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Rapamycin Destabilizes Interleukin-3 mRNA in Autocrine Tumor Cells by a Mechanism Requiring an Intact 3′ Untranslated Region

ROLF BANHOLZER, ASHA P. K. NAIR, HANS H. HIRSCH, XIU-FEN MING, AND CHRISTOPH MORONI*

Institute for Medical Microbiology, University of Basel, 4003 Basel, Switzerland

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We analyzed the effect of rapamycin on autocrine mast cell tumor lines with abnormally stable interleukin-3 (IL-3) transcripts due to a defect in mRNA degradation. Rapamycin inhibited IL-3 mRNA expression specifically, while transcripts of IL-4 and IL-6 were not affected. As indicated by the use of the transcriptional inhibitor actinomycin D or by reporter constructs, inhibition was posttranscriptional and resulted from destabilization of the mRNA. Transcripts from transgenes lacking the AU-rich 3′ untranslated region were refractory to drug-induced degradation, suggesting that these 3′ sequences contain the target of the rapamycin effect. Rapamycin did not promote IL-3 mRNA degradation in cells of a tumor variant lacking expression of FKBP12, the binding protein of rapamycin. Experiments with wortmannin indicated that rapamycin does not act via p70S6 kinase. FK-506, another ligand of FKBP12 affecting the phosphatase calcineurin, did not antagonize but shared the effect of rapamycin. Our data fit a model whereby both FKBP12 and calcineurin target an unknown regulator of IL-3 mRNA turnover.

Lymphokines and their related signal transduction pathways play an important role in the control of cell activation, proliferation, and oncogenesis. These processes can be conveniently studied in murine PB-3c mast cells, which are interleukin-3 (IL-3) dependent but progress to autocrine IL-3-producing tumors after transformation by the v-H-*ras* oncogene (35). In primary mast cells, IL-3 production follows activation of the immunoglobulin E receptors by a mechanism involving a rise in the intracellular calcium concentration (44, 57). Treatment of PB-3c mast cells with calcium ionophores induces IL-3 expression by stabilization of the intrinsically short-lived mRNA (58). IL-3 transcripts carry in their 3′ untranslated region (3′UTR) an AU-rich element (ARE) directing in *cis* rapid deadenylation and degradation (see reference 11 for references). The ARE of IL-3 contains eight canonical AUUUA motifs (9), generating two consensus nonamers [UUAUUUA(U/A)(U/A)] involved in the decay process (28, 59). Mutagenesis of three critical AUUUA motifs of an IL-3 transgene rendered the transcripts stable in mast cells and at the same time abolished induction by calcium, indicating that stabilization by calcium involves inactivation of ARE-directed mRNA degradation (52).

Stabilization of IL-3 mRNA was also found to play a role in the oncogenic transformation of mast cells by the v-H-*ras* oncogene, as suggested by previous work in which we identified PB-3c-derived autocrine tumor lines with a defect in IL-3 mRNA degradation (21). The underlying mechanism is not known, but it appears to be recessive in nature. Accordingly, fusion of tumor cells with parental PB-3c cells downregulated IL-3 expression, restored rapid IL-3 mRNA decay, and led to partial tumor suppression (15, 20a, 22). Furthermore, mRNA from transfected IL-3 constructs decayed slowly in tumor cells, but rapidly in parental cells (21). Taken together, these findings suggested that during tumor progression, a tumor suppressor gene that functions to promote IL-3 mRNA decay has become defective. Evidence for a similar oncogenic mechanism has been previously provided by Schuler and Cole (51) with an autocrine tumor line of monocyte origin. In these cells, granulocyte-macrophage colony-stimulating factor mRNA decayed with very slow kinetics, and transfection experiments similarly revealed that the defect occurred in *trans*.

We have recently reported that the abnormally stable IL-3 transcripts expressed in mast cell tumor lines with a decay defect became destabilized upon treatment with the immunosuppressive drugs cyclosporine (CsA) and FK-506. Transfection experiments with IL-3 transgenes revealed that deletion of ARE-containing 3′UTR sequences rendered the transcripts drug resistant, indicating that sequences at or near the ARE are a target of the effects of CsA or FK-506 (36).

In T cells, CsA, upon forming a complex with cyclophilin A or B, binds to and inhibits the calcium-activated Ser-Thr phosphatase calcineurin. FK-506, complexed to FKBP12, similarly inhibits calcineurin. This antagonizes the translocation of the transcription factor NFAT to the nucleus, a prerequisite for induction of IL-2 transcription upon antigenic stimulation (4, 14, 18, 31, 42). Rapamycin (RAPA), a third immunosuppressive drug, suppresses T cells not by affecting IL-2 transcription, but by blocking the cell cycle in G_1 (2, 17, 32) and by inhibiting translation (3, 7, 23, 39, 41, 54). RAPA shares the binding protein FKPB12 with FK-506, but the complex inhibits another target, called FRAP or RAFT (5, 49), the mammalian homolog of TOR (mTOR), a kinase originally identified in yeast cells (26). A downstream target of FRAP/RAFT is the p70S6 kinase (S6K), a regulator of translation and an important yet indirect target of RAPA inhibition (6, 13, 27, 45). RAPA inhibits preferentially the translation of mRNA species with a 5′ polypyrimidine track (23, 54). It also inhibits serum-induced phosphorylation of 4E-BP1, a binding protein of initiation factor eIF-4E, which blocks initiation of translation (3, 23, 30, 56).

Because FK-506 and RAPA exert their differential effects via the common binding protein FKBP12, excess of either drug

* Corresponding author. Mailing address: Institute for Medical Microbiology, University of Basel, Petersplatz 10, 4003 Basel, Switzerland. E-mail: moroni@ubaclu.unibas.ch.

has been reported to outcompete the effect of the other (4, 16). In tumor lines, however, we have unexpectantly found that RAPA does not antagonize but rather shares the destabilizing effect of FK-506. We therefore examined the mechanism by which RAPA downregulates IL-3 mRNA in those tumor lines. Here, we report that the drug acts at the posttranscriptional level, involves the AU-rich 3′UTR, but is independent of inhibition of S6K. Furthermore, RAPA was inactive in a tumor variant lacking FKBP12 expression.

MATERIALS AND METHODS

Cells. V2D1, V3D6, V3D8, and 15V4T2 are autocrine tumor lines derived from PB-3c mast cells expressing the v-H-*ras* oncogene after inoculation into syngeneic mice (35, 36). V2D1-M1 and V2D1-M1ΔAU are derived by electroporation of V2D1 with the constructs pIL3-M1 and pIL3-M1ΔAU, respectively (see below). X63-IL-3 cells were used as a source of IL-3 (24). HB 10652 (obtained from the American Type Culture Collection) is an anti-IL-3-secreting hybridoma cell line (1).

Constructs. pIL3-M1 is an 8.6-kb genomic IL-3 construct including 5.5 kb of 5′ flanking sequence (9, 36). The construct contains all of the 5′ and 3′ flanking sequences necessary for ionomycin induction and stabilization of the transcripts. In exon 3, two silent point mutations have been introduced as described previously (36) to make exogenous transcripts distinguishable from endogenous transcripts by an RNase A-T_1 protection assay. pIL3-M1ΔAU, lacking the ARE, was generated from pIL3-M1 by a 216-bp *Nco*I/*Sty*I deletion in the 3′UTR, containing eight AUUUA boxes (36). Both constructs carry the cDNA for *hph*, a gene selectable by hygromycin B that was also used as an internal control for measuring gene expression.

To generate the alkaline phosphatase (AP) reporter constructs containing the 5′UTR, the secretory signal sequence, the 3′-UTR, and the polyadenylation signal of IL-3, the pSEAP-Basic vector (Clontech) was modified in the following way. The pSEAP-Basic plasmid DNA was used as a template, and a PCR fragment with a size of 267 bp was generated with the primers M982 (5′-ACA AGCTTCCCTGGGCATCAT-3′) and M981 (5′-ATTGTATGTCTTGGACAG AG-3′). Primer M982 introduced a novel *Hin*dIII site which allowed the replacement of the sequence encoding the secretory leader of the AP with the one from IL-3 by ligation to the *Hin*dIII site present in the first exon of the IL-3 gene. The PCR fragment was cut with *Hin*dIII and *Nde*I and replaced the corresponding fragment in the pSEAP-Basic vector, yielding pMut1-AP. The *Bgl*II fragment of the genomic murine IL-3 gene containing the the 3′UTR and the polyadenylation signal was placed into the *Cla*I site downstream of the AP gene by blunt-end ligation (pMut1-AP-wtIL3). Subsequently, the *Hin*dIII-*Xba*I fragment containing the AP coding region and part of the fifth exon of IL-3 was taken to replace the genomic IL-3 gene in the hygromycin-selectable vectors pMx-IL3 and pMx-ΔAUIL3 (52), yielding pMx-AP-wtIL3 and pMx-AP-ΔAUIL3. Replacement of the Moloney murine leukemia virus enhancer with the 1.9-kb *Hin*dIII fragment containing the IL-3 promoter (9) yielded pPIL3-AP-wtIL3. The AP probe was obtained by SP6 transcription with the PCR fragment obtained with the primers M982 and M1004 (5′-CGATTTAGGTGACACTATAGAATGTCTGGCACAT GTTTGTCTAC-3′) with the pMut1-AP DNA as a template.

Methylcellulose cloning. Methylcellulose cloning was performed as previously described (35, 36). Briefly, cloning was performed in a 1-ml final volume in 35-mm-diameter tissue culture dishes. The cloning mixture contained complete Iscove's modified Dulbecco's medium (IMDM) complemented with 1.2% (wt/vol) methylcellulose, 1.0% (wt/vol) bovine serum albumin, 20% (vol/vol) fetal calf serum (FCS), 300 μg of iron-saturated human transferrin per ml, 1 mg of G418 per ml, and, where indicated, 0.8% conditioned medium of X63-IL-3 cells as an IL-3 source. The final concentrations of FK-506 and RAPA were 20 ng/ml of growth medium. Monoclonal anti-IL-3 antibodies were obtained from HB 10652 cells; 1% of the supernatant of confluent cells was used. For a control, normal mouse serum was used. Colony formation was scored after 10 to 15 days.

Suspension culture assay. Approximately 1,000 or 5,000 cells/ml were plated in 1 ml of complete IMDM supplemented with 10% (vol/vol) FCS in 24-well culture plates. Various concentrations of RAPA were added (0, 0.02, 0.2, 2, or 20 ng/ml of medium, respectively). Where indicated, exogenous IL-3 was added (0.8% [vol/vol]). The cell number was counted in duplicate at the indicated time points, and the average value was plotted.

Northern blot analysis. Approximately 10^5 cells/ml in 150 ml of complete IMDM supplemented with 10% FCS and IL-3 were incubated overnight with drug concentrations as indicated, usually at 20 ng/ml of growth medium. Cytoplasmic RNA was isolated by the method of Gough (19). Poly(A) mRNA was prepared with oligo(dT) cellulose, and hybridizations were performed as described previously (37). The following riboprobes were used: pSP65-multi-CSF, containing a 368-bp *Hin*dIII/*Xba*I IL-3 cDNA fragment (25), and pSPT18-IL-4, containing the 373-bp *Rsa*I IL-4 cDNA fragment (40). A random-primed probe for IL-6 and a riboprobe for chicken β-actin were prepared as described previously (36). Prior to rehybridization, filters were washed in 70% formamide for 30 min at 65°C, followed by rinsing in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature.

Quantification of the IL-3, AP, and β-actin mRNA hybridization signals was performed with a PhosphorImager (Molecular Dynamics) with ImageQuant software. The IL-3 and AP signals were normalized to the respective β-actin reference signal after subtraction of the filter background. The normalized IL-3 values at time zero of the time course were taken as 100%. The actin-normalized AP expression levels found with the IL-3 promoter (pPIL3-AP-wtIL3 construct in Fig. 4a) were set as 1.0. Correspondingly, the Moloney murine leukemia virus long terminal repeat (LTR)-driven AP constructs had values of 3.3 and 6.3 without and with the ARE deletion and 1.1 and 5.3 after RAPA treatment, respectively.

RNase A-T_1 protection assay. RNase A-T_1 protection assays were performed with tumor cells transfected with IL-3 constructs as described previously (36, 52). Twenty micrograms of cytoplasmic RNA was hybridized with a nonlimiting amount of each ^{32}P-labelled RNA probe at 45°C for at least 18 h. Nonhybridized RNA was digested with RNase A (40 μg/ml) and RNase T_1 (1,000 U/ml) at 25°C for 45 min.

Reverse transcription PCR. RNA (5 μg) isolated by the method of Gough (19) was reverse transcribed, and PCRs were performed as described previously (37), but with 1 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, and 20 pM primer as the final concentration. For FKBP12, we used 500 ng of cDNA, and for actin we used 5 ng. The amplification procedure was carried out at the following temperatures: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The actin primers were described in reference 34, and the FKBP12 primers were designed on the basis of reference 38: upstream primer (20-mer), GGAGTGCAGGTG GAGACCAT; downstream primer (20-mer), TCCAGTTTTAGAAGCTCCAC.

Kinase assay. For S6K assays, 20 μg of protein from cell lysates was immunoprecipitated with the S6K M5 antibody and assayed as described previously (29).

RESULTS

Effects of RAPA and FK-506 on tumor cell proliferation. To study the effects of RAPA on mast cell tumor lines with a defect in IL-3 mRNA degradation, we first analyzed cell proliferation and included FK-506 for comparison. Both drugs inhibited proliferation of the prototypic autocrine tumor mast cell line V2D1 (21, 35), as measured by colony formation in semisolid medium. By this technique, we confirmed that V2D1 cells grow via an autocrine loop, because anti-IL-3 antibody inhibited colony formation (Fig. 1a). While both RAPA and FK-506 were inhibitory in the absence of exogenous IL-3 (Fig. 1a), RAPA inhibited growth even in its presence (Fig. 1b). This inhibition of paracrine growth is consistent with the fact that RAPA blocks the cell cycle as well as postreceptor signalling of many cytokines (17, 47). A dose-dependent inhibitory effect of RAPA on autocrine (Fig. 1c) and paracrine (Fig. 1d) growth was also observed in suspension culture, where 2 ng/ml but not 0.2 ng/ml was effective.

RAPA inhibits IL-3 mRNA expression. To analyze the effect of RAPA on IL-3 expression, we examined IL-3 mRNA levels in V2D1 cells following overnight incubation. Figure 2a shows downregulation of IL-3 mRNA expression detectable already at a concentration of 0.05 ng/ml, i.e., at a slightly lower concentration than that needed to inhibit proliferation. This effect was confirmed with two other PB-3c-derived autocrine tumor lines, V3D6 and V3D8, which were also sensitive to FK-506 (Fig. 2b). Neither RAPA nor FK-506 affected the expression of two other lymphokines, IL-4 and IL-6, to a comparable degree (Fig. 2c). This indicated a mechanism specific for IL-3.

RAPA acts at the posttranscriptional level. CsA and FK-506 do not inhibit transcription of IL-3 in V2D1 cells but act at the posttranscriptional level (reference 36 and unpublished data). When we compared the decay rates of IL-3 mRNA in the presence of actinomycin D (Act D) or RAPA, we obtained $t_{1/2}$ values of 7 and 4 h, respectively, arguing for a posttranscriptional effect of RAPA. As reported for CsA (36), combination of RAPA with Act D abolished the destabilizing effect (data not shown). To seek corroborating evidence for a posttranscriptional mechanism, we designed experiments which might link the RAPA effect to the 3′UTR, a region known to regulate IL-3 mRNA decay via an ARE (52). Consequently, we tested

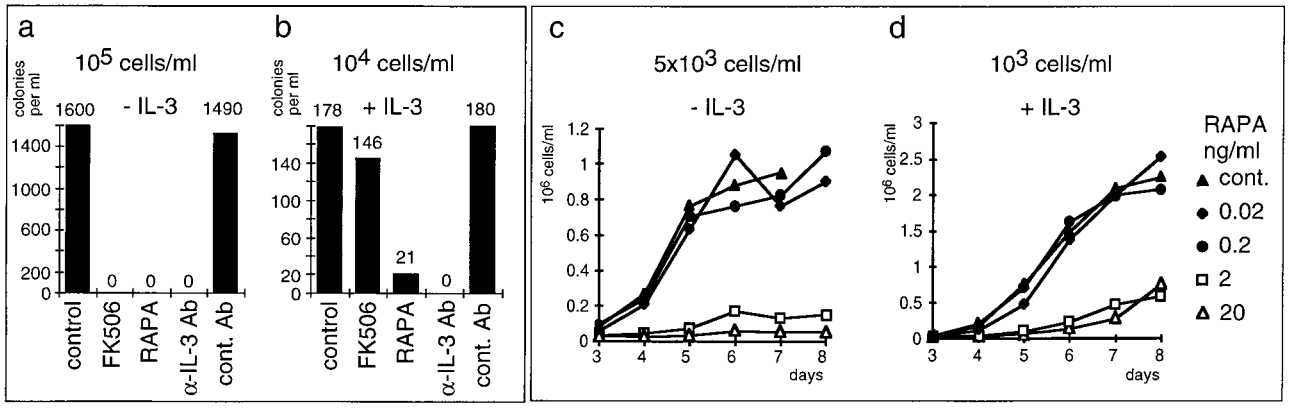

FIG. 1. RAPA and FK-506 inhibit cell proliferation. V2D1 cells were plated in methylcellulose (a and b) or in suspension culture (c and d) at the indicated concentrations. In panels b and d, the medium was supplemented with exogenous IL-3. FK-506 and RAPA were used at concentrations of 20 ng/ml (a and b) or as indicated (c and d). Colony formation was scored after 10 to 15 days of incubation. The dose-dependent inhibition of V2D1 growth in a 1-ml suspension culture is shown in panels c and d. In the absence of IL-3, 5,000 cells/ml were used as the initial concentration for optimal autocrine proliferation. The numbers above each bar in panels a and b represent the number of colonies per milliliter. cont., control; α, anti; Ab, antibody.

V2D1 cells transfected with genomic IL-3 wild-type sequences or with the same plasmid lacking the ARE; the resulting cell lines are called V2D1-M1 and V2D1-M1ΔAU, respectively. The constructs contain two silent point mutations in the third exon, which allows an RNase protection assay to distinguish endogenous from exogenous transcripts because the former generates a 368-bp fragment and the latter generates two fragments 209 and 168 bp in length. Following RAPA treatment, endogenous and exogenous wild-type IL-3 transcripts decayed with very similar kinetics (Fig. 3b, left panel). In contrast, mRNA from the transgene with ARE deleted in V2D1-M1ΔAU was not significantly affected by RAPA (Fig. 3b, right panel). The transcripts containing the ARE deletion lack 216 nucleotides and are therefore distinguishable from endogenous mRNA by poly(A) Northern blot analysis. Again, RAPA destabilized the endogenous transcripts, whereas the mRNA with the ARE deletion remained unaffected. The same effect was seen in FK-506-treated cells; both drugs acted with similar kinetics (Fig. 3c).

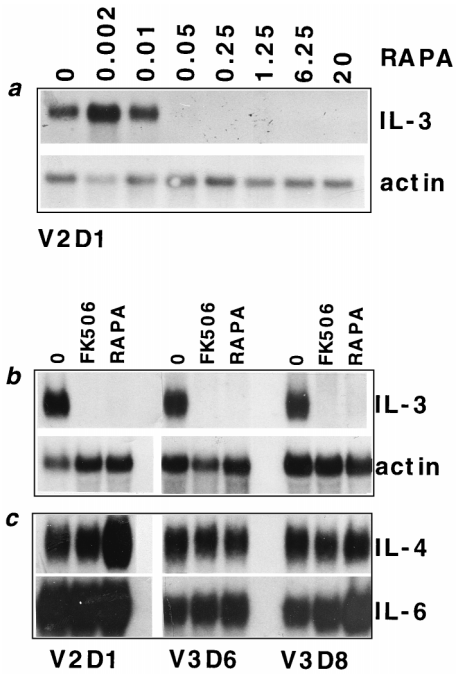

FIG. 2. RAPA and FK-506 specifically inhibit IL-3 mRNA expression in autocrine tumor lines. Cells were incubated overnight with 20 ng of RAPA or FK-506 per ml as indicated. Poly(A) mRNA was prepared from 500 μg of cytoplasmic RNA. (a) Dose-dependent inhibition of IL-3 mRNA expression in V2D1. (b) Effect of FK-506 and RAPA on V3D6 and V3D8, two additional autocrine mastocytoma lines. (c) Rehybridization of the same blot with IL-4 and IL-6 probes.

As a next crucial step, we examined whether the 3′UTR of IL-3 would confer sensitivity to a heterologous transcript. We designed three AP reporter constructs (Fig. 4a) carrying the 3′UTR of IL-3 either with (ΔAUIL3) or without (wtIL3) a deletion of the 3′ ARE-containing sequences. Transcription was driven either by the Moloney murine leukemia virus LTR, which had been found to be insensitive to RAPA treatment (data not shown), or by IL-3 promoter sequences. As shown in Fig. 4b, AP expression driven by the IL-3 promoter was detectable by Northern blotting and after quantification and normalization to β-actin was taken as 1.0 for comparison. Treatment with RAPA reduced AP expression from 1.0 to 0.3 (Fig. 4b, lanes 7 and 8). When IL-3 promoter sequences were replaced by the strong viral LTR, a 3.3-fold-higher expression resulted, which was reduced after RAPA treatment (lanes 3 and 4). The deletion of sequences containing the ARE-instability determinant from the 3′UTR (construct pMx-AP-ΔAUIL3) further increased the steady-state expression level to about sixfold and resulted in RAPA resistance (lanes 5 and 6), supporting the data shown in Fig. 3. The use of the latter construct also confirmed that transcription from the Moloney murine leukemia virus LTR is not sensitive to RAPA. We concluded that RAPA inhibits IL-3 mRNA expression by a posttranscriptional mechanism and that a 216-nucleotide sequence in the 3′UTR of IL-3 containing the ARE-instability determinant is required for the effect.

The role of the FK-506 binding protein FKBP12. When screening different PB-3c-derived tumor lines for RAPA sensitivity, we identified a tumor line (15V4T2) (36) which was RAPA resistant but CsA sensitive in the colony-forming assay (Fig. 5a). IL-3 expression in this line was inhibited by CsA (Fig. 5b), but not by the two drugs which share FKBP12 as binding protein, suggesting that this line fails to express FKBP12. Reverse transcription PCR analysis indeed revealed that the

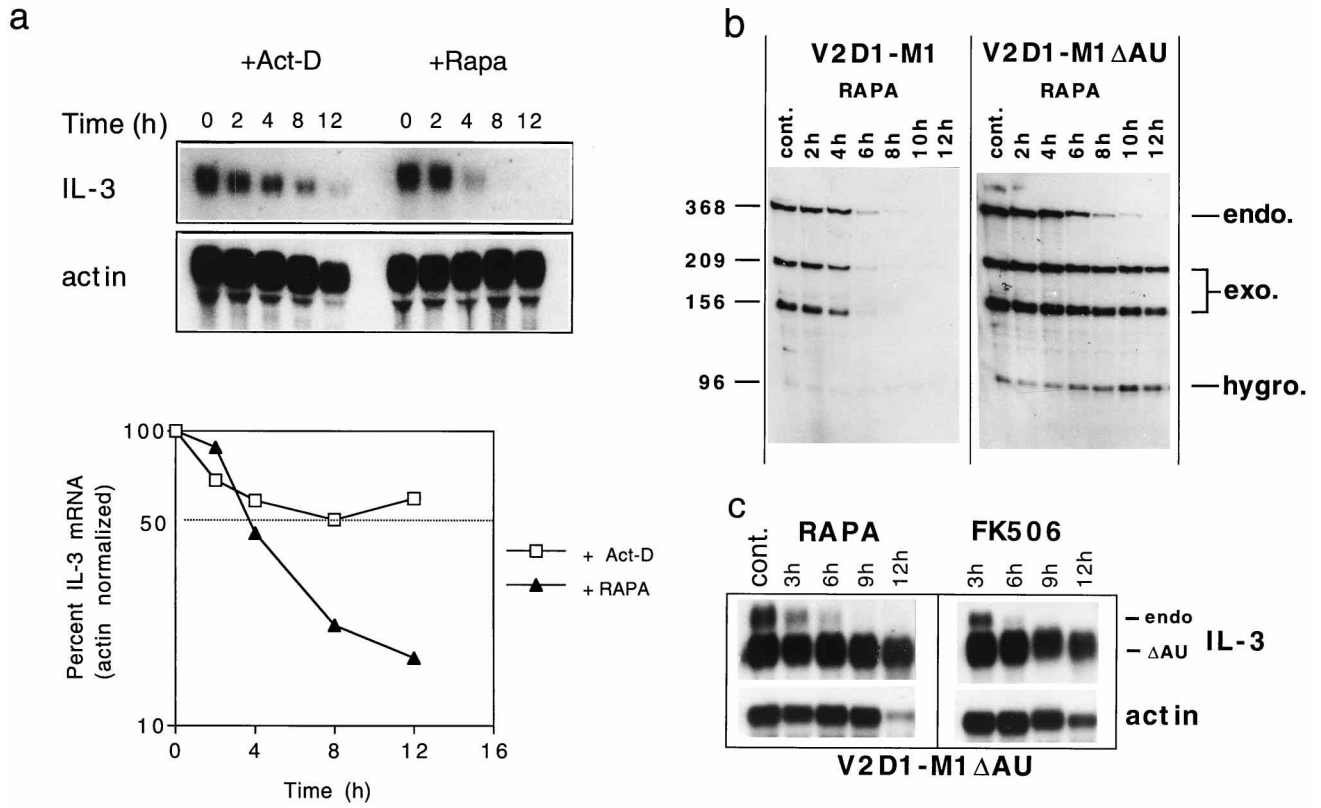

FIG. 3. Downregulation of IL-3 mRNA expression following drug treatment. (a) Time course of IL-3 mRNA levels after treatment of V2D1 cells with Act D (5 μg/ml) or RAPA (20 ng/ml). Total RNA was analyzed by Northern blot hybridization to IL-3 and to β-actin. The signals were quantitated by PhosphorImager (lower panel), where the actin-normalized values at time zero were taken as 100%. (b) Effect of RAPA on endogenous (endo.) and exogenous (exo.) IL-3 transcripts shown by RNase protection assay. Endogenous IL-3 transcripts gave a 368-bp protected fragment, corresponding to the total length of the IL-3 probe. The hybrids with the exogenous transcripts were enzymatically cut into two bands 209 and 156 bp in length due to the silent point mutations in the third exon of the transgene (see Materials and Methods). Hygromycin (hygro.) served as a loading control (96 bp). The cells were treated for the indicated times with 20 ng of RAPA per ml in the presence of IL-3. (c) Effect of RAPA and FK-506 on V2D1-M1ΔAU cells shown by poly(A) Northern blot analysis. The upper band (endo) represents the endogenous transcripts, and the lower band (ΔAU) represents the exogenous ΔAU transcripts with an ARE deletion.

FKBP12 mRNA, while expressed in V2D1, was absent in 15V4T2 (Fig. 5c). This strongly argued that the effect of RAPA and FK-506 described here is dependent on FKBP12 expression.

Role of S6K. We next performed experiments to evaluate the role of S6K activation, which is known to be sensitive to RAPA (13, 27, 45, 46). First, the effect of RAPA on S6K activity in 15V4T2 and V2D1 cells was determined by measurement of phosphorylation of S6 protein. As expected, RAPA inhibited S6K activity in V2D1 cells but not in FKBP12-negative 15V4T2 cells (Fig. 6b). In contrast, FK-506 did not inhibit S6K, but still effectively downregulated IL-3 mRNA expression in V2D1 cells (Fig. 6). These results are consistent with the notion that FK-506, like CsA, acts via the calcineurin pathway independently of S6K, while RAPA inhibits S6 via mTOR (6, 7, 50). This experiment did not indicate, however, whether inhibition of S6K activity was required for RAPA to destabilize IL-3 mRNA. To address this question, we took advantage of the phosphoinositide 3-kinase inhibitor wortmannin (55), which inhibits S6K activity independently of mTOR (12). As shown in Fig. 6b, wortmannin inhibited S6K activity in both lines tested, but this did not significantly influence IL-3 mRNA levels, even at a concentration of 1 μM (Fig. 6a). This suggested that the effect of RAPA on IL-3 mRNA expression in autocrine tumor cells is not mediated by S6K.

DISCUSSION

In this report, we provide evidence that RAPA, a well-studied immunosuppressive agent with inhibitory effects on S6 phosphorylation, translational initiation, and the cell cycle, also promotes degradation of IL-3 transcripts. RAPA has been known to inhibit mainly the postreceptor effects of lymphokines rather than synthesis (17, 47). In the mast cell tumor lines studied here, however, RAPA blocked the expression of IL-3, as previously reported for the calcineurin inhibitors CsA and FK-506 (36). The mechanism was found to act at the posttranscriptional level, as indicated by mRNA decay measurements and transfection experiments. The expression of an IL-3 transgene was inhibited with the same kinetics as those of the endogenous IL-3 mRNA; however, transcripts lacking the ARE in the 3′UTR were resistant to RAPA treatment (Fig. 3b and c). In addition, experiments with AP constructs (Fig. 4) showed that IL-3 3′UTR sequences could confer RAPA sensitivity to reporter transcripts, provided that the 3′UTR sequence was intact. We concluded that the promoter as well as the IL-3 coding region could be displaced without significantly affecting the sensitivity to the drug and that the 3′UTR, possibly the ARE localized within this region, is required for the RAPA effect reported here.

AREs are *cis* elements contained within the 3′UTR of many

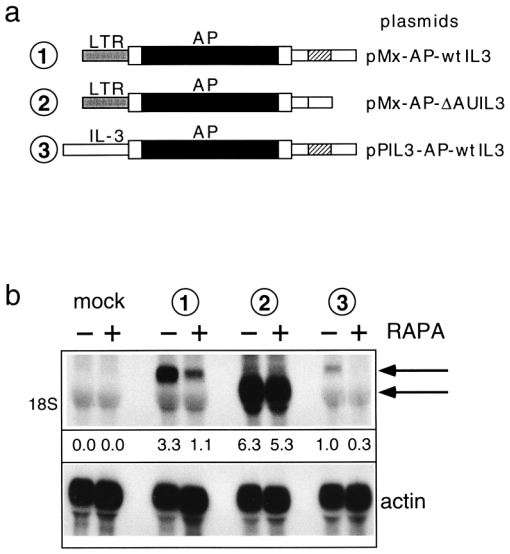

FIG. 4. 3′UTR of IL-3 confers RAPA sensitivity to transcripts from reporter constructs. (a) Schematic representation of the AP reporter constructs. At the 5′ end, the shaded box represents the Moloney leukemia virus LTR, and the narrow open boxes are genomic IL-3 promoter sequences. The wide bars represent the open reading frame starting with the secretory sequences of IL-3 (open boxes), followed by the AP coding sequence (solid boxes) linked to the entire 3′UTR of IL-3 (narrow open boxes) with or without the 216-bp fragment containing the AU-rich element (hatched box) (see Materials and Methods). Given on the right are the names of the plasmids used for transfection. (b) Northern blot analysis with an AP probe. Control and transfected lines were treated with RAPA (20 μg/ml) where indicated for 16 h. The upper arrow indicates the position of the AP-wtIL3 transcripts. The smaller AP-ΔAUIL3 transcripts (lower arrow) partially overlap with the position of the 18S rRNA. Quantitation of the AP signals was achieved after normalization to β-actin expression, with activity of pPIL3-AP-wtIL3 set to 1.0 for comparison (see Materials and Methods).

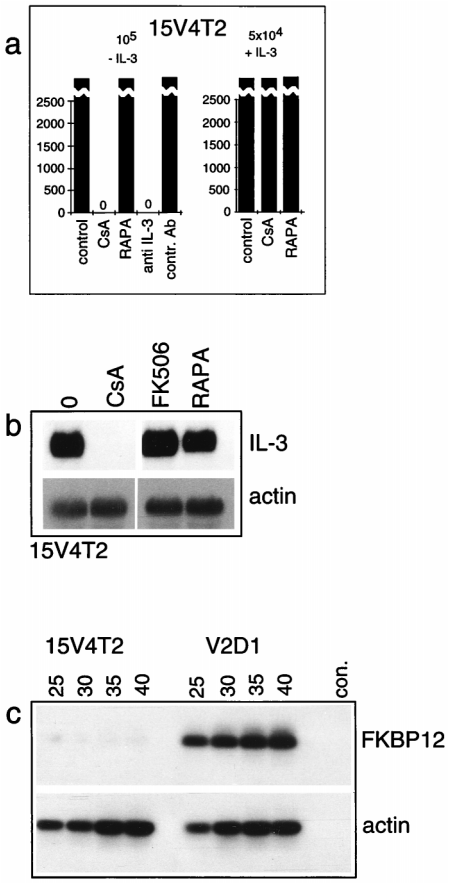

FIG. 5. FKBP12 expression is required for RAPA and FK-506 effects. (a) RAPA does not affect colony formation in 15V4T2. The cells were plated in methylcellulose at 10^5/ml in the absence of IL-3 or at 5×10^4/ml in the presence of IL-3 as indicated. Bars with broken lines are shown where plates contained too many colonies and thus could not be counted. In the presence of CsA or anti-IL-3 antibody, no colonies were formed. contr. Ab, control antibody. (b) Poly(A) Northern blotting, performed as described in the legend to Fig. 2. (c) A reverse transcription PCR experiment indicates the expression of FKBP12 expression in V2D1 but not in 15V4T2. The numbers of PCR cycles performed are indicated above the lanes. Actin amplification was used as a PCR control (con.).

short-lived mRNAs and constitute repetitive AU motifs, which target the transcripts for rapid degradation after deadenylation (see reference 11 for review). Several AU-binding proteins have been described (for review, see reference 48). These proteins are thought to be involved in the control of ARE-dependent mRNA turnover. In analogy with the model of transferrin receptor mRNA (10, 33), they may prevent the binding of a nuclease. It is possible that AU-binding regulatory proteins, similar to transcription factors, are regulated by an interplay of kinases and phosphatases and are sensitive to the corresponding enzyme inhibitors. Inspection of ARE sequences from a variety of growth factors and proto-oncogenes has revealed that significant structural differences exist, and these have been defined into two different classes (11, 21). Class I members contain one to three copies of scattered AUUUA motifs near a U-rich sequence, while class II AREs contain at least two overlapping pentamers with the consensus nonamer UUAUUUA(U/A)(U/A) (28, 59). It is interesting that the ARE of IL-3 with eight pentamer motifs (9) falls into class II, while the AREs of IL-4 (43) and IL-6 (53), two lymphokines not destabilized by RAPA (Fig. 2), fall into class I. Further work will be required to identify the detailed sequence specificity involved in the drug effects described here.

The relatively slow time course observed for mRNA destabilization seems to be in conflict with the idea that IL-3 mRNA turnover in the tumor lines studied is under direct control of a signal transduction pathway. Both RAPA and FK-506 required about 3 to 6 h to downregulate the transcripts, whereas kinase or phosphatase inhibitors regulate their primary target within minutes. However, it should be kept in mind that the drugs destabilized constitutively expressed IL-3 mRNA in tumor lines in which the decay is slow because of an unknown defect in IL-3 mRNA degradation (21) which may preclude a faster response. The relatively slow kinetics of the RAPA effect might suggest that mRNA decay is secondary to cell cycle inhibition. However, cell cycle arrest of V2D1 cells by RAPA, when measured by fluorescence-activated cell sorter analysis, proceeded at a slower rate. In addition and importantly, the methylxanthine compound SQ20006 inhibited the cell cycle without inhibiting IL-3 mRNA expression (data not shown).

The finding that IL-3 mRNA in the tumor variant 15V4T2, which does not express FKBP12, was refractory to both RAPA and FK-506 but was still sensitive to CsA treatment (Fig. 5) is a strong argument that IL-3 mRNA destabilization by RAPA, FK-506, and CsA involves the known RAPA-FKBP12, FK-506–FKBP12, and CsA-cyclophilin complexes involved in immunosuppression. RAPA inhibits phosphorylation of the ribosomal protein S6 (13, 27, 45) and thus translation of mRNAs rich in 5′ polypyrimidine tracks (23, 39, 54). Because protein synthesis and mRNA degradation are interdependent processes, it seemed possible that the RAPA effect might be linked to S6K inhibition. In vivo, RAPA leads to dephosphor-

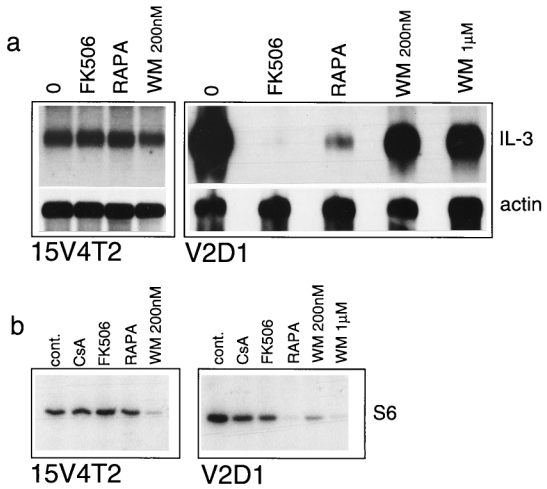

FIG. 6. Inhibition of p70S6K activity does not correlate with IL-3 mRNA destabilization. (a) Poly(A) northern analysis, performed as described in the legend to Fig. 2. Note that due to overexposure, a residual signal is still detected in RAPA-treated cells. (b) S6K assay with S6 as a substrate. The cells were treated with CsA (500 ng/ml), FK-506 (20 ng/ml), RAPA (20 ng/ml), and wortmannin (WM [200 nM or 1 μM]) for 30 min. Lysates were prepared and immunoprecipitated with anti-S6K antiserum. Immunocomplexes were subjected to an in vitro kinase assay with 40S ribosomes used as a substrate. cont., control.

ylation of S6K on a well-defined subset of sites, and wortmannin, a specific inhibitor of phosphoinositide 3-kinase activity (55), indirectly affects the same sites (20). Comparison of the effects of both drugs on FKBP12-negative (15V4T2) and FKBP12-positive (V2D1) tumor lines (Fig. 6) revealed that RAPA inhibited constitutive S6K activity and destabilized IL-3 mRNA only in V2D1 cells, while wortmannin inhibited S6K activity in both lines, but exerted no significant effect on IL-3 mRNA expression. This indicated that inhibition of S6K activity is not relevant for the effect of RAPA on IL-3 mRNA in the tumor lines studied.

The immunosuppressants RAPA, FK-506, and CsA act by targeting their respective receptor proteins as complexes to third components, which are then inhibited. RAPA-FKBP12 complexes affect mTOR, an upstream regulator of translation and probably of the cell cycle (5–7, 26, 49). FK-506–FKBP12 and CsA-cyclophilin complexes inhibit the phosphatase calcineurin and affect its substrates. It was unexpected that these three drugs, acting through different pathways, should all share the effect of downregulating IL-3 mRNA in the tumor lines studied. One possible explanation is that an unknown downstream target of both mTOR and calcineurin is a regulator of IL-3 mRNA turnover. That calcineurin is a phosphatase and mTOR a kinase is not inconsistent with this model. An alternative and more unifying hypothesis is suggested by the recent report by Cameron et al. (8), who provided evidence for an unexpected link between FKBP12 and calcineurin. These workers showed that the IP_3 receptor and the ryanodine receptor, two calcium channels, each form trimolecular complexes with FKBP12 and calcineurin. Both FK-506 and RAPA displaced FKBP12 from the complexes, which led in turn to the release of calcineurin. Thus, RAPA was shown for the first time to exert a biological effect, that being regulation of calcium flux, via calcineurin. Based on this precedent model, it is tempting to speculate that FKBP12 and calcineurin bind similarly to an unknown regulator of IL-3 mRNA turnover and that this molecule is regulated by the phosphatase activity of calcineurin. By analogy, RAPA, FK-506, and CsA could affect IL-3 mRNA stability by acting via calcineurin. This model, while quite speculative at this time, allows the prediction that regulators of mRNA turnover may be identified by their property of binding to both FKBP12 and calcineurin.

Autocrine tumor cells such as the lines studied here arise from premalignant cells by expressing a growth factor for which they have a functional receptor. Autocrine loops of tumor cells appear to provide attractive target mechanisms for pharmacological intervention. Functional interruption might be feasible at the level of transcription, splicing, mRNA transport and stability, and translation, as well as at the level of proteins and ligand-receptor interactions. Because corresponding interfering mechanisms are of potential therapeutic interest, the further characterization of the effects reported here, in particular the identification of the effector molecules, should be worthwhile goals for future research.

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