Ras-dependent, Ca\(^{2+}\)-stimulated Activation of Nuclear Factor of Activated T cells by a Constitutively Active Cbl Mutant in T Cells*  

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T cell receptor (TCR) stimulation induces rapid tyrosine phosphorylation of cellular proteins, including Cbl, a protooncogene product whose function remains unclear. As a first step toward elucidating the function of Cbl in TCR-initiated signaling, we evaluated the ability of wild-type Cbl or a transforming Cbl mutant (70Z/3) to induce transcriptional activation of a nuclear factor of activated T cells (NFAT) element derived from the interleukin 2 (IL2) promoter in transiently cotransfected Jurkat-TAg T cells. 70Z/3, but not Cbl, caused NFAT activation which was significantly enhanced by stimulation with calcium ionophore, and was drastically reduced by cyclosporin A pretreatment. A point mutation of a potential phosphatidylinositol 3-kinase (PI3-K) binding site (Y\(^{731}\)EAM to Y\(^{731}\)EAC) in 70Z/3 disrupted the association of PI3-K with 70Z/3, but did not reduce the induction of NFAT activity, suggesting that the interaction between Cbl and PI3-K is not required in the 70Z/3-mediated induction of NFAT. Additional mapping studies indicated that defined deletions of C-terminal 70Z/3 sequences affected to a variable degree its ability to stimulate NFAT activity. Strikingly, deletion of 346 C-terminal residues augmented this activity, whereas removal of 20 additional residues abolished it. Coexpression of dominant negative Ras abrogated the basal or ionomycin-stimulated, 70Z/3-mediated NFAT activation, suggesting a functional Ras is required for this activation. These results implicate Cbl in Ras-dependent signaling pathways which lead to NFAT activation.

The c-cbl protooncogene is the cellular homologue of v-cbl, a transforming gene of the Cas NS-1 retrovirus. The v-cbl oncogene was generated as a result of a rearrangement between Cas-Br-M virus and a sequence encoding the 355 N-terminal residues of the c-cbl product. This oncogene induces pro-B cell lymphomas and myeloid leukemias in mice (1). The 120-kDa product of c-cbl consists of a highly basic N-terminal region, a zinc Ring finger, multiple proline-rich sequences, and several potential tyrosine phosphorylation sites (2). While the transforming v-cbl product can be detected both in the cytoplasm and the nucleus, c-Cbl is exclusively cytosolic and lacks transforming activity (3). Another c-cbl mutant isolated from the murine 70Z/3 pre-B cell lymphoma encodes a protein with an internal deletion of 17 amino acids near the zinc Ring finger.

This mutation (70Z/3) also activates the transforming potential of c-cbl, and the corresponding protein displays enhanced tyrosine phosphorylation (4).

Several recent findings support the notion that Cbl is involved in signal transduction pathways: first, the proline-rich domain of Cbl mediates constitutive associations with SH3 domains of adaptor signaling proteins, i.e., Grb2 (5–8) and Nck (9); second, engagement of different receptors, including the epidermal growth factor receptor (10–14) and cytokine receptors (7, 14–16) causes an increase in the phosphotyrosine (Tyr(P))\(^{1}\) content of Cbl, and tyrosine-phosphorylated Cbl associates with SH2 domains of the regulatory subunit (p85) of phosphatidylinositol 3-kinase 3 kinase (PI3-K) (5–7, 13, 17, 18) and Crk (19–21) in an activation-dependent manner. Third, we found recently that in T cells, Cbl interacts with 14–3–3 proteins, and that this association is increased upon TCR stimulation (22). 14–3–3 proteins are thought to participate in signal transduction pathways via their association with different oncogene and protooncogene products (23, 24). Despite all of these findings, however, the physiological function of mammalian Cbl remains unclear.

Cbl is also likely to participate in signaling pathways initiated by the T cell antigen receptor (TCR)-CD3 complex and other immune recognition receptors, i.e., the B cell antigen receptor (18, 25), and the IgG-Fc receptor (14, 26). TCR-CD3 ligation by processed antigenic peptides or anti-receptor antibodies leads to activation of protein tyrosine kinases of the Src and Syk families (27, 28) which, in turn, phosphorylate multiple cellular proteins. These early events trigger signal transduction cascades which lead to T cell activation, lymphokine production, and proliferation. Cbl was recently identified as a prominent protein tyrosine kinase substrate in TCR-stimulated T cells (5, 6, 21, 29), and was found to associate with two protein tyrosine kinases, i.e., Fyn (5, 29, 30) and ZAP-70 (30), in activated T cells. Furthermore, ZAP-70 causes increased tyrosine phosphorylation of Cbl in an Lck- and Fyn-dependent manner (30), indicating that Cbl may couple ZAP-70 to downstream signaling events during T cell activation.

In order to begin to address the role of Cbl in TCR-CD3-mediated signaling, we evaluated the effects of wild-type or a mutated form (70Z/3) of Cbl on the transcriptional activation of nuclear factor of activated T cells (NFAT), using transient transfection assays in Jurkat T cells. NFAT is a transcription factor complex that plays a critical role in the induction of interleukin 2 (IL2) and other cytokine genes, and the two signal requirement for its activation mimicks the requirement for optimal proliferation and IL2 production in TCR-CD3-activated T cells (31, 32). NFAT-reporter gene activation in transiently

\(^1\) The abbreviations used are: Tyr(P), phosphotyrosine; TCR, T cell antigen receptor; PI3-K, phosphatidylinositol 3-kinase; NFAT, nuclear factor of activated T cells; PMA, phorbol myristate acetate; mAb, monoclonal antibody; HA, hemagglutinin; IL, interleukin.
transfected T cells has, therefore, been extensively used as a physiologically relevant assay for analyzing molecular events associated with T cell activation. We report that transient overexpression of 70Z/3 (but not wild-type Cbl) induces an increase in the basal activity of NFAT, which is further enhanced synergistically by calcium ionophore. This NFAT induction is dependent on both calcineurin and functional Ras. These results provide evidence that Cbl is involved in Ras-dependent cytokine gene activation.

MATERIALS AND METHODS

Antibodies—The anti-Cd33 monoclonal antibody (mAb), OKT3, was purified from culture supernatants of the corresponding hybridoma by protein A-Sepharose affinity chromatography. Polyclonal rabbit anti-Cbl antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The 4G10 anti-Tyr(P) mAb and a rabbit anti-ph55 antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-hemagglutinin (HA; 12CA5) or anti-Grb2 mAbs were from Boehringer Mannheim and Transduction Laboratories (Lexington, KY), respectively. Horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse IgG (F(ab')2) fragments were from Amersham Corp.

Plasmids—The cDNAs encoding human c-Cbl, v-Cbl, or the homologue of murine 70Z/3 (representing an internal 17-amino acid deletion of Cbl) were subcloned from the pGEM-2z vector into a mammalian expression vector, pEFneo (33) at the EcoRI and XhoI sites to generate pEFneo-Cbl, pEFneo-v-Cbl, and pEFneo-70Z/3, respectively. A sequence encoding an HA tag epitope has been added to the 5'-end of the 70Z/3 cDNA by a standard polymerase chain reaction. A methionine to cysteine mutation at a potential PI3-K-binding motif in Cbl (Y731EAM) was introduced by polymerase chain reaction amplification of a cDNA fragment encoding the Y731EAM mutation and ligation of the product into the TA cloning vector (InVitrogen). The sequence was verified by nucleotide sequencing, and the cDNA fragment digested with SnaBI and XhoI was ligated into pEFneo-70Z/3 which has been digested with the corresponding enzymes to create the pEFneo-Y731EAM expression vector. To construct expression plasmids encoding 70Z/3 proteins with successive deletions of their 114, 176, 346, 366, or 456 C-terminal residues, internal restriction sites or a point mutation were used to generate plasmids Δ1 (deletion of an EcoRV-XbaI fragment), Δ2 (deletion of a SnaBI-XhoI fragment), Δ3 (point mutation of the proline-560 codon to a stop codon by polymerase chain reaction), Δ4 (deletion of a BglII-XbaI fragment), and Δ5 (deletion of a ScaI-XhoI fragment), respectively (see Fig. 5A). These deletions removed distinct proline-rich sequences of the protein. The deletion mutants were ligated into the pEFNeo vector.

The luciferase reporter plasmids, NFAT-Luc and IL2-Luc, were generous gifts from Dr. G. Grabbée; AP-1-Luc and NFκB-Luc were kindly provided by Dr. M. Karin. The dominant negative H-RasN17 plasmid in the pcDNA3 vector was obtained from Dr. H. Wang.

Cell Culture and Transfection—Simian virus 40 T antigen (Tag)-transfected human leukemic Jurkat T cells (Jurkat-Tag) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN), L-glutamine, and antibiotics. Cells in a logarithmic growth phase were transfected with the pEFneo vector. Aliquots of the lysates (30–50 μg) were normalized to the same protein concentration were mixed with 100 μl of assay buffer (17.5 mM glycyl-glycine, pH 7.8, 10 mM MgCl₂, 5 mM ATP, 0.135 mM coenzyme A, 0.235 mM luciferin). Luciferase activity was determined in triplicate and expressed as arbitrary units (AU). The standard deviation among triplicates was ±10%, and each experiment was repeated at least three times.

RESULTS

Overexpression of 70Z/3 Induces NFAT Activity Which Is Synergistic with Ionomycin Stimulation—To explore whether Cbl or 70Z/3 can transduce signals leading to IL2 gene transcription, we evaluated the effects of cotransfected Cbl or 70Z/3 cDNA on the transcriptional activity of an IL2 promoter-reporter plasmid (IL2-Luc), or reporters corresponding to defined response elements in the IL2 promoter, i.e., NFAT-Luc, NFκB-Luc or AP-1-Luc, in Jurkat-TAg cells. Cell lysates were assayed for luciferase activity following culture in low serum-containing medium and stimulation with ionomycin and/or PMA. As shown in Fig. 1A, when the full-length Cbl was transfected into unstimulated Jurkat T cells, no increase (or a slight decrease) in NFAT activity was observed by comparison with the empty control vector. Similarly, Cbl did not activate NFAT in ionomycin- or PMA-stimulated cells. In contrast, transient overexpression of 70Z/3 induced a ~3–4-fold increase of NFAT activity in unstimulated cells, and stimulation of the 70Z/3-transfected cells with ionomycin which was minimally active by itself (~2-fold activation), but not with PMA, resulted in a synergistic activation (~7-fold NFAT activation). Cbl or 70Z/3 did not cause detectable activation of AP-1, and 70Z/3 overexpression led to only a slight induction of the IL2 promoter and NFκB under unstimulated or stimulated conditions (data not shown).

To rule out the possibility that the observed difference between Cbl and 70Z/3 was due to different expression levels of the two proteins, we assessed protein expression by immunoblotting cell lysates with an anti-Cbl antibody. As shown in Fig. 1B, transient transfection of Cbl or 70Z/3 increased the intracellular level of a 120-kDa protein which was recognized by anti-Cbl antibody to a similar degree. The 70Z/3 protein could also be detected with an anti-HA antibody, since an HA tag was added to its N terminus (Fig. 1B). Collectively, these results indicate that 70Z/3 is constitutively active in T cells in terms of its ability to induce NFAT-dependent transcription, and synergizes with a Ca²⁺-dependent signal in this regard. The former observation is consistent with the finding that 70Z/3 can transform fibroblasts (4).

Calcineurin, a calcium-calmodulin dependent phosphatase which plays a critical role in NFAT induction, is a major target for the immunosuppressive effect of cyclosporin A and FK506 (35, 36). To determine whether 70Z/3-induced NFAT activation is dependent on calcineurin, the cells were pretreated with cyclosporin A. As shown in Fig. 1C, cyclosporin A blocked the ionomycin-stimulated increase in NFAT activity in cells transfected with the empty vector, as well as the synergistic effect of 70Z/3 plus ionomycin. This suggests that functional calcineurin is required for the 70Z/3-mediated signal which leads to the transactivation of NFAT.

v-Cbl Fails to Activate NFAT—Cbl was originally isolated as a product of a transforming viral oncogene (v-cbl). Since both v-Cbl (1) and 70Z/3 (4) possess transforming activity, we wished to determine whether v-Cbl shares with 70Z/3 the ability to activate NFAT. When cells expressing the NFAT-Luc reporter plasmid were cotransfected with 70Z/3, an increase of 3- and 60-fold in NFAT activity was detected in the absence or presence of ionomycin stimulation, respectively, consistent with the results shown in Fig. 1. In contrast, cotransfection with the v-Cbl expression vector failed to stimulate the basal or ionomycin-induced NFAT activity (Fig. 2A). This difference
Fig. 1. **NFAT activation by 70Z/3.** A, Jurkat-TAg T cells were cotransfected with an NFAT-Luc reporter plasmid plus either empty pEFneo vector, or pEFneo vectors expressing Cbl or 70Z/3. Following culture and stimulation as indicated, the cells were lysed, and extracts were assayed for luciferase activity. Bars represent the mean of triplicate samples. B, aliquots of the same lysates were subjected to SDS-12.5% polyacrylamide gel electrophoresis, and Cbl expression was assessed by immunoblotting with anti-Cbl (left) or anti-HA (right) antibodies. Molecular mass (MW) standards are shown (× 1000). C, aliquots of the transfected cells were pretreated with cyclosporin A (CsA) prior to ionomycin stimulation, and luciferase activity was measured as in A. The data shown in A and C are representative of three experiments.

The expression of 70Z/3 and v-Cbl was not due to different expression levels of 70Z/3 and v-Cbl, since immunoblotting with an anti-HA antibody revealed similar expression levels of the two transfected proteins (Fig. 2B). Therefore, despite the fact that both v-Cbl and 70Z/3 display transforming activity, only 70Z/3 is capable of activating NFAT, suggesting that transformation by the two proteins involves different mechanisms.

**70Z/3-mediated NFAT Activation Is Independent of PI3-K Association with 70Z/3**—TCR-CD3 cross-linking causes rapid tyrosine phosphorylation of Cbl which, in turn, leads to an association of Cbl with PI3-K in an activation-dependent manner (5, 6, 17, 29). PI3-K has been implicated in TCR-CD3- and Ras-dependent, Ca^{2+}-stimulated Activation of NFAT

Next, we determined the effect of the 70Z/3 Y^{731}EAM mutation on the 70Z/3-mediated activation of NFAT. The mutant plasmid or 70Z/3 was cotransfected with the NFAT-Luc reporter plasmid and pEFneo or pEFneo-v-cbl, left unstimulated or stimulated with ionomycin as indicated, and lysates were assayed for luciferase activity. Bars represent the mean of triplicate samples. B, samples of the same lysates were separated by SDS-12.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with a mouse anti-HA antibody. Molecular mass (MW) standards are shown (× 1000).

Fig. 2. **v-Cbl does not activate NFAT.** A, Jurkat-TAg cells were cotransfected with the NFAT-Luc reporter plasmid plus pEFneo, pEFneo-70Z/3, or pEFneo-v-cbl, left unstimulated or stimulated with ionomycin as indicated, and lysates were assayed for luciferase activity. Bars represent the mean of triplicate samples. B, samples of the same lysates were separated by SDS-12.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with a mouse anti-HA antibody. Molecular mass (MW) standards are shown (× 1000).
times higher luciferase activity both in resting and ionomycin-stimulated cells than that from 70Z/3-transfected cells. Similar results were consistently observed in a number of repeated experiments. The expression levels of the transfected plasmids were determined by immunoblotting and no apparent difference was detected between 70Z/3 and Y731EAC (Fig. 4B). These data strongly suggest that an optimal interaction between PI3-K and 70Z/3 is not required for NFAT activity induced by the latter.

**The Effects of C-terminal Deletions on 70Z/3-mediated NFAT Activation**—In order to further define the region in 70Z/3 which is responsible for NFAT activation, we focused on the C-terminal region of the protein, since v-Cbl, which corresponds to the 361 N-terminal residues of human c-Cbl, lacked any NFAT-stimulating activity (Fig. 2). The C-terminal region of c-Cbl contains a proline-rich region and several potential tyrosine phosphorylation sites (2). Expression vectors encoding 70Z/3 proteins with successive C-terminal deletions were generated (Fig. 5A) and assessed for their ability to activate NFAT in Jurkat-TAg cells by transient cotransfection assays (Fig. 5B). As shown earlier (Figs. 1 and 2), 70Z/3 caused a 3-fold increase in the basal NFAT activity and stimulated it by 70-fold in the presence of ionomycin, whereas v-Cbl was inactive. Similarly, mutation of the major PI3-K-binding motif (Y731EAC) caused some increase in the corresponding activity. Deletion of 114 or 176 C-terminal residues (D1 and D2, respectively) reduced the activity by 50% relative to the untruncated 70Z/3. These results suggest that the C-terminal 114 amino acids of 70Z/3 contribute to maximal NFAT activation. Surprisingly, the D3 construct, in which the 346 C-terminal 70Z/3 residues have been deleted, induced higher basal or ionomycin-stimulated NFAT activity than the untruncated 70Z/3 construct, and deletion of additional 20 (D4) or 110 (D5) residues essentially abolished this activity (Fig. 5B). The differences in biological activity among the distinct 70Z/3 constructs did not reflect differences in their expression levels since immunoblotting lysates of the transiently transfected cell lines with an anti-HA tag antibody revealed similar expression levels of the corresponding proteins (Fig. 5C).

**A Functional Ras Is Required for the 70Z/3 Function**—The finding that 70Z/3 synergizes with ionomycin to induce NFAT activation was reminiscent of the effect of constitutively active Ras which can also cooperate with ionomycin to induce NFAT or IL2 promoter activity (40-43). To examine whether NFAT activation by 70Z/3 is dependent on functional Ras, we cotransfected Jurkat-TAg cells with combinations of 70Z/3 and/or a dominant negative Ras expression vector (or with the corresponding empty vectors), and assessed the degree of NFAT activation. The 70Z/3-induced NFAT activation was nearly completely inhibited by RasN17 in either resting or ionomycin-stimulated cells.
stimulated cells (Fig. 6A). The expression level of 70Z/3 was, however, similar in cells cotransfected with the control (pcDNA3) or RasN17 expression vector (Fig. 6B). These results suggest that functional Ras is required for the 70Z/3-mediated activation of NFAT.

**DISCUSSION**

In the present study, we have demonstrated that 70Z/3, a mutated version of the Cbl protooncogene product, but not wild-type Cbl or v-Cbl, functions in a constitutively active manner to induce NFAT activation in Jurkat T cells and, furthermore, that 70Z/3 acts in synergy with calcium ionophore in this regard. Cyclosporin A, which selectively inhibits the function of calcineurin (35, 36), as well as dominant negative Ras, blocked both the basal and the ionomycin-stimulated induction of NFAT by 70Z/3. On the other hand, a mutation that markedly reduced the association of 70Z/3 with PI3-K did not impair the activation of NFAT and, in fact, seemed to increase it. These results suggest that Cbl is involved in the TCR-mediated signaling pathways which ultimately lead to the activation of NFAT, one of the critical transcription factor complexes involved in induction of IL2 and other cytokine genes (31, 32).

Optimal activation of NFAT and the IL2 promoter requires two signals which can be mimicked by agents that increase the free intracellular calcium concentration and activate the protein kinase C/Ras pathway in T cells, respectively (40–43). Constitutively active calcineurin, which can replace the calcium signal, or ionomycin, can each synergize with phorbol ester or constitutively active Ras to induce NFAT activation (41, 42). Thus, calcineurin is a major target for the calcium signal involved in NFAT activation and IL2 gene transcription. Our finding that 70Z/3 synergized with ionomycin (but not with PMA) to induce NFAT activation, and that this effect was blocked by cyclosorin A pretreatment (Fig. 1), strongly suggest, therefore, that this constitutively active Cbl can cooperate with calcineurin to provide the requisite signals for NFAT activation. 70Z/3 mimicks in this respect the effects of constitutively active Ras (41, 42). The similarity with Ras also extends to the finding that, like Ras (41, 42), 70Z/3 synergized with ionomycin (Fig. 1), but not with an anti-CD3 mAb (data not shown), to activate NFAT. This connection between Cbl and the Ras signaling pathway is supported by our finding that dominant negative Ras inhibited almost completely the 70Z/3-induced activation of NFAT, indicating that functional Ras is necessary for NFAT induction in the context of 70Z/3. This finding does not allow us to conclude at present whether Ras acts downstream of 70Z/3, or in a parallel pathway which interacts with a Cbl-dependent pathway, to induce NFAT activation.
Recent studies have indicated that, in hematopoietic (5, 6, 17) and other (13) cells, tyrosine-phosphorylated Cbl recruits PI3-K, an interaction that may be mediated by tyrosine-containing YXXM motifs in Cbl and the SH2 domain of p85 (17). Since PI3-K may be involved in signaling pathways leading to IL2 production (37, 38), it was of interest to examine whether PI3-K association with Cbl is required in order for 70Z/3 to activate NFAT. We found that a point mutation of methionine to cysteine in the Y731EAM motif of 70Z/3 reduced the association of 70Z/3 with PI3-K to a minimum, indicating that this motif is largely responsible for the interaction of 70Z/3 with PI3-K. However, this mutation increased the activation of NFAT by 70Z/3, suggesting that activation-dependent recruitment of PI3-K to Cbl is not required for the induction of NFAT.

A striking observation in the present study is that the 70Z/3 Δ3 mutant, from which 346 C-terminal amino acid residues were deleted, exhibited the highest NFAT-inducing transcriptional activity. Further deletion of only 20 additional amino acid residues (Δ4) completely abrogated basal or ionomycin-stimulated NFAT induction. One potential explanation is that the region represented by residues 541–560 in 70Z/3 interacts directly or indirectly with an essential effector involved in NFAT activation, and that removal of more proximal residues (i.e. residues 561–906) facilitates this interaction, perhaps by abolishing other interactions with a negative regulator(s). Alternatively, differences in biological activity among the various constructs represent conformational changes in the structure of 70Z/3, in which case Δ3 may have a more favorable conformation for mediating the effector function of Cbl. Since the 70Z/3 deletions remove distinct proline-rich sequences which have been implicated in binding to SH3 domains of other proteins (5–8, 44), it is possible that interactions with SH3-containing signaling proteins are a major target for the functional effects of these deletions. This possibility is currently being analyzed.

What is the molecular mechanism by which the constitutively active Cbl mutant, 70Z/3Δ3, affects the transcriptional regulation of NFAT? The finding that 70Z/3, but not wild-type Cbl, can mediate this event suggests that amino acid residues 366–383 (which have been deleted in 70Z/3Δ3) regulate negatively the function of Cbl or its association with effector proteins, and that their removal unmasks the ability of Cbl to activate NFAT. This is compatible with the findings that the same deletion activates the transforming potential of Cbl (4). The notion of a negative regulatory activity associated with Cbl is supported by the recent finding that a Cbl homologue, Sli-1, abolishing other interactions with a negative regulator(s). Additional studies are required to establish the precise role of 70Z/3 in the regulation of signaling pathways initiated by immune recognition receptors.
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