Immunosuppressants FK506 and rapamycin have different effects on the biosynthesis of cytoplasmic actin during the early period of T cell activation

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FK506 and rapamycin are immunosuppressants that interfere with T cell activation. FK506 inhibits early events of T cell activation such as the induction of cytokine transcription, whereas rapamycin inhibits later interleukin 2 signalling events. However, both reagents either directly or indirectly reduce protein synthesis. Therefore a kinetic study was conducted in human primary T lymphocytes examining increased synthesis of proteins stimulated by either ionomycin + phorbol myristate acetate (PMA) or PMA alone. Three patterns of protein expression were observed. Synthesis of one group of proteins had enhanced synthesis with FK506, but reduced synthesis with rapamycin. A second group had reduced synthesis with rapamycin and either no change or a slight reduction with FK506 and a third group had reduction with both FK506 and rapamycin. One major protein of the first group, p42, had a rapid increase in

synthesis that decreased by 8 h. Its synthesis was strongly enhanced by FK506, but reduced by rapamycin after ionomycin + PMA stimulation. In contrast, this protein was strongly induced by PMA alone in these cells and not affected by FK506 treatment, but still reduced by rapamycin. p42 was identified as cytoplasmic actin. mRNA levels of both γ - and β -actin were found to be enhanced with FK506 treatment suggesting that regulation of actin was at a transcriptional or post-transcriptional level. Results with actinomycin D indicated that FK506 is regulating actin biosynthesis at the post-transcriptional level. Rapamycin, however, appeared to be operating at the level of translation.

Key words: ionomycin, lymphocytes, phorbol myristate acetate, protein synthesis, signalling.

INTRODUCTION

T cell activation is a complicated biochemical process that is started by the interaction of the T cell receptor with a foreign antigen–MHC complex presented by an antigen-presenting cell and a co-stimulatory signal [1]. These events trigger a cascade of signal pathways resulting in increased gene expression and protein synthesis. Immunosuppressant FK506 or rapamycin can inhibit T cell activation [2–5] and partially inhibit protein synthesis [4]. Although these two immunosuppressants bind the same FK506 binding proteins (FKBPs), they have very different mechanisms of action [2,6]. The binding of FK506 to FKBP12 prevents the activation of the phosphatase calcineurin and the subsequent dephosphorylation of the transcription factor NFAT (nuclear factors of activated T cells) [7]. As a result, NFAT is prevented from translocating from the cytoplasm to the nucleus and cannot activate interleukin 2 (IL-2) and other NFAT responsive genes [8]. In contrast, the rapamycin–FKBP bound complex inhibits activity of a cellular protein, RAFT (rapamycin and FKBP12 target; also called mTOR or FRAP), which in turn prevents the activation of p70S6K and IL-2 signalling [2,9]. Treatment of human peripheral blood T lymphocytes (T cells) reduces their immune response and prevents subsequent cellular proliferation. Although FK506 has been shown to affect transcriptional regulation of IL-2 and other cytokine genes [10], and rapamycin has been suggested to affect translation of polypyrimidine-tract containing mRNAs [11], it is possible they affect

post-transcriptional or translation regulation of other genes not already described. It is useful to identify these other affected genes and the level at which they are regulated by these reagents.

Human primary lymphocytes, which are in a natural resting or quiescent (G_0) state, were used in this study. We used electrophoretic methods to analyse the incorporation of [³⁵S]methionine into proteins *in vivo* for one hour before harvesting. This method of analysis examines the ongoing synthesis of proteins that is occurring in the T cells at the time of harvest. Monitoring the expression of these proteins actually represents the cumulative effect of the transcriptional, post-transcriptional and translational regulation of the gene. This paper examines the regulation of one of the gene targets of FK506 identified in the initial screen. p42 was identified as having its synthesis enhanced with FK506 treatment, but reduced by rapamycin treatment.

p42 was isolated and identified as cytoplasmic actin, one of the most abundant proteins in T cells. The recent interest in cytoskeletal organization during the process of T cell activation makes the study of actin biosynthesis during the early period of activation particularly important [12]. Possible mechanisms for the effects of FK506 and rapamycin on actin biosynthesis were subsequently examined and will be discussed.

MATERIALS AND METHODS

Cell culture reagents were obtained from Life Technologies (Gaithersburg, MD, U.S.A.) and foetal bovine serum was from Hyclone (Logan, UT, U.S.A.). Ionomycin, PMA and cyclo-

Abbreviations used: PMA, phorbol myristate acetate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; FKBP, FK506 binding protein; IL-2, interleukin 2; NFAT, nuclear factors of activated T cells; UTR, untranslated region.

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heximide were from Sigma and nylon wool was from Robbins Scientific (Sunnyvale, CA, U.S.A.).

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 [13]. Cells were grown in RPMI 1640, 10% (v/v) foetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and 100 µg/ml glutamine, at 37 °C in 5% CO₂.

Activation of T cells, labelling of proteins *in vivo* and analysis by SDS/PAGE

T cells (2×10^6 cells/ml) were stimulated by incubation with 0.25 µM ionomycin + 10 ng/ml PMA. One hour before harvesting 20 µCi of [³⁵S]methionine was added. Cells were harvested, washed and frozen at -70 °C. T cells were thawed and lysed with 100 µl of SDS loading buffer [62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.05% Bromophenol Blue] and 50 µl samples (containing equal numbers of cells) were subjected to SDS/PAGE (12% gel) according to BioRad Protean II procedures [14]. Gels were stained with Coomassie Blue to determine that there was equal loading of protein per sample. Autoradiography was performed with XAR or BioMax film (Kodak). Densitometer scanning was performed using a Molecular Dynamics Personal Densitometer and data analysed using ImageQuant (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

In vitro translation of polysome-associated mRNAs

T cells were activated with 0.25 µM ionomycin + 10 ng/ml PMA. Cells were harvested, washed and then lysed with cell lysis buffer (10 mM Tris/HCl, pH 7.4, 10 mM MgCl₂, 0.1 mM KCl, 1 mM dithiothreitol and 0.2% Nonidet P40) based upon modifications of the procedures of Bag and Pramanik [15]. The lysed cells were incubated on ice for 10 min and then centrifuged for 10 min in an Eppendorf centrifuge (14000 g), to pellet nuclei and unlysed cells. Supernatants were then centrifuged at 125000 g in a TL100 (Beckman) for 50 min and separated into post-ribosomal supernatants and polysome pellets. Polysome pellets were stored at -70 °C. For *in vitro* translation, these pellets were thawed, resuspended in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and translated with nuclease-treated rabbit reticulocyte lysates (Promega) plus [³⁵S]methionine according to the manufacturer's instructions and analysed by SDS/PAGE (12% gel).

Synthesis *in vitro* with biotinylated lysine

Polysome-associated mRNAs were translated *in vitro* with nuclease-treated rabbit reticulocyte lysate in the presence of biotinylated lysine (Promega, Madison, WI, U.S.A.) or with a 2 mM amino acid mixture lacking methionine and lysine, if the synthesis was conducted with [³⁵S]methionine. Biotinylated protein products were incubated with streptavidin magnetic beads (Dyna A/S, Oslo, Norway) for 30 min at 4 °C in binding buffer (50 mM Tris/HCl, pH 7.5, 0.5 mM EDTA and 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 analysed by SDS/PAGE (12% gel).

Isolation of protein product and protein sequencing

Ponceau S-stained poly(vinylidene fluoride) blots were subjected to proteolytic digestion with trypsin *in situ* (modified sequencing grade; Promega) according to the method of Fernandez et al. [16]. Digests were separated on a narrow-bore (2.1 mm × 250 mm) Vydac 218TP52 column and guard column (Separations Group, Hesperia, CA, U.S.A.) at 35 °C on a System Gold HPLC equipped with a Model 507 autosampler, Model 126 programmable solvent module and Model 168 diode array detector (Beckman, Fullerton, CA, U.S.A.). Samples were eluted at 0.25 ml/min according to the described gradient [16]. Column effluent was monitored at 215 and 280 nm. Fractions were collected at 30 s intervals and stored at -70 °C. Fractions (125 µl) were applied to a Biobrene-treated glass-fibre filter (Applied Biosystems, Foster City, CA, U.S.A.) and dried prior to amino acid sequencing on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyser (Applied Biosystems). Data were collected and analysed on a Model 610A data analysis system (Applied Biosystems). Amino acid sequences were searched in the Genetics Computer Group (GCG)-Swiss Protein Database (Genetics Computer Group, University of Wisconsin, Madison, WI, U.S.A.).

Northern blot analysis

T cells (1×10^8 cells in 50 ml) were treated with either ionomycin + PMA or PMA alone in the absence or presence of FK506 or rapamycin for various time periods and then the RNA was isolated (RNeasy, Qiagen). Treatment with 10 µg/ml cycloheximide was used for inhibition of protein synthesis in the actin mRNA stability studies and 5 µg/ml actinomycin D was used for inhibition of transcription. Northern blot analysis was performed as described previously [13]. cDNAs from γ-actin, c-Fos or glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were labelled using [α-³²P]dCTP (random-primed; BRL). Isotype-specific probes for β- and γ-actin were prepared from the 3'-untranslated regions (3'-UTR) of each cDNA [17] using the 322 bp *HinfI* fragment from β-actin and the 403 bp *HinfI*-*Fnu4HI* fragment from γ-actin. Densitometer scanning was performed using a Molecular Dynamics Personal Densitometer and data analysed using ImageQuant.

RESULTS

Effect of FK506 or rapamycin on synthesis of proteins *in vivo* in T cells activated with ionomycin + PMA

Human peripheral blood T cells were activated with ionomycin plus the phorbol ester, PMA, over an 8 h time period. Corresponding sets of activated T cells were either untreated, or treated with FK506 or rapamycin. After metabolically labelling the cells with [³⁵S]methionine for 1 h before harvest, cells were lysed and analysed by SDS/PAGE followed by autoradiography (Figure 1). Increased protein synthesis was monitored by densitometry scanning, with results identified as peak areas corresponding to the peak number (peak #; indicated on the right side of Figure 1) plotted in Figure 2. Increased protein synthesis by this method started slightly at 1–2 h with the increased synthesis of a few observable proteins, then greater synthesis at 4 h and especially at 8 h (Figure 3). By 8 h of activation there were large numbers of proteins being synthesized, with 6 major proteins (peaks 2, 3, 10, 11, 16 and 17) accounting for the majority of the increased protein synthesis (Figure 2). Treatment of T cells with either FK506 or rapamycin during activation

Figure 1 Synthesis of proteins *in vivo* in T cells activated with ionomycin + PMA in the absence or presence of FK506 or rapamycin

T cells were activated with 0.25 μ M ionomycin + 10 ng/ml PMA in the absence or presence of 10 nM FK506 or 10 nM rapamycin for 0, 0.5, 1, 2, 4 or 8 h. T cells were labelled with [35 S]methionine for 1 h before harvesting. Harvested cells were solubilized in SDS/PAGE sample buffer and analysed by SDS-PAGE (12% gel). Lanes 1–6, no immunosuppressant (—); lanes 7–12, cell were treated with FK506; lanes 13–18, cells were treated with rapamycin. Position of molecular mass standards in kDa are to the left of the gel. Data are representative of seven experiments. The peak numbers given on the right refer to Figures 2 and 3.

reduced protein synthesis by 25% and 50% respectively (Figure 3). This reduction at 8 h could be visualized by plotting the peak areas (Figures 2 and 3). The effect of the inhibitors on protein synthesis could be grouped into three categories (see chart in Figure 3). One group had enhanced synthesis with FK506, but reduced synthesis with rapamycin (peaks 17 and 19). A second group had synthesis, which was slightly reduced by FK506, but strongly reduced by rapamycin (peaks 4, 6, 11, 14, 15, 21), and finally a third group that contained the majority of proteins, the synthesis of which was reduced with both FK506 and rapamycin (peaks 1–3, 5, 7–10, 12, 13, 16, 18, 20 and 21). Furthermore, more precise separation of individual proteins by two-dimensional PAGE will need to be conducted and the identification of these proteins performed before any meaningful conclusions can be made concerning the gene regulation of these proteins.

Activation of T cells by PMA alone strongly induced synthesis of p42, which was not enhanced by FK506

T cells can be induced to proliferate with ionomycin + PMA [18–20], but stimulation with PMA alone will not induce proliferation [21]. We have shown that ionomycin + PMA will maximally stimulate protein synthesis rate, but although PMA alone will initially stimulate protein synthesis, this increase levelled off at 8 h. (results not shown). Therefore a comparison between the two conditions of activation, ionomycin + PMA versus PMA alone was performed over an 8 h time period, conducted in the

absence or presence of FK506 or rapamycin (Figures 4A and 4B). Results showed that PMA strongly stimulated protein synthesis (Figure 4B). Synthesis of p42 (peak 17 in Figure 1) in particular was strongly induced and unlike ionomycin + PMA stimulation was not reduced by 8 h (compare Figure 4A, lane 11, with Figure 4B, lane 11). Synthesis of p42 was not affected by FK506 treatment, but was reduced with rapamycin treatment. Rapamycin reduced synthesis of most proteins in PMA-stimulated T cells (Figure 4B, lane 13) to a greater extent than in the ionomycin + PMA stimulated T cells. These results suggest that down-regulation of p42 synthesis stimulated by ionomycin + PMA is dependent upon Ca^{2+} signals that are sensitive to FK506, which are not present in PMA-stimulated cells.

The effect of FK506 and rapamycin on the synthesis of p42 from ribosome-associated mRNAs *in vitro* resembles the *in vivo* effect

Although results from synthesis of proteins *in vivo* in T cells indicated an effect by FK506 and rapamycin on p42 synthesis, it was necessary to isolate and identify the protein before mechanisms controlling its gene regulation could be studied or its biological relevance ascertained. To generate enough p42 for identification, synthesis of ribosome-associated mRNAs *in vitro* was performed supplemented with the nuclease-treated rabbit reticulocyte lysate and [35 S]methionine. First p42 was synthesized *in vitro* from ribosome-associated mRNAs isolated from T cells activated in a time-course experiment and quantified by densitometry scanning (Figure 5). Results showed that synthesis of p42 from ribosome-associated mRNAs *in vitro* initially increased at 1–2 h and then decreased at 8 h with ionomycin + PMA stimulation. Treatment with FK506 showed enhanced p42 synthesis *in vitro* and treatment with rapamycin reduced p42 synthesis at 2 h, although at 4 h and 8 h, reduction by rapamycin was small. With stimulation by PMA alone, however, there was strong synthesis of p42 that was not affected by FK506, but reduced by rapamycin at 8 h. These results suggest that results from the synthesis of p42 *in vitro* can mimic results from the *in vivo* studies in T cells and enable us to scale up synthesis of p42 for isolation and sequencing.

Identification of p42 as cytoplasmic actin

In vitro translation of the polysome-associated mRNAs enabled us to synthesize sufficient quantities of proteins for identification. Previously, the difficulty was to isolate newly synthesized proteins from the large pool of proteins already synthesized. With synthesis *in vitro*, synthesized proteins can be separated from proteins of the rabbit reticulocyte lysate and other associated proteins of the ribosomal pellet by using biotinylated lysine during their synthesis *in vitro*. With the use of streptavidin-magnetic beads, we were able to isolate enough p42 for sequencing. p42 synthesized *in vitro* was separated by SDS/PAGE, transferred to poly(vinylidene fluoride) membranes and the protein band was excised. The excised protein was digested from the membrane with trypsin and the peptide fragments were subjected to automated Edman degradation. The following sequence was determined, (R)VAPEEHPVLLTEAPLNPK, which proved to be 100% identical to cytoplasmic actin.

FK506 treatment affects p42 (actin) gene expression differently from rapamycin during activation of T cells

Having identified the p42 protein as actin, regulation of actin biosynthesis by FK506 and rapamycin was studied. Northern blot analysis using a ^{32}P -labelled cDNA probe prepared from γ -

Figure 2 Quantification of protein synthesis *in vivo* in activated T cells

Lanes 6, 12 and 18 from Figure 1 were scanned by laser densitometry and the peak areas were measured and plotted as described in the Materials and methods section. The peak numbers (#) refer to the protein bands indicated in Figure 1. Data are means $\pm$ S.E.M. for two to four experiments. Top panel, no immunosuppressants; middle panel, treated with FK506; bottom panel, treated with rapamycin.

Figure 3 Effect of immunosuppressants on protein synthesis *in vivo* in activated T cells

(Top panel) Peak areas from Figure 2 are expressed as a percentage of the peak area of the ionomycin + PMA peaks from Figure 2, top panel. Open bars, no immunosuppressants; hatched bars, treated with FK506; filled bars, treated with rapamycin. I + P, ionomycin + PMA. (Bottom left panel) The peaks from Figure 2 are divided into three categories: (1) enhanced by FK506 (FK), reduced by rapamycin (rapa); (2) reduced by rapamycin, but not by FK506; (3) reduced by both FK506 and rapamycin. (Bottom right panel) Total protein synthesis was determined by laser densitometry scanning of each lane from Figure 1 as a summation of all of the proteins synthesized. DU, arbitrary densitometer units; ■, no immunosuppressant; ●, treated with FK506; ▲, treated with rapamycin.

actin was performed on RNA isolated from peripheral blood T cells activated with either ionomycin + PMA or PMA alone for 1, 2 or 4 h in the presence or absence of FK506 or rapamycin (Figure 6A). Results showed that by the first hour after activation, mRNA levels of actin increased 6-fold over unactivated or G_0 , with a greater increase observed with PMA activation (12–13-fold over G_0). By 2 h after ionomycin + PMA activation, actin mRNA levels were still increasing, but by 4 h, the amount of actin mRNA decreased to G_0 levels. With PMA activation, actin mRNA levels increased by greater than 25-fold over G_0 after 2 h, and then decreased by 4 h to 10-fold greater than G_0 levels. FK506 treatment resulted in higher actin mRNA levels when

compared with untreated, ionomycin + PMA-activated T cells. FK506 treatment of PMA-activated T cells showed little effect. At 4 h, FK506 treatment continued to enhance actin mRNA levels by 3–4-fold over G_0 levels in ionomycin + PMA activated T cells, while rapamycin had little or no effect on the already low levels of actin mRNA. In contrast, with PMA activation of T cells, actin mRNA levels were still relatively high (9-fold over G_0 levels), and were not affected by either FK506 or rapamycin treatment. These data, therefore, indicated that the up-regulation and down-regulation of p42 (actin) synthesis, which was observed in both *in vivo* and *in vitro* studies correlated well with the levels of actin mRNA and with FK506 treatment of ionomycin + PMA-

Figure 4 Activation of T cell protein synthesis by PMA in the absence or presence of FK506 or rapamycin

T cells were activated with either (A) ionomycin + PMA or (B) PMA alone in the absence (—) or presence of 10 nM FK506 (F) or 10 nM rapamycin (R) for either 1, 2, 4 or 8 h. T cells were labelled with [35 S]methionine for 1 h before harvesting. Harvested cells were solubilized in SDS/PAGE sample buffer and analysed by SDS/PAGE (12% gel). Go, resting, unactivated T cells. Data are representative of four experiments.

Figure 5 Effect of FK506 and rapamycin on the synthesis of p42 in an *in vitro* translation system using ribosomal fractions from T cells

T cells were activated with either ionomycin + PMA or PMA alone for 0, 0.5, 1, 2, 4 or 8 h. Ribosome fractions were prepared and translated *in vitro* with nuclease-treated rabbit reticulocyte lysate and [35 S]methionine as described in the Materials and methods section. Synthesized proteins were analysed by SDS/PAGE and autoradiography. Data from densitometric scans of the p42 protein bands for activated T cells with no immunosuppressant (filled bar), treated with FK506 (hatched bar) or treated with rapamycin (open bar). Data are representative of four experiments. DU, arbitrary densitometer units.

activated T cells. If T cells were treated simultaneously with both FK506 and rapamycin during activation with ionomycin + PMA, actin mRNA was enhanced, similar to the results from treatment with FK506 only, showing that the effect of FK506 on

actin mRNA has preference over the effect of rapamycin (results not shown).

Cycloheximide can stabilize actin mRNA during T cell activation, but does not block the effect of either FK506 or rapamycin on actin mRNA levels

Since cycloheximide treatment has been shown previously to superinduce actin mRNA [22], we asked whether the enhancement of actin mRNA by FK506 in ionomycin + PMA activated T cells could be affected by cycloheximide treatment. Northern blot analysis was performed of either unactivated T cells, or those activated for 2 h with either ionomycin + PMA or PMA alone, both in the absence or presence of FK506 or rapamycin and with or without cycloheximide (Figure 7A). Results were analysed by densitometer scanning and plotted in Figure 7(B). Actin mRNA was superinduced by cycloheximide in both ionomycin + PMA and PMA-treated cells after treatment with either FK506 or rapamycin (Figures 7A and 7B). Superinduction and/or stabilization of actin mRNA by cycloheximide and FK506 was clearly evident in lane 11. The Northern blot (Figure 7A) was stripped and reprobed with specific probes to γ -actin or to β -actin or G3PDH (Figure 7C). mRNAs for both isoforms increased with either ionomycin + PMA or PMA activation of T cells. However, PMA activation (Figure 7C, lanes 13–18), showed greater actin mRNAs for both β -actin and γ -actin isoforms. FK506 treatment resulted in higher levels of both actin isoforms in ionomycin + PMA activated T cells. Treatment with cycloheximide increased mRNA of both isoforms of actin. Cycloheximide also superinduced/stabilized both isoforms of actin mRNA by FK506.

Figure 6 The effect of FK506 on actin mRNA levels in T cells activated with ionomycin + PMA or PMA alone

T cells were activated with either ionomycin + PMA (I + P) or PMA alone (P) in the absence (—) or presence of FK506 (FK) or rapamycin (rapa) for 1, 2 or 4 h. After harvest, total RNA was isolated and 10 μ g/lane run on a 3% (v/v) formaldehyde, 0.8% agarose gel, transferred to Nytran, and probed with a 32 P-labelled cDNA probe for γ -actin (**A**). The arrow on the right indicates the position of actin mRNA. (**B**) Data from densitometric scans of the Northern blot probed for γ -actin (upper panel) and G3PDH (lower panel) mRNA. Data are means $\pm$ S.E.M. for two to four experiments. DU, arbitrary densitometer units.

Figure 7 Effect of cycloheximide on actin mRNA levels in T cells activated with ionomycin + PMA or PMA alone in the absence or presence of immunosuppressants

T cells were either unactivated or activated with ionomycin + PMA (I + P) or PMA alone for 2 h in the absence (—) or presence of FK506 (FK, F) or rapamycin (rapa, R) with or without (—) 10 μ g/ml cycloheximide (CHX). RNA from these cells was analysed by Northern blot analysis using a 32 P-labelled γ -actin cDNA probe (**A**). (**B**) Data from densitometric scans of the Northern blot probed for γ -actin (filled bars) and G3PDH (hatched bars) mRNA. (**C**) The Northern blot was stripped and reprobed with specific probes for γ -actin (top panel), β -actin (middle panel) and G3PDH (bottom panel) mRNA.

Figure 8 Effect of inhibiting transcription on actin mRNA levels in ionomycin + PMA or PMA activated T cells in the absence or presence of FK506 or rapamycin

T cells were activated with either ionomycin + PMA (I + P) or PMA alone (P) for 2 h in the absence (—) or presence of FK506 (FK, F) or rapamycin (rapa, R). These cells were untreated (—), treated simultaneously with actinomycin D (ActD) or pretreated for 2 h with actinomycin D before activation. (**A**) Northern Blot analysis with 32 P-labelled γ -actin cDNA. (**B**) Data from densitometric scans of the Northern blot probed for γ -actin (filled bars) and G3PDH (hatched bars) mRNA.

Actinomycin D treatment indicates that FK506 may be stabilizing actin mRNA

To determine whether regulation of actin mRNA levels by FK506 during ionomycin + PMA activation of T cells was occurring at transcriptional or post-transcriptional levels, actinomycin D was used to inhibit transcription during the activation process. T cells were either incubated simultaneously with actinomycin D or preincubated with actinomycin D for 2 h, before activation for 2 h with ionomycin + PMA or PMA alone in the absence or presence of FK506 or rapamycin. The total RNA was isolated and Northern blot analysis was performed (Figure 8A). Results from the Northern blot were analysed by densitometer scanning and the results plotted in Figure 8(B). Increases in actin mRNA induced in T cells by activation with either ionomycin + PMA or PMA alone was not prevented when simultaneously incubated with actinomycin D. However pretreatment with actinomycin D for 2 h before treatment with FK506 and activation by ionomycin + PMA reduced actin mRNA levels to those of the untreated or rapamycin treated samples. PMA activation strongly induced actin mRNAs (2–3-fold higher than ionomycin + PMA activation) which were unaffected by simultaneous incubation with actinomycin D, but reduced by pretreatment for 2 h with actinomycin D. The results of these experiments and the cycloheximide studies suggest that FK506 affects post-transcriptional regulation of cytoplasmic actins, most likely by stabilizing or reducing degradation of the transcripts.

DISCUSSION

One of the interesting results of examining the effect of FK506 or rapamycin on the synthesis of proteins *in vivo* after mitogenic activation was the effect of FK506 on the synthesis of p42, identified as cytoplasmic actin. Instead of inhibiting actin synthesis, FK506 appeared to potentiate its expression in the early time period after activation. We would like to know whether the increase in actin gene expression is important for the immunosuppressive activity of FK506. Actin is one of the abundant and highly conserved proteins in cellular evolution [23]. Mammalian non-muscle cells synthesize two types of actin, β and γ , which differ only by four conserved amino acid substitutions in the N-terminus of the protein and by their 3'UTRs [17]. The data obtained from the sequence of our sample of p42 did not distinguish between the two species; however, subsequent analysis for each specific mRNA for β - and γ -actin determined that both increased with activation by either ionomycin + PMA or PMA alone. mRNAs for both isoforms appeared to be stabilized by FK506 treatment after ionomycin + PMA activation.

Our subsequent studies to determine how immunosuppressants affected actin gene expression demonstrated that actin levels were rapidly up-regulated and then down-regulated with ionomycin + PMA stimulation within the first 8 h of activation. Actin mRNA was also strongly induced with PMA, but not as quickly down-regulated, suggesting that the down-regulation occurring in ionomycin + PMA-stimulated T cells was through a FK506-sensitive ionomycin-induced Ca^{2+} -activated pathway. Treatment with FK506 appeared to prevent the down-regulation of actin mRNA in ionomycin + PMA-treated cells, but had no effect in PMA-treated cells. Goodier and Kay [24] also showed that actin gene expression in T cells is down-regulated by another ionophore, A23184, and that this negative regulation could be antagonized by cyclosporin A. Presently, the best known target of cyclosporin and FK506 action in T cells is calcineurin [7,25]. It is, therefore, the most likely effector for the down-regulation of actin gene expression in ionomycin + PMA treated T cells. Calcineurin regulates NFAT activity in T cells, but in neurons calcineurin has been shown to be associated with the cytoskeleton, and it may regulate tau phosphorylation and modulate other steps required for the determination of polarity [26]; these activities were blocked by cyclosporin A. Calcineurin also has been implicated in exocytosis of secretory granules of basophiles [27,28] and neutrophil chemokinesis [29]. These results imply that calcineurin may be associated with the cytoskeleton, motility and movement of vesicles.

The effect by FK506 on actin synthesis was not regulated by translation since the change in mRNA levels correlated with protein levels suggesting a transcriptional or post-transcriptional regulation. Also cycloheximide treatment *in vivo* did not inhibit the effect of FK506. Instead, cycloheximide treatment super-induced actin mRNA levels, which also provides evidence that the effect of FK506 on actin biosynthesis is post-transcriptional, by stabilizing or reducing the degradation of its mRNA. Cycloheximide in low concentrations has been shown to induce expression of some genes, which include β -actin [22]. In our study, however, cycloheximide by itself did not induce actin.

Results from the treatment of T cells with actinomycin D also indicated that FK506 was affecting actin mRNA degradation/stabilization. The mechanism by which FK506 affects actin biosynthesis may be dependent on Ca^{2+} signals stimulated by ionomycin that are inhibited by FK506. These signals may be missing in T cells activated by PMA alone, explaining why there is less degradation of actin mRNA at 2 h in PMA-activated samples when compared with ionomycin + PMA activated T cell

samples. Previous reports from Goodier and Kay [24] and White et al. [30] also concluded that actin mRNA was regulated post-transcriptionally.

Rapamycin reduced actin synthesis, but also reduced synthesis of the majority of proteins, demonstrating a global effect on protein synthesis for these genes. The reduced synthesis of p42 by rapamycin, therefore, is indicative of reduced global translation, most likely affecting initiation. Rapamycin inhibits phosphorylation of the translation initiation factor eIF4E binding protein, 4E-BP1 (also known as PHAS-I in the rat). eIF4E binds to the m7G cap of mRNAs and along with eIF4A and eIF4G forms the eIF4F complex, which is important for the pre-initiation phase of translation. Unphosphorylated 4E-BP1 binds eIF4E, but upon phosphorylation releases eIF4E [31]. Rapamycin will also inhibit the phosphorylation of p70S6kinase [11]. Synthesis of the majority of proteins induced by PMA alone was also reduced by rapamycin, also indicating a global effect on translation in these cells. Actin mRNAs levels were not reduced by rapamycin although the protein levels were reduced after either ionomycin + PMA or PMA stimulation suggesting that biosynthesis of actin was translationally inhibited by rapamycin.

The biological relevance of an increase in actin biosynthesis during the early period of T cell activation is also not known. Actin is one of the most abundant proteins in the cell. Therefore, the contribution of new actin synthesis to the total actin content of the cells is probably small. But Riddle et al. [32] demonstrated in fibroblasts that new actin synthesis, which declined after the cell reached S phase, is necessary for the transition from quiescence (G_0) to S phase. Singer and colleagues found that in fibroblasts, mRNA for β -actin was selectively translocated via sequence elements in its 3'UTR to the cell membrane, where it may be involved in active polymerization of actin filaments, spatial positions of mRNAs or transport of translational machinery to the periphery [34,35]. Recently, actin polymerization and cytoskeletal reorganization in response to T cell signals, which may involve clustering of signalling molecules at the site of T cell receptor/tyrosine phosphorylation, have been demonstrated. This clustering is inhibited by treatment with cytochalasin D, and results in blocking IL-2 production and proliferation [12]. It remains to be shown whether the effect of FK506 on actin synthesis that we have seen in this study is important for the localization of mRNAs or is involved in cytoskeletal rearrangement and transport or signalling molecules. However, FK506 has been shown to inhibit the infiltration of T lymphocytes into the allograft [36] *in vivo*, which might indicate that it does affect cytoskeletal function and actin biosynthesis.

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