up-regulated IL-2 production. Expression of c-Jun/Y170F mutant significantly enhanced IL-2 production from c-Abl–/– cells to a level that was comparable with that produced from wild-type T cells. These results indicate that inhibition of c-Abl-mediated phosphorylation and Itch interaction of c-Jun rescue the activation of c-Abl–/– T cells.

DISCUSSION

In this report, we have demonstrated an essential role of c-Abl tyrosine kinase in T-cell activation. Tyrosine phosphorylation of the transcription factor c-Jun by c-Abl protects c-Jun...
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from Itch-mediated ubiquitination and degradation. Loss of c-Abl expression results in elevated c-Jun degradation and therefore reduced T-cell activation. These findings uncovered, at least partially, the molecular mechanisms regarding why the loss of function of c-Abl causes immune deficiency of mice (17).

Protein ubiquitination and phosphorylation are two of the most common systems of posttranslational modifications that are involved in many different facets of cellular regulations (1). Extracellular stimuli often affect ubiquitin-dependent protelysis by inducing target protein phosphorylation, which confers recognition by E3 ligases. We have demonstrated that JNK regulates Itch-dependent c-Jun and JunB polyubiquitination and turnover by phosphorylation of Itch (5). A recent study revealed JNK activates Itch E3 ligase by the phosphorylation-induced conformational change (29). Tyrosine phosphorylation within the PPXY motif has been identified to prevent its reorganization by WW domains of many proteins (30). Tyrosine phosphorylation of p53-binding protein negatively regulates its interaction with a WW domain-containing Yes-associated protein during DNA damage (31). Here we found that c-Abl-mediated tyrosine phosphorylation of c-Jun protects c-Jun from Itch-mediated ubiquitination and degradation. HECT domain-containing E3 ligases, with a few notable exceptions (such as E6-AP), contain multiple (generally 3–4) WW domains (32). Therefore, inhibition of the interaction between the WW domains and the PPXY motifs by this tyrosine phosphorylation event may be a common regulatory mechanism in HECT-type E3-mediated protein ubiquitination.

c-Abl has both nuclear localization and nuclear export sequences. The nuclear translocation of c-Abl is required for phosphorylation of c-Jun at the tyrosine residue. Several groups have reported that c-Abl localizes to both the nucleus and the cytoplasm. Cytoplasmic c-Abl associates with F-actin bundles and focal adhesions, which act as peripheral docking sites for the F-actin bundles. Nuclear c-Abl regulates cell cycle progression and cellular responses to genotoxic stress. Nuclear translocation of c-Abl is required for c-Jun phosphorylation, whereas the molecular mechanisms in regulating c-Abl nuclear translocation remain unknown. It has been reported that c-Abl transits from the nucleus to the cytoplasm following fibroblast adhesion to fibronectin (33). More recently, Yoshida et al. (38) reported that c-Abl is sequestered into the cytoplasm by binding to 14-3-3 proteins. Activation of the JNK induces phosphorylation of 14-3-3 proteins and their release from c-Abl that allows c-Abl to translocate into nucleus in response to DNA damage (34). Our findings here demonstrated that the nuclear translocation of c-Abl requires TCR stimulation and plays important roles in T-cell activation. TCR stimulation activates JNK; it will be interesting to investigate whether a similar mechanism exists in T cells in regulating c-Abl nuclear translocation.

Previous studies have suggested that c-Abl is required for full activation of T and B cells in mice (25, 35). Targeted disruption of the c-abl gene in mice results in increased susceptibility to bacterial infections in lung and intestine that cause death of these mice at a weaning age (16). We found that the activation of c-Abl−/− T cells was impaired significantly during the prolonged cultivation (supplemental Fig. S2). In humans, some chronic myeloid leukemia patients treated with c-Abl inhibitor STI-571 become immuno suppressed and develop varicella zoster virus infection (36). Further investigation showed that slightly decreased numbers and reduced activation of both T and B lymphocytes are likely responsible for the immunodeficiency of c-Abl-deficient mice and humans receiving STI-571 (37). Recent studies by Zipfel et al. (18) demonstrated that Abl kinases regulate T-cell activation by directly phosphorylating Zap70 and the transmembrane adaptor linker for activation of T cells. These findings support our data that sequestration of the constitutively active form of c-Abl still partially activates AP-1 reporter. Therefore, both the cytoplasmic and the nuclear forms of c-Abl are involved in T-cell activation. Once translocated into the nucleus by TCR stimulation, c-Abl protects c-Jun by the phosphorylation of the tyrosine 170 and inhibits Itch-mediated ubiquitination.

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REFERENCES

1. Gao, M., and Karin, M. (2005) Mol. Cell 19, 581–593
2. Fang, D., Elly, C., Gao, B., Fang, N., Altman, Y., Joozeiro, C., Hunter, T., Copeland, N., Jenkins, N., and Liu, Y. C. (2002) Nat. Immunol. 3, 281–287
3. Fang, D., and Kerppola, T. K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 14782–14787
4. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
5. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004) Science 306, 271–275
6. Wertz, I. E., O’Rourke, K. M., Zhang, Z., Dornan, D., Arnott, D., Deshaies, R. J., and Dixit, V. M. (2004) Science 303, 1371–1374
7. Nateri, A. S., Riera-Sans, L., Da Costa, C., and Behrens, A. (2004) Science 303, 1374–1378
8. Barila, D., Mangano, R., Fonlioni, S., Kretzschmar, J., Moro, M., Bohmann, D., and Superti-Furga, G. (2000) EMBO J. 19, 273–281
9. Wang, J. Y. (2004) Nat. Cell Biol. 6, 3–7
10. Hantschel, O., and Superti-Furga, G. (2004) Nat. Rev. Mol. Cell. Biol. 5, 33–44
11. Mayer, B. J., Jackson, P. K., and Baltimore, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 627–631
12. Pendergast, A. M., Muller, A. J., Hlavik, M. H., Maru, Y., and Witte, O. N. (1991) Cell 66, 161–171
13. Wong, S., and Witte, O. N. (2004) Annu. Rev. Immunol. 22, 247–306
14. Kharbanda, S., Pandey, P., Morris, P. L., Whang, Y., Xu, Y., Sawant, S., Zha, L. J., Kumar, N., Yuan, Z. M., Weichselbaum, R., Copeland, N., and Pandita, T. K., and Kufe, D. (1998) Oncogene 16, 1773–1777
15. Van Etten, R. A. (1999) Trends Cell Biol. 9, 179–186
16. Schwartzberg, P. L., Stall, A. M., Hardin, J. D., Bowdish, K. S., Humaran, T., Boast, S., Harbison, M. L., Robertson, E. J., and Goff, S. P. (1991) Cell 65, 1165–1175
17. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) Cell 65, 1153–1163
18. Zipfel, P. A., Zhang, W., Quiroz, M., and Pendergast, A. M. (2004) Curr. Biol. 14, 1222–1231
19. Perry, W. L., Hustad, C. M., Swing, D. A., O’Sullivan, T. N., Jenkins, N. A., and Copeland, N. G. (1998) Nat. Genet. 18, 143–146
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20. Morita, S., Kojima, T., and Kitamura, T. (2000) Gene Ther. 7, 1063–1066
21. Echarri, A., and Pendergast, A. M. (2001) Curr. Biol. 11, 1759–1765
22. Yang, C., Zhou, W., Jeon, M. S., Demydenko, D., Harada, Y., Zhou, H., and Liu, Y. C. (2006) Mol. Cell 21, 135–141
23. Yamamoto, K. K., Gonzalez, G. A., Biggs, W. H., III, and Montminy, M. R. (1988) Nature 334, 494–498
24. Banerjee, A. (1999) Biochemistry 38, 5438–5446
25. Hardin, J. D., Boast, S., Schwartzberg, P. L., Lee, G., Alt, F. W., Stall, A. M., and Goff, S. P. (1996) Cell. Immunol. 172, 100–107
26. Deleted in proof
27. Kraft, A. S., and Anderson, W. B. (1983) Nature 301, 621–623
28. Bennett, J. P., Cockcroft, S., and Gomperts, B. D. (1979) Nature 282, 851–853
29. Gallagher, E., Gao, M., Liu, Y. C., and Karin, M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 1717–1722
30. Sudol, M., and Hunter, T. (2000) Cell 103, 1001–1004
31. Espanel, X., and Sudol, M. (2001) J. Biol. Chem. 276, 14514–14523
32. Ingham, R. J., Gish, G., and Pawson, T. (2004) Oncogene 23, 1972–1984
33. Lewis, J. M., Baskaran, R., Taagepera, S., Schwartz, M. A., and Wang, J. Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15174–15179
34. Yoshida, K., and Miki, Y. (2005) Cell Cycle 4, 777–779
35. Hardin, J. D., Boast, S., Schwartzberg, P. L., Lee, G., Alt, F. W., Stall, A. M., and Goff, S. P. (1995) Cell. Immunol. 165, 44–54
36. Mattiuzzi, G. N., Cortes, J. E., Talpaz, M., Reuben, J., Rios, M. B., Shan, J., Kontoyiannis, D., Giles, F. J., Raad, I., Verstovsek, S., Ferrajoli, A., and Kantarjian, H. M. (2003) Clin. Cancer Res. 9, 976–980
37. Peng, B., Hayes, M., Resta, D., Racine-Poon, A., Druker, B. J., Talpaz, M., Sawyers, C. L., Rosamilia, M., Ford, J., Lloyd, P., and Capdeville, R. (2004) J. Clin. Oncol. 22, 935–942
38. Yoshida, K., Yamaguchi, T., Natsume, T., Kufe, D., and Miki, Y. (2004) Nat. Cell Biol. 7, 278–285