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Polypyrimidine Tract-Binding Protein Is Critical for the Turnover and Subcellular Distribution of CD40 Ligand mRNA in CD4+ T Cells

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CD40L (CD154) is regulated at the posttranscriptional level by an activation-induced process that results in a highly stable transcript at extended times of T cell activation. Transcript stability is mediated by polypyrimidine tract-binding protein (PTB)-containing complexes (complex I and II) that bind to three adjacent CU-rich sequences within the 3′ untranslated region. To assess the role of PTB in the expression and distribution of CD40L mRNA, PTB was targeted using short hairpin RNA in both primary T cells and a T cell line that recapitulates the stability phase of regulated CD40L mRNA decay. PTB knockdown resulted in a marked decrease in the mRNA stability that resulted in lowered CD40L surface expression. PTB was also critical for appropriate distribution of CD40L mRNA between the nucleus and cytoplasm and in the cytoplasm between the cytosol and the translating polysomes. The activation-induced formation of PTB-specific ribonucleoprotein complexes was observed only with cytoplasmic and not nuclear PTB indicating functional differences in the protein defined by cellular localization. Finally, we observed that cytoplasmic and nuclear PTB isoforms were differentially modified relative to each other and that the changes in cytoplasmic PTB were consistent with activation-induced phosphorylation. Together this work suggests that differentially modified PTB regulates CD40L expression at multiple steps by 1) retaining CD40L mRNA in the nucleus, 2) directly regulating mRNA stability at late times of activation, and 3) forming a ribonuclear complex that preferentially associates with translating ribosomes thus leading to an enhanced level of CD40L protein. The Journal of Immunology, 2011, 186: 2164–2171.

The near constitutive nature of CD40 expression on APCs necessitates that regulation of signaling pathways occurs primarily through modulated CD40L expression. Although CD40L transcription is rapidly induced after T cell activation, posttranscriptional processes also have a critical role in regulating CD40L gene expression throughout T cell activation (reviewed in Ref. 8). Because lymphocyte activation is characterized by transitions between different checkpoints, diversification at the level of mRNA stability provides a unique mechanism to regulate the expression of a number of responding genes (8, 9). The stability of the CD40L message in both human and mouse CD4+ T cells significantly increases with activation either through prolonged signaling through the TCR or via exposure to PMA/ion (10, 11). Transcript stability is mediated by two complexes (complex I and II) that bind to cis-acting elements within the 3′ untranslated region (3′UTR) (8, 12, 13). The major binding protein in complex I and II is the polypyrimidine tract-binding protein (PTB) or hnRNP I, which belongs to a family of ribonuclear proteins (RNP)s displaying diverse roles in multiple aspects of RNA metabolism (14). PTB shuttles between the nucleus and cytoplasm and is important for regulating alternative splicing, IRES-mediated translation initiation, and enhanced stability of a number of transcripts (15). Cellular localization and/or interactions with specific factors appear to be critical for PTB specificity (15, 16), which is consistent with its role in CD40L stabilization where two additional RNA binding proteins, nucleolin and hnRNP L, have been identified as additional components of complex I/II (17–19). Although the relationship between PTB and CD40L mRNA stability has been firmly established, how PTB-mediated regulated decay influences the expression of CD40L protein through T cell activation is less clear.
In this study, the effect of downregulating PTB on CD40L mRNA expression and distribution was analyzed both in an activated T cell line (Jurkat/D1.1) and in primary CD4+ T cells. We found that PTB-mediated regulation of CD40L mRNA stability had a considerable effect on levels of surface CD40L in both primary CD4+ T cells and cell lines. In addition, PTB influenced the cellular distribution of CD40L transcript between the nucleus and cytoplasm as well as within the polysomal cytoplasmic fraction. Finally, PTB was differentially modified as a function of its distribution within the nucleus and cytoplasm and in response to stimulation, suggesting a link between T cell activation, PTB modification, and CD40L expression.

Materials and Methods

Abs and cell lines

The anti-human-PTB mAb (ATCC CRL-2501) was purified as previously described (20). Anti-β-actin (C-11), anti-poly(ADP-ribose) polymerase, and anti-nucleolin (C23 Ab 4E2) mAbs were purchased from Santa Cruz Biotechnology. Anti-S6 ribosomal protein (54D2) mAb was purchased from Cell Signaling. Anti-DDK (Flag Ab 4C5) mAb was purchased from Origene. Anti-human-CD154 (21–28) was purchased from Biologend. The Jurkat/D1.1 cell line was obtained from Dr. S. Lederman (Columbia University, New York, NY) and cultured as previously described (12). The Flag-PTB-Jurkat-D1.1 line was generated by the stable expression of the vector pCDNA.3 expressing PTB-1 downstream of the Flag epitope.

Short hairpin RNA knockdown and analysis of PTB

The plV-CTRL and plV-PTB together with virus production techniques have been previously described (29). Briefly, the following pairs of primers were cloned into the HindIII and EcoRI sites of the pSilencer2.1-U6 hygro (Ambion): U6-shPTB: 5'-GAT CCA ACT TAT ACC AAT CAG TGG AAA-3' (forward) and 5'-AGC TTT TCC AAA AAA AAC TTC CAT CAT TCC AGA GGA GAA GCA AGT TCT CGT GAA TGG AAG TG-3' (reverse); and U6-shCTRL: 5'-GAT CCA ATC AGA CGT GGA CCA CAG GAG AGA TCT TCT GGT GAA TGA TGG AAG-3' (forward) and 5'-AGC TTT TCC AAA AAA AAC TTC CAT CAT TCC AGA GGA GAA GCA AGT TCT CGT GAA TGG AAG TG-3' (reverse). The PCR products were cloned into the plVTHM vector (Addgene plasmid 12247).

Viral particles were packaged by transfection of 293T cells with pLVTHM-U6-shPTB and pLVTHM-U6-CTRL together with the virus packaging plasmids psPAX2 (Addgene plasmid 12260) and pCI-VSVG (Addgene plasmid 1733), both obtained from D. Trono (Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland), using FuGene. A total of 5 × 10^6 Jurkat/D1.1 cells were incubated with lentivirus for 24 h followed by replacement of the conditioned medium with RPMI-complete (RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 mM l-glutamine at 37°C), and 48 h postinfection, cells were checked for GFP expression by flow cytometry.

Infection of primary human CD4+ T cells

PBMCs were isolated from freshly drawn blood and CD4+ T cells isolated using Miltenyi beads. Concentrated control (plV-CTRL) and PTB (plV-PTB) lentiviruses were cocultured with 1 × 10^6 CD4+ T cells with 0.1 μg/ml PolyU (Calbiochem) and 100 U/ml Rl-2 in RPMI-complete. Cells were expanded by adding 100 U/ml Rl-2 at 3 and 50 U/ml Rl-2 every other day following. At 10 days postactivation, the cells were placed into RPMI-complete medium minus Rl-2, and 3 to 4 days later, cells were activated for 2 h with 1 ng/ml PMA and 1 μg/ml ionomycin. Cells were stained with anti-CD40L mAb and analyzed by flow cytometry. Intracellular surface mobilization assay was performed as previously described (30). Briefly, CD4+ T cells were infected and prepared as above. Thirty minutes prior to activation, cells were treated with 10 μg/ml cycloheximide. Activation was with 1 μg/ml ionomycin, 10 ng/ml PMA with 10 μg/ml cycloheximide for 30 min.

Steady-state expression and decay analyses of the CD40L mRNA

RNA stability was measured by incubating 5 × 10^6 Jurkat/D1.1 cells with 50 μg/ml 5,6-dichlorobenzimidizole-1-β-D-ribofuranoside (DRB) and aliquots collected every 30 min for 2 h. Approximately 1 μg of RNA was reverse transcribed and real-time PCR performed on an ABI HT7000 PCR Cycler. For quantification of CD40L, and the β-actin transcripts, the following primer sets were used: CD40L 5'-TTC CAC CCT GCT CCT AGT TC-3' (forward) and 5'-CTG GAA ACA ATG GAG ACT GC-3' (reverse); and β-actin: 5'-GCA TCC TCA CCC TGA AGT A-3' (forward) and 5'-TGT GGT GCC AGA TTT GGT CC-3' (reverse).

Cell fractionation and protein immunoblots

Total, nuclear, and cytoplasmic extracts were prepared as previously described (29) and analyzed using immunoblotting with anti-PTB Abs at a dilution of 1:3000 and anti-Flag (DDK) Abs, anti-S6 ribosomal protein Abs, anti-actin Abs, and anti-nucleolin Abs at a dilution of 1:1000. HRP-conjugated secondary Abs were used for detection by ECL. To obtain the non-polysomal and polysomal proteins and RNA, cell fractionation was performed essentially as previously described (31).

RNA electromobility shift assay and RNA immunoprecipitation

Isolation of CD4+ T cells, CD3 activation of T cells, and preparation of cytoplasmic fractions and RNA electromobility shift assays were carried out as previously described (18). To analyze the CD40L mRNA bound by PTB, 5 × 10^6 Jurkat/D1.1 cell equivalents of cytoplasmic and nuclear extracts were incubated overnight at 4°C with agarose-A/g beads bound to anti-PTB Abs in NT2 buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM MgCl2, and 0.05% Nonidet P-40). The beads were washed six times with NT2 buffer and resuspended in 100 μl NT2 buffer with 0.1% SDS and 30 μg proteinase K, followed by incubation at 55°C for 3 h. RNA was extracted from the supernatant, reverse transcribed, and the cDNA amplified by PCR.

Two-dimensional gel electrophoresis and phosphatase assay

Cytoplasmic (20 μg) and nuclear (5 μg) extracts from differentially activated CD4+ T cells were dialyzed against DeStreak RSC G2 GTP Solution (GE Healthcare) for 2–4 h. Isoelectric focusing (IEF) was carried out on samples using 7 cm Immobiline DryStrip Gels with immobilized pH gradient range 6–11. IEF was performed in an IPGphor according to the protocols provided. After IEF, strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol with 1% DTT for 15 min, followed by 15 min in the same buffer without DTT but with 4% iodoacetamide. Equilibrated strips were run through 4–20% linear gradient polyacrylamide gels, transferred to PVDF membranes, and immunoblotted with anti-PTB Abs. Twenty micrograms from Jurkat/D1.1 cytoplasmic extracts were mock-treated or treated with 800 U phosphatase in 20 μl final volume of 50 mM HEPES (7.5), 100 mM NaCl, 2 mM DTT, 0.01% Brij 35, 2 mM MnCl2, and 1% PIC for 4 h. The samples were subsequently dia lyzed and focused as described.

Results

PTB is critical for maintaining steady-state levels of CD40L mRNA

To determine the effect of attenuated PTB expression on surface CD40L levels, Jurkat/D1.1 cells expressing a highly stable CD40L transcript were infected with a modified pLVTHM-U6 lentiviral vector expressing GFP and either an shRNA against PTB (pLV-PTB) or a scrambled-sequence control (pLV-CTRL). Infection efficiency was consistently between 70 and 100% (GFP+) using both viruses (data not shown). To control for off-target effects, a Jurkat/D1.1 subclone was generated that stably expressed FLAG-tagged PTB-1 from a cDNA lacking the small interfering RNA target sequence within the 3' UTR. This subclone was similarly infected with pLV-PTB and pLV-CTRL viruses and analyzed in parallel with the infected, noncomplemented populations. Two days after infection, Jurkat/D1.1 and Flag-PTB-Jurkat/D1.1 cytoplasmic and nuclear fractions were prepared and analyzed for PTB expression using immunoblotting. Cells expressing shPTB had decreased PTB in both fractions with a particularly sharp drop.
in the cytoplasmic level (20% of shCTRL), which may reflect a preferential distribution of PTB in the nucleus prior to its redistribution into the cytoplasm. In contrast, PTB expression in Flag-PTB-shPTB Jurkat cells was 70 and 60% of that observed for the Flag-PTB-shCTRL cytoplasmic and nuclear fractions, respectively (Fig. 1A). Overall, the steady-state levels of CD40L mRNA were reduced more than 50% in cells expressing shPTB, whereas cells complemented with Flag-tagged PTB had higher (Flag-PTB-shCTRL) or equal (Flag-PTB-shPTB) levels of transcript compared with those of the parent cell line (Fig. 1B).

To confirm that the change in PTB levels affected the decay rate of CD40L mRNA, infected Jurkat/D1.1 cells were treated with the transcriptional inhibitor DRB and decay measured over a 2-h period. 

**FIGURE 1.** PTB directly regulates the stability of the CD40L transcript. A, Extracts were prepared from $2 \times 10^6$ cell equivalents of Jurkat/D1.1 (lanes 1–4) or Flag-PTB-Jurkat/D1.1 (lanes 5–8) cells infected with pLV-CTRL (lanes 1, 2, 5, and 6) or pLV-PTB (lanes 3, 4, 7, and 8) and assayed for PTB expression by immunoblotting with anti-PTB mAb. Membranes were stripped and rehybridized with anti-Flag and anti-nucleolin Abs. Numbers represent the fraction of PTB in the indicated lanes relative to the appropriate CTRL lane.

**FIGURE 2.** PTB regulates CD40L surface expression in both Jurkat and primary T cells. A, CD40L expression (x-axis) of Jurkat/D1.1 (left panel) or Flag-PTB-Jurkat/D1.1 (right panel) cells infected with either pLV-CTRL (gray line) or pLV-PTB (black line). The MFIs are given for the individual populations, and results are representative of five independent experiments. B, Expression of GFP (x-axis) and CD40L (y-axis) was monitored in primary CD4+ T cells infected with pLV-CTRL (top graphs) or pLV-PTB (bottom graphs). Cells were either nonactivated (left graphs) or activated (right graphs) with PMA/ion for 2 h prior to analysis. Within a specific graph, uninfected cells are present in the left quadrants and infected cells are in the right quadrants. Numbers in each quadrant represent MFI for representative populations.

C, CD4+ T cells infected with either pLV-CTRL (top graphs) or pLV-PTB (bottom graphs) were treated with 10 μg/ml cycloheximide for 30 min prior to analysis for CD40L expression without activation (left graphs) or after activation with PMA/ion for 30 min (right graphs). Cells in left quadrants are uninfected, and those in the right quadrants are infected. Numbers in each quadrant represent MFI for representative populations.
time course (Fig. 1C). In cells infected with the pLV-PTB virus, the CD40L mRNA decayed with a \( t_{1/2} \) of \( \sim 45 \) min, which represented a 50% decrease in stability compared with that of cells infected with control virus. Assessment of Flag-tagged PTB-expressing cells harboring either shPTB or shCTRL revealed degradation profiles very similar to that seen with cells expressing the control shRNA confirming that the Flag-tagged PTB was complementing the decreased stability seen with shPTB.

CD40L surface expression is regulated by PTB

We next analyzed the effect of PTB on CD40L protein expression in the different Jurkat/D1.1 populations by comparing the mean fluorescence intensity (MFI) 2 d after infection with virus. Jurkat/ D1.1 cells expressing shPTB were found to have \( \sim 65\% \) less CD40L on the surface than that of control cells (MFI = 314.5 versus 104.5; Fig. 2A, left panel). Analysis of the Flag-PTB-Jurkat populations showed that the clonal Flag-PTB-Jurkat/D1.1 cells expressed an overall lower level of surface CD40L compared with that of the parent Jurkat/D1.1 cells (Fig. 2A, right panel). However, the difference in MFI between the shCTRL and shPTB was still evident but greatly reduced, indicating that PTB was influencing the surface expression of CD40L (Fig. 2A, right panel).

Because Jurkat/D1.1 cells have an activated phenotype and constitutively express PTB-containing stability complexes, we sought to confirm our findings using primary CD4\(^+\) T cells in which the effect of PTB downregulation could be assessed in both resting and activated cells. To this end, primary CD4\(^+\) T cells were infected with either virus and surface expression monitored after PMA/ion activation, conditions that promote a highly stable CD40L degradation profile (top panels). As shown in Fig. 2A, in cells expressing shCTRL, a 50% decrease in stability compared with that of cells expressing shPTB was observed in T cells infected with pLV-PTB both in the resting and activated states (Fig. 2C, Table I). Whereas these data support a role for PTB stabilization of CD40L mRNA during T cell activation, they also clearly indicate that PTB also influences CD40L expression through an activation-independent mechanism.

The distribution of CD40L mRNA between the nuclear and cytoplasmic fractions and within the polysome non-polysome fraction is PTB dependent

To identify an additional role for PTB in CD40L expression, we used our shRNA expression system to analyze the compartmentalization of CD40L mRNA under targeted PTB expression. Nuclear and cytoplasmic extracts were prepared from virus-infected Jurkat/D1.1 cells or Flag-PTB-Jurkat/D1.1 cells and cytoplasmic extracts further fractionated into non-polysomal (S130 and free ribosomes) and polysomal fractions. Experimental procedures are described in Fig. 3A, in cells expressing shCTRL, \( \sim 65\% \) of the CD40L transcript was found in the nucleus and 40\% associated with the cytoplasm. In contrast, downregulating PTB resulted in a reversal in CD40L mRNA distribution with 45 and 55\% of the transcript found in the nuclear and cytoplasmatic fractions, respectively. Evaluation of the Flag-tagged Jurkat cells revealed a pattern of CD40L mRNA close to that seen with shCTRL confirming that PTB plays a role in appropriately partitioning the CD40L transcript between the nucleus and cytoplasm.

Further analysis of the distribution of CD40L transcripts in the cytoplasm revealed that the vast majority associated with translating polysomes; however, under conditions of reduced PTB, there was a 20\% increase in association with the non-polysomal fraction (Fig. 3B). This skewing was observed in the presence or absence of cycloheximide but was significantly more pronounced with cycloheximide treatment (data not shown). Infection of Flag-PTB-Jurkat/D1.1 cells revealed no difference in the distribution of CD40L mRNA among the non-polysomal and polysomal fractions (Fig. 3C).

Table I. PTB is critical for CD40L expression in both resting and activated CD4\(^+\) T cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>MFI (CD40L)</th>
<th>Percentage Change in MFI (Uninfected to Infected in Same Population)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Infected</td>
</tr>
<tr>
<td>shCTRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonactivated</td>
<td>33.9 ± 0.10</td>
<td>35.32 ± 1.2</td>
</tr>
<tr>
<td>Activated</td>
<td>245.7 ± 40.3</td>
<td>260.6 ± 29.1</td>
</tr>
<tr>
<td>shPTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonactivated</td>
<td>43.1 ± 4.3</td>
<td>39.33 ± 3.06</td>
</tr>
<tr>
<td>Activated</td>
<td>271.3 ± 24.9</td>
<td>204.5 ± 17.1</td>
</tr>
<tr>
<td>shCTRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonactivated</td>
<td>38.95 ± 0.15</td>
<td>40.0 ± 0.22</td>
</tr>
<tr>
<td>Activated</td>
<td>46.4 ± 0.4</td>
<td>52.1 ± 0.7</td>
</tr>
<tr>
<td>shPTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonactivated</td>
<td>38.3 ± 0.15</td>
<td>36.1 ± 1.3</td>
</tr>
<tr>
<td>Activated</td>
<td>46.9 ± 1.65</td>
<td>38.4 ± 1.3</td>
</tr>
</tbody>
</table>

*Primary CD4\(^+\) T cells were infected with either pLV-THM-shCTRL or pLV-THM-shPTB virus in the presence of IL-2 and PHA. Ten days later, cells were either untreated (top rows) or treated with cycloheximide for 30 min (bottom rows). Cells were then left unactivated for 2 h or activated with PMA/ion for 2 h (top rows) or 30 min (bottom rows) and analyzed for GFP (infected) and CD40L (activated) expression. The MFI's represent the average of three (top rows) and two (bottom rows) independent experiments, and the numbers in the right column represent the percentage change in the average MFI between the uninfected and infected subpopulations in the same culture.
PTB is actively involved in the distribution of CD40L mRNA between the nucleus and cytoplasm. A, Levels of CD40L mRNA in the cytoplasm (gray bars) and nucleus (black bars) of pLV-CTRL and pLV-PTB infected Jurkat/D1.1 and Flag-PTB-Jurkat/D1.1 cells as determined by quantitative PCR. Results represent the average and SEM of three independent experiments. B, Quantitative PCR analysis of CD40L mRNA from the non-polysomal (white bars) and polysomal (black bars) fractions of pLV-CTRL and pLV-PTB infected Jurkat/D1.1 and Flag-PTB-Jurkat/D1.1 cells. C, Western blot analysis using cytoplasmic (2 × 10^6 cell equivalents), nuclear (2 × 10^6 cell equivalents), and polysomal (1 × 10^6 cell equivalents) extracts of Jurkat/D1.1 (lanes 1–6) and Flag-PTB-Jurkat/D1.1 (lanes 7–12) cells infected with pLV-PTB or pLV-CTRL were analyzed for expression of PTB and nucleolin in the different cellular fractions by Western blotting. Membranes were probed with Abs against β-actin and the cytoplasmic ribosomal protein S6 to validate the efficient separation of nuclear, cytoplasmic, and polysomal fractions. The membrane was further hybridized with anti-Flag Abs to confirm expression of the Flag-PTB in the different fractions. Boxed lanes show the effect of PTB downregulation on the different proteins, in particular PTB and nucleolin.

To extend these findings, we analyzed the expression of PTB protein in the nuclear, cytoplasmic, and polysomal fractions of cells expressing shPTB and found decreased levels in all three fractions (Fig. 3C, lanes 4–6). Because nucleolin is a second component of complex I, we also examined its distribution under conditions where PTB levels were reduced. Surprisingly, much less nucleolin was associated with the polysomes under conditions of targeted PTB (Fig. 3C, compare lanes 3 and 6), and this decrease was not observed when PTB was complemented by Flag-PTB (Fig. 3C, compare lanes 9 and 12). This finding strongly suggests that the association of nucleolin with the translating polysomes is dependent, in part, on PTB expression.

Complex I binding is distinct in cytoplasmic and nuclear fractions from resting and activated CD4+ T cells

The presence of substantial levels of PTB in both the nucleus and cytoplasm compelled us to ask whether PTB from both fractions complexed to CD40L mRNA. Binding experiments were carried out with a probe spanning the CD40L stability element (Xba-Hae) and hybridized with nuclear and cytoplasmic extracts from Jurkat/D1.1 cells and differentially activated CD4+ T cells. We found that strong complex I binding was observed in both fractions from Jurkat/D1.1 cells (Fig. 4A, lanes 3–5). Similarly, a high level of binding activity was observed in the nuclear fractions from resting and activated T cells from all time periods (Fig. 4A, lanes 6–9). In contrast and as previously observed, only primary T cells stimulated for 48 h, and not resting or early activated cells, contained a measurable level of complex binding (Fig. 4A, lanes 10–13). The specific requirement for PTB in complex I binding was demonstrated by inhibiting complex I formation with the addition of anti-PTB Abs to the binding reaction (lane 15).

To further confirm that both nuclear and cytoplasmic PTB binds CD40L mRNA and to quantify the binding relative to levels of PTB, RNA immunoprecipitation was carried out with Jurkat/D1.1 nuclear and cytoplasmic extracts. After incubation with anti-PTB mAb cross-linked agarose beads, one half of the eluate was used in Western blotting to analyze PTB binding, and the other half was used to isolate RNA for RT-PCR. As shown in Fig. 4B, the pattern of PTB was similar between the total and immunoprecipitated fractions. However, the majority of CD40L mRNA binding activity was associated with the cytoplasmic fraction as evidenced by the amount released from the complex. This was unexpected because the levels of both PTB and CD40L mRNA were significantly higher on a per cell basis in the nuclear fraction. Together these results confirm that the binding of nuclear PTB to CD40L mRNA occurs in the absence of activation and is present in CD4+ T cells at all times after activation. However, based on our in vivo binding studies, PTB–CD40L binding activity is highest in the cytoplasmic fraction. Thus, the complexing of CD40L mRNA with PTB in CD4+ T cells is influenced by both the subcellular localization of complex components and by signals that mediate T cell activation.

Cytoplasmic PTB is modified in response to activation

The lack of complex I binding activity in the cytoplasmic fraction from resting and early activated CD4+ T cells could be explained by either the lack of PTB in the cytoplasm or the inability of cytoplasmic PTB to bind transcript due to the absence of specific modifications. To distinguish between these two possibilities, cytoplasmic and nuclear extracts were prepared from resting and CD3-activated CD4+ T cells and analyzed by immunoblotting for PTB expression. As predicted from our binding studies, PTB was present in all nuclear fractions over the activation time course (Fig. 5A, lanes 6–8). Surprisingly, PTB was also readily present in the cytoplasmic fractions of unstimulated, 2 h-, and 48 h-stimulated cells (Fig. 5A, lanes 2–4). This finding suggested that...
The lack of PTB–CD40L mRNA complexing in resting and early-activated T cells was not due to an absence of cytoplasmic PTB but to a potential modification in PTB that favored complex formation.

To examine if PTB was differentially modified in response to activation, nuclear and cytoplasmic extracts were prepared from CD4+ T cells stimulated for different time periods and separated using two-dimensional gel electrophoresis. After Western blotting with anti-PTB Abs, we found that the pattern of nuclear and cytoplasmic PTB isoforms was clearly different (Fig. 5B). Specifically, in resting T cells, there were several cytoplasmic isoforms that were spread over a wide pH range and the major bands shifted to the negative pole upon activation. In contrast, the PTB isoforms in the nucleus were more closely aligned, were shifted more toward the positive pole, and did not change position over the time course of activation.

To test whether phosphorylation was contributing to the different cytoplasmic isoforms, extract from Jurkat/D1.1 cells was untreated or treated with 800 U λ phosphatase for 4 h prior to separation by two-dimensional gel electrophoresis across a pH gradient of 6–11. PTB was visualized by immunoblotting.

**FIGURE 5.** PTB from the cytoplasm and nucleus is differentially modified and mediated by activation. A, Cytoplasmic (lanes 1–4) and nuclear (lanes 5–8) extracts were prepared from Jurkat/D1.1 cells (lanes 1 and 5) and CD4+ T cells that were unstimulated (lanes 2 and 6) or stimulated with anti-CD3 mAb for 2 h (lanes 3 and 7) or 48 h (lanes 4 and 8). PTB expression was assessed by SDS-PAGE and immunoblotting with anti-PTB Abs. Membranes were stripped and rehybridized with Abs against S6 and poly(ADP-ribose) polymerase (lower two panels) to verify the separation of cytoplasmic and nuclear fractions, respectively. B, Two-dimensional gel electrophoresis was carried out across a pH gradient of 6–11 using cytoplasmic (left panels) and nuclear (right panels) extracts from CD4+ T cells activated for 0, 2, 24, and 48 h with anti-CD3 mAb. Black arrows indicate the major isoforms in each fraction. C, Cytoplasmic extract from Jurkat/D1.1 cells was either left untreated or treated with 800 U λ phosphatase for 4 h prior to separation by two-dimensional gel electrophoresis across a pH gradient of 6–11. PTB was visualized by immunoblotting.
Accordingly, appropriate steady-state levels of CD40L are defined by a still unique posttranscriptional control mechanism involving ribonuclear complexing at defined regions within the 3′UTR at discrete times of T cell activation (8). Previous work identified a role for PTB in CD40L mRNA stability, and our current work extends these findings by demonstrating that PTB levels directly impact the surface expression of the protein. Importantly, examination of how CD40L mRNA is apportioned within the cell revealed that optimal PTB levels are required for maintaining the appropriate distribution of both the cytoplasmic and the polysomal subpopulations. Therefore, these findings highlight a role for PTB in CD40L expression beyond its role in regulating transcript stability.

A large body of work has focused on understanding ARE-regulated mRNA decay, a mechanism used by a vast number of genes involved in the inflammatory response that results in the rapid turnover of ARE-containing transcripts (i.e., TNF-α, GM-CSF, IL-2, and IL-10) (25–28). Conversely, a program of posttranscriptional regulation that further extends the t1/2 of relatively long-lived transcripts represents an energy-efficient mechanism to sustain expression of a particular protein (32). The PTB-dependent phase of CD40L expression falls into this second type of regulation and coincides with processes that occur at more advanced stages of the humoral response, such as germinal center formation and the generation of differentiated plasma cells and memory B cells (33). Similar to what has been shown with BCR signaling (34–38), duration and quality of CD40 signals appear to directly affect differentiation outcomes of Ag-selected B cells as well as other APCs (34, 39–43). Thus, the level and duration of CD40 signaling is critical for fate decisions in responding populations, and PTB is directly linked to these decisions by its ability to regulate CD40L expression at extended times of T cell activation.

The fact that PTB-containing complexes are constitutively active in the nucleus but active only at late times of activation in the cytoplasm is consistent with the different subsets of complexes being functionally distinct. It has previously been shown that PTB acts as a chaperone for nuclear-associated viral RNAs transported to the nuclear pore (44, 45). If PTB functions in a similar way and is required to bring CD40L mRNA to the nuclear pore for export, one prediction would be that reducing levels of nuclear PTB would result in an increased accumulation of nuclear CD40L mRNA. However, our findings indicate that decreasing nuclear PTB results in an increase in cytoplasmic CD40L transcripts, suggesting that PTB functions to retain CD40L mRNA in the nucleus and that a drop in PTB nuclear levels results in an influx of CD40L mRNA into the cytoplasm. This RNA may be highly susceptible to degradation especially under conditions where PTB is limiting. These results complement other findings showing that shuttling of PTB between the nucleus and cytoplasm is independent of RNA binding (46).

Our finding that decreased levels of PTB also resulted in reduced association of CD40L mRNA with the polysomal fraction suggests that another function of PTB is to enhance the translational efficacy of specific transcripts. This finding is consistent with the known role of PTB in viral and endogenous IRES translation (15) as well as the fact that binding of hnRNPL, which is a component of complex II that binds to both PTB and site C in the stability element (18), alters the translation of the CD40L transcript (19). The fact that reducing PTB in the cell also resulted in a decreased level of nucleolin associated with the polyosomes suggests a requirement for interaction between these two factors in translation, a finding extending previous reports of PTB and nucleolin interactions during posttranscriptional processes (17, 47).

From our data, we hypothesize that levels of PTB regulate crucial nuclear and cytoplasmic events leading to optimal CD40L expression (Fig. 6). Specifically, at early times of T cell activation when transcriptional levels of RNA are at their highest, nuclear-specific PTB complexes retain only a portion of the CD40L message in the nucleus, whereas the vast majority is transported into the cytoplasm in the absence of complex. Although the turnover of this “naked” transcript is rapid, the high level of transcription along with the release of preformed protein stores (30, 48) lead to significant levels of CD40L expressed on the cell surface. Continued activation results in specific modifications of cytoplasmic PTB that lead to the formation of complex I/II ribonuclear complexes in the cytoplasm. Precedence for functional modifications comes from work on both PTB and other RNA binding proteins. Whereas the nucleocytoplasmic distribution of PTB has been linked to direct protein kinase A-dependent phosphorylation of Ser16 (49, 50), the stability of many ARE-regulated transcripts appear to be controlled by signaling through p38 MAPK-specific phosphorylation events that result in the cellular redistribution of specific RNA binding proteins including tristetraprolin (9). Thus, pathways involved in mRNA transport, localization, turnover, and potentially translation intersect with PTB expression, and ultimately these PTB-mediated events may be highly dependent on pathways induced by T cell activation.

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Disclosures

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References


