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RNA Stability of the E2A-Encoded Transcription Factor E47 Is Lower in Splenic Activated B Cells from Aged Mice

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We have demonstrated previously that DNA binding and protein expression of the E2A-encoded transcription factor E47 are lower in nuclear extracts of activated splenic B cells from old mice. In the present study, we address how E47 protein expression is regulated in aging. Results herein show that E2A mRNA levels were decreased in stimulated splenic B cells from old as compared with young mice. RNA stability assays showed that the rate of E2A mRNA decay was accelerated in stimulated splenic B cells from old mice, but E47 protein degradation rates were comparable in young vs aged B cells, indicating that the regulation of E47 expression in activated splenic B cells occurs primarily by mRNA stability. The rates of decay of other mRNAs showed that the increased mRNA degradation in aged splenic activated B cells is not a general phenomenon but restricted to a subset of mRNAs. We next investigated the signal transduction pathways controlling E2A mRNA expression and stability and found that p38 MAPK regulates E2A mRNA expression through increased mRNA stability and is down-regulated in aged activated B cells. Results show that inhibition of p38 MAPK significantly reduces E2A mRNA stability in both young and old B cells, further stressing the role of p38 MAPK in E2A RNA stabilization. These studies demonstrate that the transcription factor E2A, critical for many aspects of B cell function, is regulated by a novel mechanism in aging.

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Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Miami, FL 33101

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2 Address correspondence and reprint requests to Dr. Bonnie B. Blomberg, Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101. E-mail address: bblomber@med.miami.edu

3 Abbreviations used in this paper: bHLH, basic helix-loop-helix; AID, activation-induced cytidine deaminase; CSR, class switch recombination; ARE, adenylate/uridylate-rich element; UTR, untranslated region; MAPKAPK-2, MAPK-activated protein kinase-2; COX-2, cyclooxygenase-2; TRAF2, TNFR-associated factor 2; Act D, actinomycin D; CHX, cycloheximide; mRNA, microRNA.
unknown features for their destabilizing function. The E2A mRNA belongs to the last group of AREs. The p38 MAPK and its downstream effector MAPK-activated protein kinase-2 (MAPKAPK-2) have been described to be involved in the regulation of the stability and/or the translation of several mRNAs, including those for TNF-α, cyclooxygenase-2 (COX-2), IL-6, IL-8, GM-CSF, and c-fos (24, 29–31). Therefore, we investigated whether the p38 MAPK signal transduction pathway may also be involved in the control of E2A mRNA expression and stability and whether it was also regulated during aging. Results indicate that p38 MAPK and its substrate MAPKAPK-2 regulate E2A mRNA expression through increased mRNA stability and are reduced in activated splenic B cells from old mice.

Materials and Methods

**Mice**

Male and female young (2–4 mo of age) and old (24–27 mo of age) BALB/c were purchased from the National Institutes of Aging and maintained in our animal facilities. Most of the experiments have been done with females. A few experiments have been done with males. No significant differences between females and males were seen.

**Splenic B cell enrichment**

B cells were isolated from the spleens of young and old mice. Briefly, cells were washed twice with medium (RPMI 1640; Invitrogen Life Technologies) and incubated (106 cells/ml) for 20 min at 4°C with 100 μl of anti-B220 Microbeads (Miltenyi Biotech), according to the MiniMacs protocol (Miltenyi Biotech). Cells were then purified using magnetic columns. At the end of the purification procedure, cells were found to be almost exclusively (90%) B220 positive by cytofluorimetric analysis. After the isolation procedure was ended, cells were maintained in serum-free medium for 3 h at 4°C to minimize potential effects of anti-B220 Abs on B cell activation.

**B cell culture**

B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 10 μg/ml gentamicin, 2 × 10−3 M 2-ME, and 2 mM t-glutamin). Cells (2 × 106 in 200 μl of complete medium) were stimulated in flat-bottom 96-well culture plates with purified anti-mouse CD40 Abs and LY294002 (PI3K), both purchased from Sigma-Aldrich, and were used in DMSO (controls). The inhibitors were as follows: SB203580 (p38 MAPK), SB202190 (p38 MAPK), and are reduced in activated splenic B cells from old mice. Fos (COX-2), IL-6, IL-8, GM-CSF, and c-fos are inhibited E2A mRNA expression and DNA binding. After treatment with fms (COX-2), IL-6, IL-8, GM-CSF, and c-fos inhibited E2A mRNA expression and DNA binding. After treatment with

**Preparation of the DNA probes**

The μE5 and STAT-6 DNA probes were prepared as follows: 100 μl of each single-strand (26 bp for μE5 and 28 bp for STAT-6), at a concentration of 100 ng/μl, were annealed v/v at the following temperatures: 85°C (2 min), 65°C (15 min), 37°C (15 min), 25°C (15 min), and on ice (15 min) and then end-labeled for 40 min at 37°C, using T4 DNA polynucleotide kinase in the presence of 1 μl of [γ-32P]ATP. The probes were then purified over a G-25-50 Sepharose column. The sequences of the probes were as follows: 5′-TCGAGAAGACCTGTCGACGCTG3′ (μE5, present in the IgH intronic enhancer) (34); and 5′-GATGCTGTCTCTCCTCCAGGACTCTAAGT-3′ (STAT-6) (35).

**EMSA**

The gel mobility shift assay to determine DNA binding of E47, STAT-6, or ku 70/80 was performed as follows. The radiolabeled DNA probe was incubated with 10 μg of nuclear extract in the presence of polydeoxyribo- nosinic-deoxyribonucleic acid as unspecific competitor for μE5 and STAT-6 or with circular pUC19 as unspecific competitor for ku. The reaction was performed at room temperature (blocking solution). Filters were incubated with T4 DNA polynucleotide kinase in the presence of 1 μl of [γ-32P]ATP, then incubated with the complementary oligonucleotide, at a concentration of 100 ng/μl, at 85°C for 5 min and subsequently cooled at room temperature. The probe was then purified over a G-50-80 Sepharose spin column. The sequence of the probe was as follows: 5′-GATCAGCATGGAGGTTGGCCACTCCCTCTCGCGCTCGCTCACTG3′ (STAT-6) (35).

**Preparation of cytoplasmic and nuclear extracts and total cell lysates**

For the evaluation of E47 in splenic B cells, total cell lysates at equal protein concentration were denaturated by boiling for 4 min in sample reducing agent (NP0004; NuPAGE) and in sample buffer (LDS NP0007; NuPAGE) and then subjected to SDS-PAGE using a 4–12% polyacrylamide gel under reducing conditions (NP0335; NuPAGE). Proteins were then electrotransferred onto nitrocellulose filters (175 V for 3 h at room temperature. The gels were dried on Whatman 3MM paper and exposed to Kodak x-ray films overnight at −80°C. Films were scanned and analyzed using Scion Image for Windows (Scion). Integrated areas under the densitometric curves for each band were used as estimates of DNA binding.

**Western blotting**

For the evaluation of E47 in splenic B cells, total cell lysates at equal protein concentration were denaturated by boiling for 4 min in sample reducing agent (NP0004; NuPAGE) and in sample buffer (LDS NP0007; NuPAGE) and then subjected to SDS-PAGE using a 4–12% polyacrylamide gel under reducing conditions (NP0335; NuPAGE). Proteins were then electrotransferred onto nitrocellulose filters (175 V for 3 h at room temperature. The gels were dried on Whatman 3MM paper and exposed to Kodak x-ray films overnight at −80°C. Films were scanned and analyzed using Scion Image for Windows (Scion). Integrated areas under the densitometric curves for each band were used as estimates of DNA binding.

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cheluminescence and exposed to CL-XPosure Film (Pierce). Films were scanned and analyzed using Scion Image for Windows (Scion). Integrated areas under the densitometric curves for each band were used as estimates of protein expression.

**RNA extraction and RT-PCR**

Total RNA was isolated from 0.5 × 10^7−10^9 unstimulated or anti-CD40/IL-4-stimulated splenic B cells using the TRizol reagent (Invitrogen Life Technologies), according to the manufacturer’s protocol, eluted into 100 μl of distilled water and stored at −80°C until use. Alternatively, mRNA was extracted from limited numbers (0.5 × 10^5−10^6) of B cells using the MACS mRNA isolation kit (Miltenyi Biotec), according to the manufacturer’s protocol, eluted into 75 μl of preheated elution buffer, and stored at −80°C until use. RT-PCR was performed in a Mastercycler Eppendorf machine. Two microliters of RNA at the concentration of 0.5 μg/μl were used as template for cDNA synthesis in the RT reaction. After an initial 4-min denaturation at 95°C, the cDNA was amplified for 30 cycles (35 for ku80 and c-jun; 36 for Blimp-1). Annealing temperatures were as follows: 60°C (E2A, GAPDH), 55.5°C (ku80, Blip1), 66°C (Bof1), 64.5°C (μ), 55.5°C (c-jun), and 56.9°C (c-fos). At the end of the annealing process, an elongation phase of 2 min at 72°C took place, followed by a single extension phase of 3 min at 72°C.

Primers for PCR amplification were as follows: E2A forward, 5′-GCCTGACCGAGTGAGTCCTG-3′; E2A reverse, 5′-CAGGTGAGTCCTGAGGAGG-3′; GAPDH forward, 5′-ACCAGCTCTCCAAGTGACGG-3′; GAPDH reverse, 5′-CTTGTTGGAGGAGG-3′; ku80 forward, 5′-CTTACCCACCATTTGCTGTATA-3′; ku80 reverse, 5′-CGGAAAAGGCTGACACATG-3′; Bof1 forward, 5′-CAAGCTCTGCCCCAAAGGGCAAAAGG-3′; Bof1 reverse, 5′-CTTGCTGTCGCAGAGATGAACCCCAAGGGGGAAG-3′; μ forward, 5′-CTGGCCAGGAAATTCAGAAGCTGAGGAGGACGGGCGCCCTCTATAGGGGCCTCG-3′; c-jun forward, 5′-GGGGCCGCGCCAAGCTTGAGTGTACTTCAGGCTGAGG-3′; c-jun reverse, 5′-CACCTGTCTTTCGTAGGATGT-3′; c-fos forward, 5′-ATGATGTTCTCGGGTTTCAACG-3′; c-fos reverse, 5′-CAGGTGACCGAGTGAGTCCTG-3′; Blimp1 forward, 5′-32P-CTTACTCCTTGGAGGCCATG-3′; Blimp1 reverse, 5′-GAGAGATACGCGCCGAGCCAAGAG-3′; and GAPDH reverse, 5′-CTTACCTTGGAGGCGCCATG-3′.

**Results**

Aging decreases expression and stability of E2A mRNA in activated splenic B cells

We have demonstrated previously (13, 32, 38) that DNA binding and protein expression of E47 are lower in nuclear extracts of activated splenic B cells from old mice. In the present study, we investigated the molecular mechanism for this decrease, whether it be transcriptional, posttranscriptional, or posttranslational. Both protein and RNA stability were initially measured. For E2A mRNA stability studies, cells were stimulated with anti-CD40/IL-4 for 3, 6, and 24 h or left unstimulated. After these times, cells were harvested, RNA was extracted, and RT-PCR was performed. Unstimulated B cells express almost indiscernible levels of E2A mRNA. Stimulation of purified B cells from young and old mice with anti-CD40/IL-4 induced a marked increase in E2A mRNA expression at all the stimulation times, the levels of mRNA in old mice being lower as compared with the young mice (Fig. 1A).

Assurance of comparison of samples in the linear range for PCR was accomplished by simultaneous amplification of three 4-fold serial dilutions of the RT mixes from young and old samples and shown in Fig. 1B. Northern blot results confirmed the 24 h kinetic data (Fig. 1C) of a 3- to 4-fold reduction in E2A mRNA in aged stimulated B cells.

The amount of mRNA is controlled not only by de novo transcription but also by the stability of the mRNA. We next asked whether the age-related difference in E2A mRNA expression following anti-CD40/IL-4 stimulation could result from different stability of E2A mRNA. We used an inhibitor of transcription, Act D, which was added to the cells at the end of the 3, 6, and 24 h of stimulation with anti-CD40/IL-4 for 10, 45, and 90 min. Results in Fig. 2A, top panel, show that the stability of E2A mRNA is only slightly decreased at 24 h in B cells from young mice after 90 min in the presence of Act D, whereas it is significantly decreased at this time of stimulation in B cells from old mice. In B cells from young mice, moreover, the stability of E2A mRNA is not modified after 3 and 6 h of stimulation, whereas it progressively decreases with the increasing times of stimulation in B cells from old mice (data not shown); for the kinetics of E2A expression at these time points.

**In vitro incubation of mRNAs and proteins**

mRNAs from young and old B cells were extracted from 0.5 × 10^7−10^9 B cells using the μMACS mRNA isolation kit (Miltenyi Biotec) after 24 h activation with anti-CD40/IL-4. Same amounts of mRNAs from young and old B cells (0.025–0.1 μg) were incubated for 15 min at room temperature with total lysates from young and old B cells (24 h activated) at different mRNA:protein ratios (1:1, 1:10, and 1:20). The micrograms of mRNAs were calculated as 1% of the expected total RNA extracted from 0.5 × 10^7−10^9 B cells (10−20 μg), after 24 h of activation with anti-CD40/IL-4. Other time points from 5 to 30 min and temperatures of incubation were also tested (data not shown). After this time, mRNAs were extracted with the μMACS mRNA isolation kit (Miltenyi Biotec) and RT-PCR performed.
FIGURE 1. E2A mRNA expression is decreased in activated splenic B cells from old mice. A. Purified splenic B cells (10^6-10^7 cells/ml) were stimulated with anti-CD40/IL-4 for 3, 6, and 24 h or left unstimulated. After these times, cells were harvested, RNA was extracted, and RT-PCR was performed as in Materials and Methods. Undiluted RT-PCR are shown. Vertical columns represent the densitometric analyses (arbitrary units) of E2A mRNA expression, normalized to GAPDH, ± SE from four pairs of young (□) and old (■) mice (values of unstimulated and 24 h stimulated cells are from eight pairs of mice). Values are compared with young, unstimulated controls, taken as 1. Young values are as follows: 1 (unstimulated), 4.5 ± 0.29 (3 h), 11.75 ± 1.65 (6 h), and 18.75 ± 4.11 (24 h). Old values are as follows: 0.5 ± 0.2 (unstimulated), 0.78 ± 0.23 (3 h), 2.96 ± 0.47 (6 h), and 5.0 ± 0.5 (24 h). Fold differences were 2 (unstimulated), 5.8 (3 h), 4.0 (6 h), and 3.8 (24 h). The difference between young and old mice is significant at p < 0.05, as determined by the two-tailed Student’s t test, at all time points. B. Titration of RT mixes from young and old samples (three 4-fold serial dilutions) were performed for each time point to allow comparison of samples in the linear range for PCR. C. Northern blot of E2A mRNA in splenic B cells stimulated with anti-CD40/IL-4 for 24 h. Vertical columns represent the densitometric analyses (arbitrary units) of E2A mRNA expression, normalized to GAPDH, ± SE from two pairs of young and old mice. Young values: 21.75 ± 3.45; old values: 6.66 ± 1.44. The difference between young and old mice is significant at p < 0.05, as determined by the two-tailed Student’s t test.

points see Fig. 1B). The t_{1/2} of the E2A mRNA in RT-PCR experiments was 138 ± 11 and 21 ± 6 min in young and old B cells, respectively. Thus, the reduced stability of E2A mRNA in old mice could be by itself explain the different kinetics of mRNA accumulation and expression seen above, although we cannot formally exclude a transcriptional component to the regulation of E2A mRNA expression.

Real-time PCR experiments confirmed these results (Fig. 2A, bottom panel). In real-time PCR experiments, the t_{1/2} of the E2A mRNA in RT-PCR experiments was 255 ± 60 and 31 ± 10 min in young and old B cells, respectively.

We further wanted to address the issue whether the different degradation profiles of splenic activated B cells from young and old mice could result from different starting amounts of E2A transcripts (before Act D). To this purpose, we used a cell-free system to combine mRNA and protein from young and old B cells extracted after 24 h of activation with anti-CD40/IL-4 (see Materials and Methods). Briefly, mRNA from young and old B cells were incubated in vitro with total lysates from young and old B cells (24 h activated) for 15 min at room temperature. After this time, mRNA was extracted and RT-PCR performed. Results in Fig. 2B show that a protein lysate from young B cells induced the degradation of E2A mRNA at all mRNA:protein ratios, the maximum effect being at 1:20 ratio with 17% of remaining mRNA. The effect of mixing young mRNA with old total protein lysates was even more pronounced (10% of remaining mRNA at 1:20 ratio). Similar results as above were obtained when protein lysates from young or old splenic B cells were incubated with mRNA from old B cells for 15 min at room temperature. Protein lysates from young B cells induced maximum degradation of old E2A mRNA (18% at 1:20 ratio), whereas in the presence of protein lysates from old B cells 13% of old E2A mRNA was left (at 1:20 ratio). These results altogether suggest that at least part of the decreased stability of E2A mRNA seen in aged B cells is mediated by aged proteins. In these experiments, poly(A) minus RNAs (including small RNAs) were removed, and only mRNA was present in the mixture with proteins. Thus, at present, we do not know the contribution of microRNAs (miRNAs) to the degradation of E2A mRNA.

To further address whether the rapid degradation profile seen in old splenic B cells could result from lower E2A transcripts, we also ran RT-PCR where we loaded 3-fold more RNA from old B cells as compared with young B cells. Results (data not shown) again confirm that the E2A mRNA was degraded very rapidly in old B cells, whereas it was stable in young B cells.

Comparable E47 protein degradation in activated splenic B cells from young and old mice

Our results clearly show that the stability of E2A mRNA is decreased in aged activated splenic B cells. This is sufficient to explain the decreases we have seen in E47 protein expression (Western blot analysis) and activity (EMSA), although this would not exclude that protein degradation events might also take place. E47 is indeed highly unstable, with a half-life of 55 min in vivo, as calculated in NIH 3T3 cells transfected with plasmids encoding full-length E47, pulsed with [35S]methionine and harvested at time 0 or after a 2-h chase with cold methionine (43, 44). Its instability could be dependent on its primary amino acid sequence, which is rich in PEST residues (proline, glutamic acid, serine, threonine) common to degradation domains (45). We next looked at the amount of E47 protein degradation in splenic B cells from young and old mice in vitro stimulated with anti-CD40/IL-4 for 48 h or left unstimulated. CHX was added to cultures in the last 1–5 h of
stimulation. Results in Fig. 2A show that E47 protein degradation rates were comparable in young vs aged B cells. When splenic B cells were stimulated with lower doses of IL-4 (20 ng/ml), again no age-related differences in E47 protein degradation rates were seen, but the kinetics of degradation were faster as compared with cultures set up in the presence of high IL-4 doses (data not shown).

**FIGURE 2.** E2A mRNA, but not protein, stability is decreased in activated splenic B cells from old mice. A, Splenic B cells (10^6 cells/ml) were stimulated with anti-CD40/IL-4 for 3, 6, and 24 h. After these times, RNA transcription was blocked in cultures of anti-CD40/IL-4-stimulated splenic B cells by Act D (10 μg/ml). After 10, 45, and 90 min, cells were harvested, RNA was extracted, and RT-PCR was performed. Vertical columns represent the densitometric analyses of E2A mRNA expression, normalized to GAPDH, ± SE from six pairs of young (□) and old (■) mice. Results are expressed as percentages of the samples untreated with Act D. Unadjusted young vs old values at 24 h of stimulation are as follows: 100 vs 31 ± 7 (no Act D), 96 ± 1 vs 13 ± 1 (Act D, 10 min), 92 ± 2 vs 9 ± 2 (Act D, 45 min), and 65 ± 4 vs 5 ± 2 (Act D, 90 min). Some samples shown in the upper panel of A were also run in real-time PCR. Vertical columns represent the densitometric analyses of normalized E2A mRNA expression ± SE from two young (□) and four old (■) mice for the 0 and 90 min time points and one each for the intermediate time points. Results are expressed as percentages of the samples untreated with Act D. Unadjusted young vs old values at 24 h of stimulation are 100 vs 32 (no Act D), 107 vs 20 (Act D, 10 min), 93 vs 11 (Act D, 45 min), and 83 vs 5 (Act D, 90 min). B, Similar amounts of total mRNAs from young and old splenic B cells, 24 h activated with anti-CD40/IL-4, were incubated in vitro with total lysates from young and old B cells (24 h activated) for 15 min at room temperature. To better compare the rates of degradation of young vs old mRNA, due to the addition of young or old proteins, we took the mRNA alone values as 100 and calculated the remaining mRNA after addition of the proteins. Values are RNA young alone, 100; RNA young + proteins young, 50 (1:1), 30 (1:10), and 17 (1:20); RNA young + proteins old, 29 (1:1), 15 (1:10), and 10 (1:20). RNA old alone, 100; RNA old + proteins young, 72 (1:1), 41 (1:10), and 18 (1:20); RNA old + proteins old, 37 (1:1), 22 (1:10), and 13 (1:20). These results are representative of five independent experiments. In the experiment shown, old mRNA was 3-fold more than the young (but mRNA:protein ratios were maintained). C, Splenic B cells (10^6 cells/ml) were stimulated with anti-CD40/IL-4 for 48 h, the optimum for protein expression (see Ref. 13). Cells were harvested, counted, and aliquoted in five samples (10^6 cells/ml) and treated with 200 μg/ml CHX for 1, 2, 3, 4, and 5 h. Whole cell lysates were prepared and run in Western blots. Vertical columns represent the densitometric analyses (arbitrary units) of E47 protein expression, normalized to β-actin, ± SE from three pairs of young (□) and old (■) mice. Values are compared with young controls without CHX, taken as 100. Young vs old values are 100 vs 42 ± 11 (no CHX), 112 ± 7 vs 47 ± 13 (1 h, CHX), 145 ± 10 vs 58 ± 18 (2 h, CHX), 142 ± 23 vs 60 ± 18 (3 h, CHX), 73 ± 2 vs 31 ± 7 (4 h, CHX), and 61 ± 6 vs 30 ± 11 (5 h, CHX).
The $t_{1/2}$ of E47 protein (calculated from the point 3 h in the presence of CHX) is 58 and 54 min in young and old B cells, respectively. Thus, mRNA stability seems to be the major mechanism, which regulates E47 in activated splenic B cells, independently of the dose of IL-4 used.

**Decreased mRNA stability in aged activated B cells is limited to select mRNAs**

We then looked at the expression and stability of other mRNAs similar to or different from E2A in terms of ARE sequences. In particular, we looked at the following mRNAs: c-jun mRNA, a class III ARE (like E2A), and c-fos mRNA, a class I/II ARE, encoding an early-activating transcription factor (46, 47); Blimp-1 mRNA, a class I ARE, encoding a transcriptional repressor involved in the terminal differentiation of B cells to plasma cells (39, 48); ku80 mRNA, a class I ARE, encoding a DNA repair enzyme involved in the nonhomologous end joining processes (49, 50); Bob-1 mRNA, a class I ARE, encoding the B cell-specific factor known as Bob.1, OBF-1, or OCA-B that acts as a coactivator for Ig gene transcription (51–53); and the mRNA for the secreted Ig $\mu$-chain.

Both the expression and the stability of the mRNA for ku80, Bob-1 and $\mu$ (Fig. 3A) are not affected by aging. Fig. 3B shows also that c-jun and c-fos mRNA are comparable in activated

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**FIGURE 3.** Only select mRNAs have decreased stability in aged splenic activated B cells. A. Splenic B cells ($10^6$ cells/ml) were stimulated with anti-CD40/IL-4 for 3, 6, and 24 h. RNA transcription was blocked by Act D (10 $\mu$g/ml). After 10, 45, and 90 min, cells were harvested, RNA was extracted, and RT-PCR was performed. Results are representative of four (ku80), one ($\mu$), and three (Bob-1) independent experiments. B. Splenic B cells ($10^6$ cells/ml) were stimulated with anti-CD40/IL-4 for 1, 3, and 6 h. Results are representative of four (c-jun) and three (c-fos) independent experiments. C. Splenic B cells ($10^5$ cells/ml) were stimulated with LPS (10 $\mu$g/ml) for 1–4 days. At the end of the activation time, Act D was added for 90 min and then RNA was extracted. Results are representative of three (Blimp-1) and three (E2A) independent experiments. In the experiment shown here for E2A, to see the degradation of its mRNA in old B cells after the peak of expression (i.e., at days 2 and 4), double the amount of cDNA was used in the PCR, and 2X PCR products were loaded on the gel for both young and old mice. We have titrated the RT-PCR for young B cells stimulated with LPS for 24 h and then treated with Act D for 10, 45, or 90 min and shown that we are in the linear part of the curve (data not shown in the article).
The expression of mRNA for Blimp-1 is induced by LPS and suppressed by IL-4 (54). Therefore, we first investigated the kinetics of Blimp-1 expression after activation of splenic B cells with 10 μg/ml LPS. We have shown previously that LPS stimulation of B cells also shows less Ig class switch (to IgG3) and E2A mRNA, is extremely unstable, being already reduced to 30% after 10 min with Act D (Fig. 3B).

The increased mRNA degradation seen for E2A in old B cells is not a general phenomenon as shown to stabilize many mRNAs (29, 55–57), we looked whether it could also be involved in the regulation of E2A mRNA expression. Splenic B cells were treated with inhibitors of the p38 MAPK or PI3K signal transduction pathways and then stimulated with anti-CD40/IL-4. SB 203580 and LY294002 were used as specific inhibitors of p38 MAPK (58) and PI3K (59), respectively. Fig. 4A shows the results of both RT-PCR and real-time PCR experiments, indicating that E2A mRNA expression was inhibited by pretreatment of splenic B cells with the p38 MAPK inhibitor but not with the PI3K inhibitor. In both RT-PCR and real-time PCR experiments E2A mRNA expression was reduced more in old than in young mice with SB 203580 (p < 0.05). Thus, p38 MAPK is involved in regulating E2A mRNA expression in stimulated splenic B cells in both young and old mice.

We then investigated whether ku80 mRNA expression was also inhibited by treatment with the p38 MAPK inhibitor. Ku, composed of the 70 kDa (ku70) and 86 kDa (ku80) proteins, is the DNA-targeting subunit of the DNA-dependent serine/threonine kinase (DNA-PK), a PI3K family member, which plays a crucial role in DNA double-strand break recognition and repair in mammalian cells (49, 50). Results in Fig. 4B show that the expression of ku80 mRNA was inhibited by pretreatment of splenic B cells with the PI3 inhibitor but not with the p38 MAPK inhibitor, as shown previously (60). The reduction in ku80 mRNA expression was not significantly different in old (to 46%) and young (to 66%) mice.

Data obtained with the mRNA were extended to the functional transcription factor proteins by EMSA. Briefly, nuclear extracts of splenic B cells, pretreated with p38 MAPK or PI3K inhibitors and then activated with anti-CD40/IL-4 for 48 h, were run in EMSA with the μE5 probe or with the ku probe. Results in Fig. 4C show that the p38 MAPK inhibitor decreased DNA binding of E47 to the μE5 probe in both young and old mice, but it was ineffective on ku80 binding to the 56-oligomer probe. Conversely, the PI3K inhibitor decreased DNA binding of ku to the 56-oligomer in both young and old mice, which express the same level of ku DNA-binding (13), but it was ineffective on E47 binding to μE5 (Fig. 4D). These results altogether extend our observation that E2A mRNA expression in splenic activated B cells is controlled by the p38 MAPK signaling (34).

p38 MAPK controls the stability of E2A mRNA in activated splenic B cells from both young and old mice

To examine whether the inhibitors of the p38 MAPK or PI3K signal transduction pathways affect E2A mRNA stability, splenic B cells from old and young mice. The kinetics of degradation for the c-jun mRNA seems to be faster than that of other mRNA (Fig. 3, A and B) and c-fos mRNA, as compared with c-jun mRNA, is extremely unstable, being already reduced to 30% after 10 min with Act D (Fig. 3B).

The stability of E2A mRNA are highest at day 1 as compared with days 2 and 4 of LPS stimulation and higher in young as compared with old splenic B cells. The stability is also comparable between young and old B cells at all time points. Because Blimp-1 mRNA stability was evaluated in B cells stimulated with LPS, we also looked at the stability of Blimp-1 and E2A mRNAs after LPS stimulation. Results in Fig. 3C, top panel, confirm our preliminary results that Blimp-1 expression is higher at day 4 as compared with days 2 and 3 of LPS stimulation and is comparable in young and old splenic B cells. The stability is also comparable between young and old B cells at all time points. Because Blimp-1 mRNA stability was evaluated in B cells stimulated with LPS, we also looked at the stability of E2A after 1–4 days of stimulation with LPS. Results in Fig. 3C, bottom panel, show that both the expression and the stability of E2A mRNA are highest at day 1 as compared with days 2 and 4 of LPS stimulation and higher in young as compared with old splenic B cells at all days. The stability of E2A mRNA after 1 day of LPS and 90 min with Act D only slightly decreased in B cells from young mice, whereas it dramatically decreased to 9% in B cells from old mice, confirming once again the results obtained with anti-CD40/IL-4-stimulated B cells (Fig. 2A). Results from Figs. 3, A–C, and 2A are summarized in Table I.

Table I. The increased mRNA degradation seen for E2A in old B cells is not a general phenomenon

| mRNA | Young B Cells | Old B Cells |
|------|--------------|-------------|
|      | Act D (min)  |             |
|      | 0  | 10 | 45 | 90 | Act D (min)  | 0  | 10 | 45 | 90 |
| E2A  | 24 | 100 | 96 ± 1 | 93 ± 2 | 65 ± 4 | 31 ± 7 | 13 ± 1 | 9 ± 2 | 5 ± 2 |
| ku80 | 24 | 100 | 100 ± 4 | 101 ± 4 | 69 ± 8 | 100 | 100 ± 7 | 103 ± 13 | 78 ± 6 |
| μc  | 24 | 100 | 100 | 95 | 80 | 99 | 101 | 102 | 75 |
| Bob-1 | 24 | 100 | 100 ± 6 | 101 ± 5 | 65 ± 4 | 95 ± 5 | 97 ± 5 | 98 ± 4 | 59 ± 7 |
| c-jun | 6 | 100 | 68 ± 3 | 33 ± 3 | 9 ± 1 | 42 ± 4 | 26 ± 3 | 14 ± 2 | 3 ± 1 |
| c-fos | 6 | 100 | 41 ± 6 | 14 ± 3 | 4 ± 1 | 96 ± 3 | 42 ± 4 | 15 ± 2 | 4 ± 1 |
| E2A' | 24 | 100 | 98 ± 13 | 108 ± 3 | 70 ± 5 | 38 ± 5 | 25 ± 4 | 19 ± 1 | 9 ± 3 |
| Blimp-1 | 96 | 100 | 65 ± 4 | 40 ± 8 | 28 ± 4 | 93 ± 9 | 69 ± 2 | 36 ± 7 | 24 ± 5 |

* Splenic B cells (10⁵ cells/ml) were stimulated with anti-CD40/IL-4 (b) or with LPS (c) for the times indicated. RNA transcription was blocked by Act D (10 μg/ml). After 10, 45, and 90 min were harvested, RNA extracted, and RT-PCR performed. All values are compared with young, untreated controls, taken as 100. Results are densitometric analyses from 6 (E2A) and 4 (E2A') in Fig. 2A; 3 (E2A') in Fig. 3C; 4 (ku80); 1 (μc); and 3 (Bob-1) in Fig. 3A; 4 (c-jun); and 3 (c-fos) in Fig. 3B; and 3 (Blimp-1) in Fig. 3C, independent experiments. The hₜ of the different mRNAs in young and old B cells were as follows: 146 and 162 min (ku80); 160 and 162 min (μc); 135 and 125 min (Bob-1); 19 and 6 min (c-jun); 8 and 7 min (c-fos); and 55 and 50 min (Blimp-1).
B cells from young mice were pretreated with the inhibitors and then stimulated for 24 h with anti-CD40/IL-4. Act D was added to the cells at the end of the 24 h of stimulation for 10, 45, and 90 min. Because the effect of the SB inhibitor was dramatic in old splenic B cells (data not shown), we set up the experiment using a lower dose of the SB inhibitor (2 vs 20 μM). Results in Fig. 5 show that the SB inhibitor was able to reduce E2A mRNA levels in both young and old B cells. The fact that E2A mRNA was...
inhibited similarly in young and old B cells suggests that a protein activated through p38 MAPK signaling was inhibited to the same final level in young and old B cells. These results altogether suggest that E2A is regulated, at least in part, by p38 MAPK through the regulation of mRNA stability. However, we cannot exclude a p38-mediated control of a protein or proteins, which may regulate E2A either transcriptionally or posttranslationally.

p38 MAPK activation is lower in activated splenic B cells from old as compared with young mice

The stability of labile mRNA may be controlled by the p38 MAPK and its downstream effectors. MAPKAPK-2, in particular, has been described to be phosphorylated by p38 MAPK, thus regulating the stability and/or the translation of several mRNA, including those for TNF-α, COX-2, IL-6, IL-8, GM-CSF, and c-Fos (24, 29–31). Because of its relevant role in mRNA stabilization, we next examined both p38 MAPK and MAPKAPK-2 expression and activation by Western blot analysis in total extracts of young and old splenic activated B cells. Results in Fig. 6 show that aging decreases the level of phospho-p38. Moreover, in both young and old mice the levels of phospho-p38 MAPK are reduced in total extracts of splenic activated B cells treated with the p38 MAPK inhibitor, but not with the PI3K inhibitor, as compared with the untreated controls. Also the levels of phospho-MAPKAPK-2 are reduced in total extracts of splenic activated B cells from old mice. In one experiment, the total MAPKAPK-2 was found to be decreased in old activated B cells, suggesting that the age-related decrease in the activation of this crucial molecule may result not only from reduced phospho-p38 MAPK but also from reduced total MAPKAPK-2 (data not shown). Moreover, phospho-MAPKAPK-2 levels were decreased by the p38 MAPK inhibitor but not by the PI3K inhibitor, as compared with the untreated controls. Conversely, the levels of total p38 MAPK, as well as those of TRAF2, are unaffected by aging or by treatment with the specific inhibitor, and for this reason it has been used as the loading control. E47 protein levels were also significantly reduced in total extracts of splenic activated B cells from old as compared with young mice. Pretreatment of splenic B cells with the p38 MAPK inhibitor, moreover, but not with the PI3K inhibitor, significantly reduced the amounts of E47, in both young and old B cells.

Discussion

In this article, we show that E2A mRNA levels are decreased in anti-CD40/IL-4-stimulated splenic B cells from old as compared with young mice due to increased E2A mRNA decay. In contrast to splenic activated B cells, we have demonstrated recently that in vitro IL-7-expanded pro-B/early pre-B cells from old mice have unaltered E2A mRNA expression and stability, but E47 protein degradation is increased (61). This indicates that the reduced expression of E2A proteins in aged B cell precursors and in activated mature splenic B cells from aged mice occur by distinctly different molecular mechanisms. This is not the only difference between bone marrow B cell precursors and mature B cells in terms of E2A activity. We have indeed demonstrated that in splenic mature B cells only E47/E47 complexes bind DNA, whereas in bone marrow B cell precursors E47/E12 complexes participate in DNA binding. Moreover, only nuclear extracts of splenic mature B cells, whereas both nuclear and cytoplasmic extracts of bone marrow B cell precursors, exhibit DNA binding. Nonetheless, although accomplished by different mechanisms, the levels of E2A DNA binding in the spleen correlate with those in IL-7-stimulated bone marrow for individual mice (13) and are lower in aged mice.
In the experiments herein, the regulation of E47 seems not to be dependent on protein degradation, which is comparable in splenic activated B cells from young and old mice. Others, however, have reported increased amounts of ubiquitinated E47 protein in splenocytes stimulated with anti-IgM, which activate MAPKs and Notch-signaling pathways in B cells (62). The discrepancy between these results and ours can be attributed to the different purity of the cell population examined (B220-enriched splenic B cells here vs whole splenocytes containing both B and T cells, which differentially control their MAPKs), different culture conditions, and kinetic time points.

The specificity of the mRNA degradation process is still unknown. Belonging to a certain class of AREs does not automatically predict the stability of the mRNA. It was expected that having more ARE sequences, regardless of the class, would have created less stability in the mRNA. However, our results herein indicate that several mRNAs with multiple ARE sequences in the 3′-UTR were stable in our experiments. For example, Bob-1 mRNA, which we would have predicted to be unstable due to its 3′-UTR structure containing three ARE motifs, is a stable mRNA. The major point of this article is that E2A mRNA is less stable in aged stimulated B cells. The c-jun mRNA, a class III ARE such as E2A, is not preferentially degraded in aged stimulated splenic B cells, although it is more unstable than other transcripts in both young and old cells. Blimp-1, a class I ARE mRNA, also considered to be unstable due to its 3′-UTR structure containing 6 ARE motifs, is not degraded faster in old as compared with young B cells. Therefore, although the precise mechanisms are not yet known, E2A mRNA appears to be selectively degraded in aged activated B cells.

The c-fos mRNA, containing two single plus two overlapping AREs, is highly unstable in both young and old B cells. The differences in the stability of c-jun, c-fos, and E2A mRNAs in young B cells probably reflect the different sensitivity of different class III ARE mRNAs to the inhibitor of transcription Act D. In fact, it has been shown that Act D is able to efficiently block the transcription of both class I and class II AREs (i.e., c-fos), but it seems to have less effects on class III AREs mRNA (i.e., E2A and c-jun) (47). This indicates that we probably are seeing an underrepresentation of the amount of E2A mRNA degraded in splenic activated B cells.

mRNA turnover mediated by all classes of AREs is characterized by rapid shortening of the poly(A) tail followed by rapid decay of the mRNA. In addition, the in vivo degradation processes also involve different ARE-binding proteins. A number of such proteins have already been identified (28, 63), which can simplistically be classified into two categories. The first in which proteins bind to the AREs and result in a rapid degradation of the transcript prevent message accumulation and keep the resulting mRNA-encoding protein levels low. Secondly, proteins, which bind to the ARE-containing transcripts, result in stabilization of the transcript, effectively allowing for a rapid increase in the mRNA-encoding protein levels.

ARE-containing mRNAs can be stabilized in response to external stimuli, which activate different signal transduction pathways. MAPK family members are essential for the signal transduction of a variety of cellular functions in response to several stimuli, including CD40 (64–67). Three major MAPK subfamilies have been identified and extensively characterized: ERKs, JNKs, and the p38 MAPK. Activation of p38 MAPK has been postulated to stabilize mRNAs not only through the activation of proteins, which interact with AREs and induce mRNA stabilization (29, 55, 56), but also through the inhibition of deadenylation (57). A well-characterized substrate for p38 MAPK, the MAPKAPK-2, has indeed been demonstrated to stabilize the mRNAs for TNF-α, COX-2, IL-6, IL-8, GM-CSF, and c-Fos (24, 29–31). The contribution of MAPKAPK-2 to mRNA stabilization is demonstrated by the observation that a constitutively active mutant of MAPKAPK-2 induced the stabilization of the mRNA for IL-6 and IL-8, whereas a kinase-dead mutant of it interferes with their stabilization (31). Moreover, some recent work has shown that tristetraprolin, a RNA-binding protein that promotes decay of ARE-containing mRNA, can be directly phosphorylated by either p38 or MAPKAPK-2, losing its ARE-binding activity (68, 69). Alternatively, p38 may also phosphorylate ARE-stabilizing proteins that could compete with destabilizing proteins, as suggested for the regulation of IL-3 mRNA (70). When we investigated the signal transduction pathways controlling E2A mRNA expression and stability, we found that the p38 MAPK regulates E2A mRNA expression by increasing its mRNA stability.

It has been shown recently that phosphorylation of p38 and of MAPKAPK-2 is reduced in LPS-stimulated macrophages from old mice (71), but so far nothing was known on the effects of aging on p38 phosphorylation in splenic B cells. Our results show that total levels of p38 are comparable between young and old B cells, whereas phosphorylation of p38 and of MAPKAPK-2 is reduced in B cells from old as compared with young mice. These results suggest that, at least for B cells, the decreased levels of p38 MAPK activation are not a function of the reduced total p38 with age. The age-related reduction in the phosphorylation of p38 and of MAPKAPK-2 could help explain why E2A mRNA expression is always reduced more in old than in young B cells by pretreatment with the p38 MAPK inhibitor. TRAF2 has been reported to be involved in CD40-mediated activation of the Cε and perhaps Cγ1 promoters (72, 73), as well as AID, and has also been reported to be involved in p38 activation by CD40 (66). The levels of TRAF2 were found here to be comparable in splenic B cells from young and old mice. In contrast, TRAF-2 mRNA and protein levels have been reported to be reduced in whole cell lysates of activated T lymphocytes from aged subjects (74). We are now investigating what regulates the activation of MAPK in aging B lymphocytes in addition to TRAF2.

Although the effects of p38 MAPK on the stability of mRNAs have already been shown to be mediated by MAPKAPK-2 (29, 56, 57), the relevant substrates of MAPKAPK-2 remain to be conclusively characterized. It is likely that MAPKAPK-2 phosphorylates proteins that directly bind to the mRNA modulating its stability and translation. We don’t know yet which protein/proteins is/are responsible for the stabilization of E2A mRNA. We are currently performing RNA EMSA experiments to demonstrate that the 3′-UTR of the E2A mRNA interacts with a number of proteins, which are supposed to be involved in RNA stabilization processes. Characterization of these complexes is needed to better define the mechanisms of E2A mRNA destabilization occurring in old B cells.

In conclusion, the results herein show that aging decreases E2A mRNA levels in stimulated splenic B cells due to decreased mRNA stability. To understand the mechanisms of E2A mRNA degradation, we initially checked the structure of its 3′-UTR and found that it is a class III ARE mRNA. We also looked at other mRNAs to see whether RNA degradation was correlated to the number of ARE sequences present on the 3′-UTR of a given mRNA. Our results clearly indicate that the presence of “degradation” sequences in the 3′-UTR of a given mRNA is a necessary but not sufficient condition for its degradation. Additional experiments are needed to better understand ARE-dependent, posttranscriptional mechanisms involved in mRNA stabilization, such as characterize the specificity of the interactions of mRNA/proteins, which regulate mRNA stability, and define the organization of...
proteins required for mRNA decay and their coupling to other cellular processes. Recently, miRNAs have been described and considered very important in controlling the expression of key regulatory genes by binding to mRNA (75, 76). miRNAs are short noncoding single-strand RNA species found in a large variety of organisms. They cleave miRNAs or prevent their translation into protein. To fully characterize the fine specificity of the degradation of E2A mRNA, we are also considering miRNA-mRNA interactions, as well as the interaction of miRNA complexes with proteins.

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Disclosures

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