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CD28 Mediates a Potent Costimulatory Signal for Rapid Degradation of IκBβ Which Is Associated with Accelerated Activation of Various NF-κB/Rel Heterodimers

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Optimal activation of T cells requires at least two signals delivered by the T-cell receptor complex and costimulatory molecules such as CD28. The CD28 signaling participates in the transcription of the interleukin-2 gene through activation of an enhancer termed the CD28-responsive element (CD28RE). Stimulation of CD28 enhances mitogen-mediated induction of CD28RE-binding proteins including members of the NF-κB/Rel transcription factor family, although the underlying mechanism remains elusive. In this report, we show that CD28 costimulation leads to biphasic induction of NF-κB/Rel heterodimers, including early-phase induction of p50/RelA and c-Rel/RelA and late-phase induction of p50/c-Rel. Interestingly, activation of these NF-κB/Rel complexes by the CD28 signal is associated with the rapid degradation of both IκBα and IκBβ, two major cytoplasmic inhibitors of NF-κB/Rel. Although IκBα degradation can be induced by phorbol ester alone, degradation of IκBβ is largely dependent on the CD28 costimulatory signal. We further demonstrate that CD28-mediated transactivation of the CD28RE enhancer is potently inhibited by an N-terminal truncation mutant of IκBβ that is incapable of responding to the degradation signals. Together, these results suggest that the CD28 costimulatory signal augments activation of NF-κB/Rel by promoting degradation of IκBβ as well as enhancing degradation of IκBα and that induction of NF-κB/Rel serves as an essential step in the signal-mediated activation of the CD28RE enhancer.

Optimal activation of T cells requires both the engagement of T-cell receptor (TCR) complex and the stimulation of certain T-cell surface accessory molecules that deliver costimulatory signals (29). One major costimulatory signal is mediated by the interaction between the CD28 accessory molecule on the surface of T cells and its cognate ligand B7 on antigen-presenting cells (20, 23, 30). Ligation of CD28 alone has no detectable effect on the activation of T cells. However, in synergy with various mitogenic agents such as monoclonal antibodies against the CD3 chains (anti-CD3), the lectin phytohemagglutinin (PHA), and phorbol 12-myristic 13-acetate (PMA), the CD28 signal markedly induces production of cytokines including interleukin-2 (IL-2), leading to the proliferation of the stimulated T cells (39). The stimulatory effect of CD28 signal on the induction of IL-2 is at least partially due to the enhanced transcription of the IL-2 gene (12). A CD28-responsive element (CD28RE) has been identified in the promoter of the IL-2 gene and shown to be essential for the transcriptional induction of this gene through the CD28 signaling pathway (12). Although the precise mechanism underlying the activation of the CD28RE enhancer remains elusive, recent studies have clearly demonstrated that the CD28RE is bound by several members of the NF-κB/Rel family of transcription factors (14). Furthermore, treatment of human T cells with a monoclonal antibody against CD28 (anti-CD28) enhances PMA-induced nuclear expression of the NF-κB/Rel proteins (9, 14).

The NF-κB/Rel family includes a number of dimeric complexes composed of a set of structurally related polypeptides, including p50 (NF-κB1), p52 (NF-κB2), RelA (previously named p65), RelB, and the proto-oncoprotein c-Rel (reviewed in references 15 and 32). In resting T cells, these NF-κB/Rel complexes are sequestered in the cytoplasm as latent precursors by physical association with a family of ankyrin motif-rich inhibitory proteins (5) including IκBα (2, 18) and IκBβ (40). IκBα appears to play a major role in the regulation of the transient nuclear expression of RelA/p50 NF-κB heterodimer that accompanies cellular activation by various stimuli including mitogens like PMA and cytokines such as tumor necrosis factor alpha (TNF-α). Such cellular activation induces the phosphorylation of IκBα, which in turn targets this inhibitor for proteolysis via the ubiquitin pathway (6, 8, 10, 11, 41; reviewed in references 3, 38, and 42). However, since the nuclear NF-κB activates the expression of the IκBα gene, the depleted IκBα pool can be rapidly replenished through the enhanced de novo synthesis of IκBα protein, and the newly synthesized IκBα functions to prevent further nuclear translocation of NF-κB/Rel. In contrast to that of IκBα, the degradation of IκBβ occurs only in cells stimulated with certain inducers, such as the bacterial lipopolysaccharide (LPS), IL-1, and the Tax protein of the human T-cell leukemia virus (HTLV) (16, 24, 40). Moreover, since the expression of the IκBβ gene appears not to be induced by NF-κB, the degraded IκBβ protein cannot be rapidly replenished through de novo protein synthesis. Thus, degradation of IκBβ is associated with persistent nuclear expression of NF-κB/Rel (16, 24, 40).

Although the CD28 signaling pathway has been shown to accelerate mitogen-induced nuclear expression of various NF-κB/Rel transcription factors, the underlying molecular mechanism remains unclear. A recent study (21) has suggested that CD28 costimulation prolongs PMA-induced degradation of IκBα, leading to the sustained nuclear expression of NF-κB/

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Rel. However, it is unknown how the CD28 signal enhances the initial level of NF-κB/Rel activation. We report here that ligation of CD28 initiates a potent costimulatory signal leading to the rapid and persistent degradation of IκBβ. In addition, the CD28 signal also markedly enhances PMA-induced transient degradation of IκBα. These effects together may contribute to both the accelerated and the prolonged activation of NF-κB/Rel mediated through the CD28 costimulatory signal.

MATERIALS AND METHODS

Cells, reagents, and antibodies. Jurkat T cells (American Type Culture Collection) and Jurkat cells expressing the simian virus 40 large T antigen (Jurkat Tag [26]) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Human peripheral blood T cells were prepared from partially purified human blood (Biological Specialty Corporation, Colmar, Pa.) with a Ficoll-Hypaque gradient (Pharmacia Biotech) and then subjected to negative selection using human T-cell enrichment immunocolumns (Biotex Laboratories Inc., Edmonton, Alberta, Canada). The murine T-cell clone 16B-2 (kindly provided by Daniel Mueller) was cultivated as previously described (25). PHA, PMA, and MG132 were purchased from Sigma, LC Laboratories (Woburn, Mass.), and ProScript, Inc. (Cambridge, Mass.), respectively. The monoclonal antibody for human CD28 (clone 9.3) was provided by Bristol-Myers Squibb Pharmaceutical Research Institute and used at a 1:10,000 dilution (or 0.3 μg/ml). The monoclonal antibodies for murine CD28 (clone 37.51) and CD3 (clone 145-2C11) were purchased from PharMingen (San Diego, Calif.) and immobilized onto tissue culture plates (1 μg/ml) at 4°C overnight before use. The antibody against the influenza virus hemagglutinin (HA) epitope tag (anti-HA) was obtained from Boehringer Mannheim. The peptide-specific antisera for IκBα and various NF-κB/Rel components were gifts from Warner Greene (The Gladstone Institute of Virology and Immunology, San Francisco, Calif.). Anti-IκBβ (C-20) was purchased from Santa Cruz Biotechnology, Inc.

Nuclear extract preparation and electrophoresis mobility shift assay (EMSA). Jurkat T cells were treated for the indicated time periods with the indicated inducers and then collected by centrifugation at 800 × *g* for 5 min. Nuclear extracts were prepared as previously described (28) and immediately subjected to EMSA. EMSA was performed by incubating the nuclear extracts (~5 μg) with a ^{32}P-radiolabeled probe, covering the CD28RE (AAAGAAATTCCAAAGAGT) from the human IL-2 gene promoter, at room temperature for 10 min, and then resolving the DNA-protein complexes on native 4% polyacrylamide gels. For antibody supershift assays, 1 μl of each of the indicated antisera (prediluted for threefold) was added to the EMSA reaction 5 min before electrophoresis.

Plasmid constructs. The plasmid TaxM22 encodes a mutant form of HTLV type 1 (HTLV-1) Tax that is deficient in NF-κB activation but is still capable of activating the HTLV-1 long terminal repeat (LTR) (33). pCMV4HA-IκBβ was constructed by inserting three copies of the HA epitope tag (YPYDVPDYA) upstream of the translational initiation codon of the mouse IκBβ cDNA (kindly provided by Dr. Sankar Ghosh, Yale University [40]) cloned in the pCMV4 expression vector (1). IκBβ 19A/23A was generated by substituting serine-19 and serine-23 with alanines by site-directed mutagenesis (ClonTech, Inc.). IκBβΔ5-27, which lacks amino acids 5 to 27, was also generated by site-directed mutagenesis. Both of these IκBβ mutants were tagged with the HA epitope and cloned into the pCMV4 vector. To construct the reporter plasmid CD28RE-luc, a *Hin*dIII-*Xho*I insert (containing four copies of the human IL-2 CD28RE motif linked in front of a thymidine kinase promoter) of the plasmid 4xCD28RE-CAT (gift from Paritosh Ghosh and Howard Young, National Cancer Institute, Frederick, Md. [14]) was transferred into the pGL2-basic luciferase plasmid (Promega). The luciferase reporter driven by HTLV-1 LTR (HTLV-1 LTR-luciferase) was constructed by transferring the insert of HTLV-1 LTR-CAT (13), containing the full-length HTLV-1 LTR (35) linked to a heterologous TATA box, to pGL2-basic.

Transient transfection and immunoblotting. Jurkat Tag cells (5 × 10^6) were transfected by using DEAE-dextran (19) with the indicated amounts of IκBβ expression vectors. Between 40 and 48 h posttransfection, the cells were incubated with 8 μg of cycloheximide per ml for 30 min to block further synthesis of the transfected IκBβ and then stimulated for the indicated time periods. The cells were then subjected to nuclear and cytoplasmic extract preparation as previously described (28). For immunoblotting analyses, protein samples were fractionated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and then analyzed for immunoreactivity with various specific antisera by an enhanced chemiluminescence detection system (ECL; DuPont, NEN).

Luciferase reporter gene assays. Jurkat T cells were transfected with DEAE-dextran with the indicated luciferase reporter plasmids and cDNA expression vectors. After 40 to 48 h of transfection, the recipient cells were either left untreated or incubated with PHA (1 μg/ml), PMA (10 ng/ml), and anti-CD28 (1:10,000 dilution) for 8 h and then subjected to extract preparation using a reporter lysis buffer (luciferase reagent; Promega) at about 40 μl/10^6 cells. Luciferase activity was detected by mixing 5 μl of extract with 25 μl of luciferase substrate (Promega) and measured with a single photon channel of a scintillation counter (Beckman).

RESULTS

Biphasic induction of CD28RE-binding NF-κB/Rel heterodimers in cells treated with PMA and anti-CD28. Prior studies have shown that CD28 costimulation enhances the level of PMA-induced nuclear expression of various NF-κB/Rel subunits (14), although it remains unclear how these subunits bind to CD28RE, especially at different time points of cellular stimulation. To address this question, human Jurkat T cells were stimulated for different time periods with PMA, either in the absence or in the presence of a CD28-specific monoclonal antibody (anti-CD28, clone 9.3), and the nuclear CD28RE-binding proteins were analyzed by EMSA. As shown in Fig. 1A, stimulation of the cells with PMA for 15 min led to the moderate induction of a CD28RE-binding complex (Fig. 1A, lane 2, C2), which reached the maximum level at 30 min (lane 3) and diminished after 2 h (lane 4) of cellular stimulation. Prolonged stimulation of the cells (8 h) led to the appearance of another complex that migrated slightly more rapidly than C2 (lane 5, C3). This pattern of kinetics was not due to experimental variation, since it was consistently detected in our repeated experiments (data not shown). More importantly, the level of both the early-induced C2 and late-induced C3 complexes was significantly enhanced when the cells were costimulated with anti-CD28 (Fig. 1A, lanes 7 to 10). Furthermore, CD28 costimulation also led to the marked induction of a CD28RE-binding complex distinctive from both C2 and C3 in mobility (lanes 8 and 9, C1). Following cellular stimulation, this complex appeared rapidly and reached maximum level at 30 min (lanes 7 and 8). However, the level of this C1 complex declined after 2 h and diminished around 8 h, which coincided with the appearance of the C3 complex (lanes 9 and 10). Densitometry quantitation of the DNA-protein complexes revealed that the C1 complex was also induced by PMA alone, albeit at a very low level (Fig. 1C, C1). Antibody supershift analyses of the early-phase (30 min) protein complexes revealed that the C1 complex was supershifted by antibodies against RelA (Fig. 1B, lane 3) and c-Rel (lane 4) but not by a preimmune serum (lane 5), anti-p50 (lane 2), or antisera for any other known NF-κB/Rel subunits including p52 and RelB (data not shown). On the other hand, formation of the C2 complex was abrogated by both anti-p50 (lane 2) and anti-RelA (lane 3) but not by anti-c-Rel (lane 4). Supershift analyses of the late-phase complexes (8 h) revealed that C3 immunoreacted with antibodies for both p50 (lane 7) and c-Rel (lane 9) but not RelA (lane 8). Thus, the C1, C2, and C3 CD28RE-binding protein complexes appeared to be composed of c-Rel/RelA, p50/RelA, and p50/c-Rel, respectively.

Similar antibody supershift assays showed that the C2 and C3 complexes induced in cells treated with PMA alone (Fig. 1A, lanes 1 to 5) contained the same NF-κB/Rel components as those detected from the CD28-costimulated cells (lanes 6 to 10). Together, these results suggested that the CD28 signaling induces not only the p50/RelA and p50/c-Rel prototypical NF-κB/Rel complexes but also a RelA/c-Rel heterodimer.

Activation of NF-κB/Rel through the CD28 signaling pathway is associated with degradation of both IκBα and IκBβ. To investigate the molecular mechanism underlying the activation of NF-κB/Rel through the CD28 signaling pathway, the effect of CD28 costimulation on the fate of various NF-κB/Rel inhibitors was examined. For these studies, human Jurkat T cells were stimulated with the mitogen PMA either in the absence or in the presence of anti-CD28 and then the steady level of

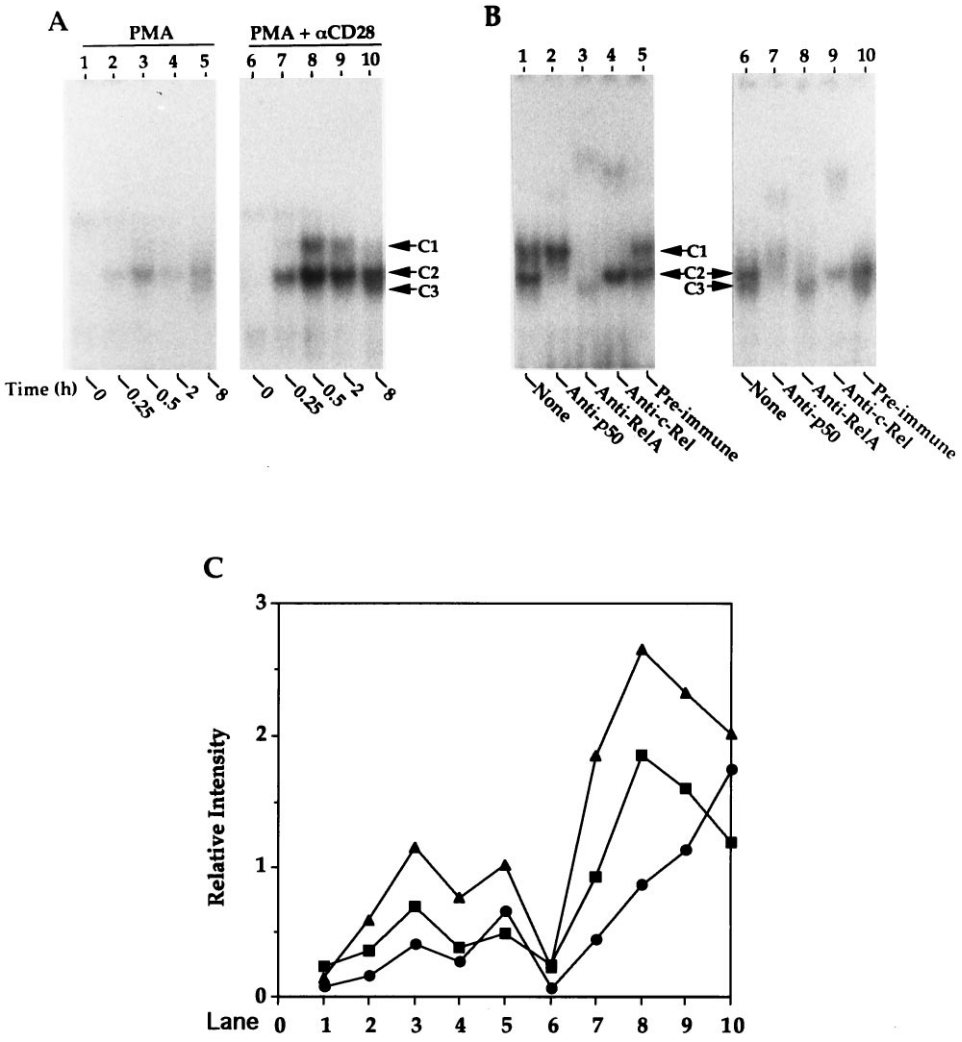

FIG. 1. CD28 costimulation enhances mitogen-mediated activation of NF-κB/Rel heterodimers. (A) Human Jurkat T cells were stimulated for the indicated time periods with either PMA (10 ng/ml) alone (lanes 1 to 5) or PMA together with anti-CD28 (1:10,000 dilution) (lanes 6 to 10) and then collected for preparation of nuclear extracts. The nuclear extracts were subjected to EMSA with a ^{32}P-labeled CD28RE oligonucleotide probe. (B) Antibody supershift analyses of nuclear extracts isolated from Jurkat cells stimulated with PMA plus anti-CD28 for 30 min (lanes 1 to 5) or 8 h (lanes 6 to 10). The nuclear extracts were subjected to EMSA either in the absence (none) or in the presence of the indicated immune or preimmune sera. (C) Densitometry quantitation of the DNA-protein complexes. The intensities of the DNA-protein complexes C1 (■), C2 (▲), and C3 (●) of lanes 1 to 10 in panel A were quantitated by a densitometer.

IκBα and IκBβ was analyzed by immunoblotting (Fig. 2A). As previously demonstrated (8, 31, 36), stimulation of the cells with PMA for 30 min led to the significant, although not complete, loss of IκBα, which was followed by the rapid replenishment of the lost IκBα protein (Fig. 2A, lower panel, lanes 1 to 5) through its de novo synthesis (8, 31, 36). Costimulation of the cells with anti-CD28 markedly accelerated and enhanced the degradation of IκBα, although this treatment did not significantly prolong the degradation process (Fig. 2A, lower panel, lanes 6 to 10). Unlike IκBα, IκBβ was not appreciably degraded in cells treated with PMA alone (Fig. 2A, upper panel, lanes 1 to 5), which was consistent with a prior study (40). However, when the cells were costimulated with anti-CD28, IκBβ was rapidly lost (lanes 6 to 10). The loss of IκBβ could be detected as early as 15 min following cellular stimulation (upper panel, lane 7), and unlike IκBα, the depleted IκBβ was not rapidly replenished (lanes 6 to 10). To test whether the disappearance of IκBβ from the cytoplasmic compartment was due to its nuclear translocation or proteolysis, nuclear extracts isolated from these stimulated cells were subjected to immunoblotting analyses. As expected, the 45-kDa IκBβ protein was not detected from the nuclear extract of unstimulated cells (Fig. 2B, lane 2) although it was readily detected from the cytoplasm of these cells (lane 1). In the nuclear extract of unstimulated Jurkat cells, a protein band migrating more slowly than IκBβ could be detected with the IκBβ antibody (Fig. 2B, lane 2, arrowhead). However, this nuclear protein did not seem to be a phosphorylated form of IκBβ since its mobility could not be altered by treatment with calf intestinal alkaline phosphatase (data not shown). More importantly, the amount of this nuclear protein was not changed along with the loss of cytoplasmic IκBβ when the cells were stimulated with PMA and anti-CD28 (compare Fig. 2A, lanes 7 to 10, and Fig. 2B, lanes 3 to 6). Thus the signal-induced loss of IκBβ did not seem to result from its nuclear translocation. We then examined whether IκBβ underwent proteolytic degradation by analyzing the effect of a proteasome inhibitor, MG132, on the inducible loss of IκBβ in cytoplasm.

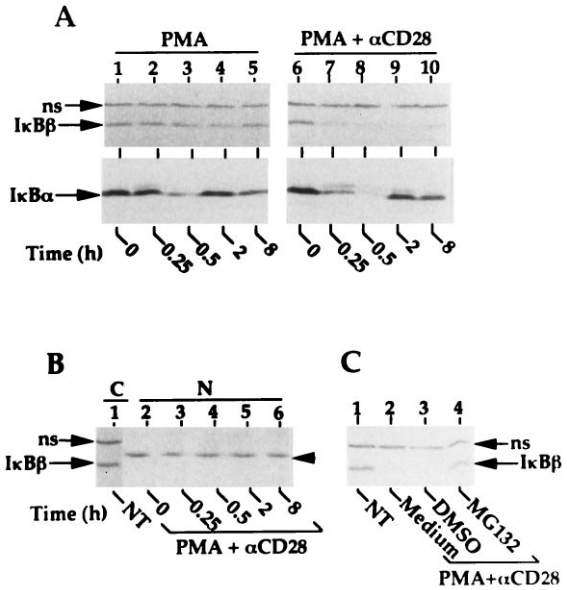

FIG. 2. CD28 mediates a potent costimulation signal for rapid degradation of both IκBα and IκBβ. (A) Jurkat cells were incubated for the indicated time periods with either PMA alone or PMA plus anti-CD28 at the concentrations indicated in Fig. 1. The cytoplasmic extracts isolated from these cells were subjected to immunoblotting analyses using antibodies specific for either IκBβ (upper panel) or IκBα (lower panel). ns is a nonspecific band that cross-reacts with the anti-IκBβ antibody. (B) Immunoblotting analysis of the nuclear extracts isolated from cells stimulated with PMA plus anti-CD28. Lane 1 is a control showing the cytoplasmic IκBβ in untreated cells. The arrowhead indicates a nuclear protein that cross-reacts with the anti-IκBβ antibody. (C) Jurkat cells were preincubated with either proteasome inhibitor MG132, the solvent dimethyl sulfoxide (DMSO), or medium for 30 min and then further incubated with PMA and anti-CD28 for 30 min. Cytoplasmic extracts were analyzed by immunoblotting using anti-IκBβ.

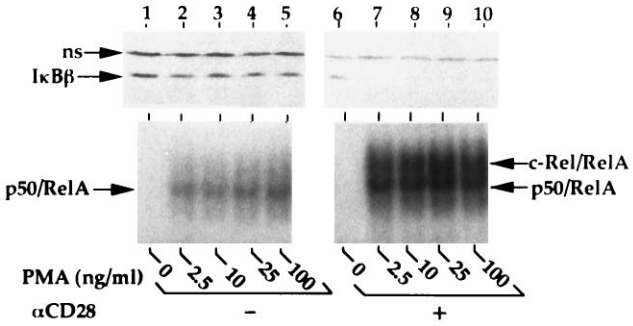

FIG. 3. PMA alone fails to induce the degradation of IκBβ even at high concentrations. Jurkat cells were stimulated for 30 min with the indicated concentrations of PMA either in the absence (lanes 1 to 5) or in the presence (lanes 6 to 10) of anti-CD28 (1:10,000 dilution). The cytoplasmic and nuclear extracts were subjected to immunoblotting using anti-IκBβ (upper panel) and EMSA (lower panel), respectively, to detect the degradation of IκBβ and activation of CD28RE-binding proteins.

As shown in Fig. 2C, the inducible loss of IκBβ was efficiently blocked by MG132 (25 μM, lane 4) but not by the solvent dimethyl sulfoxide (lane 3). Thus, as seen with IκBα (3), the signal-induced loss of IκBβ likely resulted from its proteolytic degradation. However, unlike that of IκBα, degradation of IκBβ is critically dependent on the CD28 costimulatory signal.

To examine whether the failure of PMA to target the proteolysis of IκBβ was due to a low dose of PMA used, the fate of IκBβ was analyzed in cells treated with increasing amounts of PMA. As shown in Fig. 3, no appreciable IκBβ degradation was detected when the cells were stimulated with up to 100 ng of PMA per ml (Fig. 3, upper panel, lanes 1 to 5). On the other hand, when the cells were costimulated with anti-CD28, as little as 2.5 ng of PMA per ml was sufficient to induce the degradation of IκBβ (lanes 6 to 10). Furthermore, as shown in Fig. 1 and 2A, the degradation of IκBβ was correlated with the induction of the c-Rel/RelA complex as well as enhancement of p50/RelA (lower panel, lanes 6 to 10). Together, these results suggested that the CD28 costimulation triggers a potent costimulatory signal that, in synergy with PMA, induces rapid and persistent degradation of IκBβ as well as enhanced transient degradation of IκBα, and this specific activity is associated with the induction of various NF-κB/Rel heterodimers including p50/RelA, c-Rel/RelA, and p50/c-Rel.

CD28 costimulation also induces the degradation of IκBβ in nontransformed human and mouse T cells. To address the physiological relevance of the above findings made with Jurkat cells, the effect of CD28 costimulation on the degradation of IκBβ was examined with freshly purified human peripheral blood T cells. In these cells, PMA alone again was inefficient in the induction of IκBβ degradation. After 2 h of cellular stimulation, only moderate degradation of IκBβ was detected (Fig. 4A, upper panel, lane 2). As expected, when the cells were costimulated with anti-CD28, almost the entire intracellular pool of IκBβ was depleted (lane 3). Furthermore, the degradation of IκBβ was well correlated with the induction of CD28RE-binding NF-κB/Rel factors (Fig. 4A, lower panel).

We then examined whether the CD28 costimulatory signal also synergizes with the TCR-mediated primary signal in the induction of IκBβ degradation. For these studies, we used an antigen-specific murine T-cell clone, 16B-2. These $CD4^+$ cloned T cells had been extensively used in the study of T-cell activation and anergy and shown to require both the TCR and CD28 signals for their functional activation (22, 25). As shown in Fig. 4B, IκBβ was readily detected from the resting murine T cells (lane 1). Upon stimulation of the TCR complex with anti-CD3, only moderate degradation of IκBβ was detected (lane 2). However, when the cells were costimulated with anti-CD28, the degradation of IκBβ was markedly enhanced (lane 3). No degradation of IκBβ was detected in cells treated with anti-CD28 alone (data not shown). Thus, CD28 also provides

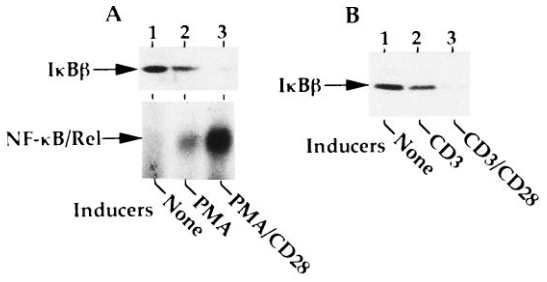

FIG. 4. Induction of IκBβ degradation by the CD28 costimulatory signal in human and murine primary T cells. (A) Induction of IκBβ degradation and activation of NF-κB/Rel in human T cells. Freshly purified human peripheral blood T cells were incubated with either PMA (10 ng/ml) or PMA together with anti-CD28 (1:10,000 dilution) for 2 h and then subjected to subcellular extract preparation. The cytoplasmic and nuclear extracts were then analyzed by immunoblotting for IκBβ degradation (upper panel) and EMSA for the activation of CD28RE-binding NF-κB/Rel factors (lower panel), respectively. (B) Synergy of anti-CD28 with anti-CD3 in the induction of IκBβ degradation in murine T cells. Murine 16B-2 T cells were incubated for 3 h in either an untreated control tissue culture plate (lane 1) or plates that had been pretreated with anti-CD3 (lane 2) or anti-CD3 plus anti-CD28 (lane 3). Cell extracts were prepared as described for panel A and subjected to immunoblotting using the anti-IκBβ antiserum.

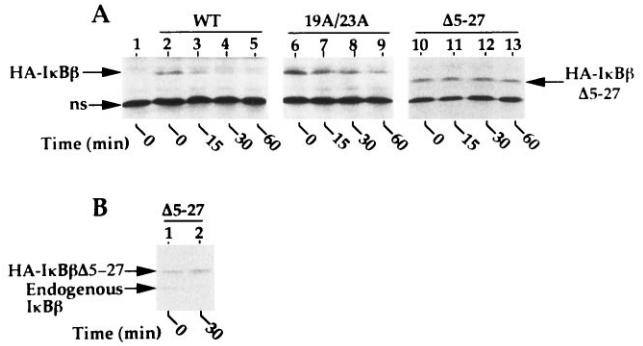

FIG. 5. The N-terminal region of IκBβ is required for its degradation induced by the CD28-PMA signals. (A) Jurkat Tag cells (5×10^6) were transfected with either an empty vector (5 μg, lane 1) or the same amounts of cDNA expression vectors encoding the indicated HA-tagged wild-type (WT) or mutant IκBβ. At 40 h posttransfection, the recipient cells were incubated with cycloheximide (8 μg/ml) for 30 min (to stop further protein synthesis) and then stimulated with PMA (10 ng/ml) plus anti-CD28 (1:10,000) for the indicated time, and the cell extracts were subjected to immunoblotting using an HA-specific monoclonal antibody (anti-HA). The wild-type and mutant HA-IκBβ are indicated. ns is a nonspecific band. (B) Cell extracts from lanes 10 and 12 of panel A were subjected to immunoblotting using the anti-IκBβ antibody. The HA-tagged IκBβΔ5-27 and the endogenous IκBβ are indicated.

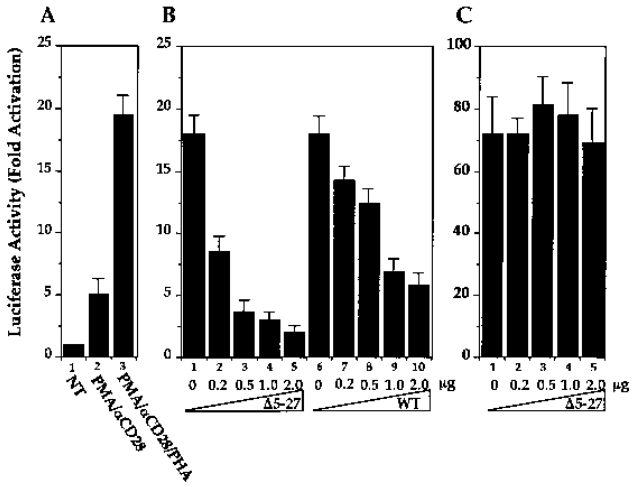

FIG. 6. A degradation-resistant IκBβ mutant potently inhibits activation of a CD28RE reporter gene by the CD28-mitogen signals. (A) Jurkat cells (5×10^6) were transfected with 6 μg of the CD28RE-luciferase reporter plasmid, and the recipient cells were either not treated (NT) or incubated with the indicated inducers (10 ng/ml for PMA, 1 μg/ml for PHA, and 1:10,000 for anti-CD28) for 8 h and then subjected to luciferase assay (Promega). Luciferase activity is presented as fold induction relative to the basal level measured in untreated cells. The values shown in this assay, as well as in panels B and C, represent the mean fold induction of three independent experiments. (B) Jurkat cells were transfected with 6 μg of the CD28RE-luciferase reporter either alone (columns 1 and 6) or together with the indicated amounts of cDNA expression vectors encoding either the N-terminal deletion mutant (Δ5-27) or wild type (WT) of IκBβ. The recipient cells were stimulated with anti-CD28 together with PMA and PHA at the concentrations indicated in panel A. The cell extracts were subjected to luciferase assay, and the luciferase activity is presented as in panel A. (C) The degradation-resistant IκBβ mutant has no effect on the NF-κB-independent induction of HTLV-1 LTR by a Tax mutant (TaxM22). Jurkat cells were transfected with 2 μg of HTLV-1 LTR-luciferase reporter together with 2 μg of TaxM22 along with the indicated amounts of IκBβ(Δ5-27). The recipient cells were subjected to luciferase assay 40 h after transfection. Luciferase activity is presented as fold activation relative to the basal level measured in cells transfected with the reporter plasmid alone.

a potent costimulatory signal for degradation of IκBβ under physiological conditions.

The N-terminal sequences of IκBβ are required for its inducible degradation through the CD28 signaling pathway. Recent studies have shown that degradation of IκBα is regulated by phosphorylation at two N-terminal serines (serine-32 and serine-36) (6, 7, 11, 41). Although IκBβ differs from IκBα in responding to stimulation signals, the two inhibitory proteins have a striking similarity in the sequence surrounding the two N-terminal serines of IκBα (located at amino acids 19 and 23 within IκBβ), and these two serines appear to be required for degradation of IκBβ induced by TNF-α and HTLV-1 Tax protein (11, 24). To examine the functional significance of these two potential phosphorylation sites in CD28-mediated degradation of IκBβ, we constructed cDNA expression vectors encoding HA-tagged wild-type or mutant IκBβ that either carries site mutations at S-19 and S-23 (IκBβ19A/23A) or lacks amino acids 5 to 27 (IκBβΔ5-27). These constructs were transiently transfected into human Jurkat Tag cells which were then subjected to stimulation with PMA together with anti-CD28 (Fig. 5). As seen with degradation of IκBα (41), the overexpressed IκBβ was only partially degraded following cellular stimulation (data not shown). However, when the recipient cells were preincubated with the protein synthesis inhibitor cycloheximide, the transfected wild-type HA-IκBβ was efficiently degraded upon stimulation with PMA plus anti-CD28 (Fig. 5A, lanes 2 to 5). Importantly, in contrast to the results observed with TNF-α and Tax, mutation of S-19 and S-23 to alanines failed to completely block the degradation of IκBβ, although these mutations indeed markedly delayed the degradation process (lanes 6 to 9). When an N-terminal region covering amino acids 5 to 27 was deleted, the generated IκBβ mutant became completely resistant to CD28-mediated degradation signals (lanes 10 to 13). Under these conditions, the endogenous IκBβ was efficiently degraded (Fig. 5B and data not shown). These results may suggest that S-19 and S-23 as well as some other amino acid residues located at the N-terminal region of IκBβ are required for the inducible degradation of this inhibitory protein through the CD28 signaling pathway.

A degradation-resistant IκBβ mutant potently inhibits the activation of a CD28RE-driven luciferase reporter gene. We have recently found that optimal activation of CD28RE is associated with its binding by both NF-κB/Rel and some other transcription factors that are induced by stimulators of the TCR complex, such as PHA and anti-CD3 (23a). As shown in Fig. 6A, the CD28RE-driven luciferase reporter (CD28RE-luc) was activated by PMA and anti-CD28 (column 2) and the luciferase activity was further enhanced in the presence of PHA (column 3). To examine the functional importance of IκBβ degradation in the activation of CD28RE enhancer, the effect of the degradation-resistant IκBβ mutant (IκBβΔ5-27) on the activation of CD28RE was assessed by a luciferase reporter gene assay. For these studies, Jurkat cells were transfected with the CD28RE-luc reporter together with increasing amounts of cDNA expression vectors encoding either IκBβΔ5-27 or the wild-type IκBβ and then stimulated with anti-CD28 together with PMA and PHA (Fig. 6B). Activation of the CD28RE-luc was potently inhibited by the degradation-resistant IκBβΔ5-27 (columns 2 to 5), suggesting that the NF-κB/Rel heterodimers indeed play an important role in the activation of the CD28RE enhancer through the CD28 costimulatory pathway. The partial inhibition of CD28RE-luc by the wild-type IκBβ (Fig. 6B, columns 7 to 10) was most likely due to the low degradation efficiency of transiently transfected IκB proteins (reference 41 and data not shown). Indeed, to achieve efficient inducible degradation, the transfected cells

had to be pulsed by incubation with the protein synthesis inhibitor cycloheximide (Fig. 5). Nevertheless, the wild-type IκBβ was significantly less efficient than IκBβΔ5-27 in inhibiting the CD28RE activation (Fig. 6B, compare columns 2 to 5 with columns 7 to 10), which was consistent with the finding that the wild-type IκBβ could be degraded in response to the CD28-mitogen signals (Fig. 5). To further confirm that the inhibitory effect of IκBβΔ5-27 on the activation of CD28RE was specific, a control reporter gene assay was performed with an NF-κB-independent system that involved the activation of HTLV-1 LTR by a Tax mutant, TaxM22. This Tax mutant had previously been shown to be able to activate the HTLV-1 LTR but was deficient in NF-κB activation (33). As previously reported (33), cotransfection of TaxM22 with the HTLV-1 LTR-luciferase reporter led to the marked induction of the reporter gene expression (Fig. 6C, column 1). More importantly, expression of IκBβΔ5-27 in these cells did not cause any appreciable inhibition of the induction of this κB-independent reporter (columns 2 to 5). Thus, the inhibitory effect of IκBβΔ5-27 on CD28RE appeared to be specific.

DISCUSSION

Optimal activation of the IL-2 gene requires not only the TCR-mediated primary signal but also a costimulatory signal that can be delivered by CD28. The role of CD28 signal in the activation of the IL-2 gene has been studied largely in combination with T-cell mitogens since this signal alone has no detectable effect on the transcription of the IL-2 gene. Prior studies have shown that, together with mitogens, a stimulating anti-CD28 monoclonal antibody is able to induce the formation of a nuclear protein complex that binds to CD28RE (12, 43). More recently, it has been shown that the CD28RE-binding protein complex contains NF-κB/Rel subunits (14). Although the mitogen PMA alone is able to induce the nuclear expression of NF-κB/Rel, CD28 costimulation leads to a heightened level of nuclear expression of various NF-κB/Rel factors (14, 21). However, it seems that the CD28 signal enhances the general increase of nuclear NF-κB complexes rather than the production of the CD28RE-specific population of NF-κB factors. Indeed, the CD28-induced NF-κB/Rel also binds to the κB enhancer present in the LTR of human immunodeficiency virus type 1 (43). In the present study, we have found that NF-κB/Rel binds to CD28RE as various complexes, including not only the prototypical p50/RelA and p50/c-Rel but also RelA/c-Rel heterodimers. Of note, the RelA/c-Rel heterodimer does not appreciably bind to the palindromic κB sequence (κB-pd [4]) that exhibits high affinity to the p50/RelA and p50/c-Rel heterodimers (data not shown), thus suggesting that the RelA/c-Rel dimer preferentially binds to certain κB-like sequences. In support of this notion, a previous report has shown that the RelA/c-Rel heterodimer also binds to a κB-like sequence in the promoter of the urokinase gene but not the κB sequence of the Igκ gene (17). Induction of the different forms of NF-κB/Rel may be mediated by different cellular stimuli. We have found that the binding of the RelA/c-Rel heterodimer to CD28RE is hardly detectable in Jurkat T cells treated with PMA alone but can be markedly enhanced when the cells are costimulated with anti-CD28 (Fig. 1A and C). We believe that formation of this heterodimer may result from the accelerated nuclear translocation of c-Rel in the costimulated cells (data not shown and reference 9), although it may also be facilitated by the posttranslational modification of c-Rel (9).

The mechanism by which the CD28 signal enhances PMA-induced nuclear expression of NF-κB/Rel has remained elusive. A recent study has shown that CD28 costimulation appears to prolong PMA-induced degradation of IκBα by preventing the rapid replenishment of the depleted IκBα protein pool (21). Although this potential mechanism may contribute to the sustained nuclear expression of c-Rel (9), it does not explain why anti-CD28 enhances the NF-κB/Rel DNA binding activity as early as 15 min following cellular stimulation (Fig. 1). We demonstrate here that CD28 costimulation initiates a potent costimulatory signal that, in synergy with PMA, induces rapid degradation of both IκBα and IκBβ. This specific activity may contribute to both the accelerated and the sustained induction of NF-κB/Rel in the costimulated cells.

IκBα and IκBβ are two major cytoplasmic inhibitors of NF-κB/Rel, and both appear to exhibit regulatory function for RelA as well as c-Rel. However, while most known NF-κB/Rel inducers target the proteolysis of IκBα, degradation of IκBβ requires more specific and probably also more potent signals, such as LPS and IL-1 (40). Thus, activation of the IκBβ-sequestered fraction of NF-κB/Rel occurs only under certain cellular stimulation conditions. Consistent with a prior study (40), we have shown that PMA alone is not efficient in the induction of IκBβ degradation in Jurkat T cells, even at high concentrations (up to 100 ng/ml) (Fig. 2 and 3). However, in the presence of the CD28 signal, as little as 2.5 ng of PMA per ml is sufficient to induce the proteolysis of IκBβ, which strongly suggests that CD28 provides a potent costimulatory signal required for IκBβ degradation. This mechanism appears to be physiologically relevant since the CD28 costimulatory effect on IκBβ degradation was also demonstrated in human peripheral blood T lymphocytes and murine antigen-specific T cells (Fig. 4). As observed with IL-1 and LPS (40), the CD28-mediated loss of IκBβ lasts at least until 8 h following cellular stimulation (Fig. 1). However, degradation of IκBβ through CD28 signaling appears to involve more rapid kinetics in that significant loss of IκBβ can be detected as early as 15 min after cell stimulation (Fig. 1). The rapid degradation of IκBβ is well correlated with the early induction of both the p50/RelA and c-Rel/RelA heterodimers. Thus, it is conceivable that CD28 costimulation may lead to the nuclear translocation of a pool of NF-κB/Rel factors that are normally sequestered in the cytoplasmic compartment through physical interaction with IκBβ. In support of this notion, we have recently found that the rapid degradation of IκBβ in cells stimulated with the phosphatase inhibitor calyculin A also leads to enhanced binding of NF-κB/Rel to CD28RE (17a). The persistent degradation of IκBβ in CD28-costimulated cells likely contributes to the sustained nuclear expression of NF-κB/Rel factors, especially the c-Rel subunit (reference 9 and data not shown). Interestingly, the Tax protein of HTLV-1, a potent activator of NF-κB/Rel (34), has been shown to induce slow but persistent degradation of IκBβ (16, 24). In HTLV-1-infected human T cells that express a high level of Tax protein, little or no IκBβ is detectable, and consistently, these infected T cells constitutively express a high level of active NF-κB/Rel proteins (16, 24).

Our results on the degradation of IκBα are not in full agreement with a prior study showing that CD28 costimulation prolongs PMA-induced depletion of IκBα (21). We could not detect a sustained downregulation of IκBα when the cells were treated with PMA together with anti-CD28 (Fig. 2). This discrepancy is likely due to the much higher concentration of PMA (50 ng/ml) and anti-CD28 (1:2,000 dilution) used in the previous study compared with the present study (10 ng/ml for PMA and a 1:10,000 dilution for anti-CD28). We selected the lower dose since it gave optimal stimulation of the CD28RE reporter gene (Fig. 6 and data not shown). Nevertheless, our results clearly demonstrate that the CD28 costimulatory signal

accelerates and enhances PMA-induced transient degradation of IκBα (Fig. 2).

Recent studies have demonstrated that signal-mediated degradation of IκBα is regulated through its phosphorylation at serine-32 and serine-36 (reviewed in reference 3). The phosphorylation appears to promote ubiquitination of IκBα via two N-terminal lysine residues, which in turn target IκBα for proteasome-mediated degradation (11, 27). IκBβ also contains the two homologous serines and a lysine in its N-terminal region. It is unclear whether the two N-terminal serines of IκBβ are also phosphorylated upon cellular stimulation. We could not detect a mobility change of IκBβ on SDS gels (Fig. 2), as shown for the phosphorylation of IκBα (3). However, since the mobility shift is not a unique feature of protein phosphorylation, it still remains possible that inducible phosphorylation does occur at the two serines of IκBβ but does not cause a mobility shift. Nevertheless, we and others have shown that mutation of these potential phosphorylation sites markedly inhibited the degradation of IκBβ (references 11 and 24 and this study). We have also found that deletion of an N-terminal region covering the potential sites of both inducible phosphorylation (serine-19 and serine-23) and ubiquitination (lysine-9) produced a more dramatic effect on the degradation of IκBβ compared with the mutation of the phosphorylation sites (Fig. 5). These results also indicate that a basal level of ubiquitination and degradation may occur in the absence of inducible phosphorylation. It may also be possible that the CD28 signaling induces phosphorylation of IκBβ at additional sites that constitute part of the signal for targeting ubiquitination and degradation of this inhibitory protein. A definitive answer awaits phosphopeptide analyses of IκBβ.

The mechanism by which the CD28 costimulatory signal promotes degradation of IκBα and IκBβ remains unknown. One possibility is that the CD28 signal enhances mitogen-induced phosphorylation of the IκB molecules. This hypothesis is consistent with the notion that phosphorylation of IκBα and IκBβ may be mediated by the same protein kinase and that the phosphorylation of IκBβ is probably less efficient than that of IκBα (11). It is likely that CD28 costimulation provides a strong signal for the activation of the IκB kinase, and under this condition, both IκBα and IκBβ are efficiently phosphorylated and degraded. This would explain why the CD28 signaling induces degradation of both IκBα and IκBβ, whereas the mitogen PMA, presumably a weak activator of IκB kinase, induces phosphorylation and degradation of only the efficient substrate IκBα. Another possibility is that the CD28 signal enhances the ubiquitination or proteolysis of IκB. In this regard, it still remains a question whether the IκB kinase is the only target of the various NF-κB inducers. Evidence against this notion is provided by studies using the phosphatase inhibitor calyculin A (37). In human Jurkat T cells, calyculin A induces rapid and efficient phosphorylation of IκBα; however, the phosphorylated IκBα is not significantly degraded until 1 h after cellular stimulation. The slow kinetics of degradation of the phosphorylated IκBα is not due to inhibition of the proteolysis machinery by calyculin A, since rapid degradation of IκBα resumes when TNF-α is added together with calyculin A. As such, the synergistic action of the CD28 signal in IκB degradation may be mediated by either elevation of IκB phosphorylation or enhancement of the subsequent proteolytic reactions or both mechanisms.

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