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Regulation of CD154 (CD40 Ligand) mRNA Stability During T Cell Activation

Gregory S. Ford, Bryan Barnhart, Scott Shone, and Lori R. Covey

The CD154 protein (CD40 ligand), which is critical to the regulation of both humoral and cellular immune responses, is expressed transiently on the surface of activated CD4+ T cells. To determine whether control of mRNA stability contributes to the highly regulated expression of CD154 during T cell activation, CD4+ T cells were isolated from human peripheral blood and stimulated for various lengths of time with plate-bound anti-CD3 mAb. At early times after anti-CD3 activation, the CD154 message was found to be very unstable, however, the stability measurably increased after 24–48 h of activation. Similar analyses of TNF-α and c-myc mRNA decay throughout a time course of T cell activation revealed patterns of regulation that were distinct from CD154. Similar to the effect on TNF-α mRNA, stimulation of T cells with PMA + ionomycin greatly increased the stability of CD154 message. However, CD154 message stability was only modestly increased in T cells coactivated with anti-CD3 and anti-CD28 at 5 h and not increased by costimulation at 24 h. Finally, an analysis of both mRNA and surface protein expression over a time course of T cell activation with anti-CD3 revealed a rapid induction of expression early after activation. This induction was followed by a more gradual decrease in expression over the next 48 h. Together, these data support a role for posttranscriptional regulation in the control and overall expression of CD154 in activated T cells. The Journal of Immunology, 1999, 162: 4037–4044.

Signaling between Th cells and APCs during a specific immune response initially involves TCR recognition of an antigenic peptide associated with class II MHC molecules expressed on the surface of the APC. One important consequence of this interaction is the up-regulation of activation-specific effector molecules, including the ligand for CD40, CD154, on the surface of the CD4+ T cells. The interaction between CD154 and its receptor CD40, expressed primarily on the surface of B cells and other APCs, has been shown to be critical for the development of both humoral and cellular immune responses (reviewed in Refs. 1–3). Although the functional analysis of CD154 has been extensive, little is known about the mechanisms controlling CD154 expression in activated T cells. Regulating the overall expression of CD154 is critical for restricting T helper activity since CD40 is constitutively expressed on the surface of both Ag-selected and nonselected B cells. Unrestricted CD154 expression could potentially lead to the activation of nonantigen-specific B cells and result in autoimmunity (4, 5).

Studies examining the kinetics of CD154 expression have shown maximal levels of surface expression 6–24 h after T cell activation with various stimuli. The amount, as well as the kinetics of CD154 expression over a time course of activation, appear to depend on the type of activation stimuli and on costimulatory interactions provided by B cells or APCs (6–15). For example, T cell contact with CD40-expressing cells clearly down-regulates CD154 surface expression on activated T cells by increasing receptor-mediated endocytosis (12). But, decreased surface expression through CD40 contact does not correlate with a reduction in the steady-state level of CD154 mRNA (15). Also, costimulation through CD28 appears to augment the expression of CD154, which is accompanied by an increase in CD154 mRNA (11). However, the absolute requirement for costimulation on the induction of CD154 expression is debatable (16–18). Together, these results point toward CD154 expression being controlled by multiple factors. These factors may be contingent on the process by which T cells become activated and the extent to which the cell has received additional signals through costimulatory and/or adhesion molecules. Also, the time after activation is an important variable in the expression of both CD154 mRNA and protein.

Posttranscriptional control of messenger RNA stability is a particularly important component of the regulation of many human genes, such as cytokines and proto-oncogenes, that are expressed in response to specific cellular signals. Rapid changes in overall mRNA levels can be more easily achieved when both transcription rate and message stability are altered (reviewed in Ref. 19). The rapid and transient expression of CD154 during T cell activation suggest that a layer of control may be at the posttranscriptional level.

Several observations suggest that CD154 may be regulated at the level of message stability. First, the CD154 gene shares considerable sequence homology with its family member TNF-α, which has been shown to be regulated in part by control of message stability (20–22). Second, the CD154 message, like TNF-α, contains multiple copies of the AUUUA sequence in its 3'-untranslated region (3'-UTR)3. This element has been identified as a sequence motif that affects the message stability of many mRNAs (23–25). The mRNA stability of several cytokines is correlated with RNA-binding activities that selectively bind to AUUUA multimers in the 3'-UTR of these cytokine messages during T cell activation (21, 26).

3 Abbreviations used in this paper: UTR, untranslated region; ARE, AU-rich elements; ion, ionomycin; Act D, actinomycin D.
POSTTRANSCRIPTIONAL REGULATION OF CD154 mRNA

Total RNA was isolated from 5 × 10^6 to 1 × 10^7 cells using the Trizol reagent (Life Technologies, Gaithersburg, MD). RNA (6–15 μg) was run in 1.2% agarose/formaldehyde gels and transferred to supported nitrocellulose. Membranes were hybridized to random-primed 32P-labeled probes overnight at 42°C and then washed three times at 68°C for 15 min with 2× SSC + 0.5% SDS. After hybridization with the 28S rRNA probe, membranes were washed three times at 68°C for 15 min with 1× SSC + 0.5% SDS, followed by a single wash for 1 h at 68°C with 0.1× SSC + 0.1% SDS. Membranes were exposed to XAR-film or scanned using the Storm PhosphorImager system (Molecular Dynamics, Sunnyvale, CA) for signal quantification.

DNA and RNA probes

The human CD154 cDNA, isolated from the D1.1 Jurkat T cell line (7, 30), was used as a probe on Northern blots. For the analysis of TNF-α expression, a 1.1-kb human TNF-α cDNA was excised from the pE4 plasmid clone (American Type Culture Collection, Manassas, VA). For the analysis of c-myc expression, a 1.6-kb human genomic fragment was isolated from a plasmid containing the 3′ half of the v-myc gene from the avian myelocytomatis virus. All probes were generated by random-primer labeling according to standard protocols. The human 28S rRNA-specific probe was generated by in vitro transcription of the pT7285 plasmid (Ambion, Austin, TX) in the presence of [γ-32P]UTP.

Determination of mRNA half-lives

mRNA t1/2 were determined by the linear regression of semilogarithmic plots of fraction mRNA remaining after Act D addition vs time after Act D addition according to previously described methods (31). Fractions of mRNA remaining were normalized to levels of the 28S rRNA to control for loading error.

Determination of CD154 surface expression

After various periods of stimulation with plate-bound anti-CD3 mAb, 0.5 × 10^6 cells were stained with either a FITC-labeled anti-human CD154 mAb (Ancell, Bayport, MN) or a corresponding FITC-labeled mouse IgG1 isotype control (Ancell). After activation, cells were washed once with 1× PBS and incubated with 1:50 dilution of either Ab. Cells were incubated for 45 min at 4°C, and, after washing once with 3.5 ml of FACS wash (MEM + 12 mM HEPES (pH 7.2), 0.2% NaHCO3), were fixed in 1% paraformaldehyde. Fluorescence was measured using an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL).

Results

CD154 and TNF-α mRNAs show similar kinetics of up-regulation in response to T cell activation

To establish the kinetics of CD154 mRNA expression in response to conditions of anti-CD3 mAb, purified CD4+ T cells were positively selected and stimulated over a time course of activation. In multiple experiments, the purity of the selected population was >97% CD3+/CD4+ and <1% CD19+HgM− cells (Fig. 1A, and data not shown). This level of purity in the T cell population insured that the analysis of CD154 expression was being conducted virtually in the absence of other cell types. When the kinetics of CD154 mRNA expression were analyzed, we found that expression was maximal 1–3 h postactivation, followed by a gradual decrease in expression over the next 2 days. In addition, a comparison of CD154 and TNF-α mRNA expression in activated T cells showed very similar kinetics except for a more gradual decline in CD154 mRNA levels after 2 h of stimulation (Fig. 1B). Together, these results demonstrate a highly regulated pattern of CD154 mRNA expression over a time course of T cell activation that is very similar to the expression of TNF-α mRNA over the same course of stimulation.

Materials and Methods

CD4+ T cell isolation

CD4+ T cells were removed from 50 cc human leukocyte preparations provided by the New Brunswick Affiliated Hospitals Blood Center (New Brunswick, NJ). Briefly, the leukocyte preparation was spun through histopaque (Sigma, St. Louis, MO) to isolate PBMC. CD4+ T cells were removed from total PBMC by biomagnetic separation using anti-CD4-coated magnetic beads, following protocols provided by Dynal (Lake Success, NY). To determine the purity of CD4+ T cell preparations, isolated T cells were dual stained with FITC-labeled mouse anti-human anti-CD3 mAb plus R-phycocerythrin-labeled mouse anti-human anti-CD4 mAb (Sigma) and analyzed for two-color fluorescence on an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL).

T cell activation and culture conditions

For stimulation of T cells with an anti-CD3 mAb, 10-cc tissue culture plates were coated overnight at 4°C with 4 μg/ml of the anti-CD3 mAb clone HIIT3a (PharMingen, San Diego, CA) in 4 ml 1× PBS. After overnight incubations, coating solutions were removed, and plates were washed gently with 1× PBS to remove unbound Ab. CD4+ T cells (~4 × 10^7) were added to separate Ab-coated plates for 2, 8, 24, or 48 h. After activation, cells were transferred to new plates and treated with 10 μg/ml of the transcriptional inhibitor Actinomycin D (Act D; Sigma) for different lengths of time.

For stimulation with PMA and ionomycin (ion), isolated CD4+ T cells (3 × 10^6) were cultured with 10 ng/ml PMA and 1 μg/ml ionomycin in 10 ml of RPMI 1640 complete medium. After various times of stimulation, cells were washed once with PBS, resuspended in 10 ml of RPMI 1640 + 10% FCS, 100 U/ml of penicillin, 100 μg/ml streptomycin, 2 mM glutamine (complete) and treated with 10 μg/ml Act D. After various times of Act D treatment, aliquots of cells were removed for RNA isolation.

For experiments assessing the effects of costimulation on CD154 mRNA stability, isolated CD4+ T cells were cultured on 10-cc plates precoated with anti-CD3 mAb as above and 4 μg/ml of the anti-CD28 mAb clone 9.3 (a gift from Dr. Carl June, Uniformed Armed Services University, Bethesda, MD) for 5 or 24 h. For stimulation with soluble anti-CD28, the 9.3 mAb (4 μg/ml) was added directly to the T cells that were being cultured on anti-CD3-coated plates. After incubation, cells were treated with Act D, as described above.

Analysis of steady-state levels of RNA were conducted on ~4 × 10^7 CD4+ T cells at each indicated time point. Cells were incubated either on anti-CD3-coated plates or with PMA/ion for the indicated period of time. Cells were then removed and frozen for RNA.

More recent studies have directly demonstrated functional relationships between AU-rich element (ARE)-binding trans-acting factors and the control of mRNA stability. For example, Hel-N1, a protein isolated on the basis of homology to the drosophila embryonic lethal abnormal vision gene (ELAV) gene, appears to stabilize the glucose transporter (GLUT-1) mRNA when overexpressed in 3T3 cells by binding to AU-rich 3′-UTR sequences (27). Another member of the ELAV-like family, HuR, binds in vitro to ARE found in several mRNAs like IL-3 and c-fos (28). Interestingly, this protein has in vitro RNA-binding specificity to different AU-rich sequences that correlates with the ability of those sequences to direct in vivo mRNA decay (28, 29). Stabilizing or destabilizing RNA-binding factors that recognize AU-rich 3′-UTR sequences could regulate CD154 message stability during T cell activation.

To investigate whether mRNA stability contributes to the overall regulation of CD154 expression, we have examined CD154 mRNA stability under different conditions of T cell activation. We have found that CD154 message stability in CD4+ T cells is dependent on the pathway and duration of T cell activation. Also, costimulatory signals did not significantly alter the stability of the CD154 message. In relating these findings to CD154 surface expression, CD154 was detected on the surface of purified CD4+ T cells at both early and later times following activation with an anti-CD3 mAb. Together, these findings suggest that the posttranscriptional regulation of mRNA stability plays a role in the control of CD154 expression during T cell activation.

DNA and RNA probes

The human CD154 cDNA, isolated from the D1.1 Jurkat T cell line (7, 30), was used as a probe on Northern blots. For the analysis of TNF-α expression, a 1.1-kb human TNF-α cDNA was excised from the pE4 plasmid clone (American Type Culture Collection, Manassas, VA). For the analysis of c-myc expression, a 1.6-kb human genomic fragment was isolated from a plasmid containing the 3′ half of the v-myc gene from the avian myelocytomatis virus. All probes were generated by random-primer labeling according to standard protocols. The human 28S rRNA-specific probe was generated by in vitro transcription of the pT7285 plasmid (Ambion, Austin, TX) in the presence of [γ-32P]UTP.

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Results

CD154 and TNF-α mRNAs show similar kinetics of up-regulation in response to T cell activation

To establish the kinetics of CD154 mRNA expression in response to conditions of anti-CD3 mAb, purified CD4+ T cells were positively selected and stimulated over a time course of activation. In multiple experiments, the purity of the selected population was >97% CD3+/CD4+ and <1% CD19+HgM− cells (Fig. 1A, and data not shown). This level of purity in the T cell population insured that the analysis of CD154 expression was being conducted virtually in the absence of other cell types. When the kinetics of CD154 mRNA expression were analyzed, we found that expression was maximal 1–3 h postactivation, followed by a gradual decrease in expression over the next 2 days. In addition, a comparison of CD154 and TNF-α mRNA expression in activated T cells showed very similar kinetics except for a more gradual decline in CD154 mRNA levels after 2 h of stimulation (Fig. 1B). Together, these results demonstrate a highly regulated pattern of CD154 mRNA expression over a time course of T cell activation that is very similar to the expression of TNF-α mRNA over the same course of stimulation.
Extended activation of T cells with an anti-CD3 mAb selectively stabilizes the CD154 mRNA

To determine how mRNA stability influences the overall control of CD154 expression during a time course of T cell activation, purified CD4\(^+\) T cells were stimulated for different lengths of time (2, 8, or 24 h) with plate-bound anti-CD3. Message stability was analyzed by treating cells with the transcriptional inhibitor, Act D, from 1–4.5 h. Under these conditions of transcriptional activation and inhibition, we found that the CD154 message was very unstable after 2 and 8 h of contact with anti-CD3 (Fig. 2A). This finding was very similar to results obtained for both the TNF-\(\alpha\) and c-myc transcripts at these time periods postactivation (Fig. 2, A and B).

Surprisingly, when we assayed CD154 message stability after 24 h of continuous activation with anti-CD3 mAb, we found that the stability of the CD154 transcript had markedly increased. Under identical conditions and throughout a time course of T cell activation, both the TNF-\(\alpha\) and c-myc mRNA decay rates remained relatively unchanged and very unstable compared with the CD154 transcript (Fig. 2, A and B).

The CD154 mRNA half-life increases significantly after 24–48 h of anti-CD3 stimulation

As a consequence of the rapid decay of the CD154 mRNA by 1 h of Act D treatment, we were unable to establish an mRNA \(t_{1/2}\) in our initial experiments. Therefore, to determine the \(t_{1/2}\) of the CD154 message after several periods of anti-CD3 stimulation (2, 8, 24, and 48 h), CD4\(^+\) T cells were treated with a shorter time course of Act D treatment (0, 15, 30, and 60 min). Again, we found that the CD154 message became measurably more stable after 24 h of anti-CD3 stimulation compared with 2 h poststimulation with \(t_{1/2}\) of 84 min vs 40 min, respectively (Fig. 3A and Table I). This time course of activation also allowed us to assess CD154 mRNA stability beyond 24 h of anti-CD3 stimulation. At 48 h, the CD154 mRNA became increasingly stable with a calculated \(t_{1/2}\) of 132 min (Fig. 3A and Table I). The Northern blot analysis data was normalized to the 28S rRNA signals and presented graphically in Fig. 3B. This representation reveals the clear difference in stability between the CD154 and TNF-\(\alpha\) mRNAs at later times postactivation. The \(t_{1/2}\) value of the c-myc message remained relatively unchanged after prolonged anti-CD3 signaling (Fig. 3A).

Our observations that 1) the greatest level of CD154 steady-state expression occurs at a time when the mRNA is being rapidly degraded and 2) message stability occurs when steady-state levels have decreased, suggest that CD154 mRNA expression is controlled by both transcriptional mechanisms and message stability throughout T cell activation. Also, the “regulated instability” pathway observed over a time course of T cell activation appears to function specifically to control the expression of CD154. This is highlighted by the fact that the stability of the TNF-\(\alpha\) or c-myc transcripts does not significantly change over the same time period under the identical conditions of activation (Table I).
**CD28 costimulation does not significantly affect the stability of the CD154 transcript**

The difference in the pattern of mRNA stability between the CD154 transcript at early and late times post anti-CD3 activation led us to examine whether this pattern of regulated instability was a consequence of costimulatory signals working in combination with anti-CD3-responsive pathways. CD154 surface expression and associated “helper” activity for B cells has previously been shown to increase in response to costimulatory interactions in the presence of CD3 signaling (11, 17). Also, in the presence of costimulation there is a significant increase in the stability of the TNF-α transcript (20). As shown above, our population of CD4 T cells was very homogeneous. However, it was possible that costimulatory interactions between T cells or with a small number of contaminating B cells or macrophage were influencing CD154 message stability at later times postactivation. Alternatively, a lack of costimulation might lead to a stabilization of mRNA that is associated with the anergic state of the T cells. Therefore, to establish if CD28-mediated signals are influencing CD154 mRNA stability at early or late times postactivation, we costimulated purified CD4 T cells with anti-CD3 mAb in the presence or absence of immobilized anti-CD28 mAb. CD4 T cells were activated according to previously described conditions for the stabilization of the TNF-α transcript (20) at time points that corresponded to an unstable (5 h) or stable (24 h) CD154 transcript. Decay of CD154 mRNA was analyzed by Act D treatment over a 1-h period. 

After CD154 mRNA expression was normalized to 28S rRNA expression, we observed a modest increase in CD154 mRNA stability from 45 to 52 or 54 min with immobilized anti-CD28 at 5 h postactivation (Fig. 4A). We observed a similar level of stabilization when T cells were costimulated by soluble anti-CD28 mAb (Table I, and data not shown). This level of increase correlated with the level of stabilization we observed for the TNF-α transcript under the same conditions (Table I). Similarly, when T cells were activated for 24 h in the presence or absence of costimulation, we observed no significant difference in the decay pattern of CD154 mRNA (Fig. 4B). To insure that, under our conditions of activation, costimulatory signals were being transduced in response to CD28 contact, steady-state levels of CD154 and IL-2 mRNA were analyzed in the presence and absence of costimulation. In accordance with previous findings (11, 32, 33), we found IL-2 and

<table>
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<th>Activation Condition</th>
<th>CD154 mRNA half-life (min)</th>
<th>TNF-α mRNA half-life (min)</th>
<th>c-myc mRNA half-life (min)</th>
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<tr>
<td>+ Anti-CD3 2 h</td>
<td>40</td>
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<td>44</td>
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<td>8 h</td>
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<tr>
<td>+ Anti-CD3 (5 h)</td>
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<td>+ Anti-CD3 (24 h)</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>+ (i) α-CD28</td>
<td>74</td>
<td>N/A</td>
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Table I. mRNA half-lives after T cell activation

The t₁/₂ values were determined from semilogarithmic plots of fraction mRNA remaining after Act D addition according to the following formula: t₁/₂ = ln(0.5) dy/dt + ln(0.5)/t₁/₂(28S), where the t₁/₂(28S) = 40 h. Values were calculated from data presented in Figs. 2–4, as well as in experiments not shown. N/A, not assayed.
CD154 mRNA expression to be up-regulated in response to co-stimulatory signals (~10-fold and 2-fold, respectively). Therefore, costimulatory signals were being actively transduced to CD4+ T cells under the conditions of our assay. However, signaling resulted in only an ~20% increase in CD154 mRNA stability at 5 h and no measurable increase in stability at 24 h. These data suggest that the regulated decay of CD154 mRNA throughout T cell activation is uncoupled to the process of T cell costimulation by CD28 signaling.

The CD154 mRNA is stable in T cells activated with PMA + ion

Previous analyses revealed that TNF-α expression is regulated at the level of mRNA stability during T cell activation and that stability is increased after PMA/ion activation (20–22, 34, 35). Because of the structural and regulatory similarities between the two genes, it seemed quite possible that CD154 mRNA stability was also altered in response to PMA/ion induction. In comparing the steady-state level of CD154 mRNA in either PMA/ion or anti-CD3-activated CD4+ T cells over a time course of activation, we found that the overall expression level was significantly greater with PMA/ion at all time points tested (Figs. 5A). This finding correlates with previous reports showing that surface expression of CD154 protein is considerably higher in PMA/ion-activated T cells over a time course of activation, we established whether the disparity in mRNA expression levels could result in only an ~20% increase in CD154 mRNA stability at 5 h and no measurable increase in stability at 24 h. These data suggest that the regulated decay of CD154 mRNA throughout T cell activation is uncoupled to the process of T cell costimulation by CD28 signaling.

The kinetics of CD154 surface expression reflect changes in steady-state mRNA levels and message stability during T cell activation

Several mRNAs require translation to “activate” the destabilization element involved in rapid decay pathways (42–46). In light of this mechanism, one possible explanation for the regulated increase in CD154 mRNA stability at later times postactivation is that the CD154 mRNA is not actively being translated. To rule out this possibility and to relate changes in message stability and mRNA levels to the overall regulation of CD154 expression, we examined the kinetics of CD154 surface expression on T cells activated with anti-CD3 mAb. CD4+ T cells were stimulated as described above and stained with either a FITC-labeled anti-CD154 mAb or a FITC-labeled isotype control mAb. As seen in Fig. 6 and in confirmation of others’ findings (6, 14, 36), the overall expression of CD154 on anti-CD3-stimulated T cells is relatively low with peak expression occurring between 8 and 24 h of stimulation. By 48 h, there is a noticeable reduction in expression, but the level is still slightly above unstimulated cells. Since the turnover rate of CD154 protein in anti-CD3-activated T cells has not been determined, it is not possible to state which mechanism is responsible for the decay of CD154 mRNA in these cells.
been shown to be ~10 h with prolonged stimulation (8), at 48 h the translation of surface CD154 protein would have occurred when the CD154 mRNA is already stabilized. Therefore, the change in CD154 mRNA stability does not appear to be related to a block in translation. The pattern of increased CD154 mRNA stability at later time points after activation corresponds to a gradual reduction or maintenance of CD154 surface expression during this same time period.

Discussion

Previous studies have emphasized the restricted nature of CD154 protein expression, both with respect to cell type and within a time course of immune response. Regulated expression is necessary to limit the exposure of CD40-bearing cells to the activated Th subset. In this study, we have shown that modulation of mRNA stability is one mechanism that controls the expression of CD154 in activated T cells.

Several novel findings with respect to the regulation of CD154 mRNA stability are reported. First, activation of T cells with either PMA/ion or anti-CD3 produces distinct effects on CD154 mRNA stability. Also, the stability of the CD154 message, but not the TNF-α or c-myc mRNAs, changes during T cell activation with anti-CD3, and this change does not appear to be related to co-stimulatory signals. Finally, changes in CD154 mRNA stability in anti-CD3-activated T cells occur during a time period when the levels of both mRNA and protein levels are declining less rapidly.

The instability of the CD154 message in response to early T cell activation with anti-CD3 is a pattern observed with several cytokine and proto-oncogene mRNAs (reviewed in Ref. 47). This pattern of mRNA decay, during the time period when CD154 message levels are at their highest, is consistent with CD154 expression being highly dependent on transcriptional mechanisms at early times postactivation.

In contrast to the rapid induction of CD154 mRNA immediately after anti-CD3 stimulation, we find that message levels steadily decline (to 50% maximal levels) by 8 h after activation and then more gradually decrease to 20% maximal levels over the next 40 h. This biphasic pattern of mRNA expression may be explained, in part, by an increase in CD154 message stability during this same time period. Increased CD154 mRNA stability during anti-CD3 activation may not necessarily result in an absolute increase in steady-state mRNA levels or surface expression, but may affect the rate of decline of the message and protein after activation. Our analysis of CD154 surface expression revealed measurable levels of protein expression 24–48 h postactivation with anti-CD3, which is consistent with previous studies (6, 14, 18, 36). Therefore, after extended T cell activation, message stabilization may allow for CD154 surface expression at a time when the steady-state levels of CD154 mRNA are relatively low. Our data, showing minor to no changes in CD154 mRNA stability with costimulation at both early and late times after activation, suggests that the "regulated decay" pathway is independent of the energized state of the T cells and argues for a physiological role for this process in CD154 expression.

One possible model for the regulation of CD154 mRNA stability during T cell activation is that TCR engagement initially leads to the transient induction of a factor that binds to elements of the CD154 mRNA, resulting in message destabilization. Subsequent loss of this destabilizing RNA-binding activity after 24–48 h of anti-CD3 stimulation would lead to the observed increase in CD154 message stability. The gain and loss of an RNA-binding activity has been previously demonstrated in the regulation of several mRNAs. For example, GM-CSF mRNA destabilization after anti-CD3 stimulation correlates with increased binding of the factor AU-B to ARE elements in the 3′-UTR region (25). Also, GM-CSF mRNA stabilization induced by PMA costimulation correlates with the loss of AU-B binding (25).

Our observation of a highly stable CD154 message after PMA/ion activation could reflect a rapid loss of a destabilizing RNA-binding activity. Alternatively, the CD154 mRNA destabilizing factor may be induced by CD3-mediated signal transduction pathways that are bypassed during PMA/ion activation. Recent data showing increased CD154 mRNA stability in PMA-stimulated mononuclear cells suggests that activation of PKC-mediated pathways has a stabilizing effect on the CD154 message (48).

Differences in the regulation of CD154 and TNF-α mRNA stability during T cell activation with anti-CD3 may be attributed to variations in mRNA sequence motifs or the RNA-binding factors that recognize these sequences. It has been previously suggested...
that the organization of ARE elements in the 3′-UTR region and the specificity of RNA-binding factors could account for differences between cytokine and c-myc message stability during T cell activation (49). ARE-sequence elements (AUUUA), which have been implicated in the control of mRNA stability, are dispersed throughout the CD154 3′-UTR (50). In contrast, these elements are tandemly arranged in the TNF-α mRNA and other cytokine messages (25). Therefore, message-specific RNA-binding factors could recognize distinct AU-rich sequences in the 3′-UTR region, or other RNA sequence motifs, in the CD154 and TNF-α mRNAs.

In summary, our results indicate that the CD154 gene is specifically regulated at the level of mRNA stability and that this regulation influences the overall control of CD154 expression during T cell activation. How this pattern might be altered or influenced by interactions with other cells in an immune response is currently being investigated. Recent studies indicate that CD154 may have critical functions in anti-tumor immune responses (51, 52), the development of autoimmune disease, and transplant rejection (reviewed in Ref. 53). Therefore, an increased understanding of factors that control CD154 expression will enhance our general knowledge of both its biological role in the regulation of immunity as well as in other clinical and disease processes.

Acknowledgments

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References