Mutational Analysis of CD28-Mediated Costimulation of Jun-N-Terminal Kinase and IL-2 Production

Corinna Barz, Thomas Nagel, Kenneth E. Truitt and John B. Imboden

J Immunol 1998; 161:5366-5372; ;
http://www.jimmunol.org/content/161/10/5366

References
This article cites 42 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/161/10/5366.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Mutational Analysis of CD28-Mediated Costimulation of Jun-N-Terminal Kinase and IL-2 Production

Corinna Barz, Thomas Nagel, Kenneth E. Truitt, and John B. Imboden

The accessory molecule CD28 delivers a costimulus that acts in concert with TCR signals to promote T cell activation. Activation of Jun-N-terminal kinases (JNK) requires simultaneous stimulation of the TCR and CD28 and, therefore, likely plays an important role in signal integration during costimulation. We investigated the effects of mutations in the 41-amino acid cytoplasmic domain of murine CD28 on its ability to deliver costimuli for JNK activation and IL-2 production when expressed in Jurkat T cells. Our results indicate that the costimulus for JNK activation requires the membrane-proximal 24 amino acids of the CD28 cytoplasmic domain and is not mediated by the tyrosine-based recruitment of signaling molecules, including phosphatidylinositol 3-kinase. Deletion of the carboxyl-terminal 17 amino acids does not affect the ability of CD28 to augment JNK activation but impairs its ability to enhance TCR-mediated production of IL-2, demonstrating that optimal costimulation of IL-2 production requires CD28 signals in addition to the activation of JNK.


The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grant AI-26644, by the Arthritis Foundation, and by the Rosalind Russell Arthritis Center. T.N. was supported by a fellowship from the Deutscher Akademischer Austauschdienst.

2 Current address: Max von Pettenkofer-Institut für Hygiene und Mikrobiologie der Ludwig-Maximilians-Universität München, Germany.

3 Current address: Medizinische Klinik III, Institut für Klinische Immunologie, Ludwig-Maximilians-Universität München, Germany.

4 Current address: Merck Research Laboratories, 126 East Lincoln Avenue, P.O. Box 2000, RY32-645, Rahway, NJ 07065.

5 Address correspondence and reprint requests to Dr. John B. Imboden, Box 0868, University of California, San Francisco, CA 94143. E-mail address: imboden@itsa.ucsf.edu

6 Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; GST, glutathione-S-transferase; hCD28, human CD28; mCD28, murine CD28; PI3-K, phosphatidylinositol-3-kinase; SH2, SH3, Src homology 2 and 3.

Copyright © 1998 by The American Association of Immunologists

0022-1767/98/$02.00
ability of CD28 to promote IL-2 production (38, 39). Instead, Tyr188 appears critical for the delivery of a costimulus in this system, but the biochemical basis for the Tyr188-dependent signal has not yet been identified (40).

Because activation of JNK depends on simultaneous stimulation of the TCR complex and CD28, it is likely that this MAPK subfamily plays a role in the costimulation of T cells. The CD28 signals that regulate coupling to JNK and the relative importance of JNK in mediating cellular responses to CD28 ligation, however, are not known. For these reasons we studied the ability of a series of CD28 cytoplasmic domain mutants to deliver costimuli for JNK activation and IL-2 production.

Materials and Methods

Abs and reagents

Murine anti-human CD3 mAbs were purchased from PharMingen (San Diego, CA). Murine anti-human CD28 mAbs (9.3) were a gift from Dr. Jeffrey Ledbetter (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA). Hamster anti-human CD28 mAbs were a gift from Dr. James Allison (University of California, Berkeley, CA). Goat anti-mouse and goat anti-hamster polyclonal Abs were purchased from Cappel (Ogano Teknika, Durham, NC). Wortmannin was purchased from Sigma (St. Louis, MO). Adenosine-5’-triphosphate (γ32P) was purchased from ICN Pharmaceuticals (Irvine, CA).

Cell culture

Jurkat cells stably transfected with mutant murine CD28 cDNAs have been described previously (38) and were passaged in RPMI 1640 medium supplemented with 10% FBS and 2 mg/ml Geneticin (Life Technologies, Gaithersburg, MD). For JNK assays as well as for IL-2 assays, cells were pelleted and resuspended in RPMI 1640 with 10% FBS at a density of 0.5 million cells/ml.

Preparation of glutathione-S-transferase (GST)-Jun fusion protein

The plasmid encoding GST-Jun (1-79) fusion protein was a gift from Dr. Roger Davis (University of Massachusetts Medical School, Worcester, MA) and was transformed into XL1-Blue Escherichia coli. Bacterial cultures were grown in CircleGrowth medium (BIO 101, Vista, CA) with 100 µg/ml ampicillin. Expression of the GST-Jun fusion protein was induced at OD600 of 0.25 mg/ml isopropl-β-thiogalactopyranoside (Boehringer Mannheim, Indianapolis, IN). Cultures were harvested after 2 h of induction. The bacteria were pelleted, resuspended in PBS, pH 7.3, and lyed by sonication. Triton X-100 (1%) was added to the lysate. After an additional spin, the supernatant containing GST-Jun fusion protein was stored at −80°C. For purification of GST-Jun fusion protein, glutathione-Sepharose beads (Pharmacia, Uppsala, Sweden) were equilibrated with PBS, pH 7.3, and incubated with the bacterial lysate for 1 h at 4°C. The beads were washed twice with PBS, pH 7.3, and four times with lysis buffer (see below) before use.

Cell stimulation and JNK assays

A total of 5 million cells were used per sample and resuspended in 200 µl of RPMI 1640 medium with 10% FBS. mAbs to CD3, human CD28 (hCD28), and murine CD28 (mCD28) were added at a concentration of 10 µg/ml. Samples were incubated at 37°C for 5 min. Goat anti-mouse or goat anti-hamster Abs were added at 100 µg/ml. Cells were incubated at 37°C. After 24 h of incubation, supernatants were harvested and analyzed for IL-2 production by ELISA (Biosource International, Camarillo, CA). Cell stimulation and IL-2 assays

A total of 0.1 million cells were used per sample and resuspended in 200 µl of RPMI 1640 medium with 10% FBS. mAbs to CD3, hCD28, and mCD28 were added at a concentration of 10 µg/ml. Samples were incubated at 37°C for 5 min. Goat anti-mouse or goat anti-hamster Abs were added at 100 µg/ml. Cells were incubated at 37°C. After 24 h of incubation, supernatants were harvested and analyzed for IL-2 production by ELISA (Biosource International, Camarillo, CA).

Results

Murine CD28 stably expressed in Jurkat cells delivers a costimulus for JNK activation and IL-2 production

Recent data implicate JNK in signal integration during costimulation of T lymphocytes (15). In an effort to define the region of the cytoplasmic tail of CD28 required for JNK activation, we studied clones of Jurkat that had been stably transfected with cDNAs encoding wild-type mCD28, mCD28 with cytoplasmic domain truncations, or mCD28 in which cytoplasmic Tyr residues had been mutated to Phe (Fig. 1) (38, 40). The different forms of mCD28 are expressed at comparable levels with the exception of the ALL F mutant, whose level of surface expression is approxi-mately threefold lower than the other mCD28s studied (Fig. 2).

The Jurkat clones express the CD3/TCR complex and the endogenous hCD28 (data not shown), which can serve as an internal positive control for CD28 function in individual clones. We have shown that hCD28 and mCD28 do not form detectable heterodimers when coexpressed in Jurkat (38).

As first reported by Su et al. (15), costimulation of Jurkat cells by the addition of mAbs to CD3 and to hCD28 enhances JNK activity threefold in comparison with stimulation through CD3 alone (Fig. 3A, compare lanes 5 and 7) and sixfold in comparison with stimulation via hCD28 only (Fig. 3A, compare lanes 5 and 7). The activation of JNK by the combination anti-CD3 and anti-hCD28 can be substantially enhanced by the addition of a secondary cross-linking Ab (Fig. 3A, lane 8). Wild-type mCD28 expressed in Jurkat also can deliver signals that act in synergy with those of the TCR to activate JNK. When cross-linked by a second-step Ab, the combination of mAbs to CD3 and mCD28 triggers a substantial increase of JNK activity in Jurkat cells that express wild-type mCD28 (Fig. 3B). The level of JNK activity achieved by cross-linking CD3 with wild-type mCD28 ranges between 50 and

![Figure 1](http://www.jimmunol.org/)  
Amino acid sequence of the cytoplasmic domain of murine CD28. The sequence of the cytoplasmic tail of murine CD28 is shown in one-letter amino acid abbreviations. Indicated are the conserved tyrosine residues (arrows above the sequence) and the truncation sites (arrows below the sequence). In the ALL F mutant, all four tyrosine residues have been substituted by phenylalanine. The Y170 mutant preserves Tyr170, whereas tyrosines Tyr188, Tyr186, and Tyr197 are replaced by Phe.
110% of that induced by the cross-linking of CD3 and the endogenous hCD28 (data not shown).

The mAbs to CD3 and hCD28 are murine and were cross-linked by goat anti-mouse Abs. The mAb to mCD28, however, is of hamster origin. Cross-linking anti-CD3 and anti-mCD28 with goat anti-hamster Ab is sufficient to increase JNK activity in our system, and thus was used for investigation of the mCD28 mutants throughout this study. The use of a mixture of both cross-linking Abs leads to a small additional increase in JNK activity, possibly due to more effective cross-linking of CD3 by goat anti-mouse Abs (Fig. 3B, lane 9).

The cross-linking of both anti-CD3 and anti-hCD28 triggers a substantial increase in IL-2 production over that observed with cross-linking anti-CD3 alone (Fig. 4A). A similar effect on CD3-mediated IL-2 production is observed when mAbs to CD3 and to wild-type mCD28 are cross-linked (Fig. 4B). The IL-2 response elicited by cross-linking CD3 and mCD28 mAbs ranges from 30 to 70% of that observed when CD3 is cross-linked with the endogenous hCD28 (Figs. 4 and 7). Therefore, wild-type mCD28 can deliver a costimulus for both the activation of JNK and IL-2 production.

The membrane-proximal 24 amino acids of the cytoplasmic domain of CD28 can mediate JNK activation upon costimulation

To define a minimal cytoplasmic domain capable of delivering a costimulus for JNK activation, we used truncation mutants of mCD28 stably expressed in Jurkat cells. Cross-linking CD3 with either the T1 or T2 mCD28 mutant induces JNK activation to a level that is comparable with that achieved upon stimulation of CD3 and the endogenous hCD28 (Figs. 5 and 6). The more proximal of these truncations, the T2 mutation, occurs after amino acid 182 and removes the carboxyl-terminal 17 amino acids (Fig. 1). Therefore, these residues, which include the distal three tyrosine residues (Tyr185, Tyr188, and Tyr197), are not necessary for mCD28 to synergize with CD3 to activate JNK. Further truncation to amino acid 174 (the T3 truncation) or deletion of all but the first four amino acids of the cytoplasmic domain (the TL mutant), however, abrogates the ability of mCD28 to deliver a costimulus for JNK activation (Figs. 5 and 6). The inability of the T3 and TL mutants to promote JNK activation is not due to distal defects in the signals that lead to JNK activation as evidenced by the intact costimulatory ability of the endogenous hCD28 in these clones (Fig. 5). Impaired coupling to JNK was observed with three independently derived clones expressing the T3 mutant (Fig. 6).

Enhancement of JNK activation is not sufficient to deliver an optimal CD28 costimulus for IL-2 production

To examine the effects of cytoplasmic domain truncations on costimulation of IL-2 production, we determined the abilities of...
mCD28 and mutants to boost IL-2 production in response to CD3 cross-linking. The boost in IL-2 mediated by the endogenous hCD28 in each clone was used as an internal standard.

When cross-linked with CD3, the two T1 truncation mutants deliver a costimulus for IL-2 production comparable with (T1D6), or greater than (T1A1), wild-type mCD28 (Fig. 7). When all experiments (n = 4-6) were analyzed, the difference between wild-type mCD28 and T1D6 did not quite reach significance (two-tailed p value = 0.06), but the boost delivered by T1A1 was significantly greater than wild-type (p < 0.01). Therefore, the carboxyl-terminal 5 amino acids, which include Tyr 197, are not essential for costimulation of IL-2 production and possibly may down-regulate the ability to promote IL-2 production. The T2 truncation mutants also can promote IL-2 production when cross-linked with CD3, but their ability to do so is impaired relative to the wild-type and to the T1 mutants (Fig. 7). The boost in IL-2 delivered by the wild-type mCD28 is significantly greater than the boosts delivered by either T2C2 or T2D5 (p < 0.01 and < 0.05, respectively; n = 5-6). Similarly, the boost delivered by either of the T1 mutants is significantly greater than that delivered by either T2 mutant (p <
0.001 for T2C2 and < 0.01 for T2D5; n = 4-7). Because both T2 mutants can costimulate JNK activity (Fig. 6), the diminished ability of T2 to enhance IL-2 production likely reflects the loss of JNK-independent signals when the mCD28 cytoplasmic domain is truncated from amino acids 194 to 182. The costimulatory ability of the T3A4 mutant (Fig. 7), other T3 mutants (data not shown), and TL mutant (Fig. 7), in contrast, is severely disrupted. The loss of costimulatory ability when the cytoplasmic domain is truncated from amino acid 182 to 174 (i.e., from T2 to T3) is of interest because this truncation also abrogates the ability of CD28 to augment JNK activity (Figs. 5 and 6).

Wortmannin does not inhibit JNK activation following stimulation of CD3 and CD28

The T2 truncation mutant retains Tyr 170 which, when phosphorylated, serves as a binding site for the Src homology 2 (SH2) domains of the p85 regulatory subunit of PI3-K (Fig. 1). We have shown that the T2 truncation does not alter recruitment of PI3-K to mCD28 (40). Tyr\textsuperscript{170} is also present in the T3 mutant, but this more severe truncation impairs the recruitment of PI3-K p85 (40). Because the ability to costimulate JNK activity also is lost when the mCD28 cytoplasmic domain is truncated from T2 to T3, we considered the possibility that PI3-K might couple CD28 to the activation of JNK. However, wortmannin, a potent inhibitor of PI3-K, enhances the ability of cross-linked CD3 and mCD28 to promote JNK activity (Fig. 8). Because 1000 nM wortmannin completely inhibits PI3-K enzymatic activity and blocks CD28-mediated production of phosphatidylinositol-3,4,5-trisphosphate when added to Jurkat (38), we conclude that PI3-K activity is not required for JNK activation in this system. Interestingly, wortmannin also enhances the ability of CD28 to promote IL-2 production by Jurkat (38).

Cytoplasmic Tyr residues are not required for the costimulatory effect of CD28 on JNK activation

To address the possibility that tyrosine phosphorylation of CD28 is critical for coupling to the activation of JNK, we studied a mCD28 mutant in which all four Tyr residues have been mutated to Phe (ALL F mutant; Fig. 1). These mutations eliminate the PI3-K- and Grb-2-binding sites at Tyr\textsuperscript{170} as well as a proposed distal secondary binding site for PI3-K (41). We have previously shown that the ALL F mutant fails to recruit PI3-K. Cross-linking CD3 and the ALL F mutant reproducibly boosts JNK activity relative to CD3 stimulation alone (Figs. 6 and 9), demonstrating that this signaling

FIGURE 7. Costimulation of IL-2 production in Jurkat cells expressing wild-type or mutated forms of mCD28. Wild-type or mutant mCD28-expressing Jurkat cells were stimulated either with mAbs to CD3, cross-linked by goat anti-mouse Abs, with mAbs to CD3 and mCD28, cross-linked by goat anti-hamster Abs, or with mAbs to CD3 and hCD28, cross-linked by goat anti-mouse Abs. After incubation of the cells for 24 h at 37°C, the supernatants were harvested and assayed for IL-2 by ELISA. The CD28-mediated boost in IL-2 is the difference in the IL-2 produced by cross-linking CD3 and CD28 vs that produced by cross-linking CD3 alone. The mCD28-mediated boost in IL-2 is presented as a percentage of the boost in IL-2 produced by cross-linking CD3 and the endogenous hCD28 in the same experiment and represents the mean ± SEM of three to seven independent experiments for each clone.

FIGURE 8. Costimulation of JNK-activity in Jurkat cells by CD3 and mCD28 is enhanced by addition of the PI3-K-inhibitor, wortmannin. Wild-type mCD28-expressing Jurkat cells (5 million cells/sample) were incubated with wortmannin at the indicated concentrations at room temperature for 5 min prior to stimulation with mAbs to CD3 and mCD28 (10 \mu g/ml each) and goat anti-hamster Abs (150 \mu g/ml) for 20 min. Following lysis, JNK-activity was determined as described in the legend to Fig. 3.

FIGURE 9. JNK activity in transfected Jurkat cells expressing wild-type or tyrosine mutants of mCD28. Jurkat cells expressing wild-type mCD28, ALL F mCD28, or Y170 mCD28 (5 million cells/sample) were stimulated for 20 min with the indicated Abs prior to lysis. JNK-activity in the lysates was assayed as described in the legend to Fig. 3.
event can be Tyr independent. Back mutation to Tyr at position 170 on the ALL F background (Y170 mutant) restores the ability of mCD28 to recruit PI3-K (38), but does not alter its ability to synergize with CD3 in the activation of JNK (Figs. 6 and 9).

**Discussion**

The recent observation that activation of JNK depends upon simultaneous stimulation of the TCR/CD3 complex and CD28 implicates this MAPK subfamily in the costimulation of T cells (15). In this study, we examined the effects of mutations in the cytoplasmic domain on the ability of CD28 to deliver costimuli for JNK activation and IL-2 production.

The CD28 cytoplasmic domain contains four tyrosine residues, at least two of which, Tyr188 and Tyr170 (murine sequence), appear to be involved in CD28-mediated signaling. The ability of CD28 to couple to JNK activation, however, appears to be independent of tyrosine-based interactions of signaling molecules. Deletion of the carboxy-terminal three tyrosines does not alter the ability of mCD28 to deliver a costimulus for JNK activation (T2 mutant; Figs. 5 and 6), and a mCD28 mutant in which all four cytoplasmic tyrosine residues have been mutated to phenylalanine delivers a potent costimulus for the activation of JNK (ALL F mutant, Figs. 6 and 9).

Studies with truncation mutants indicate that the membrane-proximal 24 amino acids of the cytoplasmic domain are sufficient for mCD28 to deliver a costimulus for JNK activation. Deletion of an additional 8 amino acids, however, abrogates coupling to JNK activation. Therefore, these 8 amino acids are either directly involved in generating a signal for JNK activation or are required for the structural integrity of the remaining cytoplasmic domain. The deleted 8 amino acids contain a Pro-Xaa-Xaa-Pro motif, which represents the minimal binding element for SH3-mediated associations and which is conserved in hCD28 (Fig. 1) (42). Therefore, one possibility, is that an interaction with SH3-domain containing molecule couples CD28 to activation of the JNK-signaling cascade.

The best-characterized early CD28-mediated signaling event is the recruitment of PI3-K. This event is triggered by the phosphorylation of Tyr170, which lies within a consensus binding motif (Tyr-Met-Asn-Met) for the SH2 domains of the p85 subunit of PI3-K (33–35). Activation of PI3-K has been implicated in a range of cellular events, including the mitogenic responses to growth factors, cytoskeleton organization, receptor trafficking, inhibition of apoptosis, and the regulation of integrin function (43). Attempts to link PI3-K to CD28-mediated costimulation of IL-2 production, however, have led to contradictory results, with mutation of the PI3-K-binding site abrogating costimulation in certain systems and having no effect in others (36–39). Based on the studies with the ALL F mutant and wortmannin presented here, it is highly unlikely that CD28-activated PI3-K delivers the costimulus for JNK activation. Interestingly, wortmannin augments JNK activation in response to the combination of ionomycin and PMA (40). The diminished ability to deliver a costimulus for IL-2 production may reflect loss of a signaling motif based on Tyr170 (40). Mutation of all four Tyr residues to Phe impairs the ability of CD28 to augment IL-2 production in response to the combination of ionomycin and PMA (40). Restoration of JNK at position 188, but not at any of the other mutated sites, restores this costimulatory ability (40). The biochemical basis for the Tyr188-dependent signal is not known. The impaired but still detectable ability of the T2 mutant to costimulate IL-2 production when cross-linked with CD3 (Fig. 7) contrasts with our earlier demonstration that a mAb to this mutant cannot augment IL-2 production in response to the combination of ionomycin and PMA (40). Interestingly, ionomycin and PMA act in synergy to activate JNK and thus may circumvent any requirements for CD28-mediated costimulation of JNK (15). The ability of CD28 to promote IL-2 production in response to this combination, therefore, may depend largely on signals other than costimulation of JNK activity, and these signals are abrogated by the T2 truncation.

Taken together with earlier studies of CD28 mutants, the results presented here indicate that there are at least three discrete structural elements involved in mCD28 signaling: Tyr170, whose phosphorylation recruits PI3-K; the Tyr188-dependent signal; and a tyrosine-independent signal for JNK activation, which maps to the membrane-proximal 24 amino acids of the mCD28 cytoplasmic domain.

**References**