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CD154 (CD40L) mRNA turnover is regulated in part at the posttranscriptional level by a protein complex (termed Complex I) that binds to a highly CU-rich region of the 3'UTR. Polypyrimidine tract-binding protein (PTB) has previously been identified as a major RNA-binding protein in Complex I. Nondenaturing gel filtration of total extract from Jurkat T cells demonstrated that the CD154 mRNA-binding activity migrates as a ~200-kDa complex, indicating the presence of multiple complex-associated proteins. We have currently undertaken a biochemical approach to further characterize Complex I and observed that it segregates over DEAE-Sepharose into two subcomplexes (termed I-L and I-U). Furthermore, nucleolin was identified as a component of both subcomplexes and was shown that it is the major RNA-binding protein in I-U. To directly demonstrate the biological significance of Complex I binding to the CD154 transcript, cytoplasm from human Jurkat cells was fractionated over a sucrose gradient and the different cellular fractions subjected to immunoprecipitation with anti-PTB and anti-nucleolin Abs. RT-PCR of the immunoprecipitated products using CD154-specific primers clearly demonstrated that nucleolin and PTB are associated with CD154 mRNA in both the ribonucleoprotein and polysome fractions. These data strongly support a model whereby nucleolin and PTB are integral to the stability of CD154 mRNA and are components of the CD154 ribonucleoprotein particle associated with actively translating ribosomes.


H umoral and cell-mediated immune responses are critically dependent on the appropriate expression of CD154 (formally known as CD40L) on activated CD4+ T cells. The interaction of CD154 with its cognate receptor CD40, which is constitutively expressed on B cells, macrophage, dendritic cells, and other APCs, directly influences the comprehensive course and outcome of the immune response. Most notably, CD154/CD40 interactions are critical for class switch recombination and somatic mutation, two molecular processes that underlie the differentiation of B cells into effector and memory cells (reviewed in Ref. 1). In addition to its role in B cell activation, CD40 signaling regulates an array of immune responses, including the rescue from apoptosis of both Ag-induced and naive B cells, and the activation of cellular immunity primarily through the interaction of activated CD4+ T cells with CD40-expressing macrophage (reviewed in Refs. 2 and 3).

The time course and extent of CD154 expression on CD4+ T cells are highly dependent on the source of stimuli as well as on costimulatory interactions provided by B cells or APCs (4–14). Early studies examining the kinetics of CD154 expression on activated CD4+ T cells revealed that it was both rapidly induced and transient in nature (6, 8, 15, 16). The transitory nature of CD154 expression on APC-activated T cells corresponds to the internalization of CD154 upon contact with CD40-expressing APCs through receptor-mediated endocytosis (10). CD154 expression can also be modulated by different cytokines such as IL-12, which greatly sustains CD154 expression on anti-CD3-activated T cells (17), and both IL-2 plus IL-15, which reinduce the expression of CD154 on effector T cells in the absence of TCR signaling (17–19). Because the kinetics and duration of CD154 expression most likely control many APC effector functions, defining the mechanisms that regulate CD154 expression on activated T cells is a critical step in understanding the global regulation of the immune response.

Both transcriptional (20, 21) and posttranscriptional (22–28) processes play key roles in regulating CD154 expression. We have previously shown that CD154 expression is regulated at the posttranscriptional level by a unique pattern of mRNA decay (23). At early times of T cell activation, CD154 mRNA is degraded with a t1/2 ≤ 40 min, which is a characteristic shared by multiple cytokine, growth factor, and cell cycle mRNAs that decay via an AU-rich element (ARE) mechanism involving the 3' untranslated region (3'UTR) (reviewed in Refs. 29 and 30). However, at extended times of activation, CD154 mRNA decays with an unusual pattern of regulated instability, which results in a 3- to 4-fold increase in CD154 mRNA t1/2 (23). This regulated pattern of decay is not observed with either the TNF-α or IL-2 transcripts in activated T cells (23, 31, 32).

We previously identified a ribonucleoprotein (RNP) complex (termed Complex I) that conferred stability on the CD154 mRNA in vitro and was present only in cytoplasmic extracts from late, but not early, anti-CD3-activated CD4+ T cells (26). This complex bound specifically to two adjacent and independent binding sites within a highly pyrimidine-rich region in the 3'UTR. A related

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Abbreviations used in this paper: ARE, AU-rich element; APP, amyloid precursor protein; DEAE-B, DEAE bound; DEAE-FT, DEAE flow through; ECM, evolutionary conserved motif; NRE, nucleolin recognition element; PTB, polypyrimidine tract-binding protein; RBD, RNA-binding domain; REMSA, RNA EMSA; RNP, ribonucleoprotein; UDG, uracil DNA glycosylase; UTR, untranslated region.
complex (Complex II) bound a third adjacent CU-rich region. We and others identified a 55-kDa RNA-binding component of complexes I and II as the polypyrimidine tract-binding protein (PTB) or heterogenous nuclear RNP-1 (27, 28). Our current work extends our previous findings and shows that Complex I can be separated into two subcomplexes distinguished by the nature of their RNA-binding components. Furthermore, our results are consistent with nucleolin being a second component of Complex I, which can bind directly to the stability element in the CD154 3'UTR. We also demonstrate that PTB and intact forms of nucleolin are expressed in the cytoplasm of activated T cells only when the CD154 mRNA is stabilized. Furthermore, PTB, nucleolin, and CD154 mRNA co-localize with the RNP and polysome fractions of Jurkat/D1.1 cells, indicating that Complex I is an integral component of the CD154 RNP particle that is associated with the actively translating ribosomes. Collectively, our findings define a critical role for nucleolin in the formation of the CD154 RNP and as an extension in controlling CD154 expression in T cell activation.

Materials and Methods

Cell cultures and preparation of protein extracts

The human D1.1 T cell line is a CD154-expressing Jurkat subclone and has been previously described (5, 33). Jurkat/D1.1 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Howard Hughes Medical Institute, University of California, Los Angeles, CA). Anti-human PTB mAb MS3 was a gift from J. Patton (Vanderbilt University, Nashville, TN), and anti-human PTB mAb BB7 was a gift from D. Black (Howard Hughes Medical Institute, University of California, Los Angeles, CA). The anti-eIF4G Abs were a gift from T. Goss Kinzy (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ).

Gel filtration analysis

Gel filtration under nonnaturing conditions was performed, as described by Rodgers et al. (34). Briefly, 1–2 mg of total cellular extract was loaded onto a Superose-12 HR 10/30 gel filtration column (Amersham Biosciences, Piscataway, NJ) pre-equilibrated with HG–40 buffer (20 mM HEPES, pH 7.9, 10% glycerol, and 40 mM KCl). Forty 0.5-mL fractions were collected and stored at −80°C.

RNA probes

The XbaI-HaeIII-poly(T) template was generated, as described previously (27). HaeIII-1816-poly(T) template includes nt 1589–1816 (in the CD154 3'UTR) and has been previously shown not to bind Complex I (26). Briefly, these templates were PCR synthesized using specific forward primers containing a T7 promoter sequence and specific reverse primers containing a 60-nt poly(T) sequence at their 5' end. [32P]-labeled RNA probes were synthesized using 0.5 μg of template DNA; 0.4 mM each of rATP, rGTP, and rCTP; 0.04 mM rUTP; 30 mM DTT; 20 U of RNasin; 1× T7 transcription buffer (40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 U/ml RNaseH); 25–40 μM of [α-32P]UTP; and 0.5 μl of T7 RNA polymerase (Promega, Madison, WI) at 37°C for 4 h, treated with RQI RNase-free DNase at 37°C for 15 min, and centrifuged through G25 columns (Amersham Biosciences) to remove the unincorporated nucleotides.

RNA EMSAs (REMSA) and Ab interference analysis

RNA EMSAs (REMSA) were performed, as described previously (27). Typically, 5.0 μg of protein extract was incubated with 4 × 107 cpm of in vitro synthesized RNA probes in 20 μl of RNA-binding buffer (10 mM HEPES, pH 7.9, 3 mM MgCl2, 40 mM KCl, 1 mM DTT, 5% glycerol, and 270 ng of Escherichia coli tRNA). In Ab interference experiments, 1–2 μl of test or control polyclonal Abs was added to the reaction for 1 h before addition of the probe. Following a 30-min incubation at room temperature, RNAse mix (40 U of RNase T1, 100 pg of RNase A, and 0.015 U of RNase V1) was added, and the reactions were incubated at 37°C for 30 min. A total of 100 μg of heparin was added to each reaction, followed by a 10-min incubation on ice. Samples were resolved on a 7% native acrylamide gel at 200 V for 2–4 h, and visualized by autoradiography. To carry out depletion experiments, 100–200 μg of Jurkat/D1.1 extract was pre-cleared with 50 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) at 4°C and incubated with 1–5 μg of Abs for 2 h at 4°C, and complexes were precipitated with protein A/G-agarose beads for 1 h.

Purification of Complex I

A total of 550 mg of Jurkat/D1.1 cell extract was loaded on four 5-ml Hitrap DEAE Fast-Flow columns connected in series (Amersham Biosciences) and pre-equilibrated with RNA-binding buffer. After extensive washing, the bound proteins were eluted by applying a 0.1–1.0 M linear gradient of KCl. Bound fractions, containing the peak Complex I-binding activity, were pooled, desalted, and concentrated. Bound and flow through fractions were separately loaded on four 1-ml Hitrap heparin high performance liquid chromatography columns connected in series (Amersham Biosciences). Elution was conducted with a linear gradient of 0.1–1.0 M KCl. Fractions containing Complex I activity were further purified by RNA affinity chromatography using an in vitro transcribed and polyadenylated CD154 (XbaI-HaeIII) mRNA immobilized onto poly(U)-Sepharose beads (Sigma-Aldrich). Proteins were eluted in RNA-binding buffer with a 0.5, 1.0, 2.0, and 3.0 M KCl step gradient, and fractions were tested for the Complex I activity by REMSA.

UV cross-linking assay

D1.1 cell extract (20 μg) and 32P-labeled, in vitro synthesized RNA probe (2 × 106 cpm) were incubated at room temperature for 20 min in 20 μl of RNA-binding buffer containing 50 μg/ml each of yeast tRNA and heparin. Unbound RNA was digested with 100 U of RNase T1 for 15 min at 37°C. UV cross-linking was conducted by exposing the reactions to 254 nm of UV light for 30 min at 0°C. After digestion with 1 μg of RNase A for 30 min at 37°C, samples were boiled for 5 min and separated by SDS-PAGE at 30 mA for 4.5 h. The gels were fixed, dried, and visualized by autoradiography.

Affinity purification of nucleolin

A total of 400 μg of anti-nucleolin mAb MS3 was coupled to CNBr-activated Sepharose 4B beads, according to standard protocols (Amersham Biosciences). After washing with 20-column volumes of 0.15 M PBS, pH 7.2, the beads were incubated with 5.0 μg of Jurkat/D1.1 cellular extract at 4°C for 2 h and packed in a 10-ml column. Specifically bound proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.5, and neutralized, and OD280 was determined. Positive fractions were pooled and dialyzed, and protein concentration was determined.

Western blotting

Proteins were resolved on 10% SDS-PAGE gels and electrophoretically transferred to the polyvinylidene difluoride membranes (Amersham Biosciences). Following overnight incubation with BLOTTO (5% dry nonfat milk in 0.15 M PBS, pH 7.2) at 4°C, the blots were incubated with specific primary Abs either for 1 h at room temperature or overnight at 4°C. The blots were washed three times with PBS + 0.05% Tween 20 (PBS-T) and incubated with a 1/4000 dilution of goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Ab (Santa Cruz Biotechnology) for 1 h at room temperature. Following incubation, the blots were washed five times with PBS-T and visualized using ECL luminescent reagent (Santa Cruz Biotechnology).

Coimmunoprecipitation

A total of 100–200 μg of Jurkat/D1.1 cell extracts was treated with 50 μl of protein G/A-agarose beads at 4°C for 30 min, and incubated with a specific Ab for 1.5 h at 4°C. A total of 50 μl of protein A/G-agarose beads were added for 1 h at 4°C, and the immune complexes were washed and separated by centrifugation. The beads were resuspended in 50 μl of 1× SDS sample buffer (100 mM Tris-HCl 6.8, 4% SDS, 20% glycerol, 12% 2-ME, bromophenol blue) and boiled for 5 min, and supernatants were analyzed by SDS-PAGE and Western blotting.
A total of 10^6 Jurkat/D1.1 cells were incubated with 100 μg/ml cycloheximide (Sigma-Aldrich) for 15 min at 37°C. Cells were washed three times with PBS containing 100 μg/ml cycloheximide and lysed in 500 μl of lysis buffer (20 mM Tris, pH 7.5, 100 mM KCl, 1 mM MgCl2, 0.5% Nonidet P-40, 100 μg/ml cycloheximide, 500 U of RNasin, and protease inhibitor mixture (Sigma-Aldrich)). The lysates were clarified by centrifugation at 10,000 rpm for 15 min at 4°C. Fifty OD 260 U of the cytoplasmic extract was loaded on a 7–20% step sucrose density gradient prepared in lysis buffer and centrifuged at 4°C for 4 h at 27K rpm in an SW28 rotor. Fractions (1.0 ml) were collected from the top of the gradient, and their OD 260 was measured. RNA was isolated from individual fractions by using TRIzol LS reagent (Invitrogen Life Technologies, Carlsbad, CA).

Preparation of CD3-activated CD4+ T cells
Preparation and activation of human, peripheral CD4+ T cells were conducted essentially as described in our previous published work (23, 26).

Results
Native Complex I is ~200 kDa
We have previously shown that PTB is the RNA-binding component of Complex I and specifically binds to distinct CU-rich sites in the CD154 3’UTR (Fig. 1A). In an effort to further characterize Complex I with respect to size and subunit composition, total cellular Jurkat/D1.1 extract was resolved on a Superose-12 gel filtration column under non-denaturing conditions that result in the separation of proteins between 1 and 300 kDa. Fractions from the column were assayed for Complex I activity using REMSA with a uniformly labeled polyadenylated RNA probe spanning the XbaI-HaeIII region (XbaI-HaeIII poly(A); see Fig. 1A). We found that a major peak of Complex I activity eluted close to or at the void volume (Fig. 1B, lanes 5–7), and a second peak eluted in fractions corresponding to ~200 kDa relative to protein standards run separately under identical conditions. These results indicate that a major form of Complex I is ~200 kDa, and that the larger form may represent higher order aggregates that are excluded from the column. Because PTB is known to be active as a 110-kDa dimer (35), the presence of a 200-kDa complex suggested that Complex I consists of multiple PTB subunits or PTB plus additional proteins.

Identification of a novel Complex I-associated activity
To purify the components of Complex I, we followed a purification scheme that involved fractionating Jurkat/D1.1 extract using two ion exchange columns (DEAE and heparin), followed by RNA affinity chromatography (Fig. 2A). Purification of Complex I was monitored using REMSA with the XbaI-HaeIII poly(A) probe. Assessment of Complex I activity in the dialyzed DEAE column fractions revealed that the majority of activity eluted in 1.0 M KCl (termed DEAE-bound (B) fraction); however, an unexpected second peak of activity was clearly present in the DEAE-flow through (FT) fraction (Fig. 2B, lanes 5 and 4, respectively). The complex from the DEAE-FT (termed I-U) comigrated with the upper edge of Complex I in the total extract (lane 3), whereas the complex in the DEAE-B fraction (termed I-L) comigrated with the lower edge of Complex I. Surprisingly, immunoblotting of PTB in the corresponding DEAE fractions revealed that PTB was nearly absent in the DEAE-FT fraction, although highly abundant in the DEAE-B fraction (Fig. 2C). This result was quite intriguing because it suggested that Complex I is composed of two unique subcomplexes: the I-L subcomplex, which clearly contains PTB as a CU-binding protein, and the I-U subcomplex, which appears to contain an unidentified CU-binding protein distinct from PTB.

To identify this putative Complex I-associated protein, the DEAE-FT fraction was concentrated, loaded on a heparin-Sepharose column, and eluted with a linear gradient of KCl, and fractions containing Complex I activity were further purified by RNA affinity chromatography. Analysis by SDS-PAGE revealed major proteins of 37- and 75-kDa apparent molecular masses that were identified by in-gel trypsin digestion and mass spectrometry as uracil DNA glycosylase (UDG) and nucleolin, respectively (Fig. 2D). Additional proteins of 40-, 47-, 90-, and 140-kDa apparent molecular masses were also detected, but they accounted for bands of minor intensity relative to nucleolin and UDG. Given the fact that fractions from the Superose 12 column that contained Complex I activity, but did not contain UDG (data not shown), and that UDG has been extensively characterized as a DNA repair enzyme (reviewed in Ref. 36), we chose not to analyze UDG further, but to focus on nucleolin and its potential role in Complex I formation.

Nucleolin is a subunit of Complex I
Nucleolin is a 709-aa protein that is a major component of the nucleolus and has multiple roles in ribosome biogenesis (reviewed in Refs. 37 and 38). It has also been previously implicated in other cellular functions, including the turnover of transcripts encoding β-amylod precursor protein (APP) (39), IL-2 (40), and poliovirus (41). Nucleolin migrates with a molecular mass of 106 kDa, but also exists as multiple cleavage products of ~97, 80, 72, 56, and 45 kDa that are generated by a closely associated catalytic activity

FIGURE 1. Identification of native Complex I as a ~200-kDa complex.
A. Shown is a schematic of the human CD154 mRNA, with the 5′-UTR, the 786-nt coding region (■), and the 982 nt of 3′UTR (□) highlighted. The three Complex I binding sites (A–C) within the XbaI to HaeIII region are denoted by hatch marks. B. A total of 1 mg of Jurkat/D1.1 T-cell extract was loaded onto a Superose-12 gel filtration column under native conditions and proteins eluted with HG-40 buffer in 0.5–ml fractions. The fractions (lanes 4–19) were analyzed for Complex I activity by REMSA using 2 μg of protein and a uniformly labeled CD154 XbaI-HaeIII poly(A) RNA probe. The activity of unfractionated Jurkat/D1.1 cell extract is shown in lane 3. Lane 1, Probe in the absence of extract and RNase; lane 2, the probe with RNase in the absence of the extract. The sizes of the molecular mass standards are shown above the bracket in kilodaltons.
that is regulated, in part, by an endonuclease inhibitor in rapidly dividing cells (42–45).

Next, the DEAE-B fraction was subjected to an identical purification scheme to determine whether nucleolin was also associated with subComplex I-L. Similar to purification of I-U, the majority of the binding activity of I-L was eluted from the RNA affinity column in 2.0 M KCl (Fig. 3A). Analysis of purified I-L revealed the presence of multiple nucleolin cleavage products and the 50- and 55-kDa PTB isoforms in total Jurkat/D1.1 extract (lane 1) and partially purified extract from the DEAE-F fraction (lane 2) and DEAE-B (lane 3) fractions using the anti-PTB mAb, BB7. D, SDS-PAGE and silver stain analysis of partially purified fractions of Complex I-U. Shown is 6 μg of protein from total Jurkat/D1.1 extract (lane 1), Complex I-positive DEAE-FT (lane 2), and heparin-Sepharose (lane 3), and 3 μg of protein from the RNA-affinity fraction (lane 4). Bands indicated by the upper and lower arrows were gel purified, eluted, analyzed by mass spectrometry, and identified as nucleolin and UDG, respectively.

Nucleolin binds directly to the CU-rich stability element

To demonstrate a direct association between nucleolin and Complex I activity, we conducted REMSA with Jurkat/D1.1 cell extracts that had been immunodepleted for either nucleolin or a control RNA-binding protein, NF-90 (Fig. 4A). These experiments revealed a marked decrease in complex binding using extract depleted of nucleolin (lane 5). Consistent with our fractionation results, we observed a loss of activity in both the I-U and I-L subcomplexes; however, the loss was most dramatic in the I-U subcomplex. In contrast, the loss of activity with NF-90-depleted Jurkat/D1.1 extract was much less obvious (lane 4). To extend this
finding, REMSA was performed with Jurkat/D1.1 extract and extract isolated from 48-h anti-CD3-activated CD4+ T cells in the presence of control Ig or polyclonal anti-nucleolin Abs (Fig. 4B). Results from this experiment demonstrate a nearly complete disruption of complex formation when anti-nucleolin Abs are added directly to the binding reaction (lanes 5 and 10), indicating that nucleolin is critical for maximal Complex I-binding activity in both Jurkat T cells and activated Primary T cells, and as an extension CD154 RNP formation.

Finally, our previous published UV cross-linking data showed that PTB was the major RNA-binding protein in Complex I. However, frequently, we would observe a weak band at $\sim75$ kDa. To reconcile these past observations with our recent data, we modified our UV cross-linking procedure to include sequential steps of RNase T1 and RNase A instead of coinubcation with these enzymes. Under these conditions, we were able to clearly detect two RNA-binding proteins of $\sim50$–$55$ kDa and a third protein of $\sim75$ kDa that bound to the XbaI-HaeIII region (Fig. 4C, lane 3). Notably, we never detected bands corresponding to the lower m.w. nucleolin cleavage products, suggesting that these forms do not participate directly in RNA binding. Together, these findings strongly suggest that nucleolin is a second component of Complex I that is capable of binding directly to the CU-rich stability site, albeit with less affinity than PTB.

PTB and nucleolin are associated with each other

We next asked whether Complex I could be reconstituted in vitro using immunopurified nucleolin. Total Jurkat/D1.1 extract was passed over an immunoaffinity column consisting of anti-nucleolin mAb, MS3, coupled to Sepharose 4B beads. After elution from the column and renaturation of the protein, increasing amounts of nucleolin were titrated into binding buffer with uniformly labeled probes corresponding to either the complete Complex I-binding region (XbaI-HaeIII) or a region lacking binding activity (HaeIII-1816). As shown in Fig. 5A, Complex I was specifically reconstituted using $1 \mu$g or more of purified protein and only with the XbaI-HaeIII probe (compare lanes 7 with lanes 15 and 16). Analysis of the nucleolin species in the purified and total fractions indicated that only a subset of the cleavage products was pulled down under non-denaturing conditions by the MS3 Ab. In particular, these forms represented the more intact species, suggesting a direct relationship between Complex I formation and the presence of the upper m.w. forms of nucleolin (Fig. 5B, lanes 1 and 2).

Surprisingly, when the same extract was immunoprecipitated with anti-PTB Abs, we observed that a fraction of PTB was pulled down with anti-nucleolin Abs (Fig. 5B, lane 4). This unexpected result indicated that in addition to nucleolin binding directly to the 3′UTR stability element, it was also interacting with PTB either through protein-protein or protein-RNA interactions.

To determine whether the interaction between PTB and nucleolin required an RNA bridge, Jurkat/D1.1 cell extract was treated with RNase A, immunoprecipitated with either anti-PTB or anti-nucleolin mAbs, and immunoprecipitated with Abs against nucleolin and PTB, respectively (Fig. 5C). In both reactions, nucleolin and PTB were co-precipitated, supporting the idea that the proteins are associating through protein-protein interactions rather than indirectly through an RNA bridge. Also, immunoprecipitation with anti-PTB Abs coprecipitated only the higher m.w. forms of nucleolin, supporting our hypothesis that the more intact species of nucleolin are involved in complex formation. Our collective results strongly indicate that within Complex I, the higher m.w. forms of nucleolin are both 1) binding directly to RNA and 2) associating directly or indirectly with PTB through protein-protein interactions.

Complex I containing PTB, nucleolin, and CD154 mRNA forms in vivo

As a way of confirming that the PTB- and nucleolin-dependent CD154 RNP complex is a valid representation of the stability complex that forms in vivo, Jurkat/D1.1 cytoplasmic extract was fractionated into different subcellular components over a sucrose density gradient, and each fraction was assayed for the physical association of nucleolin and PTB with CD154 mRNA. Initially, we confirmed the integrity of the gradient by immunoblotting the different fractions using the eL10 Ab that recognizes the 60S ribosomal subunit protein. As presented in Fig. 6A, eL10 was present only in the 60S, 80S, and polysome fractions, therefore demonstrating that the gradient successfully separated the cellular proteins. Initial assessment of the gradient by Western blotting revealed that the vast majority of the nucleolin cleavage products and the 55- and 25-kDa isoforms of PTB eluted with the free proteins, although a small, but measurable amount of the 55-kDa form of PTB and the higher m.w. forms of nucleolin was present in all fractions, including the polysomes (data not shown). To determine whether nucleolin and PTB are associated with the CD154 mRNA in actively translating ribosomes, different fractions from the gradient were immunoprecipitated either with control Ig or specific anti-PTB and anti-nucleolin Abs. The immunoprecipitated pellets were resuspended and divided into two equal fractions that were extracted either for protein or RNA. Total RNA isolated from each fraction was reverse transcribed and amplified by PCR using primers specific for CD154. As shown in Fig. 6, B and C, CD154 mRNA is associated with PTB and nucleolin in both the RNP and polysome fractions, although both proteins are found in all fractions of the gradient (free proteins, RNPs, and polysomes). The
specificity of the reactions was confirmed by showing that the CD154 transcript was not coimmunoprecipitated with control IgG (Fig. 6, B and C, lanes 2, 4, 6, and 8). Together, these data are consistent with Complex I, consisting of both nucleolin and PTB, being bound to CD154 mRNA in vivo.

**Nucleolin and PTB are differentially expressed in activated T cells**

Finally, our current findings suggest that PTB and nucleolin should only be present in activated T cells at late times of activation when message stabilization (and Complex I formation) is observed. To test this proposition, CD4⁺ T cells were isolated from peripheral blood and activated with plate-bound anti-CD3 mAb for varying time periods, and cytoplasmic extracts were prepared for Western blot analysis. As shown in Fig. 7, only the lower m.w. species of nucleolin are present both in resting and 2-h stimulated extracts, which corresponds to a time when the message is rapidly degraded (2 h). Also, there is limited cytoplasmic PTB at these time periods (lower panel). In contrast, the more intact forms of nucleolin are present beginning at 24 h, and become highly visible at 48 h after CD3 activation. These findings support our hypothesis that the more intact
forms of nucleolin are important for stabilizing the CD154 mRNA at late times of T cell activation.

Discussion
In recent years, there has been a growing appreciation for the fundamental role of posttranscriptional mechanisms in regulating global gene expression. The complexity of the immune response, with its ongoing need for rapid adjustments of cellular reactions based on internal and external stimuli, provides an ideal milieu in which to study such mechanisms. Within this immunological framework, mRNA degradation processes, mediated by different cis- and trans-acting factors, contribute to establishing appropriate levels of proteins within a cell at any given time. Our previous work revealed that CD154 mRNA is differentially stabilized at specific times after CD3-directed T cell activation by the binding of PTB-containing complexes to three CU-rich sites within the CD154 3′UTR (23, 26, 27). In our current study, we extend these findings and show that a second RNA-binding protein, nucleolin, is also an integral component of complex I.

Nucleolin is a known RNA-binding protein that interacts with RNA by directly binding to two mutually exclusive RNA sequences: the nucleolin recognition element (NRE), which is a short hairpin

**FIGURE 5.** Immunoaffinity-purified nucleolin can form Complex I in vitro. A, REMSA was conducted with uniformly labeled Xba-HaeIII poly(A) probe (lanes 3–8) or control HaeIII-1816 poly(A) probe (lanes 11–16) with 5.0 μg of Jurkat D1.1 cell extract (lanes 3 and 11) or 10 ng (lanes 4 and 12), 100 ng (lanes 5 and 13), 500 ng (lanes 6 and 14), 1 μg (lanes 7 and 15), and 2 μg (lanes 8 and 16) of immunoaffinity-purified nucleolin. Control reactions showing probe alone (lanes 1 and 9) and probe with RNase in the absence of extract (lanes 2 and 10) are included. B, 20 μg of total Jurkat/D1.1 extract (lanes 1 and 3) or 3 μg of immunoaffinity-purified nucleolin protein (lanes 2 and 4) was immunoblotted with anti-nucleolin mAb, MS3 (lanes 1 and 2), or anti-PTB mAb, BB7 (lanes 3 and 4). C, 200 μg of Jurkat/D1.1 cell extract was treated with 50 μg/ml RNase A for 15 min at 37°C, immunoprecipitated with 5 μg of control IgG (lanes 2 and 5), 50 μg of anti-nucleolin mAb MS3-conjugated beads (lane 3), or 5 μg of anti-PTB Abs (lane 6). The immunoprecipitates were probed with the MS3 mAb (upper panels) or the BB7 mAb (lower panel). Samples of Jurkat/D1.1 cell extract were the positive controls (lanes 1 and 4).

**FIGURE 6.** Nucleolin and PTB are physically associated with CD154 mRNA in actively translating polysomes. A, Jurkat/D1.1 cytoplasmic extract was fractionated on a 7–47% sucrose gradient, and the OD (254 nm) was determined for each fraction. A representative OD profile of the fractions is shown. A total of 30 μl of each fraction was resolved by SDS-PAGE and immunoblotted using anti-eL10. B and C, Total Jurkat/D1.1 cytoplasmic extract (lanes 2 and 3) and representative fractions from free proteins (lanes 4 and 5), polysomes (lanes 6 and 7), and RNP (lanes 8 and 9) were immunoprecipitated using either control mAb (lanes 2, 4, 6, and 8), anti-PTB mAb BB7 (lanes 3, 5, 7, and 9), or anti-nucleolin mAb MS3 (lanes 3, 5, 7, and 9), as described in Materials and Methods. The immunoprecipitates were evenly divided, and one-half was Western blotted to insure fidelity of the reaction (B and C, lower panels). RNA was extracted from the remainder of the precipitate, reverse transcribed, and analyzed for CD154 mRNA using PCR (B and C, upper panels). Lane 1, PCR using total cDNA from Jurkat/D1.1 cells (upper panels) and 20 μg of Jurkat/D1.1 extract run as positive control in Western blot analysis (lower panels).
binding affinity of nucleolin for this site relative to PTB. The absence of the consensus sequence is not entirely unprecedented because other identified nucleolin binding sites, including those in IL-2 (40), APP, and poliovirus RNAs, have nonconsensus binding sites that lack identity through the CCC core region. Comparison of the CD154 E1'–E5' sequence to the 29-nt APP nucleolin binding site revealed greater than 50% identity with this element (Fig. 8). Together, these findings suggest that nucleolin binds to the 3' region of the E1'–E5' RNA and that sequences 5' of this region are most likely required for PTB binding. This assumption is supported by our previous results showing that truncation of 9 nt at the 5' end of the E1'–E5' binding site severely reduced, but did not eliminate binding, whereas deletion of 20 nt from the 3' end abolished binding altogether (26). Deleting sequences in the 5' region would presumably eliminate PTB binding, but still allow nucleolin to bind sequences in the 3' end of the region. In contrast, eliminating the putative nucleolin binding site may interfere both with RNA binding and nucleolin-PTB association, resulting in an absence of Complex I binding. We are in the process of testing these assumptions experimentally.

The finding that nucleolin is a subunit of Complex I is reminiscent of other nucleolin-mediated complex interactions. For example, nucleolin has been shown to interact with several transcription factor complexes, such as LRI (48–51), α-1 acid glycoprotein (52), and E47 (53), as well as other nuclear components, including the matrix attachment region, switch DNA repeat, telomere DNA repeat, and topoisomerase I (reviewed in Ref. 44). Nucleolin also associates with multiple cellular proteins, including CK II (54); B23, a nucleolar protein involved in signaling (55); and the survival motor neuron 1 protein, which is implicated in spinal muscular atrophy (56). Also, PTB is known to physically interact with a number of proteins in determining splice-site selection (57, 58). By demonstrating that removing either PTB or nucleolin from the extract results in a loss of binding activity and that the interaction of PTB with nucleolin is not dependent on a RNA bridge, we extend our understanding of CD154 mRNA stability by confirming that protein-protein interactions are essential for Complex I formation.

That both nucleolin and PTB are components of the CD154 RNP particle is supported by our data showing copurification of these proteins with CD154 mRNA in the RNP and polysome fractions, and coimmunoprecipitation of all three components using Abs directed against either PTB or nucleolin. These findings suggest that Complex I assembles on the CD154 mRNA before association with the ribosomes, which is consistent with its function of protecting the RNA from cellular ribonucleases. Nucleolin is known to shuttle between the nucleus and cytoplasm, and this localization has been shown to occur in response to infection with poliovirus as well as to the expression of membrane-associated CD3ε chain (41, 59). Nucleolin binds to a proline-rich segment of the CD3ε cytoplasmic tail, and this association is greatly enhanced in response to CD3 signaling (59). Thus, signaling through CD3 may induce changes in the cellular distribution of nucleolin, which would promote enhanced complex assembly after T cell activation. In addition, previous work has shown that nucleolin is rapidly degraded by an autocatalytic activity in resting PBMC that can be rescued by the addition of extract from stimulated cells (42, 43). Thus, T cell activation through CD3 signaling may function to both increase the level of nucleolin in the cytoplasm and down-regulate the associated autocatalytic activity of cytoplasmic nucleolin. The net result would be a significant increase in functional nucleolin in the cytoplasm.

The extreme consequences resulting from inappropriate or lack of expression of CD154 underscore the importance of general regulatory proteins, such as nucleolin, in the control and expression of highly regulated genes. The collective findings from several groups suggest that nucleolin may function as a general regulator of RNA stability (reviewed in Refs. 37 and 38). Subsets of RNAs may be targeted by the presence of nucleolin-specific binding sites or by the association of nucleolin with other RNA-binding proteins (e.g., PTB). For example, it has been recently demonstrated that nucleolin stabilizes the Bcl-2 mRNA by interacting with a region of the 3'UTR that contains an identified ARE, yet does not bind to the AU-rich element directly. This raises the possibility that nucleolin is
interacting with ARE-binding proteins to alter the turnover rate of the transcript (60). Furthermore, RNA and protein interactions may both contribute to nucleolin’s association with a particular transcript such as is our hypothesis concerning its interaction with CD154 RNA. Future and ongoing studies are aimed at dissecting the molecular mechanisms underlying Complex I formation on the stabilization of immune-related transcripts throughout T cell activation.

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