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CD28-Mediated Regulation of mRNA Stability Requires Sequences Within the Coding Region of the IL-2 mRNA

Jack A. Ragheb, Mary Deen, and Ronald H. Schwartz

Using sequence-tagged genomic reporter constructs, we investigated the contribution of IL-2 sequences to CD28-mediated regulation of mRNA stability. We find that CD28 signaling acts transiently to stabilize the IL-2 mRNA following T cell activation. Such stabilization requires sequences within both exon 2 and the coding region of exon 4. Unexpectedly, CD28 signaling at later times enhances the decay of the IL-2 mRNA. This CD28-dependent decay of IL-2 mRNA requires sequences localized between exon 3 and the stop codon. Our findings demonstrate that the coding region of the IL-2 mRNA contains previously undefined CD28-responsive sequence elements that are critical for the regulation of mRNA stability. The Journal of Immunology, 1999, 163: 120-129.

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2 Abbreviations used in this paper: ARE, AU-rich element; UTR, untranslated region; PCC, pigeon cytochrome c; FAM, 6-carboxyfluorescein; CSA, cyclosporin A; Act D, actinomycin D; INK, c-Jun NH2-terminal kinase; RT, reverse transcriptase.

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SV40 late poly(A) region substituted for the IL-2 3’ UTR and poly(A) signal, was shuttled back into IL2X to generate IL2xpA.

To generate the IL2XpA construct, the pBluescript subclone of the 3’ portion of the IL-2 gene was linearized at the MuniHI site within exon 3 and the site was filled in; next, the plasmid was cut at a flanking BamHI site in pBluescript. The SV40 late poly(A) region from the pSI vector (Promega) was cloned into this plasmid as a Smal-BamHI fragment between the filled-in MuniHI site and the flanking BamHI site. This results in an insertion of seven codons between codon 116 in the native IL-2 sequence and the new methionine initiation codon at position 37. This was confirmed by dideoxy DNA sequencing of the construct. The 3’ portion of the IL-2 gene, now containing the SV40 late poly(A) region substituted for sequences downstream of position 384 in exon 3, was shuttled back into IL2X to give ΔIL2xpA.

Cell culture, transfection, and stimulations

A.E7 is a normal CD4+ Th1 mouse T cell clone that recognizes the pigeon cytochrome c (PCC) peptide 81–104 (29). These cells do not grow continuously; they must be induced to proliferate by exposure to PCC in the context of I-Ek. Proliferation is transient, and the cells subsequently enter a resting state. Resting A.E7 cells are passed by stimulation for 48 h with irradiated B10.A splenocytes at a 10:1 ratio of splenocytes to A.E7 with 5 μM whole PCC (Sigma, St. Louis, MO). The cells were subsequently diluted 1/20 into fresh medium (ER10) consisting of 40% RPMI 1640, 50% modified Eagle’s MEM in HBSS, and 10% FCS (Biofluids, Rockville, MD) with 10 U/ml mouse IL-2 (R&D Systems, Minneapolis, MN) and left undisturbed for 10–14 days at 37°C in a 5% CO2 atmosphere. Stable transfectants of A.E7 with the various sequence-tagged reporters were obtained by electroporation as described below. After stimulation with splenocytes and PCC, cells were harvested on a Ficoll gradient and resuspended in RPMI 1640 with 20% FCS at 4°C. One-half ml of cells was added to 50 μg of linearized DNA on ice in a 4-mm gap cuvette (BTX, San Diego, CA); electroporation was performed with a BTX 600 (BTX, San Diego, CA). Immediately afterward, cells were diluted 30-fold into fresh ER10 with 20 U/ml IL-2 and incubated overnight at 37°C in a 5% CO2 atmosphere. Cells were then Ficoll gradient-purified and resuspended in fresh ER10 at 2.5 × 106 cells/ml with 20 U/ml mouse rIL-2 and 0.3 mg/ml G418 (Life Technologies, Gaithersburg, MD). Following 7–10 days of drug selection, surviving cells were restimulated with PCC and costimulated cell samples. Cells were stimulated for the indicated length of time, after which supernatants were harvested and filtered for a determination of IL-2 concentration by a CTL-L bioassay (32) or by ELISA (R&D Systems). IL2XAC mRNA was detected and quantitated as described.

RNA isolation and quantitation

Cytoplasmic RNA was prepared by lysis of the cells in situ with a Nonidet P-40-containing buffer (Qiagen, Santa Clara, CA) containing DTT (1 mM), RNase inhibitor (5′-3′, Inc., Boulder, CO), and 5 mM EDTA, and RNA was isolated by centrifugation of the lysate through a QIAshredder column (Qiagen). Subsequent purification steps were conducted as described for cytoplasmic RNA. As an additional precaution, for those primer pairs that do not distinguish between mature mRNA and DNA (IL2XAC and IL2X24), RNA was digested with RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C according to the manufacturer’s instructions (Qiagen) before the addition of the silica gel membrane before the wash and elution steps of the protocol.

RT-PCRs were coupled and performed in the same tube using 10–100 ng of total RNA in 1X TaqMan EZ buffer, 2.5 mM manganese acetate, 300 μM of each dNTP, and 100 U/ml of recombinant Thermus thermophilus DNA polymerase in a total volume of 25 μl (Perkin Elmer Applied Biosystems, Foster City, CA). The reverse transcriptase (RT) step was primed with the IL-2-specific primer 5′-TGTTGCGTCCGTGAGC-3′ and conducted at 60°C for 30 min, preceded by a 75°C/10 min denaturation step. The PCR step consisted of 40 cycles of 94°C/15 s, 60°C/60 s, and 72°C/60 s. The IL2XAC mRNA was detected by RT-PCR as described for IL2X.

The IL2X24 mRNA was detected by RT-PCR as described for IL2X, with the exception that a different IL-2 specific primer (5′-TGTTGTAAGCAGAGGGTACATAGT-3′) was used which was also used to prime the RT step. The IL2X24 mRNA was detected and quantitated as described for IL2XLC, except that the coupled RT-PCR was performed in a single tube using Moloney murine leukemia virus RT (0.25 U/ml) and AmpliTaq Gold DNA polymerase (0.025 U/ml) in 1X TaqMan buffer A, 5.5 mM magnesium chloride, and 300 μM of each dNTP in a total volume of 25 μl (Perkin Elmer Applied Biosystems). The RT step was conducted at 48°C for 30 min followed by a 95°C/10 min denaturation step. PCR consisted of 40 cycles of 94°C/15 s → 58°C/60 s.

FIGURE 1. Structure of IL-2 reporter constructs. The DNA structure of the constructs is shown. Open boxes numbered 1–4 are IL-2 exons. Solid lines are introns or flanking genomic sequences. Letters A, B, and C indicate the introns. Closed boxes represent the late SV40 3′ UTR and poly(A) signal. The relative position of the start codon (ATG), stop codon (TAA or TGA), and mRNA instability element (ATTTA) are shown. The sizes of the various DNA components in the schematic are not proportional.
The IL2X24 mRNA was detected and quantitated as described for IL2XAC, except for the use of primers, a probe, and an amplification temperature of 60°C. The IL-2-specific back primer 5'-TTGGTTGAGATGATGCTTITGA-3' and a different IL-2 back primer (5'-CCGCGCACAGATTCC-3') and the corresponding H-2K-specific back primer 5'-CGCAGAGGTC-3' were used in the RT and PCR steps. The sequence-tag specific forward primer 5'-ATGGGACCTAGGCCCCT-3' and the probe 5'-CCTGTCCTAGGCCCCCTCGA-3' were used in the PCR.

Detection and discrimination of the endogenous IL-2 mRNA from the sequence-tagged mRNAs were accomplished through the use of a forward primer specific for the endogenous IL-2 mRNA (5'-ATGGGACCTACAGGAGGCT-3') and a different IL-2 back primer (5'-GGGCTCAGGCAGCC-3') or 5'-GAGCT-3' as described above. RT-PCR conditions were exactly the same as those used in the reactions were the same as described above for IL2X.

H-2K mRNA was used as an internal reference. The RT step was primed with the H-2K-specific primer 5'-GGGCTCAAGGCAGGCC-3' or 5'-GTAATGTATTACACATGC-3'. PCR was conducted with either the H-2K-specific forward primer 5'-AGAAGTGGCATCTGTTG-3' or 5'-CCGCGCACAGATTCC-3' and the corresponding H-2K-specific back primer used in the RT step. Detection of the amplification was achieved by dequenching a 6-FAM-labeled H-2K-specific probe (5'-TTGGGAGAGCAGATATGCAGATGC-3' or 5'-CACAAGGGCCATGGAACCGT-3') as described above. RT-PCR conditions were exactly the same as those described above.

Standard curves were generated for H-2K, IL-2, and IL-2 sequence-tagged mRNAs with total RNA from the appropriate cells costimulated with anti-CD28 for 4 h. The log of the total RNA (nanograms) plotted vs the threshold cycle number is a linear function; threshold cycle number is defined as the amplification cycle number at which the fluorescence emitted is 10× greater than the baseline fluorescence (usually the amount of fluorescence measured between cycles 3 and 15). In general, the standard curves were linear over the range of 5 pg to 100 ng of total RNA. The relative amount of mRNA in the unknown samples was determined from the standard curves. All samples were assayed in triplicate, and the arithmetic mean amount of IL-2 or sequence-tagged IL-2 mRNA was corrected for the amount of H-2K mRNA present. The corrected values were then normalized and plotted as a function of time. Normalization was usually to the corrected amount of mRNA in the 3-h sample stimulated with anti-TCR alone. In the one instance in which the amounts of IL2X and IL2XpA mRNA are directly compared, a correction is also made for the difference in transgene copy number between these stable transfectants. Transgene copy number was determined by quantitative PCR. In most of the experiments in which cyclosporin A (CSA) was added, the 3-h timepoint also represented the “zero time” from which mRNA decay was measured. In some of these experiments, there was a delay in the onset of CSA action as assessed by a failure to observe a drop in the level of IL-2 mRNA within 30 min of CSA addition in cells stimulated with anti-TCR alone (data not shown). In those cases the mRNA t_{1/2} was determined from the point at which mRNA decay was first observed in anti-TCR-stimulated cells (i.e., 30 min). When CSA was added at 2 h, the data were normalized to the 2-h timepoint because it represented the zero time for measuring mRNA decay. In those experiments in which there was not a 3-h timepoint, the data were normalized to the following timepoint. When the decay of the endogenous IL-2 mRNA and the IL-2 reporter mRNA were directly compared in the same CD28-costimulated cells, the data are normalized to the amount of mRNA in the untreated CD28-costimulated sample at 3 h. Because different primers are used to detect the endogenous IL-2 and the IL-2 reporter mRNAs, a direct comparison of the amount of these mRNAs cannot be made. Thus, each mRNA is normalized to its respective untreated 3-h control.
Results

IL-2 reporter constructs

To perform a genetic analysis of CD28-mediated regulation of IL-2 mRNA levels and to observe processes that might alter nuclear posttranscriptional events, we generated a series of sequence-tagged IL-2 genomic reporter constructs (Fig. 1). The construction of these reporters is described in detail in Materials and Methods. The wild-type reporter, IL2X, contains 1.9 kb of 5′ and 1.5 kb of 3′ flanking sequences in addition to the IL-2 exons and introns. Deletion mutants were generated by removing sequences between introns (IL2XBC and IL2XAC), between exons (IL2X24), or from the 3′ end (IL2XpA and ΔIL2XpA) of IL2X. We specifically chose to use an IL-2 promoter-driven reporter construct for our studies to selectively block IL-2 transcription with CSA during our measurement of mRNA 1/2 (34–36). Actinomycin D (Act D), a global inhibitor of transcription, was not used because of its previously reported effect on the stabilization of IL-2 and other mRNAs (15, 34, 37, 38). Although the presence of a CSA-resistant CD28 pathway for IL-2 production has been reported in human T cells, such a mechanism does not appear to be operational in mouse T cells (39, 40). Thus, when CSA is added at the beginning of T cell activation in the presence of maximal TCR and CD28 costimulation, IL-2 (and IL2X) expression is completely blocked (data not shown). The mRNA produced from the sequence-tagged IL-2 transgene was discriminated from the endogenous IL-2 mRNA and quantitated by using real time RT-PCR with a fluorescence-based detection system (33).

CD28 costimulation enhances IL-2 mRNA reporter levels

Examination of the temporal expression of IL2X mRNA reveals that its level in CD28-costimulated cells is increased both early and late in the time course relative to the IL2X mRNA level in cells that have been stimulated with anti-TCR alone (Fig. 2A). This pattern is similar to that observed previously for the native IL-2 mRNA in these cells (15). To assess the contribution of mRNA stabilization to the increased level of IL2X mRNA in CD28-costimulated cells, IL-2 transcription was blocked by the addition of CSA after 3 h of stimulation with or without anti-CD28 Ab. In the presence of CSA, IL2X mRNA levels decline rapidly in cells that are stimulated through the TCR alone (Fig. 2B, □). In cells that were costimulated with anti-CD28 Ab, IL2X mRNA exhibits a biphasic decay curve following the addition of CSA, remaining stable for ~2 h before declining (Fig. 2B, ●). In three independent experiments, IL2X mRNA decayed with a mean 1/2 of 33 min (SEM = 3 min) in CD28-costimulated cells following the initial 2-h period of stability. In cells stimulated with anti-TCR alone, the mean 1/2 was 21 min (SEM = 5 min). The somewhat slower rate of decay in costimulated cells presumably reflects a small residual effect of CD28 signaling on mRNA stability. Previously, more detailed studies had shown that IL-2 mRNA decay exhibits complex kinetics that are best fit by a logistic decay curve (15). For simplicity, in this study we have calculated mRNA 1/2 on the basis of a linear exponential decay process using a least squares fit of the data. The relative level of IL2X mRNA (●) parallels that of the endogenous IL-2 mRNA (▲) in the same CD28-costimulated cells.
mRNA coding sequences are required for responsiveness to CD28 costimulation

To explore the possibility that CD28-regulatory elements exist within the body of the IL-2 mRNA, we created internal deletions of the IL2X construct. The first of these, IL2XBC, deletes between introns B and C, removing exon 3. The mRNA produced from this construct displays a steady-state temporal profile similar to that of the wild-type construct (Fig. 3A, open symbols). IL2XBC mRNA is also stabilized in CD28-costimulated cells that have been treated with CSA (Fig. 3A, ●). The degree of stabilization, however, is not quite as great as that of the wild-type mRNA. Although the level of wild-type IL-2 mRNA typically does not change during the first 2 h of CSA treatment in CD28-costimulated cells, the IL2XBC mRNA decays with an average $t_{1/2}$ of 245 min ($n = 2$, SEM = 36 min) between 3 and 5 h. The decay of IL2XBC mRNA, compared with that of the endogenous IL-2 mRNA (▲) in the same CD28-costimulated cells, is shown in Fig. 3B. Like IL2X, following an initial 2-h period of stability in the presence of anti-CD28, the IL2XBC mRNA decays at a rate ($t_{1/2} = 39$ min) similar to that of the endogenous IL-2 mRNA. These results demonstrate that exon 3 does not contain sequences that are essential for CD28-mediated stabilization of the IL-2 mRNA.

Deletion of exon 2 along with exon 3 in the IL2XAC construct dramatically diminishes CD28-mediated mRNA stabilization. The IL2XAC mRNA in CD28-costimulated cells treated with CSA (Fig. 4, A and B, ○) does not exhibit an initial 2-h window of mRNA stability, as is seen with the parental IL2X mRNA and the mutant IL2XBC mRNA. That the IL2XAC construct is responsive to CD28 signaling, however, is apparent early on, as demonstrated by the steady-state profile of this mRNA (Fig. 4A, open symbols). Decay of the IL2XAC mRNA in cells treated with anti-TCR alone (Fig. 4A, ▲) approximately parallels that seen in CD28-costimulated cells. The average ($n = 3$) $t_{1/2}$ in cells treated with anti-TCR alone is 21 min (SEM = 4 min), which is the same as that of IL2X. The average $t_{1/2}$ of the IL2XAC mRNA in CD28-costimulated cells during the initial 2 h following the addition of CSA is 40 min (SEM = 2 min), in contrast to the 245 min $t_{1/2}$ of IL2XBC. That this alteration in the CD28-mediated stabilization of IL2XAC mRNA is a consequence of the sequences that have been deleted from this construct is illustrated by comparing the decay of IL2XAC mRNA with that of the endogenous IL-2 mRNA in the same cells. Although the relative level of the endogenous IL-2 mRNA (Fig. 4B, ▲) remains unchanged between 30 and 120 min following the addition of CSA, IL2XAC mRNA decays with a $t_{1/2}$ of 37 min in this experiment (Fig. 4B, ○). These results demonstrate that sequences within exon 2 are required for CD28-mediated mRNA stabilization and that exons 1 and 4 are not sufficient.

To determine the contribution of coding sequences within exon 4 to CD28-mediated mRNA stabilization, sequences between the 3′ boundary of exon 2 and the stop codon in exon 4 were deleted to generate the IL2X24 construct. As observed for the IL2XAC mRNA, the steady-state profile of the IL2X24 mRNA demonstrates that this construct is responsive to CD28 signaling (Fig.
which does not decay during the same interval. The slightly longer 5 min mRNA is 34 min (SEM absence of costimulation. The average indicating that IL2X24 mRNA may be slightly more stable in the presence of CD28 signaling regulates mRNA instability conferred by the 3' UTR and upstream sequences

In several systems in which IL-2 or an IL-2 reporter are constitutively expressed, mRNA stabilization is associated with deletion of the 3' UTR. Experiments with the c-fos mRNA, which like IL-2 contains AUUUA elements within its 3' UTR, have shown that the influence of the 3' UTR on mRNA stability is affected by the level and mode of gene expression (17, 37). To determine in our inducible system the contribution of the IL-2 3' UTR to the observed changes in IL2X mRNA levels, we substituted the 3' UTR and poly(A) signal from the SV40 late region for the 3' UTR and poly(A) signal of IL-2 in our genomic construct. In the absence of the IL-2 3' UTR, this construct (IL2XpA) continued to exhibit significant responsiveness to anti-CD28 (Fig. 6A), the magnitude of which was greatest at early timepoints. In cells stimulated through the TCR alone, IL2XpA mRNA (Fig. 6A, - - -) reaches higher steady-state levels and persists much longer than does IL2X mRNA (Fig. 6A, - - -). Furthermore, IL2XpA mRNA levels achieved by TCR stimulation alone reach and even exceed at later times the levels of IL2X mRNA obtained with CD28 costimulation (Fig. 6A, - - -). The dramatic difference in the temporal profile of the IL2X and IL2XpA mRNAs in cells stimulated with anti-TCR alone illustrates the destabilizing effect of the native 3' UTR on IL-2 mRNA. That this difference is due to enhanced mRNA stability is demonstrated by the decay curve for IL2XpA mRNA in TCR-stimulated cells treated with CSA (Fig. 6B, - - -). IL2XpA mRNA levels remain unchanged for 240 min following the addition of CSA, in contrast to IL2X mRNA, which decays with a t1/2 of 124 min), but at a rate considerably slower than that of IL2X.

Unexpectedly, the IL2XpA mRNA in cells treated with CSA after 3 h of CD28 costimulation (Fig. 6C, -) was less stable than in CSA-treated cells that had been stimulated through the TCR alone. In CD28-costimulated cells, the level of IL2XpA mRNA shows little change during the first 2 h following the addition of CSA, but subsequently decays with an average (n = 3) t1/2 of 144

FIGURE 5. Temporal expression and stability of IL2X24 mRNA. The DNA structure of the IL2X24 construct is shown at the top of the figure. See the legend to Fig. 1 for details. mRNA values are the arithmetic average of triplicate samples. Error bars show the SEM. Where error bars are not visible, the size of the bar was smaller than the figure symbol. A. A.E7 cells stably transfected with the IL2X24 reporter were stimulated with anti-TCR Ab alone (■) or in combination with anti-CD28 Ab (○). Results shown are from one representative experiment of six performed. B. A.E7 cells stably transfected with the IL2X24 reporter were stimulated with anti-TCR Ab with or without anti-CD28 Ab for 3 h (0 timepoint) before the addition of CSA. Subsequently, RNA was harvested at the indicated times. The relative level of IL2X24 mRNA in CSA-treated cells stimulated with anti-TCR Ab alone (■) in the same cells is also shown. In this panel, the IL2X24 and IL-2 mRNAs are normalized to their respective untreated, anti-CD28-stimulated, 3-h control. Results shown are from one representative experiment of four performed.
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FIGURE 6. Temporal expression of IL2XpA mRNA. The DNA structure of the IL2XpA construct is shown at the top of the figure. See the legend to Fig. 1 for details. mRNA values are the arithmetic average of triplicate samples. Error bars show the SEM. Where error bars are not visible, the size of the bar was smaller than the figure symbol. A, A.E7 cells stably transfected with the IL2X (dashed line) or the IL2XpA (solid line) reporter were stimulated in parallel with anti-TCR Ab alone (□) or in combination with anti-CD28 Ab (○). mRNA levels were normalized to the amount of 4 h IL2X mRNA in the presence of anti-TCR Ab alone. The relative amounts of IL2X and IL2XpA mRNA were corrected for the 1.5-fold difference in transgene copy number in the transfectants (data not shown). Results shown are from one representative experiment of three performed. B, A.E7 cells stably transfected with the IL2XpA reporter were stimulated with anti-TCR Ab alone for 3 h before the addition of CSA. Subsequently, RNA was harvested at the indicated times from untreated (□) and CSA-treated (○) cells. Results shown are from one representative experiment of three performed. For comparative purposes, results from an independently performed experiment with the IL2X reporter (shown in Fig. 2B) are reproduced in this panel (dashed line) after correction for differences in transgene copy number. C, A.E7 cells stably transfected with the IL2XpA reporter were stimulated with anti-TCR Ab alone (□) or in combination with anti-CD28 Ab (○) for 3 h before the addition of CSA. Subsequently, RNA was harvested at the indicated times from untreated (open symbols) and CSA-treated (filled symbols) cells. Results shown are the average of two independently performed experiments. D, A.E7 cells stably transfected with the IL2XpA reporter were stimulated with anti-TCR Ab alone (□) or in combination with anti-CD28 Ab (○) for 2 h before the addition of CSA. Subsequently, RNA was harvested at the indicated times from untreated (open symbols) and CSA-treated (filled symbols) cells. Results shown are from a single experiment.

Discussion

Stabilization of cytoplasmic IL-2 mRNA is a well recognized but not fully elucidated consequence of CD28 costimulation. Although cytokine mRNA 3′ UTR sequences have long been known to impart instability to a heterologous mRNA, it was only recently that the same sequences were implicated in responsiveness to CD28 signaling (20, 21). In that system, the first IL-2 exon was also required and neither the CD28 unresponsive c-fos 3′ UTR nor, surprisingly, the CD28 responsive GM-CSF 3′ UTR could substitute for the IL-2 3′ UTR (20). Those findings indicate that there is either tremendous specificity in the proteins that presumably interact with the 3′ UTR or that sequence-specific interactions between the 5′ and 3′ UTRs of a given mRNA are necessary for stabilization. Using an inducible sequence-tagged genomic IL-2 reporter, we demonstrate that additional sequences within the coding region of the IL-2 mRNA are required for stabilization of the mRNA in CD28-costimulated cells. Our results, which are summarized in Fig. 8, indicate that sequences within both exons 2 and 4 are required for CD28 responsiveness and that exons 1 and 4 are not sufficient. The latter conclusion is also supported by our observation that a rabbit β-globin reporter containing the IL-2 5′ and 3′ UTR is unresponsive to CD28 signaling (data not shown).

The requirement for multiple, topographically separated sequences in the IL-2 mRNA to participate in CD28-mediated stabilization indicates that a higher order RNA structure may be involved in the process. A similar proposal has been made for the IL-11 mRNA, the phorbol ester-induced stabilization of which has also been found to require sequences dispersed throughout the transcript (41). Such structures could be the consequence of RNA-RNA interactions or could be secondary to protein-protein interactions between trans-acting factors that bind to disparate regions of the mRNA. We are currently working to distinguish between...
these possibilities and to identify RNA binding proteins that are specific to the CD28 pathway.

The transient nature of CD28-induced IL-2 mRNA stabilization and its rather abrupt cessation suggest that this is a tightly regulated process. Control of this process is thus probably mediated by a rapidly reversible mechanism, such as protein phosphorylation. It has been reported that activation of the c-Jun NH2-terminal kinase (JNK) is up-regulated by CD28 (42). Furthermore, inhibitors of JNK activation reduce the CD28-mediated stabilization of IL-2 mRNA, suggesting that JNK is involved in this CD28 pathway (20). Chen et al. found that both a portion of the first IL-2 exon and the 3' UTR were required for JNK-dependent stabilization of a constitutively expressed chimeric IL-2 reporter mRNA in the presence of Act D (20). Deletion of the 5' UTR from the exon 1 IL-2 sequences of the chimeric reporter, however, only partially reduced mRNA stability, while completely abolishing JNK-dependent stabilization.

This last observation, along with the partial blockade of IL-2 mRNA stabilization by inhibitors of JNK, suggests that at least two mechanisms are involved in the CD28-induced stabilization of the IL-2 mRNA. One of these involves JNK and is blocked by inhibitors of JNK activation, such as CSA (42). The other(s) is independent of JNK and resistant to CSA. The use of CSA to selectively block IL-2 transcription in our experiments may consequently have resulted in a blockade of the JNK-dependent component of CD28-induced mRNA stabilization. Therefore, our observation that coding region sequences in exons 2 and 4 are required for CD28-mediated IL-2 mRNA stabilization may represent a sequence requirement that is specific to the CSA-resistant component of mRNA stabilization (Fig. 8). Alternatively, this sequence requirement may reflect other differences in the experimental systems used, such as the use of constitutive vs inducible promoters or the presence of foreign sequences and tandem 5' UTRs in the reporter of Chen et al. (20).

We chose not to use Act D in our experiments because of its previously reported stabilization of IL-2 and other mRNAs (15, 34, 37, 38, 43). A comparative analysis of Act D and CSA in human PBLs demonstrated that IL-2 mRNA decayed in CSA-treated cells with the same kinetics that were observed upon mitogen withdrawal. By contrast, IL-2 mRNA levels remained unchanged over the same time interval in Act D-treated cells (34). Others, however, have reported IL-2 mRNA decay in the presence of Act D (14, 20). Collectively, these studies suggest that there are also at least two components of IL-2 mRNA decay: one that is blocked in the presence of Act D and another that is unaffected by Act D. These two components of IL-2 mRNA decay may be differentially affected by CD28 costimulation. The enhanced decay of IL-2 mRNA in Act D-treated cells that also receive CSA suggests that this is the case (20).

Our results also indicate that there are two IL-2 mRNA instability sequence elements that are influenced by CD28 signaling in CSA-treated cells (Fig. 8). The principal element resides within the

**FIGURE 7.** Temporal expression and stability of ΔIL2XpA mRNA. The DNA structure of the ΔIL2XpA construct is shown at the top of the figure. See the legend to Fig. 1 for details. mRNA values are the arithmetic average of triplicate samples. Error bars show the SEM. Where error bars are not visible, the size of the bar was smaller than the figure symbol. A, A.E7 cells stably transfected with the ΔIL2XpA reporter were stimulated with anti-TCR Ab alone (○) or in combination with anti-CD28 Ab (□). Results shown are from one representative experiment of six performed. B, A.E7 cells stably transfected with the ΔIL2XpA reporter were stimulated with anti-TCR Ab alone (○) or in combination with anti-CD28 Ab (□) for 2 h before the addition of CSA. Subsequently, RNA was harvested at the indicated times from untreated (open symbols) and CSA-treated (filled symbols) cells. Results shown are from one representative experiment of five performed. C, A.E7 cells stably transfected with the IL2XpA or the ΔIL2XpA reporter were stimulated with anti-TCR Ab in combination with anti-CD28 Ab for 3 h before the addition of CSA. Subsequently, RNA was harvested at the indicated times. The relative amounts of the IL2XpA (●) and the ΔIL2XpA (○) mRNAs in CSA-treated, anti-CD28-stimulated samples normalized to their respective untreated 3-h controls are shown. Results shown are from one representative experiment of three performed.
IL-2 3' UTR and presumably is the AUUUA sequence that had been found there previously. Determination of the mRNA levels produced from the inducible IL2XpA and DIL2XpA genomic constructs supports previous results in which a constitutively expressed IL-2 cDNA construct with a partial IL-2 3' UTR and a rabbit β-globin poly(A) signal produced higher levels of IL-2 protein (18). Taken together, these results indicate that the instability conferred by the IL-2 3' UTR is not dramatically influenced by the mode of gene expression. One cannot conclude from this, however, that CD28 responsiveness is independent of the mode of gene expression.

Sequence instability elements have also been identified outside the 3' UTR of several mRNAs (37, 41, 44, 45). The existence of a second sequence element outside of the IL-2 mRNA 3' UTR that confers instability is suggested in our studies by the enhanced decay of the IL2XpA mRNA in CD28-costimulated cells relative to cells stimulated through the TCR alone, and by the insensitivity of the DIL2XpA mRNA to this effect. The latter demonstrates that the CD28-induced instability observed with the IL2XpA mRNA is not simply due to the presence of an SV40 3' UTR or poly(A) signal and localizes a sequence element necessary for this instability to the region between nt 384 in exon 3 and the stop codon (Fig. 8).

Precedence for CD28 down-regulatory rather than up-regulatory signaling comes from an examination of the expression of the α inhibitor of NF-κB (IκBα), CCR5, and Nil-2a levels in response to CD28 signaling (46–48). In our A.E7 T cell system, we have also observed a down-regulation of mouse CCR5 mRNA in response to CD28 costimulation (data not shown). Whereas CD28-induced IL-2 mRNA stabilization is detectable as early as 2 h and is over by 5 h, CD28-induced mRNA decay, which is mediated through coding region elements, is not apparent until after 5 h. These kinetics suggest that CD28-induced IL-2 mRNA stabilization may be counterregulated by a late acting, CD28-induced decay mechanism. The instability conferred by the IL-2 coding region element is small (2-fold) relative to that caused by the 3' UTR. Nonetheless, the effect of the second element is readily apparent in the absence of the 3' UTR, but remarkably, only in cells that have been CD28 costimulated. The dire biological consequences of a 2-fold change in mRNA t₁/₂ have recently been demonstrated for TNF-α. In this case, mice lacking an RNA binding protein that interacts with the ARE of the TNF-α mRNA develop a spectrum of autoimmune diseases that is entirely attributable to the alteration in TNF-α mRNA t₁/₂ (25).

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