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Differential Regulation of Translation and eIF4E Phosphorylation During Human Thymocyte Maturation

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Activation of peripheral blood T cells by cross-linking of CD3 results in a rapid and substantial rise in translation rates and proliferation, which coincides with an increase in the cap-binding protein, eIF4E activity. In contrast, immature $CD4^+CD8^+$ double-positive (DP) thymocytes undergo apoptosis in response to anti-CD3 mAb. We have investigated translation initiation in the response of immature thymocytes to activating signals. Activation by anti-CD3 + anti-CD4 of immature $CD4^+CD8^+$ DP thymocytes results in a rapid decrease in protein synthesis. In contrast, similar treatment of $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes results in an increase in protein synthesis. The rate of protein synthesis is linked to the phosphorylation status of eIF4E. Following anti-CD3 + anti-CD4 stimulation, eIF4E phosphorylation strongly decreases in immature DP thymocytes, whereas it increases in mature SP thymocytes. The expression of 4E-BP2, a specific repressor of eIF4E function, is high in DP cells but decreases during maturation, raising the possibility of a role for 4E-BP2 in repressing eIF4E phosphorylation. These data provide evidence for differential regulation of the translational machinery during T cell development. ***The Journal of Immunology*****, 1998, 160: 3269–3273.**

Differentiation of T cells in the thymus proceeds through an ordered sequence of events characterized in part by expression of CD4 and CD8, both of which function as cell interaction molecules for developing thymocytes and mature T cells (1, 2). The most immature population of T cells is $CD3^-CD4^-CD8^-$. As maturation ensues, cells coexpress both the CD4 and CD8 molecules ($CD4^+CD8^+$, double-positive (DP)[2] cells), and after productive TCR gene recombination, they also display the TCR/CD3 complex on their surface. Their continued maturation is dependent on a process of "positive selection," which ensures that the cell response to foreign Ag will be restricted by self-MHC molecules. Positively selected cells lose either CD4 or CD8 to become mature single-positive (SP) thymocytes ($CD3^+CD4^+$ or $CD3^+CD8^+$) (3–6). Immature thymocytes can be eliminated by an active process of apoptosis initiated through TCR stimulation, contrasting starkly with the proliferative response seen after TCR engagement of mature peripheral blood T cells or mature medullary thymocytes. The basis for differential response of immature thymocytes and mature T cells to TCR engagement is not known.

Translation rates generally increase in response to treatment with growth factors, cytokines, hormones, and mitogens (reviewed in Refs. 7 and 8). Most of the control of translation occurs at the level of initiation. Translation initiation entails the positioning of the ribosome at the AUG initiation codon. Cellular mRNAs contain a cap structure (m7G(5′)ppp(5′)*N*; where *N* is any nucleotide) at their 5′ termini (9). The multisubunit translation initiation factor eIF4F binds to the cap structure via the eIF4E subunit to promote ribosome binding (7). eIF4E, a 24-kDa polypeptide (10), is the limiting factor of this step. The phosphorylation state of eIF4E positively correlates with cell growth (7). Phosphorylation appears to enhance eIF4E activity because only the phosphorylated form of eIF4E is present in the 48S mRNA ribosome complex, and phosphorylation of eIF4E by PKC increases the ribosome-binding activity to mRNA (11, 12). Two repressors of cap-mediated translation, termed 4E-BP1 and 4E-BP2 (eIF4E-binding protein 1 and 2), or PHAS-I, have been characterized (13, 14). 4E-BP1 and 4E-BP2 are heat- and acid-stable proteins in which activity is regulated by phosphorylation (13–15). Dephosphorylated 4E-BP1 and 4E-BP2 interact with eIF4E, and these interactions result in the specific inhibition of cap-dependent translation, both in vitro and in vivo (14). Furthermore, phosphorylation of eIF4E can be regulated by the [repressor 4E-BPs] in vitro. In the presence of 4E-BP1 or 4E-BP2, phosphorylation of eIF4E by PKC is strongly reduced (16).

Activation of human peripheral blood T cells by cross-linking of TCR-CD3 results in a strong increase in translation rates and expression of initiation factors (17). An increase in eIF4E phosphorylation has been shown in response to mitogenic stimulation (18). Our goal was to investigate translation rates and eIF4E phosphorylation in the differential effects of TCR stimulation on immature and mature thymocytes.

Materials and Methods

Abs and reagents

Murine mAb to human CD3 (G19-4) and CD4 (G17-2) were generous gifts of D. Fox (American Type Culture Collection, Rockville, MD); mAb to CD28 (9.3) was a generous gift of J. Ledbetter (Bristol-Myers Squibb, Seattle, WA); and mAb to CD1a (OKT6) was purchased from Coulter Immunology (Hialeah, FL). Goat anti-mouse-coated magnetic beads were purchased from Advanced Magnetics (Cambridge, MA). PMA was purchased from Sigma (St. Louis, MO), and ionomycin from Calbiochem (San Diego, CA). All cell culture media and reagents were from Life Technologies (Grand Island, NY). Ab 11201 was raised in rabbit (Pocono Farm,

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[2] Abbreviations used in this paper: DP, double positive; SP, single positive; eIF, eukaryotic translation initiation factor; 2D, two-dimensional; pI, isoelectric point; PKC, protein kinase C.

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Canadensis, PA) against a GST-4E-BP2 fusion protein, which cross-reacts with 4E-BP1, a protein that is 56% identical to 4E-BP2. Ab 5853 against eIF4E was raised in rabbit.

Preparation of mature T cells and thymocyte subpopulations

Peripheral blood was obtained from healthy volunteer donors and mononuclear cells were isolated using Ficoll-Paque (Pharmacia Biotech, Uppsala Sweden) density gradient centrifugation.

Thymic tissue was obtained from children under age 3 undergoing corrective cardiac surgery, and a single-cell suspension was prepared using nylon mesh. Mononuclear cells were isolated using Ficoll-Paque density gradient centrifugation. Preparations of thymocytes enriched for $CD4^+CD8^+$ cells were obtained by negative selection with a mAb to CD28, as CD28 is expressed at high surface density only on SP cells (19). Unfractionated cells were incubated with 2 mg/ml of anti-CD28 mAb for 1 h at 4°C. The cells were washed three times and incubated with goat anti-mouse Ig-coated magnetic beads for 30 min on a rocker. The beads were collected using a magnet, and the separation procedure was repeated on the unbound cell fraction. Thymocytes in this fraction were typically >92% $CD4^+CD8^+$, with 4% $CD3^-CD4^-CD8^-$, 2% $CD3^-CD4/8^+$, and 2% $CD3^+CD4/8^+$ cells (20). Mature $CD3^+$ SP thymocytes were obtained by negative selection with a mAb to CD1 (anti-CD1a), as CD1 is expressed on DP thymocytes but is absent from the surface of SP thymocytes (21), and were >85% $CD3^+CD4/8^+$. Virtually all the contaminants were $CD3^-CD4^-CD8^-$ thymocytes, with <4% $CD4^+CD8^+$ cells. Since immature $CD3^-CD4/8^+$ cells express the CD1a Ag, these were removed by the isolation procedure. The most immature thymocytes, which are $CD3^-CD4^-CD8^-$, express neither CD1 nor CD28. They are present in very small numbers (1–2%) in the human postnatal thymus and do not significantly contaminate the $CD1^-$ or $CD28^-$ preparations.

Cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, at a density of 10×10^6 cells/ml. When specified, PMA was used at 5 ng/ml, ionomycin at 250 ng/ml anti-CD4 at 5 mg/ml, and anti-CD3 at 5 mg/ml.

In vivo radioactive labeling

Cells were preincubated for 1 h in methionine-free medium. [^{35}S]Methionine (100 μCi) was added. Cells were lysed in buffer containing 0.5% Nonidet P-40, 140 mM NaCl, and 30 mM Tris-HCl, pH 7.5, and radioactivity incorporated into TCA-precipitable material was measured.

[^{32}P]O_4^{3-} labeling was performed by preincubating the cells for 4 h with 250 μCi of [^{32}P]O_4^{3-} in phosphate-free culture medium. Samples were prepared for electrophoresis as described previously (22).

Two-dimensional (2D) PAGE

The procedure followed was as previously described (22). Cell pellets were solubilized in lysis buffer containing 9.5 M urea, 2% Nonidet P-40, 20 ml of ampholytes (pH 3.5 to 10), 2% 2-ME, and 0.2 mM of PMSF in distilled deionized water. After lysis, 30-μl aliquots containing 3×10^6 cells were applied to isofocusing gels. Isoelectric focusing was conducted, using pH 4 to 8-carrier ampholytes at 1200 V, followed by 16 h and 1500 V for an additional 2 h. For the second-dimension separation, an acrylamide gradient of 11.4 to 14.0 g/dl was used. Proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA) and blotted with an anti-eIF4E antiserum, or gels were silver-stained. Phosphoprotein patterns were visualized and spots quantitated by phosphorimaging technology.

SDS-PAGE and Western blotting

Cells were lysed by successive freeze-thaw cycles in 20 mM Tris-HCl, pH 7.5, buffer containing 5 mM EDTA and 100 mM KCl. The homogenate was centrifuged at $6000 \times g$ for 10 min, and the supernatant was collected. To analyze for 4E-BP2, 50 μg of proteins were dissolved in Laemmli sample buffer (23), and the samples were loaded onto an SDS-15% polyacrylamide gel. Proteins were transferred onto a 0.22-mm nitrocellulose membrane, which was blocked in 5% milk for 2 h followed by incubation for 2 h with rabbit polyclonal antiserum against 4E-BP2 (1:1000) in 10 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl. Incubation with [^{125}I]protein A (Amersham) was performed (1:1000), and the signal was quantified using phosphorimager analysis.

Results

Protein synthesis rates in unstimulated and stimulated immature DP and mature SP thymocytes

Unseparated thymocytes from children 3 yr old or less are typically 5 to 10% $CD4^-CD8^-$, 65 to 80% $CD4^+CD8^+$, 10 to 15% $CD4^+CD8^-$, and 10 to 15% $CD4^-CD8^+$. We fractionated human thymocytes into two major subtypes consisting of immature DP thymocytes and mature SP thymocytes. To study translation rates in immature DP $CD4^+CD8^+$ thymocytes, we depleted the thymocyte population of SP cells using anti-CD28 mAb. We also prepared $CD1^-$ thymocytes corresponding to mature SP $CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes.

In peripheral blood T cells, cross-linking of TCR-CD3 results in a strong increase in translation rates (17) followed by cell proliferation. In contrast, a similar treatment does not induce cell proliferation in immature DP thymocytes. We stimulated the thymocytes by adding anti-CD3 plus anti-CD4. Cross-linking CD4 with CD3 strongly enhances signal transduction via CD3 (20). The translation rate was determined by metabolic labeling of cells with [^{35}S]methionine, and incorporation rates were measured 3 h after stimulation. The relative

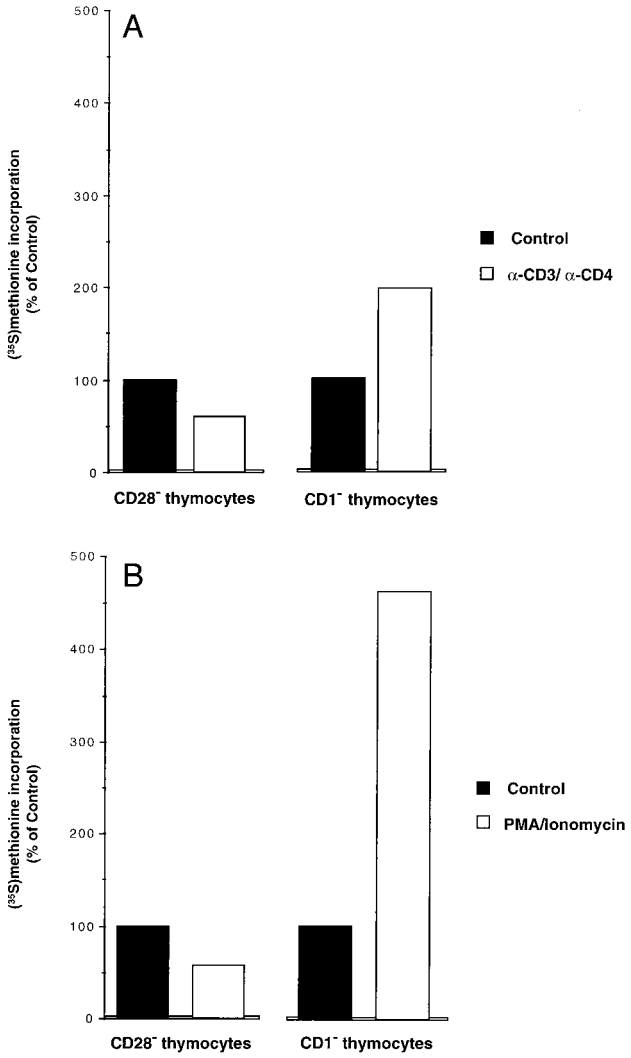

FIGURE 1. Protein synthesis in stimulated DP $CD28^-$ and SP $CD1^-$ thymocytes. $CD28^-$ thymocytes and $CD1^-$ thymocytes were preincubated for 1 h in methionine-free medium. *A*, anti-CD3 mAb (5 mg/ml) and anti-CD4 mAb (5 mg/ml) were added with [^{35}S]methionine (100 μCi). Cells were harvested after 3 h, and radioactivity incorporated into TCA-precipitable material was measured. *B*, The same experiment was performed with cells treated with PMA (5 ng/ml) and ionomycin (250 ng/ml). The relative basal rates of protein synthesis in mature SP $CD1^-$ thymocytes compared with immature DP $CD28^-$ thymocytes are similar. Incorporation of [^{35}S]methionine is expressed as percentage of the control. The experiments were conducted two times, and similar results were obtained.

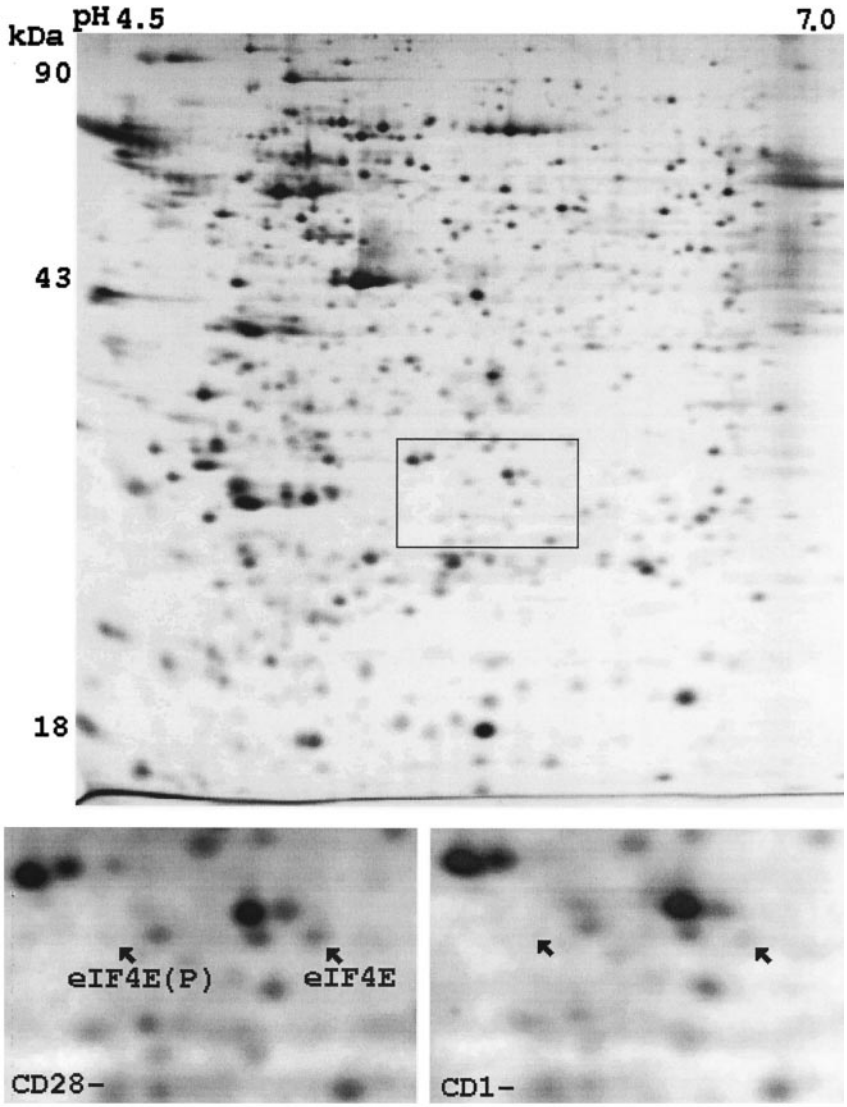

FIGURE 2. 2D-PAGE analysis of eIF4E isoforms from resting CD28$^-$ and CD1$^-$ thymocytes. Resting CD28$^-$ thymocytes and CD1$^-$ thymocytes were solubilized by a standard lysis buffer as described in *Materials and Methods*. Proteins were resolved by 2D-PAGE using pH 4 to 8-carrier ampholytes for the first dimension and an 11 to 14% polyacrylamide gradient for the second dimension. A silver-stained gel obtained from CD28$^-$ cell lysates is shown. The positions of eIF4E isoforms were identified by immunoblotting analysis using a polyclonal Ab to eIF4E; the blown sections displaying the eIF4E isoforms are shown. Arrows marked eIF4E and eIF4E(P) indicate the positions of the nonphosphorylated and phosphorylated forms of eIF4E, respectively. The corresponding region obtained from a silver-stain 2D-gel from CD1$^-$ cell lysates is shown on the *bottom right panel*.

basal rates of protein synthesis in mature SP CD1$^-$ thymocytes compared with immature DP CD28$^-$ thymocytes are similar. However, treatment of mature SP CD1$^-$ thymocytes with anti-CD3 + anti-CD4 resulted in an increase in protein synthesis (twofold); in immature DP CD28$^-$ thymocytes, the same treatment resulted in an opposite effect, a twofold reduction in protein synthesis (Fig. 1*A*). Therefore, our results on the protein synthesis rates observed in anti-CD3 + anti-CD4-treated DP and SP thymocytes are consistent with the well-described opposite proliferation status of DP and SP thymocytes following anti-CD3 treatment.

As PMA/ionomycin treatment of peripheral blood T cells was shown to mimic the effects of α-CD3 on translation and cell proliferation, we examined the effects of PMA/ionomycin treatment of thymocytes on protein synthesis rates. PMA/ionomycin treatment for 3 h caused a decrease in [^{35}S]methionine incorporation in DP CD28$^-$ thymocytes (twofold), whereas it increased [^{35}S]methionine incorporation in SP CD1$^-$ thymocytes by fivefold (Fig. 1*B*).

Phosphorylation state of eIF4E

We analyzed the phosphorylation state of eIF4E in CD28$^-$ DP and CD1$^-$ SP thymocytes treated with anti-CD3 + anti-CD4. Thymocytes were incubated with [^{32}P]orthophosphate, and at various times of treatment, cells were lysed in urea buffer and proteins were analyzed on 2D-PAGE. eIF4E is resolved by 2D-PAGE into two isoforms, a nonphosphorylated (pI = 6.3) and a phosphorylated form (pI = 5.9). The eIF4E isoforms on the 2D gels were identified by immunoblotting using an Ab against eIF4E. Figure 2 shows the position of these two isoelectric forms on silver-stained gel obtained from CD28$^-$ thymocytes and CD1$^-$ thymocytes. Phosphorylation of eIF4E was quantified by measuring the incorporation of [^{32}P]orthophosphate into the phosphorylated form of eIF4E (Fig. 3). In immature DP cells (CD28$^-$ thymocytes), treatment of the cells with anti-CD3 + anti-CD4 resulted in a rapid decrease of [^{32}P]incorporation into eIF4E, apparent as early as 5 min following the addition of anti-CD3 + anti-CD4. The decrease in eIF4E phosphorylation reached the maximum (7-fold) after 30 min. In mature SP cells (CD1$^-$ thymocytes), an opposite effect was observed, as addition of anti-CD3 + anti-CD4 increased [^{32}P]incorporation into eIF4E by ~2.5-fold (Fig. 3*A*).

Following PMA/ionomycin treatment of DP CD28$^-$ thymocytes, a decrease in [^{32}P]incorporation into eIF4E was observed but was less pronounced as compared with anti-CD3 + anti-CD4 treatment of the CD28$^-$ thymocytes (threefold decrease after 30 min; Fig. 3*B*). The phosphorylation state of eIF4E correlates well with the translation rates observed following anti-CD3 + anti-CD4 or PMA + ionomycin treatment of DP CD28$^-$ thymocytes and SP CD1$^-$ thymocytes.

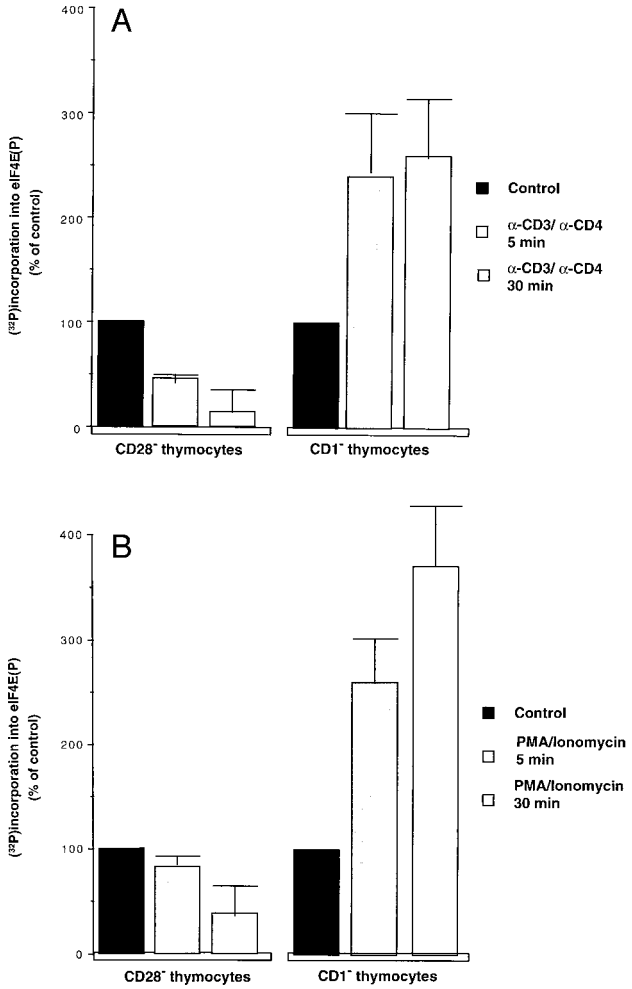

FIGURE 3. [^{32}P]incorporation into eIF4E(P) in stimulated DP CD28$^-$ and SP CD1$^-$ thymocytes. Cell extracts were prepared from CD28$^-$ thymocytes and CD1$^-$ thymocytes, following α-CD3 + α-CD4 or PMA + ionomycin treatment, and labeled with [^{32}P]orthophosphate. The experiments were conducted four times.

To determine whether the variation of [^{32}P]incorporation into eIF4E was due to the increased rate of the phosphate turnover or to an increase of phosphorylation (or dephosphorylation), we analyzed the amount of eIF4E(P) in the same samples by 2D-PAGE followed by immunoblotting using an Ab against eIF4E (Fig. 4). The amount of the phosphorylated form eIF4E(P) detected by the Ab against eIF4E could account for the variation in [^{32}P]incorporation into eIF4E(P). Thus, the variation of phosphorylation of eIF4E during treatment is not due to variation in the rate of phosphate turnover.

Expression of 4E-BP1 and 4E-BP2 proteins in thymocytes

eIF4E function and phosphorylation are regulated by the 4E-BPs translational repressors. In the presence of 4E-BP1 or 4E-BP2, phosphorylation of eIF4E by PKC is strongly diminished in vitro (16). We thus examined the expression of 4E-BP1 and 4E-BP2 in thymocytes. In whole human thymus, 4E-BP2 mRNA is more abundant than 4E-BP1 mRNA (24). We also observed a higher expression of 4E-BP2 protein than 4E-BP1 protein (data not shown); in purified thymocytes, 4E-BP1 protein was hardly detectable, whereas 4E-BP2 protein was abundant (Fig. 5*A*). Furthermore, 4E-BP2 protein is differentially regulated in immature DP thymocytes, in mature SP thymocytes, and in peripheral blood T cells. The expression decreased as thymocytes matured: the expression of 4E-BP2 protein is high in CD28$^-$ thymocytes, decreases in CD1$^-$ thymocytes, and is hardly detectable in PBL (Fig. 5*B*). No variation in 4E-BP2 expression was observed following anti-CD3 + anti-CD4 or PMA + ionomycin treatment of the cells, as compared with unstimulated mature and immature thymocytes (data not shown). The high level of expression of 4E-BP2 protein may explain the lack of phosphorylation of eIF4E during stimulation of immature DP cells.

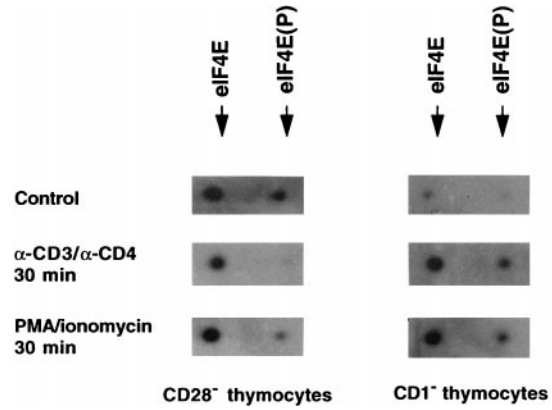

FIGURE 4. Steady state levels of eIF4E isoforms analyzed by 2D-PAGE. Cells (1×10^6 each) were solubilized with a standard lysis buffer, and proteins were separated by 2D-PAGE. Only the blown sections depicting the regions of eIF4E isoforms are shown. Unphosphorylated and phosphorylated eIF4E isoforms are indicated by arrows as eIF4E and eIF4E(P), respectively.

Discussion

The relationship between intrathymic proliferation and the processes of positive and negative selection of human thymocytes is not well

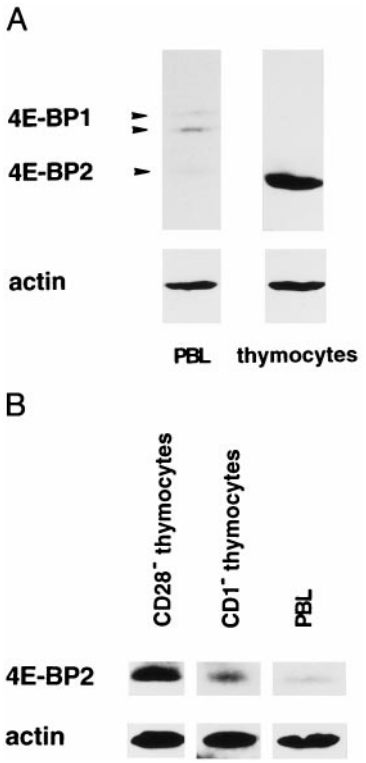

FIGURE 5. *A*, Western blot analysis of 4E-BP1 and 4E-BP2 protein expression in thymocytes. *B*, Western blot analysis of 4E-BP2 protein expression during thymocyte maturation.

understood. Immature cortical thymocytes undergo a process of selection to promote the development of cells bearing MHC-restricted TCR and to eliminate potentially autoreactive cells (25). As a result of these selective processes, >95% of all cortical thymocytes die because of either "benign neglect" or activation-induced cell death. Both phenomena, cell death in the absence of external stimuli and cell death caused by CD3 stimulation (26), contrast with the proliferative response seen after CD3 stimulation of mature peripheral blood T cells or mature medullary thymocytes. The translation rate is an important determinant of cell proliferation. Our results show rapid but contrasting effects of stimulation of immature DP vs SP human thymocytes on translation. These effects correlate with the behavior of these cells in proliferation assays. Regulation of translation is mostly at the initiation step. The initiation factor eIF4E is the cap-binding protein. eIF4E activity is regulated at different levels: 1) phosphorylation: the phosphorylated form of eIF4E binds to the cap structure; 2) expression: it is the limiting factor in the translation process and its overexpression results in an increase of cell proliferation; 3) interaction: the translational repressors 4E-BP1 and 4E-BP2 inhibit the association of eIF4E with eIF4G. Our results demonstrate a drastic change in eIF4E activity following activation of thymocytes.

The physiologic kinase of eIF4E has not been determined. PKC phosphorylates eIF4E in vitro at the physiologically relevant site, Ser^{209} (16). Recently, however, it has been shown that Mnk1, a member of a new family of serine/threonine kinases, the MAP kinase-interacting kinases, also phosphorylates eIF4E in vitro at Ser^{209} (27). Both kinase PKC and Mnk1 are candidate kinases for the responsibility for phosphorylation of eIF4E in vivo in human thymocytes, because phorbol esters, which activate PKC and Mnk1 (27, 28), induce eIF4E phosphorylation in T cells (18, 29, 30). We describe here a similar effect on eIF4E phosphorylation during activation of mature SP thymocytes. However, during activation of immature DP thymocytes, we observed an opposite effect on eIF4E phosphorylation. B lymphoid cells that were activated with LPS or phorbol esters showed an increased rate of phosphate turnover, as the increase in the amount of the phosphorylated form (2- to 3-fold) could not fully account for the enhanced labeling (50-fold) (31). This is not the case here. The interaction of eIF4E with the repressor 4E-BP2 may be sufficient to abrogate the induction of eIF4E phosphorylation. In this respect, the understanding of Mnk1 activity during human thymocyte maturation will be of great interest.

A TCR- or PMA-induced posttranscriptional mechanism that regulates early thymocyte development, specific for selective messenger RNAs, requiring protein synthesis, and itself developmentally regulated, was reported (32). TCR signals did not block the differentiation of early thymocytes by inhibiting transcription but rather, by eliminating mRNA's encoding two distinct families of molecules involved in the differentiation of early thymocytes into $CD4^+CD8^+$ cells: 1) the coreceptor molecules CD4 and CD8 that mediate critical cellular interactions in the thymus and 2) the recombination-activating genes (*RAG*)-1 and -2 that are required for rearrangement of TCR gene loci.

It is likely that the differences are accounted for, in part, by the regulation of the translational machinery via components such as eIF4E.

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