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Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells

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ABSTRACT **Differentiating 3T3-L1 cells express an immunophilin early during the adipocyte conversion program as described in this issue [Yeh, W.-C., Li, T.-K., Bierer, B. E. & McKnight, S. L. (1995) *Proc. Natl. Acad. Sci. USA* 92, 11081–11085]. The temporal expression profile of this protein, designated FK506-binding protein (FKBP) 51, is concordant with the clonal-expansion period undertaken by 3T3-L1 cells after exposure to adipogenic hormones. Having observed FKBP51 synthesis early during adipogenesis, we tested the effects of three immunosuppressive drugs—cyclosporin A, FK506, and rapamycin—on the terminal-differentiation process. Adipocyte conversion was not affected by either cyclosporin A or FK506 and yet was significantly reduced by rapamycin at drug concentrations as low as 10 nM. Clonal expansion was impeded in drug-treated cultures, as was the accumulation of cytoplasmic lipid droplets normally seen late during differentiation. Rapamycin treatment likewise inhibited the expression of CCAAT/enhancer binding protein α, a transcription factor required for 3T3-L1 cell differentiation. All three of these effects were reversed by high FK506 concentrations, indicating that the operative inhibitory event was mediated by an immunophilin–rapamycin complex.**

Rapamycin is a potent immunosuppressant that is structurally similar to FK506 (1). Both drugs share the same set of intracellular receptors, termed FK506-binding proteins (FKBPs), which exhibit peptidylproline *cis-trans*-isomerase (PPIase, also rotamase) activity (2–4). Binding of rapamycin or FK506 inhibits the enzymatic activity of FKBPs, and the drug–receptor complexes then function as specific, active moieties that bind to and inhibit secondary target proteins (5, 6). Surprisingly, target proteins and signal-transduction pathways sensitive to rapamycin–FKBP are distinct from those sensitive to FK506–FKBP. Therefore, these two drugs act as reciprocal antagonists in a number of biological assays (6–9).

In addition to suppression of T-cell activation, rapamycin has also been shown to inhibit the proliferation of a variety of mammalian cells, including T cells (1, 6, 7), B cells (10), hepatoma cells (11), Swiss 3T3 cells (12), osteosarcoma cells (13), rhabdomyosarcoma cells (14), and myogenic cells (8). By blocking the proliferation of BC3H1 myogenic cells, rapamycin was observed to induce muscle differentiation (8).

Studies pertinent to the antiproliferative properties of rapamycin have revealed specific inhibition of mitogen-activated p70 S6 kinase and the subsequent phosphorylation of ribosomal protein S6 (11, 12, 15). In both yeast and mammals, rapamycin has been shown to block cell-cycle progression by causing G_1 arrest and abrogating the activation of p34 cdc2 and p33 cdk2 kinases (13, 16, 17). However, no direct interactions between rapamycin and these preidentified kinases have been reported. A candidate target for the drug–receptor complex has been isolated as the molecule variously termed FKBP–rapamycin-associated protein (FRAP), rapamycin and FKBP12 target 1 (RAFT1), mammalian targets of rapamycin (mTOR), or RAPT1 (18–21). FKBP–rapamycin-associated protein/mammalian targets of rapamycin is the mammalian homologue of the yeast targets-of-rapamycin proteins that in turn, display considerable similarity to the 110-kDa subunit of phosphatidylinositol 3′-kinase (22). Although the specific functions of these recently identified phosphatidylinositol 3′-kinase-related proteins are yet to be defined, evidence favoring the interaction between rapamycin–FKBP and signaling pathways pertaining to cell growth and differentiation is substantial.

In the present study, we examined the effect of rapamycin on the differentiation of 3T3-L1 preadipocyte cells (23). Confluent 3T3-L1 cells typically proceed through an early phase of proliferation when induced to differentiate by an adipogenic hormone mixture consisting of insulin, fetal bovine serum, dexamethasone, and isobutylmethylxanthine (24). Here we demonstrated a pronounced inhibitory effect of rapamycin on adipose conversion. Drug-treated cultures failed to accumulate cytoplasmic fat droplets normally seen late during differentiation. Expression of CCAAT/enhancer binding protein α (C/EBPα), a hallmark of terminal adipocyte differentiation, was also inhibited by rapamycin. Interestingly, rapamycin inhibited the p30 form of C/EBPα much more severely than the full-length p42 form of the protein. Finally, all inhibitory effects of rapamycin were overcome when cells were cotreated with high FK506 concentrations, implicating a drug–receptor complex as the relevant inhibitory entity.

MATERIALS AND METHODS

Cell Culture and Differentiation. The 3T3-L1 preadipocyte cell line was provided by M. D. Lane (Johns Hopkins University School of Medicine) and maintained in Dulbecco's modified Eagle medium (DMEM)/10% calf serum. Adipocyte differentiation was induced by treating confluent cells with a hormone mixture containing 10% fetal bovine serum, insulin at 10 μg/ml (Elanco, Indianapolis), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), and 1 μM dexamethasone (Sigma). Two days later, cells were changed to DMEM supplemented with only insulin and fetal bovine serum; the medium was then replenished every other day. For the effect of drugs on adipocyte differentiation, appropriate concentrations of rapamycin, FK506, and cyclosporin A were added at the start of hormone treatment, and the same concentrations of drugs were supplemented at 2-day intervals when culture medium was replenished.

Morphological aspects of adipocyte conversion were monitored by serial photography of representative fields observed by light microscopy. Measurements of cell proliferation during adipocyte differentiation were taken by using a hemacytom-

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Abbreviations: FKBP, FK506-binding protein; C/EBPα, CCAAT/enhancer binding protein α, PPIase, peptidylproline *cis-trans*-isomerase.

eter. Results were derived from the average of three independent experiments.

Immunoblot Analysis of C/EBP Proteins. Protein extracts from total cells were harvested at appropriate time points from 3T3-L1 cells during the differentiation process as described (25, 26). Aliquots of protein samples were electrophoresed on a SDS/15% polyacrylamide gel. Proteins were transferred from the gel to a poly(vinylidene difluoride) (PVDF) membrane (Millipore) and then probed with relevant antisera against specific C/EBP isoforms (26). Immunoreactive protein species were visualized by an enhanced chemiluminescence detection system (Amersham).

RESULTS

Inhibitory Effects of Rapamycin on 3T3-L1 Preadipocyte Differentiation. Confluent 3T3-L1 preadipocytes, upon exposure to an adipogenic hormone mixture, convert into fat-laden adipocytes in 6–8 days (24). The conversion process can be monitored by the gradual accumulation of cytoplasmic fat droplets seen by light microscopy and by the expression of biochemical markers detectable by immunoblotting. To investigate the potential effects of immunosuppressive drugs on adipocyte differentiation, various concentrations of rapamycin, FK506, and cyclosporin A were added to confluent 3T3-L1 cells at the outset of the differentiation program. As shown in Fig. 1*A*, neither FK506 nor cyclosporin A affected adipocyte conversion, even when added at concentrations as high as 1 μM (FK506) or 10 μM (cyclosporin A). Rapamycin, on the other hand, inhibited adipocyte differentiation at concentrations as low as 10 nM. Microscopic inspection of rapamycin-treated cells cultured under optimal differentiation conditions revealed a significant reduction in the accumulation of cytoplasmic fat (Fig. 1*A*).

A key regulatory protein induced during the adipocyte differentiation is C/EBPα (27). Expression of this transcription factor is strictly concordant with the initial appearance of cytoplasmic fat (28, 29). Molecular biological studies have shown that C/EBPα is both necessary and sufficient for differentiation of 3T3-L1 cells (30–34). The alternative use of two initiator methionines results in the production of two molecular forms of C/EBPα (35, 36). A full-length, 42-kDa

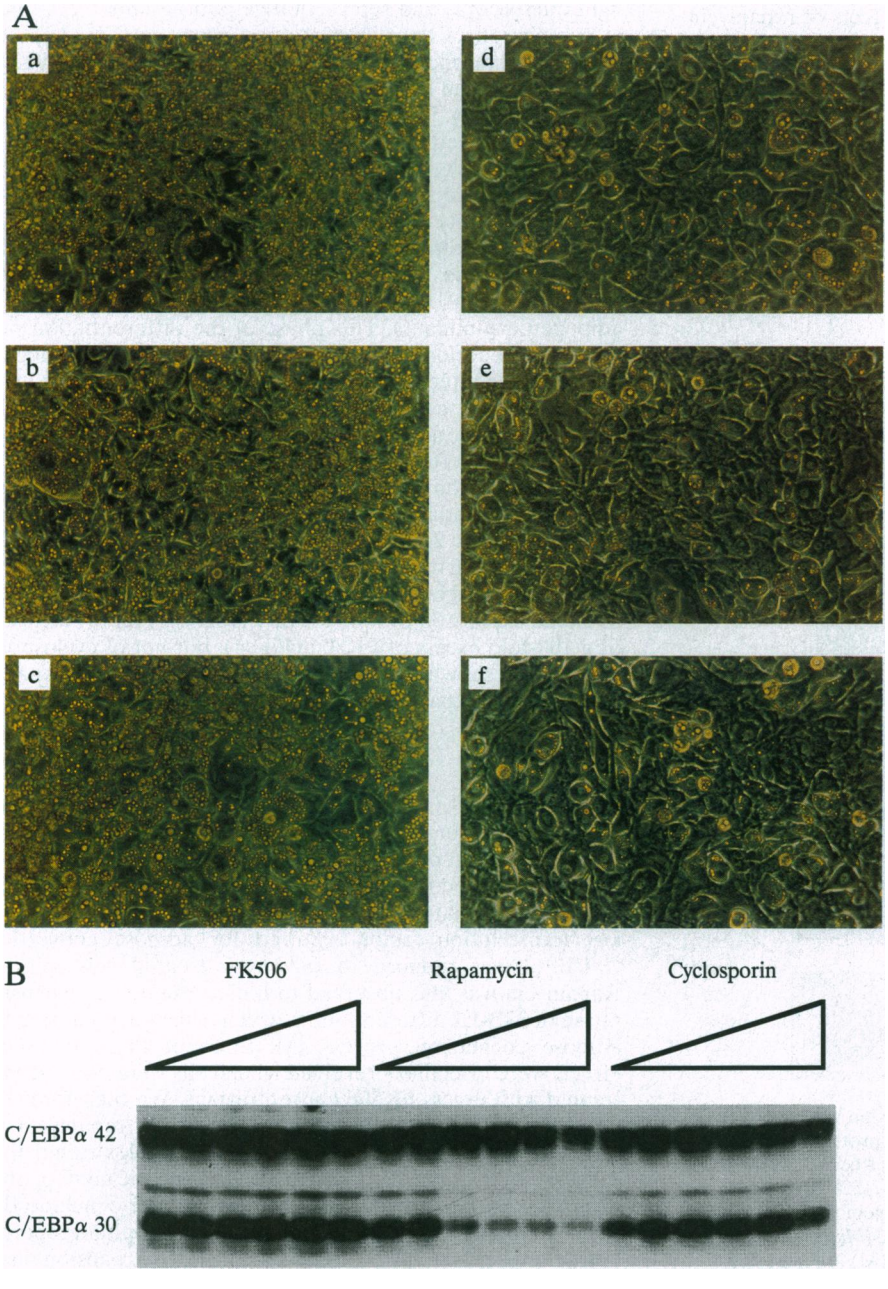


FIG. 1. Inhibitory effects of rapamycin on adipose conversion of 3T3-L1 preadipocytes. Various concentrations of FK506, rapamycin, and cyclosporin A were added to cultured 3T3-L1 cells at the outset of the differentiation program. (*A*) Morphological evidence of terminal adipocyte differentiation at day 6 of the conversion process. Photographs show control cells (*a*) as well as cells treated with 1 μM FK506 (*b*), 10 μM cyclosporin A (*c*), 10 nM rapamycin (*d*), 100 nM rapamycin (*e*), and 1 μM rapamycin (*f*). Accumulation of cytoplasmic fat (yellow, birefringent spheres) provided morphological evidence of differentiation. (*B*) Biochemical evidence of adipocyte differentiation was monitored by immunoblot analysis of C/EBPα accumulation. Total cellular protein was harvested at day 6 of the adipose conversion process from cells treated with increased concentrations of FK506, rapamycin, or cyclosporin A. FK506 and rapamycin doses varied in half-logarithmic increments from 1 to 1000 nM (left to right). Cyclosporin A doses varied in half-logarithmic increments from 10 nM to 10 μM (left to right). Protein samples were separated on an SDS/polyacrylamide gel, transferred to a poly(vinylidene difluoride) membrane, and detected by antiserum specific to C/EBPα (26).

protein (p42) is translated from the in-frame AUG codon located closest to the 5′ terminus of the C/EBPα mRNA. A smaller, 30-kDa translation product (p30) results from a translation initiation event 118 amino acid residues internal to the C/EBPα open reading frame.

To further examine the effects of immunosuppressive drugs on adipocyte conversion, immunoblotting was done with extracts prepared from cells treated under optimal differentiation conditions in the presence of various concentrations of rapamycin, FK506, and cyclosporin A (Fig. 1*B*). Neither FK506 nor cyclosporin A affected C/EBPα synthesis. Rapamycin-treated cultures did, however, show alterations in the expression of this transcription factor. Accumulation of the p42 form of C/EBPα was mildly reduced at rapamycin concentrations from 10 to 100 nM. Expression of the p30 form of C/EBPα was more significantly affected by rapamycin at drug concentrations as low as 3 nM.

Genes encoding two proteins highly related to C/EBPα, termed C/EBPβ and C/EBPδ, are expressed during the clonal expansion phase of adipocyte conversion (26). Recent evidence has indicated that one or both of these early acting relatives of C/EBPα play a critical role in the differentiation of 3T3-L1 cells (37). To assess potential effects of rapamycin on the expression of C/EBPβ and C/EBPδ, immunoblots were done with protein harvested from control and drug-treated cultures taken at 2-day intervals throughout the differentiation program. Fig. 2 shows that rapamycin did not alter the onset of expression (day 2) or abundance of C/EBPδ. Drug treatment mildly affected the expression of C/EBPβ, leading to a slight reduction in the amount of the full-length p34 form of C/EBPβ (LAP) and a somewhat more significant effect on the

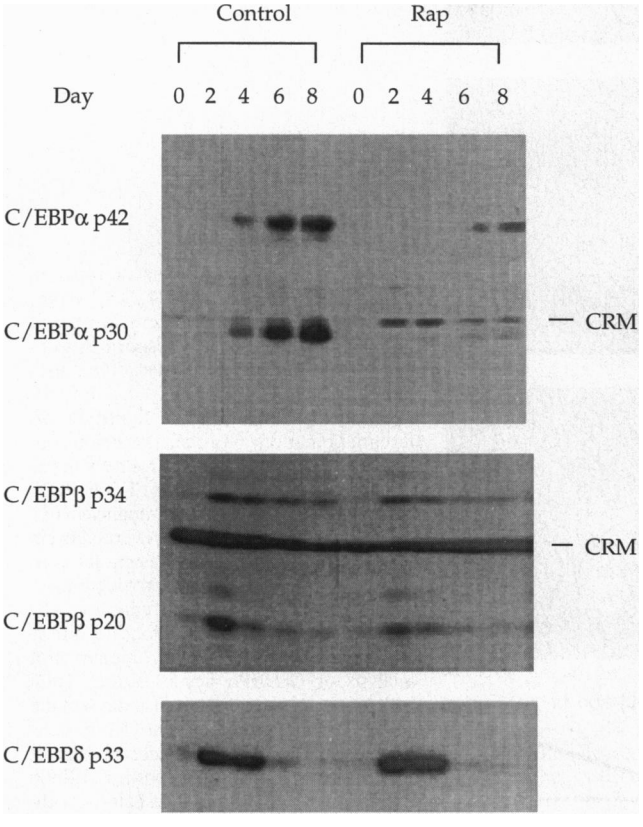


FIG. 2. Effects of rapamycin on the expression of three C/EBP isoforms during adipocyte differentiation. Total proteins harvested at 2-day intervals from the control culture as well as cells exposed to 10 nM rapamycin (Rap) were electrophoresed on a 15% SDS/polyacrylamide gel, blotted to a poly(vinylidene difluoride) membrane, and probed with antiserum specific to C/EBPα (*Top*), C/EBPβ (*Middle*), or C/EBPδ (*Lower*). Nonspecific cross-reacting materials (CRM) are indicated.

internally initiated p20 form of the protein (LIP). Finally, as observed in the dose–response study (Fig. 1*B*), inclusion of rapamycin at 10 nM significantly impeded C/EBPα expression.

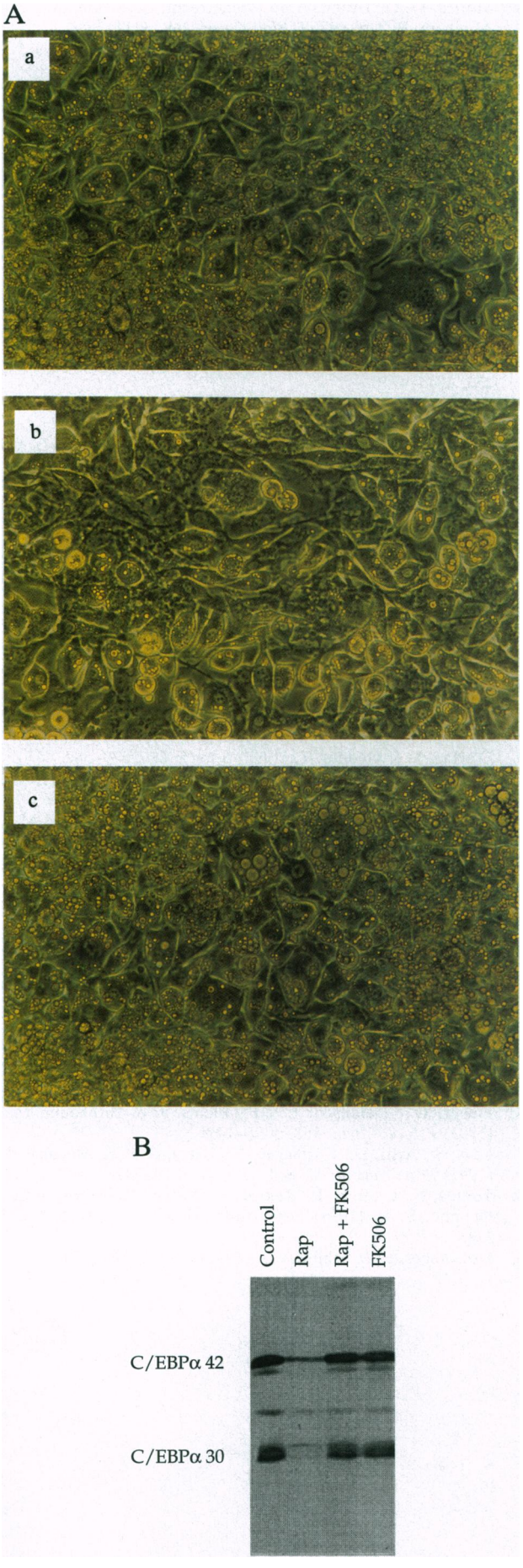
High Concentrations of FK506 Reverse the Inhibitory Effects of Rapamycin on Adipocyte Conversion. Rapamycin and FK506 bind to an overlapping set of intracellular receptors, designated FKBPs (1), yet the two drugs affected adipocyte conversion very differently (Fig. 1). Extensive studies of these two macrolides in the context of the immune response have shown that, although they indeed inhibit the enzymatic activities of their intracellular receptors (PPIases), their biological activities arise from the formation of drug–receptor complexes that affect the activities of distinct molecular targets. Because FK506 did not inhibit adipocyte conversion and yet binds to many of the same intracellular receptors as rapamycin, we tested whether it might overcome the inhibitory effects of rapamycin.

Various concentrations of FK506 were added to confluent 3T3-L1 cells along with 10 nM rapamycin. The cells were then induced to differentiate in response to the optimal differentiation regimen. Morphological changes were monitored by light microscopy, and representative photographs were taken at completion of the differentiation program (Fig. 3*A*). According to morphological criteria, a 100-fold excess of FK506 fully reversed the inhibitory effects of rapamycin on differentiation. Similarly, FK506 rescued the expression of C/EBPα in rapamycin-treated 3T3-L1 cells (Fig. 3*B*). Excessive amounts of cyclosporin A, which can interact with a different set of intracellular receptors (1, 38), did not effectively reverse the inhibitory activity of rapamycin (data not shown).

Rapamycin Inhibits the Clonal Expansion of Differentiating 3T3-L1 Cells. Several rounds of cell division occur after exposure of confluent 3T3-L1 cells to the optimal mixture of adipogenic stimulants. This phase of the differentiation program, termed clonal expansion, appeared by morphological criteria to be altered by rapamycin. Instead of stacking up as densely packed cells, rapamycin-treated cultures of 3T3-L1 cells retained the appearance of the starting, confluent culture. This perceived effect on clonal expansion was quantitated by taking hemocytometer cell counts at 2-day intervals throughout the differentiation program. Cell number in control cultures increased 2.6-fold between days 0 and 2, increased 1.7-fold between days 2 and 4, and remained constant between days 4 and 6. Inclusion of 10 nM rapamycin reduced the pace of clonal expansion by a factor of 2 (Fig. 4). Finally, inclusion of a 100-fold excess of FK506 (Fig. 4), but not of cyclosporin A (data not shown), rescued the inhibitory effects of rapamycin on clonal expansion.

DISCUSSION

In this report we provide evidence that rapamycin, a secondary metabolite secreted by *Streptomyces hygroscopicus*, acts as a potent inhibitor of adipocyte differentiation. When treated with rapamycin concentrations as low as 10 nM, 3T3-L1 cells fail to differentiate into fat-laden adipocytes. Expression of a key transcription factor required for adipose conversion, C/EBPα, was reduced in rapamycin-treated cell cultures. Rapamycin was also observed to inhibit the clonal expansion phase of 3T3-L1 differentiation, which occurs early during the adipose conversion process. All three of these inhibitory effects were effectively reversed when cells were concurrently treated with excess FK506 concentrations. We therefore conclude that the pertinent inhibitory activity of rapamycin reflects the formation of a drug–receptor complex rather than the ability of rapamycin to inhibit the enzymatic activity of its direct molecular targets (PPIases). We further conclude that the enzymatic activity of FKBP51, the immunophilin reported in this issue to be expressed during the clonal expansion phase



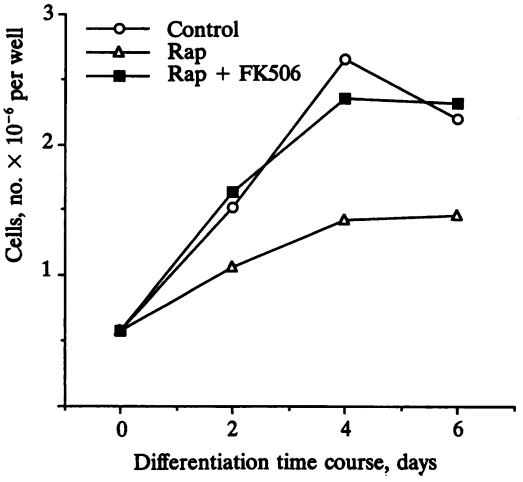


FIG. 4. Rapamycin (Rap) inhibition of clonal expansion of differentiating 3T3-L1 cells is reversed by high FK506 concentrations. Confluent 3T3-L1 preadipocytes (day 0) were induced to undergo adipose conversion. Parallel cultures of 3T3-L1 cells were harvested at day 0 (undifferentiated), day 2, day 4, or day 6 of the differentiation program in the absence of drugs or presence of either 10 nM rapamycin or 10 nM rapamycin plus 1 μM FK506. Cell numbers were determined by taking hemocytometer cell counts. Data represent the average of three independent experiments.

of adipocyte differentiation and be potently inhibited by both rapamycin and FK506 (39), is not essential for adipose conversion.

Previous studies of rapamycin–FKBP complexes have provided evidence that they inhibit secondary targets involved in specific signaling pathways (11–13). An attractive candidate for the inhibitory activity of such complexes in differentiating 3T3-L1 cells is the insulin- or insulin-like growth factor-signaling pathway. Insulin and insulin-like growth factor are required to facilitate adipose conversion (40, 41). A signaling molecule designated p70 S6 kinase, known to be involved in mediating insulin effects (11, 42), has been shown to be inhibited by rapamycin in 3T3-L1 preadipocytes, as well as in its parent cell line, Swiss 3T3 cells (12, 43). Signaling via the insulin-like growth factor receptor is also known to be inhibited by rapamycin (14). Moreover, FKBP–rapamycin-associated protein/mammalian targets of rapamycin, a direct molecular target of the rapamycin–FKBP drug complex (18–21), is significantly related at the level of primary amino acid sequence to the catalytic subunit of phosphatidylinositol 3′-kinase, which also plays a critical role in mediating insulin signaling (42). It is therefore likely that rapamycin blocks adipocyte differentiation, at least in part, by inhibiting insulin signaling.

Rapamycin-mediated inhibition of p70 S6 kinase and consequent effects on phosphorylation of the S6 subunit of 40S ribosomes have been reported to suppress the translation of mRNAs characterized by 5′-terminal polypyrimidine tracts (15, 44, 45). mRNAs of this class include ones encoding certain ribosomal proteins and translational elongation factors. It is

FIG. 3. High concentrations of FK506 reverse the inhibitory effect of rapamycin on adipocyte differentiation. (*A*) Confluent 3T3-L1 culture cells were induced to differentiate with or without immunosuppressive drugs. Morphological evidence of adipocyte differentiation was monitored by light microscopy and photographed at day 6: (*a*) control cell culture; (*b*) culture treated with 10 nM rapamycin; (*c*) culture treated with 10 nM rapamycin plus 1 μM FK506. (*B*) Biochemical evidence of adipocyte differentiation by immunoblot analysis of C/EBPα expression in control cells, cells treated with 10 nM rapamycin (Rap), cells treated with 10 nM rapamycin plus 1 μM FK506, and cells treated with 1 μM FK506 alone.

notable, in this regard, that rapamycin differentially affected the accumulation of the two molecular forms of C/EBPα. Accumulation of the internally initiated p30 form of C/EBPα was inhibited at considerably lower concentrations of rapamycin than the full-length, p42 gene product. We likewise observed a diminution of the internally initiated, C/EBPβ translation product in rapamycin-treated cultures. Recent evidence has indicated that the various C/EBPα and C/EBPβ translation products have distinct biological activities (35–37, 46). It is therefore possible that an imbalance in the ratios of these transcription factors may account, at least in part, for the inhibitory effect of rapamycin on terminal adipocyte conversion.

One overt effect of rapamycin is manifest in its antiproliferative activity (1, 6–8, 10–14). We likewise observed an antiproliferative effect of rapamycin. Drug-treated 3T3-L1 cells failed to undergo clonal expansion early during the adipogenic conversion program. It is interesting that in this particular model for terminal cell differentiation, rapamycin acts as a potent inhibitor. Similar studies of cultured myoblast cell lines have shown conversely that rapamycin stimulates the process of terminal differentiation (8). Thus, although adipocytes enter a postmitotic state upon terminal differentiation, the conversion pathway from the 3T3-L1 preadipocyte to the fully differentiated adipocyte appears to require a preparatory phase of clonal expansion.

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