Optimal T cell activation and interleukin-2 production requires a second signal in addition to antigen-mediated T cell receptor (TCR) signaling. The CD28 molecule has been demonstrated to act as an effective costimulatory molecule upon binding by B7.1 or B7.2 present on antigen-presenting cells. The CD28 signal acts in concert with the TCR signal to significantly augment activation of the NF-κB family of transcription factors. The interleukin-2 gene is regulated by NF-κB among other transcription factors, in part, via a CD28 responsive element (CD28RE) present in the IL-2 promoter. Enhanced activation of NF-κB by CD28 is mediated by rapid phosphorylation and proteasome-mediated degradation of the NF-κB inhibitory proteins IκBa and IκBβ, which allows for accelerated nuclear expression of the liberated NF-κB. Herein, we provide evidence that the catalytic activities of two recently identified IκB kinases, IKKα and IKKβ, are significantly elevated when T cells are stimulated through CD28 in addition to mitogen treatment. Catalytically inactive forms of IKKs are able to block the in vivo phosphorylation of IκBa induced by mitogen and CD28. Furthermore, CD28-mediated reporter gene transactivation of the CD28RE/AP-1 composite element is consistently attenuated by the IKK mutants. These findings suggest that cellular signaling pathways initiated at the TCR and CD28 converge at or upstream of IKK, resulting in more robust kinase activity and enhanced and prolonged NF-κB activation.

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1 The abbreviations used are: IL, interleukin; TCR, T cell receptor; CD28RE, CD28 responsive element; TNF-α, tumor necrosis factor-α; NIK, NF-κB-inducing kinase; IKKα, IκB kinase α; IKKβ, IκB kinase β; PMA, phorbol 12-myristate 13-acetate; TPCK, tosylphenylalanyl chloromethyl ketone; GST, glutathione S-transferase; MEK1, mitogen-activated protein kinase/ERK kinase kinase-1; JNK, c-Jun NH2-terminal kinase; SEK, stress-activated protein kinase/extracellular signal-related protein kinase; HA, hemagglutinin.

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accelerate TCR-induced nuclear expression of various NF-κB/Rel transcription factors, the underlying molecular mechanism remains elusive. We and others have previously demonstrated that ligation of CD28 initiates a potent costimulatory signal leading to the rapid and persistent degradation of IκBβ and enhanced degradation of IκBα (20, 23). However, it is not known if CD28 is mediating enhanced IκB kinase activity. We report here that CD28 potentiates the kinase activity of IKKα and IκKβ, which are only weakly activated by mitogen or TCR signals alone.

MATERIALS AND METHODS

Cell Culture and Reagents—Jurkat T cells (ATCC) and Jurkat cells expressing the SV40 large T antigen (Jurkat Tag) (32) 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 lymphocyte-enriched human blood (Biological Specialty Corporation, Colmar, PA) with a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech) followed by negative selection with human T cell enrichment immunocolumns (Biotex Laboratories Inc., Edmonton, Alberta, Canada). C305 (anti-clonotypic Jurkat TCR) was provided by Dr. Michael Karin (University of California, San Francisco) and used at a 1:1000 dilution. 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 (0.2 μg/ml). The antibody against the influenza hemagglutinin (HA) epitope tag (anti-HA) and protein A-agarose (Boehringer Mannheim) was added and incubated for an

CD28 potentiates the kinase activity of IKKα and IκKβ, which are only weakly activated by mitogen or TCR signals alone.

FIG. 1. IKKα kinase activity is enhanced by CD28 costimulation in Jurkat T cells and primary T cells. A, Jurkat cells were either untreated (lane 1) or treated with PMA (10 ng/ml; this concentration was used for all subsequent studies as well, lane 2), PMA plus anti-CD28 (1:10,000, lanes 3 and 6), or anti-CD28 (lane 4) for 7 min. Jurkat cells were also pretreated with 50 μM TPCK for 15 min and then incubated with PMA and anti-CD28 for 7 min (lane 5). An in vitro kinase assay was performed with Jurkat cell lysates using anti-IKKα-specific antiserum and either GST-IκBa-1–55 (upper middle panel, lanes 1–5) or GST-IκBa-1–55 (lanes 6) as a substrate. Autophosphorylation of IKKα is displayed in the uppermost panel. After autoradiography, the membrane was used for immunoblotting with IKKα-specific antiserum to ensure the presence of comparable levels of IKKα protein between different samples (lower middle panel). The same extracts used for kinase assays were also subjected to immunoblotting with an IκBα-specific antiserum (lower panel). B, CD28 also potentiates IKKα kinase activity in primary T cells. Primary T cells were isolated from peripheral blood by Ficoll-Hypaque followed by negative selection. T cells were either left unstimulated (lane 1), or treated with PMA (lane 2) or PMA plus anti-CD28 (lane 3). Whole cell lysates were subjected to in vitro kinase assays as above with the GST-IκBa-1–55 substrate. C, TCR and CD28 costimulation also augments IKKα kinase activity. Jurkat T cells were either left untreated (lane 1) or treated with C305 (1:1000, lane 2), C305 plus anti-CD28 (lane 3), or anti-CD28 alone (lane 4). Lysates were used for in vitro kinase assays with GST-IκBa-1–55 (upper panel). The membrane was subjected to immunoblotting with anti-IKKα to ensure similar protein level (lower panel).

RESULTS

The CD28 Signal Potentiates IKKα Activation in Both Jurkat and Primary Human T Cells—We and others have previously demonstrated that IκBa degradation is enhanced by CD28 costimulation (20, 23), although the underlying mechanism has remained unclear. We examined whether CD28 potentiates mitogen-mediated IKKα activation. We first performed in vitro kinase assays with Jurkat T cells utilizing GST-IκBa-1–55 as a substrate. The kinase activity of IKKα was slightly induced by PMA treatment as described previously (26) (Fig. 1A, lane 2, upper middle panel). However, when cells were treated with CD28 antibody in addition to mitogen treatment, the kinase activity of IKKα was significantly elevated (lane 3), although CD28 alone had no effect on the kinase activity (lane 4). Autophosphorylation of IKKα was also strongly induced by PMA.
plus anti-CD28 treatment (lane 3, upper panel). It should be noted that the time of each stimulation for this and subsequent experiments was 7 min, which represented maximal kinase activity as exerted by each stimulus. In a time course experiment, the kinase activity was highest at 7 min, was sustained for at least 15 min, and finally subsided by 30 min (data not shown). Pretreatment of cells with TPCK, a chymotrypsin-like protease inhibitor, known to block IkB degradation by unknown mechanisms (37), abolished all kinase activity associated with PMA and anti-CD28 treatment (Fig. 1A, lane 5, upper middle panel). To ensure that IKKα kinase activity was directed to the two N-terminal serine residues of IkBα (serine 32 and 36), we used a GST-IκB protein with alanine residues substituted for the two serines as substrate. Importantly, this substrate was not phosphorylated by IKKα in Jurkat cells treated with mitogen and anti-CD28 (lane 6). Immunoblotting of the immunoprecipitated IKKα revealed equal amounts of IKKα for all samples (Fig. 1A, lower middle panel). In addition to IKKα kinase activity, we examined the fate of endogenous IkBα from the same extracts used for kinase assays. Immunoblots of IkBα revealed that PMA only slightly induced IkBα phosphorylation as assessed by a slower migrating band on SDS-polyacrylamide gel electrophoresis gels (Fig. 1A, lane 2, lower panel). When CD28 antibody was added in addition to PMA, a strong phosphorylated band was readily observed (lane 3), and as expected, CD28 alone did not induce IkBα phosphorylation (lane 4). TPCK also inhibited the inducible phosphorylation of IkBα (lane 5). Taken together, IKKα kinase activity is enhanced by PMA and CD28 treatment and is well correlated with in vivo IkBα phosphorylation.

To confirm the physiological relevance and generality of these findings, we recapitulated our experiments in primary T cells. To this end, we purified T cells from human peripheral blood, treated with either PMA alone or PMA together with anti-CD28, and performed IKKα kinase assays with GST-IκBα as a substrate. As observed in Jurkat cells, the untreated human primary T cells exhibited no detectable IKK kinase activity (Fig. 1B, lane 1). Treatment with PMA alone induced moderate IKKα kinase activity (lane 2), which was marked enhanced in the presence of CD28 antibody (lane 3). Autophosphorylation of IKKα was also evident when the cells were treated with both PMA and anti-CD28 (lane 3). Therefore, CD28-mediated IKK kinase activation occurs in both Jurkat and primary human T cells. To confirm that these findings were not specific to the mitogen PMA but rather reflected signaling through the TCR in a more physiological manner, we also used C305 ascites (anti-clonotypic Jurkat TCR) (38). As seen with PMA, treatment of Jurkat cells with C305 resulted in a small degree of IKKα kinase activity (Fig. 1C, lane 2, upper panel). This was significantly enhanced when CD28 was present (lane 3). The levels of IKKα protein were similar in all samples as detected by immunoblotting (Fig. 1C, lower panel). We conclude that CD28 mediates a costimulatory signal resulting in enhanced IKKα-mediated IkBα phosphorylation.

Given that IκBβ is also rapidly degraded when T cells receive a costimulatory signal (23), we next examined the effect of CD28 ligation on IKKα-mediated phosphorylation of IκBβ. For these purposes, we used GST-IκBβ 1–82, which contains the two IKK phosphorylation sites at serines 19 and 23. As expected, IKKα from untreated Jurkat cells displayed no kinase activity toward IκBβ (Fig. 2, lane 1). When treated with PMA, there was slight kinase activity toward IκBβ (lane 2). Importantly, when Jurkat cells were treated with both PMA and CD28, there was a significant up-regulation of kinase activity directed toward IκBβ (lane 3). Once again, CD28 alone had no effect on IKKα kinase activity (lane 4), emphasizing the requirement for two signals to achieve maximal IKKα kinase activity. Together, it appears that CD28 potentiates IKKα-mediated phosphorylation of both IkBα and IκBβ.

CD28 Also Mediates Enhanced IκBβ Phosphorylation of IkBα and IκBβ—We next focused on the other cytokine-responsive IκB kinase IKKβ to determine if it had a similar activation response to CD28 costimulation. First, we examined the effects of endogenous IKKβ on GST-IκBα. In untreated Jurkat cells, there was no IKKβ activity toward IkBα (Fig. 3A, lane 1). Upon treatment with PMA, there was slight IKKβ activity (lane 2), which was subsequently elevated with CD28 treatment (lane 3). As expected, CD28 alone had no effect on IKKβ kinase activity (lane 4). These results suggest that IKKβ activation is reminiscent of IKKα as shown in Fig. 1. We also tested the kinase activity of IKKβ toward IκBβ. Similarly, PMA alone induces a degree of phosphorylation (Fig. 3B, lane 2), which is augmented by CD28 (lane 3). We conclude that IKKβ, in addition to IKKα, is responsive to the CD28 costimulatory signal.

A Catalytically Inactive IKKα Mutant Blocks CD28-mediated in Vivo IkBα Phosphorylation—CD28 synergizes with TCR signaling to accelerate IkBα phosphorylation and degradation (20, 23). To determine the role played by IKKα in the in vivo phosphorylation of IkBα mediated by CD28 we utilized a catalytically inactive IKKα mutant. This mutant, which has been previously demonstrated to inhibit TNF-α-induced RelA nuclear translocation (28), has a methionine substituted for a lysine at position 44, resulting in defective ATP binding. We transiently transfected Jurkat-Tag cells with an HA-tagged IkBα constructs and split the transfection into two, leaving one sample untreated and treating the other sample with a combination of PMA and anti-CD28. As expected, we observed an unphosphorylated IkBα by immunoblotting when the cells were not treated (Fig. 4, lane 2). When the cells were treated with PMA/CD28, two bands were readily detected, with the slower migrating band representing the phosphorylated form (lane 3). Interestingly, when the catalytically inactive form of IKKα was cotransfected with IkBα, the phosphorylation induced by PMA and CD28 was completely blocked (lanes 5 and 7). This result suggests that IKKα is required for CD28-mediated IkBα phosphorylation.

Catalytically Inactive Forms of IKKα and IκBβ Inhibit CD28-mediated CD28/RE/AP-1 Transactivation—It has been previously reported that CD28 responsiveness within the IL-2 promoter is mediated by a CD28/RE/AP-1 composite element (7, 8). We transfected a luciferase reporter construct driven by this composite element into Jurkat cells and either treated with PMA alone or PMA together with anti-CD28. PMA alone was unable to substantially activate this reporter gene (Fig. 5A, lane 2), but when CD28 was added in conjunction with PMA, reporter activity rose to approximately 12-fold above that observed with untreated cells (lane 3 versus lane 1). We next
Interestingly, transfection of a moderate amount (150 ng) of CD28RE/AP-1 reporter and treated it with PMA and CD28. GST-IκBα 1–55 were transfected with 2 μg of an HA-tagged IκBα cDNA together with either an empty vector (lanes 1–3), or the indicated amounts of HA-tagged IKKα K44M (lanes 4–7). Approximately 48 h later, transfected cell lysates were collected and subjected to immunoblotting with an anti-IκBα antibody.

FIG. 3. IKKβ kinase activity is potentiated by the CD28 co-stimulatory signal. A and B, Jurkat cells were treated exactly as described in Fig. 2, except that anti-IKKβ was used for the immunoprecipitation. Cell lysates were subjected to in vitro kinase assays with GST-IκBα 1–55 in A and GST-IκBβ 1–82 as substrate in B.

cotransfected the catalytically inactive IKKα with the CD28RE/AP-1 reporter and treated it with PMA and CD28. Interestingly, transfection of a moderate amount (150 ng) of IKKα K44M cDNA reduced the reporter activity by approximately 50% (Fig. 5C, lane 3). However, a higher dose of IKKβ K44A (200 ng) acted as an even more potent inhibitor of CD28-mediated reporter gene activation, reducing the activity to about 25% of the control (lane 4 versus lane 2). When both IKKα K44M and IKKβ K44A were cotransfected, the inhibition observed was only slightly greater than the inhibition seen with a high dose of IKKβ K44A alone (data not shown). The lack of complete inhibition of reporter gene activity by the dominant negative IKKs is likely due to the high sensitivity of this assay, but we cannot preclude the involvement of additional IκB kinases in CD28-mediated NF-κB activation. From these experiments we conclude that both IKKα and IKKβ are required for CD28-mediated transactivation of a CD28RE/AP-1 composite element.

DISCUSSION

The NF-κB transcription factors are activated by a diverse array of signals that target the inhibitory proteins IκBα and IκBβ for phosphorylation and proteasome-mediated degradation (10). The T cell auxiliary molecule, CD28, which provides a costimulatory signal for T cell activation and IL-2 production, is a potent inducer of NF-κB (23, 39). Although CD28 promotes the rapid degradation of both IκBα and IκBβ, the mechanism has not yet been elucidated. Possibilities include synergy of IκB kinase activity, activation of an IκB kinase unique to the CD28 pathway, or perhaps enhanced ubiquitination and/or direct degradation. In this study, we report that CD28 specifically enhances the kinase activity of IKKα and IKKβ, two recently identified cytokine-responsive IκB kinases. Specifically, we found that the kinase activities of IKKα and IKKβ were elevated for both IκBα and IκBβ when T cells were treated with mitogen and anti-CD28. In addition, a catalytically inactive mutant of IKKα effectively inhibited in vivo CD28-mediated IκBα phosphorylation. Inactive forms of IKKα and IKKβ also attenuated CD28RE/AP-1 luciferase gene reporter activity induced by PMA and CD28. This study provides strong evidence that signaling through the TCR and CD28 converge at or upstream of IKKα and IKKβ, resulting in enhanced kinase activity and NF-κB activation.

Where could the TCR and CD28 pathways possibly converge? Kinases upstream of IKK include the mitogen-activated protein kinase kinase (MEKK1), NIK, and the mitogen-activated protein kinase/ERK kinase kinase 1, MEKK1 (40). A recent report suggests that MEKK1 preferentially activates IKKβ, while NIK activates IKKα and IKKβ equally well (41). Previous studies suggest that MEKK1 is a downstream target of CD28 signaling, and that its kinase activity is up-regulated when stimulated with both anti-CD3 and anti-CD28 (42, 43). It is not likely that MEKK1 is solely responsible for the CD28-mediated up-regulation of IKK kinase activity since we consistently observed stronger kinase activity associated with IKKα rather than IKKβ. NIK is a potential candidate to mediate the signal integration between the TCR and CD28 if the signals do in fact converge upstream of IκK. NIK is already known to integrate signals from pathways initiated by IL-1 and TNF-α to activate NF-κB (30). We are currently investigating any potential role NIK may play in CD28-mediated NF-κB activation.

Besides MEKK1, several other kinases have been identified which have up-regulated kinase activity when T cells are stimulated with anti-CD3 and anti-CD28. Full activation of JNK in T cells is dependent on the integration of the two signals (44). As expected, kinases within the JNK pathway such as p21-activated kinase (43), SEK (45), and MKK7 (45) are similarly dependent on T cell costimulation for full activation. The transcriptional capacity of the c-Jun protein, one of the AP-1 components, is activated as a result of signaling through the JNK pathway (46). The vital role that AP-1 plays in IL-2 transcrip-
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Fig. 5. CD28-mediated CD28RE/AP-1 transactivation is attenuated by catalytically inactive IKK mutants. A, Jurkat T cells (5 × 10^5) were transfected with 2 μg of a CD28RE/AP-1 reporter gene. Approximately 40 h later, transfectants were split into three and either left untreated (lane 1), or treated with PMA (lane 2) or PMA plus anti-CD28 (lane 3) for 8 h. Cells were then lysed and subjected to luciferase assays. Results shown are presented as the fold induction compared with untreated cells (approximately 30,000 cpm). The error bars represent the standard error of the mean for three independent sets of transfectants. B, Jurkat T cells were transfected with 2 μg of a CD28RE/AP-1 reporter gene and either empty vector (lanes 1 and 2) or 150 (lane 3) or 300 ng (lane 4) of IKKα K44M. The cells were either untreated (lane 1) or treated with PMA and anti-CD28 (lanes 2–4). Lysates were subjected to luciferase assays with three independent experiments used to calculate the fold induction and the standard error of the mean. C, Jurkat T cells were transfected, treated, and subjected to luciferase as in B, except that 100 (lane 3) and 200 ng (lane 4) of IKKβ K44A were used in lieu of IKKα K44M.

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