Global and Specific Translational Control by Rapamycin in T Cells Uncovered by Microarrays and Proteomics

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Rapamycin has been shown to affect translation. We have utilized two complementary approaches to identify genes that are predominantly affected by rapamycin in Jurkat T cells. One was to compare levels of polysome-bound and total RNA using oligonucleotide microarrays complementary to 6,300 human genes. Another was to determine protein synthesis levels using two-dimensional PAGE. Analysis of expression changes at the polysome-bound RNA levels showed that translation of most of the expressed genes was partially reduced following rapamycin treatment. However, translation of 136 genes (6% of the expressed genes) was totally inhibited. This group included genes encoding RNA-binding proteins and several proteasome subunit members. Translation of a set of 159 genes (7%) was largely unaffected by rapamycin treatment. These genes included transcription factors, kinases, phosphatases, and members of the RAS superfamily. Analysis of [35S]methionine-labeled proteins from the same cell populations using two-dimensional PAGE showed that the integrated intensity of 111 of 830 protein spots changed in rapamycin-treated cells by at least 3-fold (70 increased, 41 decreased). We identified 22 affected protein spots representing protein products of 16 genes. The combined microarray and proteomic approach has uncovered novel genes affected by rapamycin that may be involved in its immunosuppressive effect and other genes that are not affected at the level of translation in a context of general inhibition of cap-dependent translation.

Rapamycin is a macrolide antibiotic originally isolated from Streptomyces hygroscopicus (1). It is a potent immunosuppressant with therapeutic applications in the prevention of organ allograft rejection and in the treatment of autoimmune disease (2–6). The importance of rapamycin as an immunosuppressant is not limited to T cells, since this drug inhibits the proliferation of many mammalian cell types as well as that of yeast cells (7).

Rapamycin blocks progression of the cell cycle at the G₁ phase by binding to FKBP12 (FK506-binding protein) (10, 11). The rapamycin-FKBP12 complex inhibits mTOR (mammalian target of rapamycin), also referred to as FRAP (FKBP-rapamycin-associated protein) (9). Targets of mTOR include 4E-BP1 and the 40 S ribosomal protein S6 kinase, p70s6k (12–16). tor-associated protein) (9). Targets of mTOR include 4E-BP1 and the 40 S ribosomal protein S6 kinase, p70s6k (12–16).

To determine rapamycin-sensitive transcripts, we used a methodology based on the separation of polysomes from mRNPs using sucrose gradient centrifugation followed by oligonucleotide microarray hybridization. This technology has been recently adapted for studies of translational control (21–23) and is based on the assumption that translationally inactive mRNAs are present as free cytoplasmic mRNPs, whereas actively translated mRNAs are contained within polysomes. This enables identification of mRNAs specifically mobilized from free mRNPs onto polysomes and vice versa in T cells in response to rapamycin. A complementary approach used proteomic analysis to systematically analyze gene expression in T cells in response to rapamycin.

EXPERIMENTAL PROCEDURES

Cell Culture—The human Jurkat T cell clone E6–1 (American Type Culture Collection, Manassas, VA) was grown in the presence of 10% heat-inactivated fetal calf serum, using RPMI 1640 medium supplemented with 2 mM l-glutamine, 10 mM Hepes buffer, and gentamycin (20 μg/ml). The day prior to performing the polysome profiles, the cells

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‡ The abbreviations used are: IL, interleukin; eIF4E, eukaryotic initiation factor 4E; IRES, internal ribosome entry site; MAP, mitogen-activated protein.
and total protein extract was analyzed by Western blotting using polyclonal antibody to 4E-BP1 followed by monoclonal anti-actin. 

1.5

the cell proliferation assay, cells were seeded at an initial density of 1.5 × 10⁵ cells/ml without or with 20 ng/ml of rapamycin and were cultured for the indicated times without any change of media. Viable cells were counted after 24, 48, and 72 h of culture. The shown concentrations are the mean of three separate experiments, and the error bars indicate the S.D. B, protein synthesis rates in T cells. T cells (2 × 10⁶) were preincubated 1 h in methionine-free medium. Rapamycin was added to the cells together with [35S]methionine (100 µCi). Cells were harvested at 4 and 8 h, and radioactivity incorporated into trichloroacetic acid–precipitable material was measured. The effect of rapamycin is expressed as percentage of the control. The experiment was carried out three times, and the error bars indicate S.D. C, effect of rapamycin in 4E-BP1 phosphorylation. After 1- and 4-h exposure to rapamycin, T cells were lysed, and total protein extract was analyzed by Western blotting using polyclonal antibody to 4E-BP1 followed by monoclonal anti-actin.

were seeded in fresh medium at a density of 10⁵ cells/ml. When indicated, cells were incubated with 20 ng/ml rapamycin (Calbiochem). For the cell proliferation assay, cells were seeded at an initial density of 1.5 × 10⁵ cells/ml with or without rapamycin and cultured for 3 days without any change of media. Cell proliferation was monitored every 24 h by determining cell number with a Coulter counter ZM equipped with a Coultronic 256 channelizer (Hialeah, FL).

Metabolic Labeling—Jurkat cells were preincubated at 37 °C for 1 h in methionine-free RPMI 1640 medium. Rapamycin and [35S]methionine (100 µCi; PerkinElmer Life Sciences) were added together for the indicated times, and the cells were either lysed in 20 mM Tris-HCl, pH 7.5, buffer containing 5 mM EDTA and 100 mM KCl for the measure of radioactivity incorporation rates after trichloroacetic acid precipitation or were processed for two-dimensional PAGE analysis.

Western Blotting Analysis of 4E-BP1—Untreated and rapamycin-treated Jurkat cells were rinsed twice with ice-cold phosphate-buffered saline and 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 × g for 10 min, and the supernatant was collected. Proteins (100 µg) were loaded onto a 15% polyacrylamide gel, separated, and transferred onto a 0.22-µm nitrocellulose membrane (Schleicher and Schuell). Following transfer, membranes were incubated for 2 h in blocking buffer containing 5% milk in 10 mM Tris-HCl, pH 7.5; 2.5 mM EDTA, pH 8; 50 mM NaCl. The membranes were incubated for 2 h with rabbit polyclonal antisera against 4E-BP1 (TEBU, Le Peray-en-Yvelines, France) and actin (ICN Biomedical, Aurora, OH) at a dilution of 1:1000. The membranes were then incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit antibodies, at a 1:2000 dilution. Immunodetection was realized by ECL (Amersham Biosciences).

Two-dimensional PAGE—The procedure followed was as previously described (24). Cells were solubilized in 200 µl of lysis buffer containing 9.5 M urea (Bio-Rad), 2% Nonidet P-40, 2% β-mercaptoethanol, 2% carrier ampholytes, pH 4–8 (Gallard/Schlessinger, Carle Place, NY), and 10 mM phenylmethanesulfonyl fluoride. Aliquots containing 5 × 10⁶ cells were applied onto isofocusing gels. Isoelectric focusing was conducted using pH 4–8 carrier ampholytes at 700 V for 16 h, followed by 1000 V for an additional 2 h. The first dimension gel was loaded onto the second dimension gel, after equilibration in 125 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 1% dithiothreitol, and bromphenol blue. For the second dimension separation, a gradient of 11–14% of acrylamide (Serva; Crescent Chemicals, Hauppauge, NY) was used. Gels were then either silver-stained or dried and exposed to an x-ray film. The gels were digitized at 1024 × 1024-pixel resolution using an Eastman Kodak Co. CCD camera. Spots were detected and quantified with Visage software (Genomic Solutions, Ann Arbor MI) as described (25).

RNA Isolation and Polysome Fractionation—Total RNA was isolated using Trizol reagent (Invitrogen) and quantitated by absorbance at 260 nm. Cytoplasmic RNA was obtained by lysing cells in 1 ml of polysome buffer containing 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40, and a ribonuclease inhibitor, RINasIn (500 units/ml; Promega, Madison, WI). After the removal of nuclei, the cytosolic supernatant was supplemented with 150 µg/ml cycloheximide, 665 µg/ml heparin, 20 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride. Mitochodria and membrane debris were removed by centrifugation, and postmitochondrial supernatant was applied directly to sucrose gradient for polysome separation as described previously (26). Briefly, 1 ml of postmitochondrial supernatant was overlaid onto a 15–40% sucrose gradient and spun at 38,000 rpm for 2 h at 4 °C in a SW41Ti rotor (Beckman Instruments, Inc.). Fractions (500 µl) were collected from the bottom of each gradient and deproteinized with 100 µg of proteinase K in presence of 1% SDS and 10 mM EDTA. After Trizol extraction, the amount of RNA in each fraction was determined photometrically, and RNA integrity was controlled by electrophoresis analysis on denaturing 1.2% formaldehyde-agarose gels and subsequent Northern blot. After RNA transfer to nylon membranes (GeneScreen; PerkinElmer Life Sci-
ences) and UV cross-linking, the distribution of 18 and 28 S rRNAs was visualized by methylene blue staining of the membranes (see Fig. 2). Fractions 10–19 and fractions 1–9 corresponding to polysome-bound and nonpolysome RNA, respectively, were pooled from each sucrose gradient according to the distribution profile. Poly(A+/-H11001) RNA was isolated from total and polysome-bound RNA by using oligo(dT) resin (Oligotex; Qiagen, Chatsworth, CA).

Preparation of cRNA, Gene Chip Hybridization, and Data Analysis—Preparation of cRNA, hybridization, and scanning of the HuGeneFL arrays were performed according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA) and as previously described (27). Briefly, 5 μg of poly(A+) from both total and polysome-bound RNA were converted into double-stranded cDNA by reverse transcription using a cDNA synthesis kit (Superscript Choice System; Invitrogen). Following second

| GenBank™ accession no. | Gene description | -fold change |
|------------------------|------------------|-------------|
| Up-regulated |
| Signaling/Growth control |
| Z36714 | Cyclin F | ~11.6 |
| U47414 | Cyclin G2 | 6.1 |
| U41804 | IL-1 receptor-like 1 ligand | ~4.6 |
| U53174 | RAD9 | ~3.8 |
| X61123 | BTG1 | ~3.4 |
| U68485 | Bridging integrator 1 | ~2.9 |
| U53822 | MAD 1-like 1 | 2.7 |
| Z15065 | CENPE | 2.6 |
| Z22630 | Protein-tyrosine kinase Syk | 2.4 |
| X77909 | Iκ-B-like 1 | 2.1 |
| Nuclear proteins |
| U15655 | Ets2 repressor factor | ~3.3 |
| U64675 | BS-63 | 2.5 |
| Membrane proteins |
| X83492 | Fas/Apo-1 | 3.3 |
| M58286 | Tumor necrosis factor receptor | ~3.2 |
| U97502 | Butyrophilin | 3.1 |
| X7351 | Interferon-induced transmembrane protein 2 | 2.5 |
| M62762 | Vacular H+ ATPase proton channel subunit | 2.1 |
| Metabolism/Structure |
| Z14093 | Branched chain decarboxylase α-subunit | 6.8 |
| L60704 | Dynamin 1 | ~5.9 |
| Down-regulated |
| Signaling/Growth control |
| D13639 | Cyclin D2 | ~3.3 |
| AB000449 | Vaccinia related kinase 1 (VRK1) | ~2.5 |
| M15353 | Translation initiation factor 4E (eIF4E) | ~2.4 |
| AB003103 | 26 S proteosome subunit p55 | ~2.4 |
| D21090 | RAD23B | ~2.3 |
| X83368 | Phosphatidylinositol 3-kinase γ | ~2.3 |
| AB003698 | Cdc7-related kinase | ~2.1 |
| D11428 | Peripheral myelin protein 22 (PMP22) | ~2.1 |
| M74524 | Ubiquitin-conjugating enzyme E2A | ~2.1 |
| AB003102 | 26 S proteosome subunit p44.5 | ~2 |
| AB000177 | 26 S proteosome subunit p27 | ~2 |
| Nuclear proteins |
| M85085 | Cleavage stimulation factor CSTF2 | ~3 |
| Y12393 | Karyopherin α-4 | ~2.9 |
| D88003 | RAP250 | ~2.6 |
| AB000468 | Ring finger protein 4 | ~2.3 |
| D87448 | Topoisomerase (DNA) II-binding protein | ~2.3 |
| X79200 | Synovial sarcoma, X breakpoint 2 (SSX2) | ~2.3 |
| U80669 | NKX3A | ~2.1 |
| Secreted proteins |
| M60278 | Heparin-binding EGF-like growth factor (DTR) | ~2 |

TABLE I
Transcriptionally regulated mRNAs in rapamycin-treated T cells
Rapamycin-modulated genes were classified according to their known function and -fold change and represented in clusters containing functionally related genes. ~, -fold change calculation for which the smaller value is replaced by an estimate of the minimum value for detectable transcripts.
and RNA transcript levels for different genes were determined using oligonucleotide arrays. Transcripts for ~2,800 genes (44%) of the 6,300 unique genes assessed were expressed in Jurkat T cells. We identified a small subset of genes (51) that differed in their expression levels during rapamycin treatment, by 2-fold or greater, in both experiments. The genes identified are presented in Table I, with 19 up-regulated and 32 down-regulated genes. Regulated genes included several growth-related genes that may contribute to the antiproliferative effect of rapamycin. Indeed, negative regulators of cell growth such as cyclin G2, MADI-like 1, BTG1, bridging integrator 1, Syk, and CENPE were up-regulated, with a concomitant decrease in genes involved in cell cycle progression such as cyclin D2, Cdc7-related kinase, phosphatidylinositol 3-kinase γ, CSTF2, and eIF4E. Up-regulation of IκB-like 1, Fas, and tumor necrosis factor receptor was also observed. Remarkably, expression of three subunits of the 26S proteasome was decreased.

Identification of Translationally Regulated Genes by Rapamycin, Using Oligonucleotide Arrays—To identify genes whose expression is translationally regulated, we combined a sucrose gradient separation of polysomes from mRNPs with microarray analysis. Polysome-bound mRNAs (Fig. 2, fractions 10–19) were purified from Jurkat cells untreated or treated with rapamycin for 4 h, and poly(A)− mRNAs were isolated. Two independent experiments were performed, and polysome-bound RNA transcript levels were determined using oligonucleotide arrays. Translation of the large majority of the genes was partially reduced following rapamycin treatment. However, translation of 136 genes was strongly inhibited (by 90% or more) in both experiments (Table II). Genes known to be highly repressed by rapamycin changed their expression accordingly in our analysis. This group included numerous ribosomal proteins and elongation factor proteins. However, for most of the 136 genes uncovered, their high sensitivity to rapamycin was unknown. These novel changes included other RNA-binding proteins such as translation initiation factors 4A and 5A and four genes encoding for nuclear ribonucleoproteins. Remarkably, translation of seven genes encoding proteasome subunits was fully inhibited following rapamycin treatment. Translation of prothymosin α, a gene associated with proliferation of T cells, was also strongly repressed by rapamycin. Microarray analysis of the non-polysome gradient fractions (Fig. 2, fractions 1–9) were also performed for both experiments and demonstrated that the 136 strongly repressed transcripts were not lost or degraded during rapamycin treatment or polysome separation.

Transcripts levels for 159 genes remained bound to polysome following rapamycin treatment, suggesting that translation of these genes was not affected by rapamycin. Table III lists the genes whose mRNAs were associated with polysomes from both untreated and rapamycin-treated cells. Notably, this list includes mRNAs encoding a large number of kinases and phosphatases as well as DNA-binding proteins. Transcription factors and genes involved in DNA and RNA synthesis included AR1, TFID, TIFFE, TFIIF, E2F, c-MYB, YY1, CREBP1, HSF1, Rb1, ILF1, LIM domain only 4, RNA polymerase II, DNA polymerase α-subunit, and replication factors C1 and C5. Translation of several genes encoding for kinases and phosphatases, such as four members of the mitogen-activated protein kinase family, the PI-3 kinase regulatory subunit, protein kinase C-ε, p72vck, and protein phosphatases 1, 2, and 4, was unaffected by rapamycin. Finally, transcripts for nine members of the Ras superfamily including N-Ras, Rap1a, Rap1b, Rap4, Rap5c, Rac1, and RhoG remained bound to polysomes in rapamycin-treated cells.
### Table II

| GenBank™ accession no. | Gene description | GenBank™ accession no. | Gene description |
|------------------------|------------------|------------------------|------------------|
| **Signaling/Growth control** | | | |
| D00761 | Proteasome subunit, β type, 1 | Z26876 | Ribosomal protein L38 |
| D26598 | Proteasome subunit, β type, 3 | Z12962 | Ribosomal protein L41 |
| D26600 | Proteasome subunit, β type, 4 | M14199 | Lamin receptor 1 (67 kDa, ribosomal protein SA) (LAMR1) |
| D29012 | Proteasome subunit, β type, 6 | X56997 | Ubiquitin ribosomal protein fusion product 1 |
| D38048 | Proteasome subunit, γ type, 7 | D13748 | Translation initiation factor 4A, isoform 1 (eIF4A1) |
| D00763 | Proteasome subunit, α type, 4 | J04617 | Translation elongation factor 1 α 1 |
| X59417 | Proteasome subunit, α type, 6 | X03689 | Translation elongation factor TU |
| D38047 | Proteasome 2S subunit, non-ATPase, 8 | X12517 | Small nuclear ribonucleoprotein polypeptide C (snRNP) |
| D23662 | Ubiquitin-like protein | U15008 | Small nuclear ribonucleoprotein D2 (snRNP2) |
| M31469 | RAN, RAS oncogene family member | M25372 | Small nuclear ribonucleoprotein polypeptide F (snRNP) |
| L20688 | Rho GDP dissociation inhibitor | D13413 | Heterogeneous nuclear ribonucleoprotein U (hnRPU) |
| L32866 | Apoptosis inhibitor 4 (survivin) | D28423 | Pre-mRNA splicing factor SRp20 |
| M31303 | Oncoprotein 18 (Op18), stathmin | X71428 | Fus |
| M22382 | Heat shock 60-kDa protein 1 | M68058 | Nucleolin |
| X15183 | Heat shock 90-kDa protein 1, α | M25613 | Nuclearum (nucleolar phosphoprotein B23, numatin) |
| J04988 | Heat shock 90-kDa protein 1, β | U50839 | RNA binding motif protein 6 |
| U48296 | Protein-tyrosine phosphatase, type IVA, | | |
| X52479 | Protein kinase C | | |
| M17733 | Thymosin β-4 | | |
| X52851 | Cyclophilin A | | |
| **RNA metabolism** | | | |
| M17885 | Ribosomal protein, large, P0 | U09477 | p53-binding protein |
| X17206 | Ribosomal protein S2 | M14483 | Prothymosin α member 1 |
| M54711 | Ribosomal protein S3A | D17268 | Wiln tumor-related protein |
| X55715 | Ribosomal protein S3 | J05614 | Proliferating cell nuclear antigen (PCNA) |
| E8876 | Casein kinase II | D16851 | S-Oxo-dGTPase |
| M52668 | MAP kinase kinase kinase kinase 5 (MAPK5) | U96915 | Sin3-associated polypeptide p18 (SAP18) |
| U77129 | MAP kinase kinase kinase 5 | D63874 | High-mobility group, protein 1 (HMGI) |
| M84332 | ADP-ribosylation factor 1 | D21205 | Zinc finger protein 147 |
| L38490 | ADP-ribosylation factor 4-like | U86602 | Nucleolar protein |
| M17733 | Thymosin β-4 | U18271 | Thymopoietin |
| X52851 | Cyclophilin A | | |
| **Cell surface proteins** | | | |
| M21498 | Guanine nucleotide-binding protein (G protein), β polypeptide 2-like 1 | M21498 | \(5^±\)-binding protein |
| D15057 | Defender against cell death 1 (DAD1) | S71824 | Neural cell adhesion molecule 1 (NCAM1) |
| D29963 | CD151 | CD151 | |
| M31525 | Major histocompatibility complex, class II, DN α (HLA-DNA) | M31525 | Major histocompatibility complex, class II, DN α (HLA-DNA) |
| D49824 | Major histocompatibility complex, class I, B (HLA-B) | U11370 | Proteasocerin 1 (cathderin-like 1) |
| **Secreted proteins** | | | |
| S86297 | β-2-Microglobulin | D14838 | Fibroblast growth factor 9 (FGF9) |
| U50839 | RNA binding motif protein 6 | M37435 | Colony-stimulating factor 1 (CSF1) |
| **Metabolism/Structure** | | | |
| M13934 | Laminin receptor 1 (67 kDa, ribosomal protein SA) | X73460 | Ribosomal protein L3 |
| L11566 | ATP synthase | | |
| L39522 | ATP synthase | | |
| L19739 | ATP synthase | | |
| U14971 | Ribosomal protein S9 | | |
| U19147 | Ribosomal protein S10 | | |
| L01124 | Ribosomal protein S13 | | |
| M58458 | Ribosomal protein S4, X-linked | | |
| U14970 | Ribosomal protein S5 | | |
| M77232 | Ribosomal protein S6 | | |
| Z25749 | Ribosomal protein S7 | | |
| X57247 | Ribosomal protein S8 | | |
| U14971 | Ribosomal protein S9 | | |
| U14972 | Ribosomal protein S10 | | |
| L01124 | Ribosomal protein S13 | | |
| M58458 | Ribosomal protein S4, X-linked | | |
| U14970 | Ribosomal protein S5 | | |
| M77232 | Ribosomal protein S6 | | |
| Z25749 | Ribosomal protein S7 | | |
| X57247 | Ribosomal protein S8 | | |
| **Translationally repressed mRNAs in rapamycin-treated T cells** | | | |
| **Not classified** | | | |
| D21261 | KIAA0120 | | |
| D31885 | KIAA0069 | | |
| D23673 | KIAA0864 | | |
| D23673 | KIAA0864 | | |

Translational Control by Rapamycin

22179
### TABLE III
Translational Control by Rapamycin

| GenBank™ accession no. | Gene description | GenBank™ accession no. | Genesescription |
|------------------------|------------------|------------------------|----------------|
| **Signaling/Growth control** | | | |
| X16901 | General transcription factor II F, polypeptide 2 |
| M64673 | Heat shock transcription factor 1 (HSF1) |
| M77988 | Transcription factor YY1 |
| M98853 | Transcription factor FLI-1 |
| U24576 | Translation factor LIM domain only 4 |
| M13666 | c-Myb |
| X15875 | ATF2/CREBP1 |
| M19701 | Retinoblastoma 1 |
| X64229 | DEK oncoprotein  |
| U15655 | Ets2 repressor factor |
| X26039 | Centeronin |
| M11507 | Transcription factor 20 (AR1) |
| X05276 | Tropomyosin 4 |
| M37197 | CCAAT-box-binding transcription factor |
| M11058 | 3-Hydroxy-3-methylglutaryl-coenzyme A reductase |
| X83928 | Transcription factor TFIID |
| X01691 | Annexin V |
| M34181 | cAMP-dependent protein kinase catalytic subunit |
| U58522 | Ubiquitin-conjugating enzyme E2D 1 |
| X65362 | Sodium channel 1 isoform 2 |
| D88378 | Proteasomal inhibitor subunit 8 |
| X78140 | Ubiquitin-conjugating enzyme E2 |
| M74091 | Cyclin C |
| X61587 | Rho G |
| M19845 | Bip/GPR78 |
| D38873 | Protein-tapsole phosphatase, non-receptor type 2 |
| X78140 | Ubiquitin-conjugating enzyme E2D 1 |
| M38282 | Sdc-c4 like |
| X82554 | S-phase response (cyclin-related) |
| Y08915 | Irunamgulin binding protein 1 |
| D90070 | RNA-binding protein 1 Nova |
| U18242 | Calcium-modulating cyclophilin ligand |
| D26069 | Centaurin β 2 |
| U16811 | BCL2-antagonist factor 1 (BAX1) |
| S78085 | Programmed cell death 2 |
| U85410 | Mad2 |
| X17576 | NCK-α |
| U40038 | GTP-binding protein α q |
| U23435 | Abl-binding protein 3 |
| Z38851 | S100 calcium-binding protein A10 |
| RNA metabolism | | | |
| X64707 | Ribosomal protein L13 |
| U23946 | RNA binding motif protein 5 |
| U26032 | Translation initiation factor eIF-2α |
| L19161 | Translation initiation factor eIF-2γ |
| U94855 | Translation initiation factor 3 subunit 5 |
| M75715 | Translation termination factor 1 |
| X95384 | Translation inhibitor protein p14.5 |
| X85237 | Splicing factor 3a, subunit 1 (SAP14) |
| M90104 | Splicing factor SC35 |
| U77664 | Ribonuclease P |
| M67468 | Fringe X mental retardation 1 (FRMI) |
| Y11651 | RNA 3′-terminal phosphate cyclase |
| **Nuclear proteins** | | | |
| Y08765 | Zinc finger protein 162 |
| U09825 | Zinc finger protein 173 |
| U37251 | Zinc finger protein 177 |
| X95808 | Zinc finger protein 261 |
| X38779 | Zinc finger protein, X-linked |
| M37197 | CCAT2-box-binding transcription factor |
| U19345 | Translation factor 20 (AR1) |
| X83928 | Translation factor TFID |
| X63469 | Translation factor TFIIE β |
| U15641 | Translation factor E2F4 |

**Translationally unaffected mRNAs in rapamycin-treated T cells**

- **Membrane proteins**
  - Z17227 | Interleukin-10 receptor, β |
  - M58286 | Tumor necrosis factor receptor |
  - X68397 | TPR (translocated) |
  - U05237 | Fetα/APO-1 |
  - U14680 | BRCA1 |
  - U17898 | Nuclear autoantigen |
  - X86998 | Adenovirus 5 E1a-binding protein |
  - U90547 | Ro/SBA ribonucleoprotein homolog |
  - X85133 | Retinoblastoma-binding protein 6 |
  - D80000 | SMC1-like 1 |

- **Secreted proteins**
  - M32904 | Metalloproteinase inhibitor 2 |
  - U41745 | Platelet-derived growth factor-associated protein 1 |

- **Metabolism/Structure**
  - M98045 | Polyproline synthase |
  - M34335 | Spermidase synthase |
  - X09825 | Sphingosine synthase |
  - M11507 | Transferrin receptor (CD71) |
  - X64647 | T cell receptor α |
  - U51857 | Golgi autoantigen |
  - U51840 | Lysozyme-associated membrane protein 2 |
  - U01691 | Annexin V |
  - X65362 | Sodium channel 1 |
  - U18009 | Vesicle amine transporter protein 1 |
  - S98753 | Lysosomal-associated membrane protein 2 |
  - X92396 | Synaptophysin 1 |
  - X68194 | Synaptophysin-like protein |
  - X92098 | Repression 5-phosphate isomerase |
  - L25441 | Protein geranylgeranyltransferase type I |

- **RNA metabolism**
  - X64707 | Ribosomal protein L13 |
  - U23946 | RNA binding motif protein 5 |
  - U26032 | Translation initiation factor eIF-2α |
  - L19161 | Translation initiation factor eIF-2γ |
  - U94855 | Translation initiation factor 3 subunit 5 |
  - M75715 | Translation termination factor 1 |
  - X95384 | Translation inhibitor protein p14.5 |
  - X85237 | Splicing factor 3a, subunit 1 (SAP14) |
  - M90104 | Splicing factor SC35 |
  - U77664 | Ribonuclease P |
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- **Nuclear proteins**
  - Y08765 | Zinc finger protein 162 |
  - U09825 | Zinc finger protein 173 |
  - U37251 | Zinc finger protein 177 |
  - X95808 | Zinc finger protein 261 |
  - X38779 | Zinc finger protein, X-linked |
  - M37197 | CCAT2-box-binding transcription factor |
  - U19345 | Translation factor 20 (AR1) |
  - X83928 | Translation factor TFID |
  - X63469 | Translation factor TFIIE β |
  - U15641 | Translation factor E2F4 |
Of the 19 mRNAs whose intracellular levels increased in rapamycin-treated cells, five (cyclin F, Ets2 repressor factor, Apo-1/Fas, tumor necrosis factor receptor, and Syk) were found to be greatly enriched in the polysomal fractions from rapamycin-treated cells.

**Proteomic Profiling of Rapamycin-treated T Cells**—Protein changes during rapamycin treatment of the Jurkat T cells were investigated by proteomics. Metabolic labeling was performed in untreated and rapamycin-treated Jurkat cells, and equal amounts of total [35S]methionine-labeled proteins were separated by two-dimensional gel electrophoresis. Following exposure to films, the autoradiograms were digitized, and two-dimensional protein patterns were matched by computer analysis. In this study, 830 protein spots were matched and quantitated. Whereas the overall two-dimensional patterns of untreated and rapamycin-treated cells were largely similar, some protein changes were reproducibly detected. We selected protein spots whose intensities changed in all experiments by 3-fold or greater in response to rapamycin. A set of 111 protein spots was identified, with 70 up-regulated and 41 down-regulated by rapamycin. Interestingly, prothymosinα was strongly repressed. Translation of prothymosinα was also strongly repressed by rapamycin. Interestingly, prothymosinα has been reported to enhance cell-mediated immunity as well as proliferative and cytotoxic responses of T cells (30–33). In vivo, prothymosinα has been shown to exert a potentiating effect on human CD4+ T cell proliferation in response to antigens, which was associated with a prothymosin-induced increase in IL-2 production. It was also demonstrated that prothymosinα, in combination with IL-2, can render cell to cell interactions more effective, resulting in increased killing of autologous tumors (34).

Remarkably, translation of seven genes encoding proteasome subunit members was abolished, which would explain in molecular terms the reported inhibition of proteasome activator expression and proteasome activity by rapamycin (35). The proteasome-mediated degradation pathway regulates a wide variety of cellular activities, including cell growth and immune and inflammatory responses. Within the immune system, the proteasome is essential for production of peptides for major histocompatibility complex class I antigen presentation. More recent studies have suggested a possible role for the proteasome in regulating the levels of cell surface receptors. In particular, a functional proteasome is required for optimal endocytosis of the IL-2 receptor-ligand complex and is essential for the subsequent lysosomal degradation of IL-2, possibly by regulating trafficking to the lysosome (36). In addition, several studies have implicated the proteasome in the regulation of Jak-STAT signal transduction, including IL-2-induced activation of STAT5 (37, 38). Adhesion molecules are essential in interaction between T cells and antigen-presenting cells, between T help cells and T effector cells, and between T cells and endothelial cells. It has been recently demonstrated that proteasome inhibitors repress T lymphocyte aggregation and then potentially cell-cell interactions in the immune system (39). Finally, a role of proteasomes in T cell activation, proliferation, and apoptosis has been reported (40, 41) including a requirement of the proteasome activity for T cells to progress from the G0 to S phase. Most interestingly, inhibition of proteasome activity is a common feature of immunosuppressant drugs such as cyclosporin A and FK506 (42). This raised the intriguing possibility that the proteasome is one of the common downstream targets of these drugs. In addition, our data elucidated the mechanisms by which rapamycin is inhibiting the expression of some proteasome proteins. Therefore, we identified important downstream targets of rapamycin such as prothymosinα and proteasome subunits that may modulate the immune response following rapamycin treatment and mediate the immunosuppressive effects of this drug.

**DISCUSSION**

To develop a better understanding of rapamycin’s molecular mechanism in T cells, we utilized two complementary approaches to identify specific genes regulated by rapamycin in T cells. One relies on the quantitative analysis of translated mRNAs by DNA microarrays. The other relies on quantitative analysis and identification of proteins by proteomics. In addition, we quantitated polysome-bound mRNAs as a measure of their translation efficiency (29). Ribosomal proteins and elongation factors contain a polypyrimidine tract at the 5'-end of their mRNAs and have been described as translationally repressed by rapamycin (17). Indeed, translation of a large number of ribosomal proteins and elongation factors was found to be strongly repressed by rapamycin in our study. We have uncovered a large number of additional genes. Part of the regulated genes have functions related to RNA processing and translation. Translation initiation factors 4A and 5A were strongly repressed. Translation of prothymosinα was also strongly repressed by rapamycin. Interestingly, prothymosinα has been reported to enhance cell-mediated immunity as well as proliferative and cytotoxic responses of T cells (30–33). In vivo, prothymosinα has been shown to exert a potentiating effect on human CD4+ T cell proliferation in response to antigens, which was associated with a prothymosin-induced increase in IL-2 production. It was also demonstrated that prothymosinα, in combination with IL-2, can render cell to cell interactions more effective, resulting in increased killing of autologous tumors (34).

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**TABLE III—continued**

| GenBank accession no. | Gene description |
|-----------------------|-----------------|
| U11313                | Sterol carrier protein 2 |
| M27891                | Cystatin C |
| M14219                | Decorin |
| M20469                | Chitin, light polypeptide |
| D67120                | Predichotost bone protein |
| X79537                | Glycogenin |
| U77718                | Desmosome-associated protein pinin |
| L19783                | Phosphatidylinositol glycan, class H |
| Z22551                | Kinesin receptor |
| U55946                | Secretory protein Sec10-like 1 |
| U53115                | β-crystallin-like protein |
| X90905                | UV radiation resistance-associated gene |
| D87459                | WASP family, member 1 |
| Not classified         | |
| M92439                | Leucine-rich protein |
| X99961                | Novel protein (HSNOV1) |

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Translation of the majority of eukaryotic mRNAs is initiated through a cap. Some mRNAs, however, are translated by a cap-independent mechanism, mediated by ribosome binding to internal ribosome entry site (IRES) elements located in the 5'-untranslated region. So far, only a handful of cellular IRES have been described (43). We previously demonstrated that rapamycin inhibits specifically cap-dependent translation, whereas cap-independent translation is unaffected or slightly increased (12, 20). We identified 159 genes that are still translated in the presence of rapamycin. These genes are candidates for IRES-driven mRNAs. Remarkably, these genes included...
FIG. 3. Two-dimensional profiles of T cells. A, up-regulated (white arrows) and down-regulated (black arrows) protein spots are reported on a representative silver-stained two-dimensional gel corresponding to the protein expression profile in rapamycin-treated T cells. These results are representative of three independent experiments. B, close-up sections of [35S]methionine protein labeling two-dimensional gels from untreated (left panel) and rapamycin-treated (right panel) T cells, corresponding to boxed sections in A, are shown for comparison.
translational control by rapamycin.

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