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Biochemistry

Cyclosporin A potentiates the dexamethasone-induced mouse mammary tumor virus–chloramphenicol acetyltransferase activity in LMCAT cells: A possible role for different heat shock protein-binding immunophilins in glucocorticosteroid receptor-mediated gene expression

(steroid receptors/immunosuppressant analogs/cyclophilins/FK506-binding proteins)

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ABSTRACT **As previously observed for FK506, we report here that cyclosporin A (CsA) treatment of mouse fibroblast cells stably transfected with the mouse mammary tumor virus–chloramphenicol acetyltransferase (MMTV–CAT) reporter plasmid (LMCAT cells) results in potentiation of dexamethasone (Dex)-induced CAT gene expression. Potentiation by CsA is observed in cells treated with 10–100 nM Dex but not in cells treated with 1 μM Dex, a concentration of hormone which results in maximum CAT activity. At 10 nM Dex, 1–5 μM CsA provokes an ≈50-fold increase in CAT gene transcription, compared with transcription induced by Dex alone. No induction of CAT gene expression is observed in cells treated with CsA or FK506 in the absence of Dex. The antisteroid RU 486 abolishes effects obtained in the presence of Dex. Using a series of CsA, as well as FK506, analogs, including some devoid of calcineurin phosphatase inhibition activity, we conclude that the potentiation effects of these drugs on Dex-induced CAT gene expression in LMCAT cells do not occur through a calcineurin-mediated pathway. Western-blotting experiments following immunoprecipitation of glucocorticosteroid receptor (GR) complexes resulted in coprecipitation of GR, heat shock protein hsp90 and two immunophilins: the FK506-binding protein FKBP59 and the CsA-binding protein cyclophilin 40 (CYP40). Two separate immunophilin–hsp90 complexes are present in LMCAT cells: one containing CYP40–hsp90, the other FKBP59–hsp90. Thus, both FKBP59 and CYP40 can be classified as hsp-binding immunophilins, and their possible involvement as targets of immunosuppressants potentiating the GR-mediated transcriptional activity is discussed.**

Several reports have recently indicated that a 59-kDa protein (1) is part of a large protein complex that includes the 90-kDa heat shock protein (hsp90) and an unliganded steroid receptor, in particular the unliganded glucocorticosteroid receptor (GR) (for review see refs. 2 and 3). As demonstrated by genetic and biochemical analyses (4–7), this protein is in fact an immunophilin of the FK506-binding protein (FKBP) family (4) capable of binding both FK506 and rapamycin (5, 6, 8) and is termed FKBP59. The same protein has been cloned from human cells (FKBP52) (9) and has been found sensitive to heat shock in IM9 cells (hsp56) (10). FKBP59 binds hsp90 (11) and is therefore a *h*eat shock protein-*b*inding *i*mmunophilin or HBI (7). The binding to hsp90 involves the C-terminal part of FKBP59 (12) which contains a three tetratricopeptide repeat (TPR) (13). Recently, FK506 and rapamycin were shown to potentiate the GR-mediated gene expression in mouse L929 cells stably transfected with the reporter plasmid mouse mammary tumor virus–chloramphenicol acetyltransferase (MMTV–CAT; LMCAT cells, ref. 14). In this work, we have obtained similar results‖ by treating LMCAT cells with cyclosporin A (CsA), an immunosuppressive drug that does not bind to FKBPs but binds to other immunophilins termed cyclophilins (CYPs) (15). We also demonstrate that a recently described 40-kDa cyclophilin (CYP40) (16, 17), known to be a component of inactivated estrogen receptor complexes (13), is a component of inactivated GR complexes in these LMCAT cells. Immunosuppressive analogs of CsA and FK506 were studied, and we looked for a correlation between their immunosuppressive properties, their ability to inhibit peptidylprolyl isomerase (PPIase) and calcineurin (CN) phosphatase activities. Finally, we discuss the potentiation activity of these immunosuppressive drugs on the dexamethasone (Dex)-induced CAT gene expression, which may involve their direct binding to immunophilins localized in GR complexes via hsp90.

MATERIALS AND METHODS

Drug Treatment of CAT Reporter Cell Line. The LMCAT cell line was established from L929 mouse fibroblast cells as described (14). They were grown in Dulbecco's modified Eagle's medium, containing 10% heat-inactivated (30 min at 55°C) fetal calf serum and 0.2 mg of geneticin (G418) per ml.

Cells grown to 50% confluence were exposed to immunosuppressive drugs for 4 h followed by addition of Dex or up to 0.1% ethanol vehicle for 18 h. For hormone binding-affinity measurements, LMCAT cells were grown in 850-cm^2 roller flasks.

CAT enzyme activity was measured as described (14). Briefly, duplicate samples containing 3 μg of total protein were assayed by using [^{14}C]chloramphenicol (56 mCi/mmol; 1

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Abbreviations: GR, glucocorticosteroid receptor; hsp, heat shock protein; CAT, chloramphenicol acetyltransferase; TA, triamcinolone acetonide; RU 486, (11β,17β)-11-[4-(dimethylamino)-phenyl]-17-hydroxy-17-(1-propynyl)estra-4,9-diene-3-one; CsA, cyclosporin A; CYP, cyclophilin; FKBP, FK506-binding protein; CsH, cyclosporin H; Dex, dexamethasone; MMTV, mouse mammary tumor virus; CN, calcineurin; PPIase, peptidylprolyl isomerase.

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Ci = 37 GBq; Amersham) as substrate and acetylcoenzyme A as cofactor. All experiments were repeated at least three times.

Affinity Purification of Immunophilins. After harvesting cells grown in roller flasks, cytosol was prepared by homogenization in buffer A [10 mM Hepes, pH 7.5/1 mM EDTA/10% (vol/vol) glycerol/1 mM dithiothreitol] with a Teflon/glass homogenizer, followed by centrifugation at 2°C in a Beckman Ti50 rotor (45 min at 45,000 rpm in a Beckman L2 68B ultracentrifuge). After incubation for 18 h at 0°C with [^{3}H]triamcinolone acetonide (TA) (49.5 Ci/mmol; NEN) for measurement of GR binding parameters or with [^{3}H](11β,17β)-11-[4-(dimethylamino)-phenyl]-17-hydroxy-17-(1-propynyl)estra-4,9-diene-3-one ([^{3}H]RU 486) (36.4 Ci/mmol; gift from D. Philibert, Roussel-UCLAF), cytosol was supplemented with 20 mM sodium tungstate to stabilize the heterooligomeric form of the GR (11). Portions of cytosol were treated with dextran-coated charcoal for measurement of ligand binding or were loaded onto 0.2 ml of CsA-Affigel-10 or FK506-Affigel-10 resin, each containing 1 mg of immunosuppressant per ml of resin. The samples were rotated for 18 h at 4°C, and the gels were then washed successively with 1 ml of buffer A supplemented with 20 mM sodium tungstate (buffer C), 1 ml of buffer B [0.1 M sodium phosphate, pH 7.5/10% (vol/vol) glycerol/20 mM sodium tungstate], and then three times with 1 ml each of buffer C. Bound proteins were eluted by 3–5 changes of 0.2 ml of buffer C containing 1 mM CsA or FK506, respectively. Proteins were measured with the Bio-Rad protein assay with bovine serum albumin as the standard.

Immunopurification of GR- and CYP40-Containing Complexes. Cytosolic extracts (500 μl) of LMCAT cells prepared in buffer A were incubated with 5 nM [^{3}H]RU 486 for 2 h at 0°C and then supplemented with 20 mM sodium tungstate. For GR immunoprecipitation, 10 μg of the monoclonal anti-GR antibody BuGR$_2$ (Affinity BioReagents, Neshanic Station, NJ) or of nonimmune mouse IgG was added to the cytosolic extracts and incubated for 4 h at 0°C. For CYP40 immunoprecipitation, 500 μl of cytosol was incubated under the same conditions with 20 μl of the C-terminal 63 8W rabbit antiserum against human CYP40. Protein G-Sepharose (25 μl) was then added to the samples for 1 h at 0°C. After centrifugation at 700 $\times$ *g* for 5 min at 0°C, the pellets were washed once with 1 ml of buffer C, once with 1 ml of buffer B, and then three times with 1 ml each of buffer C. The pellets were resuspended in 200 μl of Laemmli SDS sample buffer (19) and boiled prior to denaturing SDS/PAGE and Western-blotting experiments.

Measurement of TA Binding to GR. LMCAT cells were grown in roller flasks and incubated for 18 h with or without 10 μM CsA or FK506. After cells were harvested and cytosol was prepared in buffer A, duplicate aliquots (130 μl) of cytosol were incubated at 0°C with 3×10^{-11} to 5×10^{-6} M [^{3}H]TA for 2 h, supplemented with 20 mM sodium tungstate, and then incubated on ice for an additional 16 h. Radioactivity in triplicate aliquots (10 μl) was determined to measure the total steroid concentration. A dextran-charcoal suspension (50 μl) was added to duplicate samples, which were incubated on ice for 7 min and then centrifuged at 0°C for 5 min at 700 $\times$ *g*. Radioactivity in duplicate aliquots (50 μl) of the supernatants was determined, and the amount of bound steroid was calculated. Binding parameters were determined by a computer program (18).

SDS/PAGE and Western Blotting. Samples were resolved on SDS/10% PAGE according to Laemmli (19). Western blots were performed as described (11). For detection of hsp90, the blots were probed with antibody 174, a polyclonal antipeptide antibody raised in rabbits against the C-terminal amino acid sequence extending from Met-807 to Glu-823 of human hsp90. CYP40 was identified with rabbit antibody, 63 8W raised against the C-terminal part of human CYP40 extending from Asp-356 to Asp-370, and FKBP59 was detected with 173, a rabbit C-terminal antipeptide antibody (4). All the antibodies were used at a 1:1000 dilution, and staining of the antigen–antibody complexes was performed with the Vectastain ABC kit (Vector Laboratories). Purified preparations of hsp90, FKBP59 (20), and CYP40 (17), generated by recombinant protein expression in *Escherichia coli*, were used as internal positive controls.

RESULTS

Potentiation of Dex-Induced MMTV–CAT Activity by CsA and FK506. Fig. 1 shows the relative CAT activities in LMCAT cells exposed to different doses of CsA and FK506 in the presence of 10 nM Dex. A dose-dependent increase is observed for both immunosuppressants, with a maximum occurring at 5 μM. The decrease of CAT activity that occurs for CsA concentrations higher than 5 μM may be due to drug cytotoxicity (21). The extent of potentiation of the transcription (Fig. 1) induced by the two drugs is similar, and the highest levels obtained were $\approx$50 times those obtained with 10 nM Dex alone. In addition, the maximal potentiations observed at 5 μM CsA equaled the CAT activities seen in response to 1 μM Dex alone (see Fig. 2), confirming our prior report that FK506 does not increase the maximal enhancement of transcription of the GR but instead causes a shift to the left in the hormone dependency of GR activation. FK506 appears more efficient than CsA at low doses. Similar experiments performed at 100 nM Dex also result in CsA and FK506 potentiations of transactivation (data not shown). In all cases, increasing the concentration of RU 486 2-fold over that of Dex abolishes CAT activity induced by Dex alone and its enhancement by immunosuppressants. These results strongly suggest that this effect is mediated via the GR.

When CsA and FK506 are both present in the culture medium (0.25 μM or 0.5 μM each), potentiation of the 10 nM Dex-induced MMTV–CAT activity by each drug is additive (data not shown). This is not observed at 1 μM immunosuppressant.

Potentiation by CsA Analogs, FK506 Analogs, and Rapamycin. Fig. 2*A* shows that two nonimmunosuppressive analogs

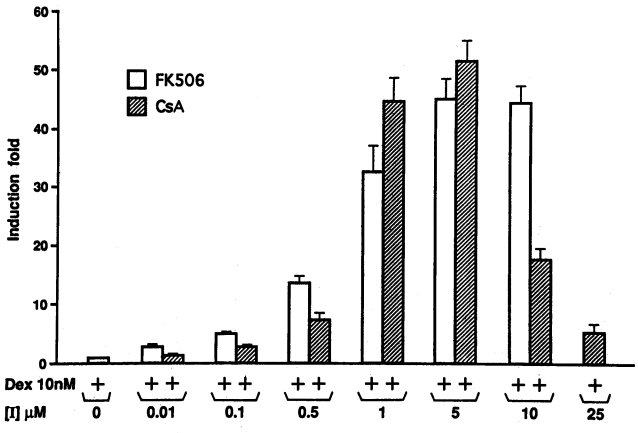

FIG. 1. CsA and FK506 potentiate Dex-induced expression of MMTV-CAT. Replicate Petri dishes (75 cm^2) of LMCAT cells were treated with 0.1% ethanol (data not shown) or 0.1% ethanol containing the indicated concentrations of FK506 or CsA. After 4 h, 10 nM Dex was added for an additional 18 h. CAT assays were performed as described in *Material and Methods* and induction values were calculated relative to the CAT activity induced by 10 nM Dex alone. Each value represents the mean $\pm$ SE of three different experiments with CAT assays performed in duplicate. No significant difference was observed when Dex and immunosuppressants were added simultaneously or if Dex was added (at 10 nM) 4 h prior to immunosuppressants. No significant increase in CAT activity was observed for the ethanol vehicle controls. I, immunosuppressant.

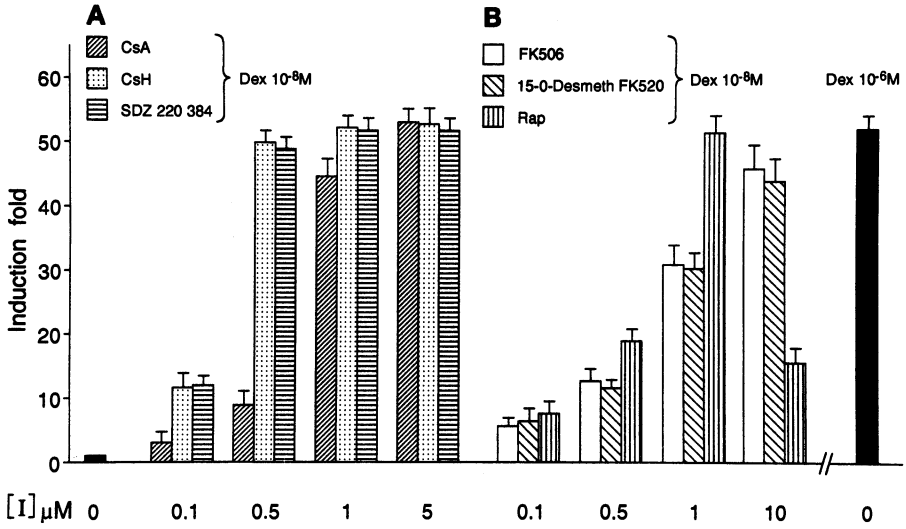

FIG. 2. Comparison of the potentiations of Dex-induced MMTV–CAT activity by different immunosuppressants and nonimmunosuppressant analogs. (*A*) CsA and nonimmunosuppressive CsA analogs. (*B*) FK506 and weak analog 15-*O*-desmethyl FK520 and rapamycin (Rap). Treatment of LMCAT cells and CAT assay were essentially as described in the legend to Fig. 1. Cyclosporin H (CsH), SDZ220384, 15-*O*-desmethyl FK520, and Rap at 0.1–10 μM were added to the cell culture medium for 4 h prior to the addition of 10 nM Dex for 18 h. Inductions of CAT activity were calculated relative to that with 10 nM Dex alone control. The response of the cells to 1 μM Dex alone is shown for comparison. I, immunosuppressant.

of CsA, CsH and SDZ220384 (*N-methyl*-Val-4-CsA), neither of which inhibits the phosphatase activity of CN (15, 22, 23), also increase Dex-induced MMTV–CAT activity in LMCAT cells. They have no effect on their own (data not shown). The maximal effect of both CsH and SDZ220384 is obtained at a concentration 1/10th the concentration of CsA giving the same potentiation effect. Similarly, 15-*O*-desmethyl FK520, an analog of FK506 that is a weak CN inhibitor, also has a strong potentiation effect (Fig. 2*B*). Rapamycin, an active immunosuppressant that binds FKBPs but does not act via the inhibition of CN phosphatase activity (24), does induce transcriptional activity (Fig. 2*B*), as already observed (14). The decrease of potentiation observed at high rapamycin doses is consistent with its well-known negative effect on cell viability (25). As with CsA and FK506, RU 486 blocks all of the potentiation effects of these analogs.

Other CsA and FK506 analogs were also tested in LMCAT cells. All of them potentiate (Table 1) the Dex-induced CAT activity, although to different extents. It is interesting to note that $MeBm_2t$-CsA, which is a potent immunosuppressant with CN inhibition activity but which does not inhibit the PPIase activity of CYP18 (26), strongly enhances transcription (Table 1). This was also found for $MeAla_6$-CsA, a nonimmunosuppressive CsA analog with strong PPIase inhibition activity but which does not inhibit CN (27).

Table 1. Comparative potentiation effects of different immunosuppressive and nonimmunosuppressive drugs on Dex-induced transcription in LMCAT cells, with regard to other functions

Drug	Immuno-suppression	PPIase inhibition	CN inhibition	Dex-induced transcription
CsA	+	+ (15)	+ (24)	+
CsH	−	− (23)	− (23)	+
SDZ220384	−	+ (22)	− (22)	+
$MeBm_2t$-CsA	+	− (26)	+ (26)	+
$MeAla_6$-CsA	−	+ (27)	− (27)	+
FK506	+	+	+ (24)	+
FK520	+	+ (26)	+ (26)	+
15-*O*-desmethyl FK520	+ (26)	+ (26)	Weak (26)	+
L685818	− (28)	+ (28)	− (28)	+
Rapamycin	+	+ (26)	− (24)	+
Deoxyspergualin	+	−	−	−

The lack of activity by the drug is indicated by −, while activity is indicated by +. Numbers in parentheses correspond to references. The extent of the increase of transcription provoked by each drug at 1 to 5 μM is greater than 20-fold the basal level induced by 10 nM Dex alone.

The FK506 analog L685818, a nonimmunosuppressant drug that does not inhibit CN (28), also potentiates the Dex-induced CAT activity, as does FK520 (ascomycin), an analog of FK506 (26).

Increase in Steroid-Binding Affinity Following Exposure of LMCAT Cells to Immunosuppressants. In cells grown in the absence of immunosuppressant, the dissociation constant (K_d) of the GR for TA is 1.45 ± 0.4 nM (N, the number of specific binding sites = 280 ± 30 fmol per mg of protein). This value decreases ≈2-fold in the presence of CsA (K_d = 0.4 ± 0.2 nM; N = 290 ± 40 fmol per mg of protein), or FK506 (K_d = 0.5 ± 0.3 nM; N = 308 ± 45 fmol per mg of protein), with no significant modification of the number of binding sites.

Immunoprecipitation of Immunophilin-Containing GR Complexes. Preliminary experiments showed that immunoprecipitation with $BUGR_2$ of the tungstate-stabilized heterooligomeric GR complexes from cells not exposed to Dex resulted in the specific retention of several proteins, as visualized by silver staining of SDS gels (data not shown). Western blot analysis of these samples revealed the presence of GR (Fig. 3*A*), hsp90 (Fig. 3*B*), FKBP59 (Fig. 3*C*), and CYP40 (Fig. 3*D*), while none of these proteins are detected under control conditions when using nonimmune mouse IgG. These data support the concept that the two immunophilins FKBP59 and CYP40 are associated with the GR in its tungstate-stabilized form(s). Measurements of radioactivity in cytosolic extracts before and after the precipitation showed that 95% of the [^{3}H]RU 486–GR complexes are present in the immunoprecipitate (data not shown). An identical experiment performed with LMCAT cytosol prepared in buffer A not supplemented with tungstate ions gave the same GR yield but no coisolation of hsp90, CYP40, and FKBP59, suggesting that these proteins dissociate from GR during the precipitation step or the high-salt washing of the immunopellet (data not shown).

Immunosuppressant-Affinity Purification of Immunophilins from LMCAT Cells. Portions of cytosol in which the GR had been labeled with [^{3}H]RU 486 were then supplemented with tungstate ions and subjected to either CsA-Affigel or FK506-Affigel affinity chromatography. SDS/PAGE analysis revealed the presence of several proteins in eluates of both the CsA-Affigel and the FK506-Affigel columns (data not shown) that may be immunophilins or immunophilin-associated proteins. The recovery of heterooligomeric GR complexes was low. Western blotting performed with the anti-CYP40 63 8W antibody detected a 40-kDa protein in the CsA-Affigel column eluates (Fig. 4*C*). The presence of hsp90 was detected in the

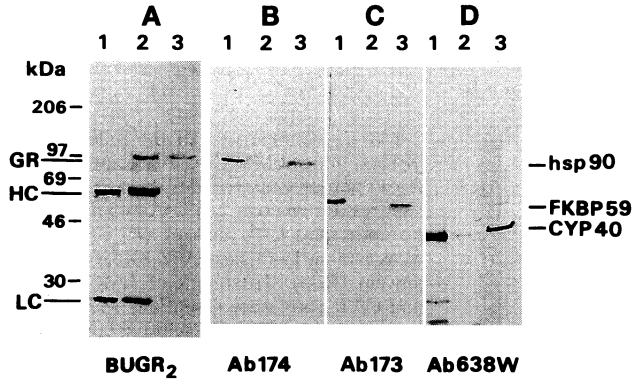

FIG. 3. Western blot of immunopurified GR complexes from LMCAT cells. Immunoprecipitation of GR heterocomplexes from LMCAT cell cytosolic extracts (500 μl) was performed as described under *Material and Methods.* Samples (70 μl) from each precipitation were resolved by SDS/PAGE and Western blotting in which the following antibodies were used as probes: $BuGR_2$, antibody against GR (*A*); Ab174, antibody against hsp90 (*B*); Ab173, antibody against FKBP59 (*C*); and Ab638W, antibody against CYP40 (*D*). (*A*) Lane 1, material precipitated with nonimmune mouse IgG; lane 2, material precipitated with $BuGR_2$; lane 3, 10 μl of LMCAT cytosol loaded directly onto gel. (*B*) Lane 1, 400 ng of purified hsp90; lane 2, material precipitated with nonimmune mouse IgG; lane 3, material precipitated with $BuGR_2$. (*C*) Lane 1, 500 ng of purified FKBP59; lane 2, material precipitated with nonimmune mouse IgG: lane 3, material precipitated with $BuGR_2$. (*D*) Lane 1, 800 ng of purified CYP40; lane 2, material precipitated with nonimmune mouse IgG; lane 3, material precipitated with $BuGR_2$. The position of size standards and that of identified antigens are indicated. HC, IgG heavy chain; LC, IgG light chain.

same eluates by antibody 174 (Fig. 4*B*). Western blotting performed with antibody 173, detected FKBP59 in the eluates of the FK506-Affigel column. As before, hsp90 was detected in the same eluates by the antibody 174 (Fig. 4*A*). It is noteworthy that FKBP59 was absent from the CsA-affinity column eluate, just as CYP40 was absent from that of the FK506-affinity column (Fig. 4*B*, lane E and Fig. 4*C*, lane E_A, respectively). Conversely, the flow-through fractions of the two immunosuppressant-affinity columns—i.e. CsA- and FK506-Affigel—contained FKBP59 and CYP40, respectively. Thus, two different populations of hsp90–immunophilin complexes appear to be present in LMCAT cell cytosol: one containing hsp90–CYP40, the other FKBP59–hsp90 (Fig. 4). Both complexes could independently associate with the GR, as suggested by the data of Fig. 3.

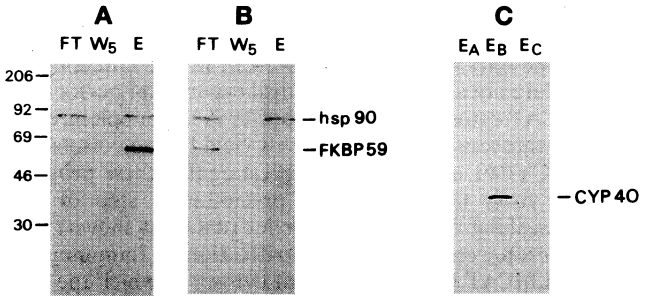

FIG. 4. Western blotting of proteins purified by FK506- and CsA-affinity chromatography. Immunosuppressant-affinity purification of immunophilins from 600 μl of cytosol was performed as described in *Material and Methods.* Proteins from samples (50 μl) of the flow-through fractions (FT), the last wash (W_5) and the pooled eluates (E) from FK506- (*A*) and CsA-affinity columns (*B*) were analyzed by SDS/10% PAGE blotted, and incubated with a mixture of antibodies 174 and 173 to reveal hsp90 and FKBP59, respectively. In *C*, eluates from FK506- (E_A) and CsA-affinity columns (E_B) and Affigel control column (E_C) were blotted with 63 8W to reveal CYP40.

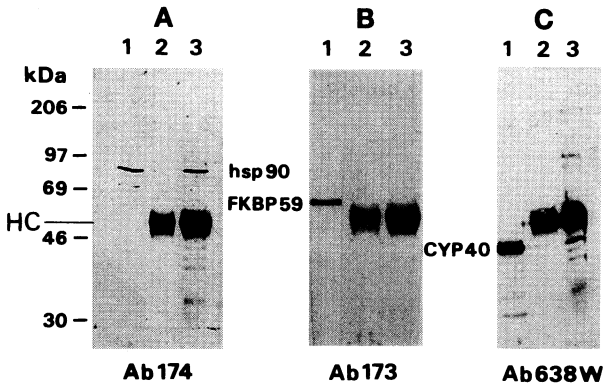

FIG. 5. Coimmunoprecipitation of CYP40 and hsp90. CYP40 was immunoprecipitated from LMCAT cell cytosolic extracts by antibody Ab638W as described in *Material and Methods.* The immunoprecipitates (40 μl) in lanes 3 were incubated with Ab174 to detect hsp90 (*A*), Ab173 to detect FKBP59 (*B*), or Ab638W to detect CYP40 (*C*). In lanes 2 of all panels 40 μl of material precipitated with nonimmune rabbit IgG was analyzed. In lanes 1, 400 ng of recombinant purified hsp90 (*A*), 500 ng of purified recombinant FKBP59 (*B*), or 800 ng of purified human CYP40 (*C*) were used as internal positive controls. HC, IgG heavy chain.

Immunoprecipitation of CYP40 with 63 8W Antibody. To demonstrate the association of CYP40 with hsp90, CYP40 was immunoprecipitated with the anti-CYP40 antipeptide antibody 63 8W. Western blots indicate that the immunoprecipitate indeed contains CYP40 and hsp90 but not FKBP59. A small amount of CYP40 was adsorbed to control nonimmune rabbit IgG–protein G-Sepharose (Fig. 5*C*, lane 2). Small amounts of [^{3}H]RU 486–GR complexes were found in the precipitate, suggesting either that GR dissociates during the experiment or that, by analogy with the excess of FKBP59–hsp90 complexes in several cell systems (3, 11, 29), an excess of CYP40–hsp90 complexes is present in LMCAT cells compared with the GR content.

DISCUSSION

As shown in Fig. 1, CsA increases the relative CAT activity induced by 10 nM Dex ≈50-fold, a potentiation effect similar to that obtained with FK506.

Both CsA and FK506 are drugs known to inhibit T-cell activation by a mechanism that involves the inhibition of the phosphatase activity of CN (21, 24, 30). Recently, it was reported that FK506 potentiates progestin-induced transcription in a yeast strain transfected with the human progesterone receptor and that this potentiation may be mediated by CN inhibition but that FKBP12 was not involved (31). This does not seem to be the case in LMCAT cells, since either rapamycin (14, 24), or CsA analogs (CsH, SDZ220384, and $MeAla_6$-CsA) (22, 23, 27), known to be devoid of CN inhibition activity, all potentiate Dex-induced CAT activity (Fig. 2). Similarly, among the FK506 analogs, 15-*O*-desmethyl FK520, an avid ligand for FKBPs but a poor inhibitor of CN (26), and L685618 (28), which is devoid of CN inhibition, also potentiate the Dex-induced transcription. Thus, the mechanism by which immunosuppressants increase Dex-induced transcriptional activity in mouse fibroblast LMCAT cells does not appear to involve CN.

In addition, the immunosuppressant $MeBm_2t$-CsA, which, like CsH, is a poor inhibitor of the PPIase activity of CYP18 (27), is also a strong potentiator of Dex-induced transcription. The inhibition of PPIase by immunophilin ligands is generally felt not to be responsible for their immunosuppressive activity (21), and thus, it may also not be associated with the increased transcription of the GR-responsive MMTV–CAT gene.

It is noticeable that our results are obtained with micromolar concentrations of drugs, contrasting with the very low concentrations (nM) of CsA and FK506 which give immunosuppressive activities (21, 25). The affinity of FKBP59 for FK506 and rapamycin is weaker than that of FKBP12. Similarly, the affinity of CYP40 for CsA is weak compared with that of CYP18 (K. Hoffmann, unpublished data). Thus, FKBP59 and CYP40 could be logical candidates for mediating the observed effects described here. Micromolar concentrations of similar CsA analogs provoke a decrease of cytotoxic resistance of a human small-cell lung cancer cell line (32). The multiplicity of immunophilins with different drug affinities, therefore, could be responsible for different cell responses to different drug concentrations.

We found that CYP40 is associated with GR from LMCAT cells not exposed to glucocorticosteroids; CYP40 has also been detected in the untransformed form of the calf uterus estradiol receptor complex (13), as well as FKBP59, known to be a common component of many unliganded receptor complexes in many species (1–3, 11, 29). FKBP59 is a 90-kDa hsp-binding immunophilin, as indicated previously (11, 12). Exposure of different receptors to FK506 or CsA *in vitro* (8, 33, 34), as well as in cells (this work), does not apparently modify the protein composition of unliganded receptors. From CsA-affinity purification experiments (Fig. 4), it is tempting to speculate that CYP40 is also able to bind hsp90. This is strongly supported by the coimmunoprecipitation of both CYP40 and hsp90 with anti-CYP40 antibodies (Fig. 5 and J. Owens-Grillo, K.H., K. A. Hutchison, A. W. Yem, M. R. Deibel, Jr., R.E.H., and W. B. Pratt, personal communication). Thus, both CYP40 and FKBP59 may belong to a new class of immunophilins, hsp-binding immunophilins. CYP40 and FKBP59 share C-terminal sequence homology, characterized by the presence of tetratricopeptide repeats (TPRs) (13). These TPRs are necessary for FKBP59–hsp90 association (12), and it is speculated that TPRs could be necessary for CYP40–hsp90 interaction. From our data, we also submit that, although CYP40 and FKBP59 can bind hsp90, they form distinct complexes with hsp90. A CYP40–hsp90–FKBP59 complex does not seem to exist.

In conclusion, the present biochemical studies demonstrate the Dex-induced potentiation by immunosuppressants and nonimmunosuppressant analogs of the MMTV–CAT reporter gene. We also suggest that this effect may be mediated by two types of hsp90–immunophilin complexes. There is no direct evidence that the enhancement of transcription is mediated by receptor-associated immunophilins. Nevertheless, a series of observations are in favor of this hypothesis. Exposure to immunosuppressants has been shown to modestly increase the hormone-binding affinities of receptors (8, 23), and hormone-induced GR translocation (ref. 14 and J. Cidlowski, personal communication). We have indicated that the affinities of FKBP59 and CYP40 for drugs are much weaker than those of FKBP12 and CYP18, respectively. An immunosuppressant, such as deoxyspergualin, that does not bind to immunophilins (Table 1) but is a ligand for hsp90 and hsp70 (35), has none of the above-mentioned effects upon transcription, receptor–hormone binding, and nuclear translocation. Work is in progress to determine the pathway(s) by which immunosuppressive drugs act to control transcriptional activities mediated by steroids in different cells.

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