Detection of early gene expression changes during activation of human primary lymphocytes by in vitro synthesis of proteins from polysome-associated mRNAs

SUZANNE MIYAMOTO,^{1,4} JUN QIN,^{2,3} AND BRIAN SAFER¹

¹Molecular Hematology Branch, Section on Protein and RNA Biosynthesis, NHLBI, Bethesda, Maryland 20892, USA

²Laboratory of Biophysical Chemistry, NHLBI/National Institutes of Health, Bethesda, Maryland 20892, USA

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Abstract

The rapid increase in protein synthesis during the mitogenic stimulation of human peripheral blood lymphocyte is the result of global and specific translational control mechanisms. To study some of these mechanisms, we examined the in vitro translatability of mRNAs associated with the polyribosome fraction. Polyribosome fractions were isolated from lymphocytes after activation with ionomycin and the phorbol ester PMA. The associated PAmRNAs were translated in the presence of mRNA-depleted rabbit reticulocyte lysate and [³⁵S]Met, and the protein products were analyzed by SDS-PAGE and autoradiography. There was little synthesis of protein from the PAmRNAs isolated from unactivated T cells, but the PAmRNAs isolated from activated T cells showed a rapid increase in translatability. Translation of the PAmRNAs was sensitive to edeine and m7GTP, suggesting their cap-dependent translation. With activation, the majority of proteins showed increasing in vitro translation, but two proteins, p72 and p33, were found to have increased synthesis within 30 min, which decreased in 1 h. Transcription inhibitors were used to ascertain if regulation of their expression was transcriptional or translational. To identify these proteins, we used biotinylated lysine during the in vitro translation reaction, and we extracted the biotinylated protein by using streptavidin magnetic beads. The protein product was analyzed by mass spectrometry. p33 was identified as a prohibitin-like protein (BAP37), but the identification of p72 was not found in the databases. The distinct up-regulation and down-regulation of their protein expression suggest their tightly controlled regulation during early T cell activation.

Keywords: Translation; gene expression; T cells; proteins; mRNA; protein synthesis; lymphocytes

Reprints requests to: Brian Safer, Molecular Hematology Branch, NHLBI, Bethesda, Maryland 20892, USA.

³Present address: Verna and Mars McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, USA.

⁴Present address: Suzanne Miyamoto, Department of Biological Chemistry, University of Davis School of Medicine, Tupper Hall, Davis, California 95616, USA; e-mail: smiyamot@ucdavis.edu; fax: (530) 752-3516.

Abbreviations: ActD, actinomycin D; CHX, cycloheximide; CID, collision-induced ionization; DRB, dichloro 1-β ribofuranoside benzimidate; EST, expressed sequence tag; FBS, fetal bovine serum; I + P, ionomycin and PMA; LC/ESI/MS/MS, liquid chromatography/electrospray ionization/mass spectrometry/mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; nRRL, nuclease-treated rabbit reticulocyte lysates; PAmRNAs, polysome-associated mRNAs; PMA, phorbol myristate acetate; RNP, ribonucleoprotein; [³⁵S]Met, [³⁵S]methionine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T cells, peripheral blood T lymphocytes.

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Translation of mRNAs into proteins is an important regulated process of the mitogenic activation of human peripheral blood lymphocytes (T cells) (Varesio et al. 1980). T cells are normally quiescent, with low rates of RNA, protein, or DNA synthesis. However, when they are activated with I + P, there is an immediate increase in protein synthesis, which is important for proliferation (Varesio and Holden 1980). This increase in translation has been suggested to be largely the result of global and specific translational control mechanisms that involve increased translation initiation and increased recruitment of mRNAs to the ribosomes. Individual mRNAs themselves are regulated by transcriptional, posttranscriptional, and translational mechanisms in addition to global regulation. The important and final outcome of gene expression is translation of the mRNA into protein.

The ribosome/polysome fraction from briefly stimulated T cells can provide valuable information about the early changes in global and specific protein synthesis. This fraction should contain mRNAs that are being actively translated. Very little is known about the physiological mechanisms of mRNA recruitment, initiation, and translation in the T cell. mRNA expression of genes changes rapidly with activation of T cells. Early mRNA expression of immediate early genes is followed by expression of intermediate and then late genes (Ullman et al. 1990). In this study, we present data demonstrating biochemical changes associated with the ribosome/polysome fractions of the early-activated T cells. We describe the detection of protein changes during the activation of T cells by *in vitro* synthesis of PAmRNAs that were contained in the ribosome/polysome fractions. These PAmRNAs with their associated factors were translated *in vitro* without purification of RNA or poly(A) RNA. *In vitro* translation of proteins in the presence of biotinylated lysine/tRNA from PAmRNAs was also performed, which allowed the quick isolation of the synthesized proteins via magnetic streptavidin bead separation. This process was scaled up to yield enough protein for mass spectrometry analysis. Identification of protein products by tandem mass spectrometry made it possible to identify some of the genes that appeared to have tightly regulated gene expression during T cell activation.

Results

In vitro translation of PAmRNAs increases with activation of T cells

Protein synthesis in human peripheral blood lymphocytes (T cells) can be stimulated by the combination of a calcium ionophore ionomycin and the phorbol ester PMA (Miyamoto and Safer 1999). One approach to determining the mechanism of translational control is to isolate the ribosome/polysome fraction from T cells during activation and examine the translatability of the associated mRNAs with the assistance of mRNA depleted by nuclease-treated rabbit reticulocyte lysates (nRRL). The addition of nRRL provides active translation components (Pelham and Jackson 1976) that might be lacking in the T cell fractions. Polyribosome fractions were isolated from T cells activated with I + P for 0, 0.5, 1, 2, 4, and 8 h. The PAmRNAs contained in these fractions were translated *in vitro* using nRRL, amino acids, and [³⁵S]Met (see Materials and Methods; Fig. 1). Results showed little synthesis of proteins from unactivated T cell PAmRNAs (0 h) (Fig. 1, lane 1) even in the presence of nRRL. However, with 0.5 h of activation (Fig. 1, lane 2), there was a large increase in the *in vitro*-synthesized protein products. Protein products showed increasing synthesis over the 8-h time period measured. Overall, protein synthesis increased by 8- to 10-fold after 8 h of activation, but the

Fig. 1. *In vitro* synthesis of proteins from PAmRNAs show protein synthesis changes with activation of human T cells. T cells were activated with I + P for 0, 0.5, 1, 2, 4, or 8 h. Ribosome pellets were isolated (see Materials and Methods), and the mRNAs associated with this fraction were translated in the presence of nRRL and [³⁵S]Met for 1 h at 30°C. Aliquots were analyzed using SDS-PAGE and autoradiography. Two proteins, p72 and p33, are indicated by arrows (see text). This experiment is representative of three or four similar experiments.

amount of synthesized protein products produced varied on an individual protein basis. Autoradiographs of the SDS-polyacrylamide gels were scanned, and protein levels were quantitated (data not shown). The majority of proteins showed continually increasing synthesis after 0.5 h. However, several proteins, one ~33 kD (p33) and another ~72 kD (p72), showed synthesis that rapidly increased and then decreased within the first hour of activation, which suggested a tightly regulated gene expression mechanism.

Translation of PAmRNAs showed early protein synthesis changes that were not detected through analysis of in vivo-synthesized proteins

Previous studies that examined protein synthesis during the early period of T cell activation by monitoring the incorporation of [³⁵S]Met into intact cells showed little change in proteins being synthesized after 1 h of activation with I + P (Miyamoto and Safer 1999). In contrast, *in vitro* translation of PAmRNAs obtained from T cells after 0.5 h of activation with I + P showed significant changes in proteins synthesized (Fig. 1). A direct comparison between these two meth-

ods was performed to show this difference using a shorter time course (60 min). In this experiment, one set of samples was activated in the presence of [35 S] Met and lysed directly in SDS loading buffer (in vivo). A second set of cells was also activated for the same time periods but without [35 S]Met. Lysates were prepared from these cells and processed into ribosome/polysome pellets and postribosome supernatant (PRS) fractions (see Materials and Methods). Aliquots from resuspended polysome and PRS fractions were incubated with nRRL and [35 S]Met, and analyzed using SDS-PAGE (Fig. 2). Unactivated T cells (0 h, no I + P added) and T cells with I + P added at harvest were included to monitor the extent of activation for the time it took to process the samples. More radiolabeled protein was detected in the in vitro-synthesized samples (Fig. 2, lanes 1–6) than in the in vivo-labeled samples (Fig. 2, lanes 7–12), showing greater synthesis for the in vitro-translated samples. Detectable changes in the synthesis of proteins were observed as early as 10 min (Fig. 2, lane 3). There was a slight increase in the 0-h sample with I + P added (Fig. 2, lane 2), indicating that during the time period of processing

mRNA was already becoming more translatable. This increase could be the result of increased mRNA levels from increased transcription, stabilization of mRNA, or increased translation of existing mRNA. Interestingly, the two proteins identified as p33 and p72 in Figure 1 showed increased synthesis occurring within 10 min of activation, which peaked at 20–30 min and decreased within 1 h (Fig. 2, lanes 3–5). In comparison, synthesis of p33 and p72 was not observed in the in vivo-radiolabeled T cells (Fig. 2, lanes 9–12). Only a prominent p42 protein, which was identified as actin in a previous publication (Miyamoto and Safer 1999), was observed being synthesized in the 60 min of activation (Fig. 2, lanes 7–12). Actin (p42) was also the prominent protein observed in Figure 1, whose synthesis increased within 1 h and then decreased after 4 h of activation.

The corresponding cytoplasmic PRS fractions (remaining after centrifugation of the ribosomal/polysome pellets) were also assayed for translatable mRNAs. These fractions would contain free mRNPs, which are not associated with the ribosomes. Results from in vitro translation of these fractions with nRRL showed a few proteins being synthesized from this fraction. There was no change in the translation of these fractions with activation of T cells for 60 min. These results suggest that translatable mRNAs in T cells are mostly associated with the ribosome fractions and not stored as free mRNPs in the PRS fraction. However, untranslated mRNPs might be part of a large complex that co-sediments with the ribosome fraction.

Fig. 2. Comparison between in vitro and in vivo synthesis of proteins shows better detection of early protein synthesis changes with in vitro translation of PAmRNAs. Corresponding sets of T cells were activated for 60 min. Polysome fractions were isolated from one set, and PAmRNAs were translated for 1 h with nRRL and [35 S]Met (lanes 1–6, left). The second set was incubated in the presence of [35 S]Met (lanes 7–12, middle). Samples from the PRS fraction were also translated in vitro with nRRL and [35 S]Met (lanes 13–18, right). Each sample of the polysome pellet-translated and in vivo-translated groups contained the equivalent numbers of cells. Equivalent volume of PRS was used for each sample. Samples were analyzed on the same gel by SDS-PAGE and autoradiography. p72 and p33 are indicated by arrows.

Isolation of total RNA from ribosome/polysome pellets and translation in vitro demonstrates the presence of translatable but repressed mRNA

Because we did not know if the low amount of protein synthesis from PAmRNAs isolated from unactivated T cells was the result of a lack of mRNA or of mRNAs that were present but not translated, we isolated total RNA from the ribosome fractions of the T cells. These RNA samples, along with corresponding samples of PAmRNAs and RNA isolated from the low-speed-nuclei-containing pellet (Fig. 3A), were translated in vitro (see Materials and Methods). Results from a short time-course of activation (1 h) showed that proteins could be synthesized from mRNA contained in the total RNA isolated from T cells (Fig. 3A, lanes 7–12). Interestingly, there was a small amount of protein that could be synthesized from RNA extracted from polyribosome fraction of the unactivated T cells (Fig. 3A, lane 7), whereas there was little or no protein synthesized from the PAmRNAs contained in the unextracted fraction (Fig. 3A, lane 1).

These results suggest that mRNAs in unactivated T cells might be repressed and not translatable, or that nucleases present in the unactivated T cells are removed through the extraction of RNA. We also observed that in vitro transla-

Fig. 3. The results of in vitro translation of PAmRNA compared with those of in vitro translation of purified RNA. (A) Comparison among in vitro synthesis of PAmRNAs (*left*), total RNA isolated from the polysome pellet (*middle*), and total RNA isolated from the low-speed pellet (nuclei and microsomes) (*right*). Samples were translated in vitro with [35 S]Met and nRRL and analyzed by SDS-PAGE. p72 and p33 are indicated by arrows. (B) Ethidium bromide stain of formaldehyde agarose gel of RNA samples. 28S and 18S ribosome subunits are indicated.

tion of higher molecular weight proteins (e.g., p72; Fig. 3A, arrow) was sometimes difficult using purified RNA samples (Fig. 3A, lanes 10–12). In contrast, these higher molecular weight proteins could be synthesized from the PAmRNA samples (Fig. 3A, lanes 4–6). These results suggest that transacting factors that were contained in these preparations helped their in vitro synthesis. In contrast, p33 could be efficiently translated from total RNA isolated from ribosome pellets, even from the unactivated T cells (Fig. 3A, lane 7). p33 synthesis increased and decreased with similar kinetics from both the RNA and PAmRNAs. These results indicated that p33 had either increased mRNA levels or increased mRNA translatability, which was independent of transacting factors.

The third set of samples contained total RNA isolated from the low-speed pellet (14,000g) of the lysed T cells. This pellet contained nuclei, microsomes, and unlysed cells. In vitro translation of these samples (Fig. 3A, lanes 13–18) showed synthesis of proteins, which suggested the presence of translatable mRNAs even in the unactivated T cells (Fig. 3A, lane 13). However, with activation, synthesis of proteins from this fraction increased only slightly, thereby supporting the concept that mRNAs might already be synthesized but unavailable for translation. It was also observed that synthesis of proteins from these samples was sometimes difficult because of nuclease activity associated with this fraction, which often degraded the RNA sample.

RNA contained in these sample sets was analyzed on a denaturing formaldehyde gel stained with ethidium bromide (Fig. 3B). Results showed the similar loading of RNA for each set of samples. Diffuse staining of RNA from the ribosome pellet (lanes 1–6) was observed, suggesting the heterogeneity of these samples (still complexed with protein). Removal of proteins from these samples by preparation of total RNA resulted in mostly 28S and 18S ribosome subunits being stained (lanes 7–12).

Importance of initiation and cap-dependent translation to in vitro translation of PAmRNAs from T cells

Because in vitro translation of PAmRNAs showed a rapid increase in translation, which was not dependent on factors of the translation machinery (provided by nRRL), we wanted to know whether in vitro translation of these mRNAs was dependent on a cap-dependent mechanism. Dependency on initiation was assessed by the use of edeine, an in vitro inhibitor of translation initiation and by inhibition of in vitro translation by the cap analog, m⁷GTP. Edeine is a strong basic linear oligopeptide excreted by *Bacillus brevis*, which inhibits initiation of translation in reticulocyte lysate with no effect on translation elongation (Obrig et al. 1971). Cycloheximide was also used, which is an inhibitor of translation elongation (McKeehan and Hardisty 1969). Cap-dependent translation of the PAmRNAs was assessed with the use of m⁷GTP, a cap analog that inhibits binding of eIF-4E to the cap structure of mRNAs, thereby preventing initiation of cap-dependent mRNA translation (for review, see Jackson et al. 1995). A time course of T cells that were activated with I + P, was performed, and ribosome fractions were isolated. PAmRNAs were translated in vitro with nRRL in the absence or presence of either edeine or CHX (Fig. 4A) or m⁷GTP (Fig. 4B). The results showed that edeine strongly reduced the amount of in vitro translation in the earlier time points and only partially at 4 and 8 h. Inclusion of CHX during in vitro translation of the PAmRNAs completely inhibited translation at any time point. Treatment with m⁷GTP (Fig. 4B) also strongly reduced in vitro trans-

Fig. 4. PAmRNAs from early activation of T cells were translated in vitro in a cap-dependent manner. In vitro translation of samples (see Fig. 1) of PAmRNAs from T cells activated for 0, 0.5, 1, 2, 4, or 8 h in the absence (A and B, left) or presence (A, middle) of edeine, CHX (A, right), or m⁷GTP (B, right).

lation of PAmRNAs but was less effective at the later time points. Treatment with translation elongation inhibitor CHX, however, totally inhibited in vitro translation, even of those resistant to inhibition by edeine and m⁷GTP. Therefore, in vitro translation of PAmRNAs that were isolated from the early activation period of T cells was dependent on cap recognition. In contrast, translation of PAmRNAs isolated from the later time period (8 h) was not as effected by the presence of edeine or m⁷GTP, suggesting a potential cap-independent mechanism.

Treatment with transcriptional inhibitors shows that some PAmRNAs might be regulated through translation and others through transcription

It is not possible to distinguish those mRNAs that are regulated transcriptionally, posttranscriptionally, or translationally by translating PAmRNAs in vitro. To address this question, we treated T cells in culture using transcription inhibitors DRB or ActD, two different inhibitors of transcription, to assess the contribution of transcription to PAmRNA levels and subsequent translation. Two inhibitors were used because each inhibitor has a different mechanism of action. ActD inhibits transcription but has been reported to inhibit initiation of protein synthesis by interfering with the binding of mRNA to ribosomes (Kostura and Craig 1986; Singer and Penman 1972). DRB inhibits synthesis of mRNA by targeting polymerase II transcription (Yankulov et al. 1995).

In culture, T cells were stimulated with I + P (0.5 h) in the

absence or presence of increasing concentrations of DRB (Fig. 5A) or ActD (Fig. 5B) and processed into polysome fractions for in vitro translation. Both DRB and ActD reduced in vitro synthesis of some but not all of the proteins. Synthesis of p72 and p33 was reduced more by DRB than by ActD. These results suggest the presence of translationally regulated mRNAs because their synthesis was not affected by DRB or ActD treatment. Results with DRB, however, suggested that p72 and p33 expression is regulated by transcription; however, results with ActD did not show the same effect, and high concentrations of ActD were necessary to inhibit p72 and p33 in vitro protein synthesis. Therefore, the actual regulation of p72 and p33 gene expression requires identification of the protein and the appropriate reagents for these proteins so that their gene expression can be studied at the transcriptional, posttranscriptional, and translational levels. The results from these studies indicated that in spite of treatment of T cells in culture with transcription inhibitors DRB or ActD, in vitro translation still occurred for some mRNAs, which suggested translational regulation for these genes.

Using biotinylated lysine during in vitro synthesis of proteins with RRL allows the isolation of synthesized products

Identification of the proteins synthesized from in vitro-translated PAmRNAs is possible through the use of biotinylated lysine/tRNA_{lys} in the in vitro translation mixture

Fig. 5. Treatment of T cells in vivo with DRB or ActD did not reduce in vitro translation of some PAMRNAs. T cells in culture were pretreated with varying concentrations of DRB (A) or ActD (B) and then activated with I + P in the presence of inhibitor for 30 min. Ribosome/polysome pellets were isolated and the PAMRNAs were translated in vitro with nRRL and [35 S]Met. Protein products were analyzed by 12% SDS-PAGE. p72 and p33 are indicated by arrows.

followed by affinity purification with magnetic streptavidin beads. This process enables the separation of translated protein products away from the cellular and RRL proteins. T cells were activated with I + P, and polysome pellets were isolated. PAMRNAs were translated in the presence of biotinylated lysine/tRNA_{lys}, [35 S]Met, and nRRL. After synthesis, samples of the reaction mixture (Fig. 6A,B, left sides) and proteins bound to streptavidin magnetic beads (Fig. 6A,B, right sides) were analyzed by SDS-PAGE. Coomassie blue staining of the gels (Fig. 6A) shows the entire protein complement (hundreds of proteins) from the reaction mixture, which contained samples of the resuspended polysome fraction and rabbit reticulocyte lysate mixture (Fig. 6A, left). In comparison, proteins eluted from the streptavidin gel were not detectable by Coomassie blue staining (Fig. 6A, right). These proteins were only detectable by autoradiography (Fig. 6B). Radiolabeled in vitro-synthesized proteins were detectable in samples from the reaction mixture (Fig. 6B, left). Radiolabeled biotinylated proteins that bound to streptavidin magnetic beads, which were not observed by Coomassie blue staining, were also observed by autoradiography (Fig. 6B, right), demonstrating the small amount of protein that was translated from PAMRNAs. Therefore, it was necessary to increase the amount of protein product by at least 10- to 100-fold to obtain enough protein for analysis by mass spectrometry (see below). The use of silver stain would allow detection of low femtomole quantities of protein. With optimized capil-

lary LC/ESI/MS/MS, it should be possible to analyze this small quantity of protein and identify these proteins.

p33 was identified as a prohibitin-like protein by mass spectrometry, but p72 was not found in the databases

Biotinylated protein products from the large-scale in vitro translation of PAMRNAs obtained from activated (30 min) human T cells (see Materials and Methods) were bound to streptavidin magnetic beads, eluted with SDS loading buffer, and subjected to SDS-PAGE. The SDS-polyacrylamide gel was stained with Coomassie blue; protein bands corresponding to p74, p72, and p33 kD were extracted and in-the-gel digestions performed (Zhang et al. 1998). Location of the correct protein product was monitored by comparison with a companion reaction containing [35 S]Met that was run on the same SDS-polyacrylamide gel; autoradiography was performed to identify the correct protein. A corresponding reaction without the PAMRNAs was run as a control for the large number of nonspecific proteins, which bound to the streptavidin magnetic beads. After in-the-gel digestion, a sample of the digested peptides from three proteins, p74, p72, and p33, was analyzed by MALDI-TOF spectrometry (Fig. 7), and the remainder of the sample was analyzed by LC/ESI/MS/MS (Zhang et al. 1998). It was not possible to make any positive identifications from the MALDI-TOF data. Therefore, LC/ESI/MS/MS was conducted on these samples. The resultant CID spectra from the LC/ESI/MS/MS were analyzed for protein identification by

Fig. 6. Extraction of biotinylated in vitro-synthesized proteins with streptavidin magnetic beads enables the isolation of protein products of the in vitro translation reactions. T cells were activated, polysomes isolated, and PAmRNAs translated in the presence of tRNA/biotinylated lysine and nRRL. Newly synthesized biotinylated proteins were extracted using streptavidin magnetic beads and analyzed using SDS-PAGE. (A) Coomassie stained gel. (B) Autoradiography, with p72 and p33 indicated by arrows.

searching databases (nucleotide, protein, and ESTs). Because of the low amount of sample, it was possible to analyze only a limited number of CID spectra. At least eight peptide CIDs were examined for p74, eight peptide CIDs were examined for p72, and six peptide CIDs were examined for p33. Peptide matches were found for p33, which identified it as a prohibitin-like protein, but matches for the p74 and p72 proteins were not found in databases for proteins or ESTs. A representative MS/MS spectrum for p33, with sequence, is presented in Figure 8. p33 was thus identified as a prohibitin-like protein that was originally cloned as a human B cell receptor-associated protein by the Lamers laboratory (Terashima et al. 1994). Trypsin-digested fragments from p72 and p74 were also analyzed by MALDI-TOF and LC/MS/MS, but the data searches conducted did not yield a protein identification or EST sequence. This might be the result of incorrect sequence in the databases, which would not allow the correct identification of the protein or EST. Insufficient amount of sample might also have made identification of this protein difficult. Protease digestion and analysis of other proteins that were also in vitro translated from PAmRNAs is currently in progress.

Discussion

Although the rapid increase in protein synthesis after mitogenic stimulation of T cells can be measured, the mechanisms of regulation are still relatively unknown. Examination of the translatability of mRNAs isolated with the ribo-

some pellet without removal of associated protein factors might represent the actual physiological state of the mRNA in the intact cell. nRRL (Pelham and Jackson 1976; Jackson et al. 1983) was included in this system to provide an efficient translation system. This eliminates any dependency on limiting components of the translation machinery that may not be present in the T cells before and during early activation of them. Efficient expression of these mRNAs after activation might be critical for the progression of the T cell from quiescence to proliferation. Different expression of mRNAs in activated T cells and other cell types can be examined by a number of methods that include SAGE (Ryo et al. 1999), cDNA arrays (Lockhart et al. 1996) and substrate libraries (Zipfel et al. 1989). However, protein levels do not necessarily correlate with mRNA levels as shown in yeast by Gygi and coworkers (Gygi et al. 1999). Therefore, examination of mRNAs that are associated with polyribosomes selects those mRNAs that are more likely to be translated.

In our system, unactivated T cells had low translation, but removal of associated proteins resulted in increased in vitro translation of these mRNAs. These results suggest that there might be a repression or silencing of the expression of these mRNAs before activation. Wallace et al. (1979) also reported that in resting T cells, mRNAs are available but not able to be translated. We take this observation a step further and find that the repression is associated with the ribosome fraction. Repression of translation in the unactivated T cell could not be relieved by the addition of nRRL to the PAmRNAs. In contrast, mRNAs isolated as total RNA was

Fig. 7. MALDI-TOF analysis of proteins isolated from *in vitro* translation of PAmRNAs. MALDI-TOF analysis was performed on p74, p72, and p33 proteins extracted from preparative *in vitro* translation of PAmRNAs isolated from 30-min I + P-activated human T cells followed by extraction of biotinylated proteins by using streptavidin magnetic beads and SDS-PAGE (see Materials and Methods). (T) Peptides of trypsin.

efficiently translated. We do not know the identity of these mRNAs, their associated protein factors, or their mechanism of increased expression that is stimulated by activation. We are working on the identity of both the protein and corresponding mRNA and have identified one protein, p33, as a prohibitin-like protein. We believe that after activation, these mRNAs may have increased translatability that may be dependent on their associated protein factors (for review, see Minich and Ovchinnikov 1992).

There were only a few translatable mRNAs in the PRS fraction of the T cell. These results support those of Jagus and Kay (1979), who determined that the majority of mRNAs in T cells were associated with the heavy (ribosome) fraction and not as free mRNPs. DRB and ActD were

used to identify mRNAs that were sensitive *in vivo* to this transcriptional inhibitor, helping us to distinguish potential transcriptional- from translational-regulated mRNAs. One example of an RNP that is repressed during *in vitro* translation is the translation elongation factor EF-1 α . This RNP, but not other RNPs, was found to be repressed. The use of *in vitro* translation relieved this repression if the particle is washed with 0.5 M KCl (McCarthy and Kollmus 1995). We want to determine if mRNAs in the quiescent T cell are under a similar repression mechanism.

Preparation of the ribosome fractions was adapted from methods described by Bag and Pramanik (1987). These methods were originally designed to separate translationally active mRNP polysomal complexes from repressed nonpoly-

Fig. 8. Mass spectrometry of p33 identifies it as a prohibitin-like protein. PAmRNAs were translated in the presence of biotinylated lysine/tRNA_{lys}; the biotinylated proteins were bound to streptavidin magnetic beads, eluted, and run on SDS-polyacrylamide gels. p33 was extracted from the gel and digested with trypsin. Capillary high-pressure LC/ESI/MS/MS was performed on the trypsin digest (see Materials and Methods). Data from three peptide fragments with their corresponding amino acid (aa) sequence are at the top of the figure. An example of the amino acid sequence for one of the peptide fragments with y and b forms of the amino acid sequence indicated is at bottom.

somal cytoplasmic (free) mRNPs. Translation of PAmRNAs either in a free or membrane-associated state has been a source of mRNAs that can be translated in vitro with the aid of rabbit reticulocyte lysate or other cell-free fractions (Aroskar et al. 1980). In vitro translation of polysomes/ribosomes has been demonstrated for liver cells (Shafritz 1974; Takiguchi et al. 1985), lung tissue (Collins and Crystal 1975), rat and human brain tissue (Ramsey and Steele 1977; Heikkila et al. 1981; Marotta et al. 1981), adenovirus-infected HeLa cells (Persson and Oberg 1977), Krebs II ascites, and 3T3 cells (Vedeler et al. 1991). In vitro trans-

lation with [³⁵S]Met allows quick assessment of the translatability of mRNAs associated with the ribosome/polysome fraction, which can be analyzed by SDS-PAGE, two-dimensional PAGE, or immunoprecipitation.

Our methods are different from those that directly isolate total or poly(A)⁺ RNA. In these procedures, mRNA is isolated from samples and translated in vitro with RRL, wheat germ cell-free extracts, or other cell-free extracts (Lee and Engelhardt 1979; Wallace et al. 1979; Kecskemethy and Schafer 1982). Interestingly, PAmRNAs from I + P-activated T cells that were translated with nRRL were not trans-

lated using wheat germ cell-free extract (pers. obser.). However, mRNA contained in the total RNA that was extracted from the same polysome fraction could be translated with wheat germ extracts. These results suggest that wheat germ extract may not be compatible with translating human PAmRNAs. Wheat germ initiation factors may not be able to translate mRNAs that are associated with mammalian ribosomes, or mRNAs may need to be associated with wheat germ ribosomes for efficient translation in the wheat germ extract.

In vitro translation of PAmRNAs also may be a more physiologically relevant system for studying translation of mRNAs rather than translating protein-depleted poly(A)+ RNA. Potentially important transacting factors (RNA-binding proteins, initiation factors) that might stabilize the mRNA and aid in its translatability are still associated with their specific mRNAs (for reviews, see McCarthy and Kollmus 1995; Spirin 1996). This system also enabled us to concentrate mRNAs, thereby increasing the sensitivity for the detection of less abundant mRNAs. Some mRNAs might be missed because not all mRNAs are able to bind oligo(dT) (the most common extraction method), the result of short poly(A) tails (Milcarek et al. 1974). Our method will enable the identification of these mRNAs and their protein products. Two proteins, p72 and p33, which had atypical in vitro protein expression kinetics in the early activation period (0–2 h), were targeted for identification by mass spectrometry. The kinetics of their expression suggested tight control of their gene expression and a potentially important role in the activation mechanism.

The use of biotinylated lysine/tRNA (Kurzchalia et al. 1988) in the reaction mixture made it possible to isolate the synthesized product with streptavidin magnetic beads. Magnetic bead isolation of the product is quick and allows for the removal from other unwanted cellular proteins and protein components of the nRRL. There were some difficulties with the procedure, however, including some nonspecific proteins binding to the streptavidin magnetic beads, and the amount of incorporation of biotinylated lysine into some proteins was low, making their isolation difficult with streptavidin magnetic beads (data not published). However, for this study we were able to synthesize enough p72 and p33 for mass spectrometry analysis. Sequences for p72 peptide fragments after LC/ESI/MS/MS were not found in the DNA, protein, or EST databases. The sequence for p33 identified it as BAP37 (also known as the IgM-associated protein or prohibitin-associated protein [Terashima et al. 1994; Lamers and Bacher 1997]). BAP37 has been found to be associated with the mitochondria in yeast and mammalian cells and may play a role in cellular senescence (Coates et al. 1997). With the tentative identification of p33 as BAP37 and the appropriate reagents (antibodies and cDNAs), we will determine if BAP37 is important during the early activation of lymphocytes and if this gene is transcriptionally,

posttranscriptionally, or translationally regulated. We are also in the process of identifying the other genes that might be translationally controlled.

Materials and methods

Cell culture reagents were obtained from Life Technologies (GIBCO BRL); FBS was obtained from Hyclone. Ionomycin, PMA, and CHX came from Sigma. Nylon wool came from Robbins Scientific. Streptavidin magnetic beads came from Dynal or Promega. nRRL, RNasin, and Transcend tRNA came from Promega.

Preparation of T cells

Mononuclear cells obtained from normal human peripheral blood by cell elutriation were further enriched for T lymphocytes by nylon wool purification as described by Miyamoto et al. (1996). Cells were grown in RPMI 1640, 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 100 μ g/mL glutamine at 37°C with 5% CO₂.

Activation of T cells, in vivo labeling of proteins, and analysis by SDS-PAGE

T cells (2×10^6 cells/mL) were stimulated by incubation with 0.25 μ M ionomycin and 10 ng/mL PMA. At 1 h before harvesting, 20 μ Ci of [³⁵S]Met was added. Cells were harvested, washed, and frozen at –70°C. T cells were thawed and lysed with 100 μ L of SDS loading buffer (BioRad Protein II procedures) and a 50- μ L sample subjected to SDS-PAGE (12%). Gels were stained with Coomassie Brilliant Blue to determine equal loading of samples. Autoradiography was performed with XAR or BioMax film (Kodak). Scanning was performed using a Molecular Dynamics Personal Densitometer, and data were analyzed using ImageQuant (Molecular Dynamics).

In vitro translation of PAmRNAs

T cells were activated with 0.25 μ M ionomycin and 10 ng/mL PMA. After activation, cells were harvested, washed, and lysed with cell lysis buffer (10 mM Tris-HCl at pH 7.4, 10 mM MgCl₂, 80 mM KCl, 1 mM DTT, 0.2% Nonidet P-40) by incubation on ice for 10 min and centrifugation for 10 min (14,000g) to pellet nuclei, microsomes, and unlysed cells. Supernatants were then centrifuged at 125,000g in a TL100 (Beckman) for 50 min and separated into postribosomal supernatants and ribosome pellets. Ribosome pellets were stored at –70°C. For in vitro translation, pellets were resuspended in TE buffer (10mM Tris-HCl at pH 8.0, 1 mM EDTA) and translated with nRRLs (Promega) and [³⁵S]Met and then analyzed using 12% SDS-PAGE.

In vitro synthesis with biotinylated lysine

PAmRNAs were translated in vitro with nRRL in the presence of biotinylated lysine and Transcend (tRNA/lys) (Promega) either according to the manufacturer's instructions (Promega) or with 2 mM amino acid mixture without methionine and lysine if the synthesis was conducted with [³⁵S]Met. Synthesized biotinylated protein products were incubated with streptavidin magnetic beads (Dynal) for 30 min at 4°C in binding buffer (0.05M Tris-HCl at pH 7.5, 0.5 mM EDTA, 1 M NaCl) with rotation. After incubation, streptavidin magnetic beads were washed three times with binding buffer. Bound biotinylated proteins were removed by incubation for 5 min in SDS-PAGE loading buffer (BioRad Protean II instructions) and analyzed using 12% SDS-PAGE.

Mass spectrometry of isolated proteins

Proteins isolated from in vitro synthesis were separated on SDS-polyacrylamide gels, and extracted protein bands were digested with trypsin. The resulting tryptic peptides were analyzed by tandem MS/MS using an electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with capillary high-pressure liquid chromatography (Magic 2202, Michrom BioResources, Auburn, CA), according to methods described by Zhang et al. (1998).

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