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Stabilization *In Vitro* of the Untransformed Glucocorticoid Receptor Complex of S49 Lymphocytes by the Immunophilin Ligand FK506

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Untransformed steroid receptors are large heteromeric complexes which have been shown to contain the mammalian heat shock proteins hsp56, hsp70 and hsp90. Based on functional and sequence homology studies, it was recently discovered that hsp56 also belongs to the FKBP class of immunophilin proteins, which are thought to mediate the actions of the immunosuppressive drugs FK506 and rapamycin. This discovery has led to the speculation that FK506 and related drugs could influence the actions of steroid receptors. In this work, we have examined the effects of FK506 on the transformation and hormone-binding properties of glucocorticoid receptors (GR) present in the cytosolic fraction of mouse S49 lymphocyte cells. Based on immunoprecipitation studies, it was found that hsp56 was indeed a component of untransformed GR complexes in S49 cytosols. It was also found that the untransformed but not the transformed GR was retained following affinity chromatography with FK506-affigel resin, reinforcing the possibility that hsp56 within the untransformed GR complex could be a target for the actions of FK506. Using a DNA–cellulose-binding assay, FK506 exhibited a 60% inhibition of dexamethasone (Dex)-induced transformation of the GR to the DNA-binding state, while sodium molybdate, a transition metal oxyanion known to stabilize GR complexes, was 100% effective. This inhibition of GR transformation by FK506 was shown to correlate with an inhibition of Dex-induced GR/hsp90 dissociation, with 10 μM FK506 preventing 48% of the GR/hsp90 complexes from dissociating. Scatchard analysis of GR hormone-binding function was performed, with FK506 treatment of cytosols causing K_d values to decrease (3.36 nM) as compared to vehicle (8.42 nM) and no-addition (9.82 nM) controls. Taken together, our results suggest that FK506 can stabilize the untransformed GR complex of S49 cells and that this stabilization in turn results in an increase in GR ligand-binding affinity. Although we speculate that these actions of FK506 on the GR complex are mediated by the associated hsp56 component, other possible mechanisms are also discussed.

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INTRODUCTION

The glucocorticoid receptor (GR) is a member of the steroid receptor family of proteins which act as ligand-regulated enhancers of specific gene transcription [1–3]. In cells grown in the absence of hormone, the "untransformed" GR is recovered in the cytosolic fraction as a large heteromeric complex containing several members of the mammalian heat shock protein family [see ref. 4 for review]. These non-steroid-binding components of the untransformed GR include hsp90 [5–8], hsp56 [9, 10] and, under certain circumstances, hsp70 [11]. A series of recent reports have shown that the GR and other untransformed steroid receptor complexes also contain members of the immunophilin class of proteins. The initial observation of this kind was made by Yem *et al.* [12] when it was shown that the NH_2-terminus of a $\approx$60 kDa protein capable of binding the immunosuppressive drug FK506 was nearly identical in amino acid sequence to hsp56 (which has also been referred to as p59 [10], FKBP59 [13], FKBP52 [14] and HBI [15]). Since then, the cDNAs of human [14] and rabbit [15] hsp56 have been cloned with each exhibiting sequence homology

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with the region of an FK506-binding protein (FKBP-12) responsible for rotamase activity; while the human form of hsp56 was shown to have rotamase activity *in vitro* that was inhibited by FK506 binding [14]. Similarly, Tai *et al.* [13] reported the existence of an FK506-binding protein in human thymus cells that was recognized by a monoclonal antibody against hsp56, and that the untransformed GR complex of human IM-9 cells could be purified via FK506 affinity chromatography, presumably through its hsp56 subunit.

In addition to hsp56, other components of untransformed steroid receptor complexes may also be immunophilins. For example, progesterone receptor complexes not only contain hsp56 but also a p54 protein which shares sequence homology with hsp56 [16, 17]; while estrogen receptor complexes contain a 40 kDa protein that is also homologous to hsp56 [18] and which is identical to a recently discovered cyclosporin A-binding protein, CyP-40 [19]. This association of immunophilins with steroid receptor complexes has fueled the speculation that the immunophilin and steroid receptor signal transduction pathways may be functionally convergent. However, very few functional studies of this kind *in vitro* or *in vivo* have been reported. In one such study, Hutchison *et al.* [20] showed that FK506 had no effect on a variety of properties of the L929 cell GR, including steroid-binding function, nuclear translocation, transcriptional enhancement and reconstitution of the untransformed receptor complex. In contrast, our laboratory has found that pretreatment of intact L929 cells with FK506 or rapamycin will cause an increase in dexamethasone (Dex)-induced GR transcription enhancement activity [21]. A more recent report by Renoir *et al.* [22] provided evidence that the hormone-binding function of rabbit uterus progesterone receptors (PR) can be increased by treatment of cytosols with FK506 and that FK506 could partially stabilize 9S PR complexes during purification.

As the principal therapeutic target of both glucocorticoid- and FK506-mediated immunosuppression are T lymphocytes, we have chosen the T cell-derived mouse S49 cell line to examine the effects of FK506 on the GR transformation process under cell-free conditions. Our results show that FK506 will increase the steroid-binding affinity of the S49 cell GR, while at the same time inhibiting Dex-induced GR transformation to the DNA-binding state by stabilizing the GR/hsp90 association.

EXPERIMENTAL

Chemicals

FK506 was obtained from the Fujisawa Pharmaceutical Company (Deerfield, IL). The FK506-affigel affinity resin was obtained from the Upjohn Company (Kalamazoo). The BuGR2 anti-glucocorticoid receptor monoclonal antibody [23] was a gift from Drs William Hendry and Robert Harrison. The UPJ56 antibody (rabbit serum) against hsp56 [24] was obtained from Martin Diebel and Karen Leach (Upjohn Company). [^{3}H]Triamcinolone acetonide (TA) at 57.90 Ci/mmol and ^{125}I-conjugated goat anti-mouse IgG (11.8 μCi/μg) were obtained from ICN Radiochemicals. Immobilon P membranes were obtained from Millipore Corp. Dexamethasone, tris, EDTA, DMSO, sucrose, protein A-Sepharose, DNA–cellulose, horseradish peroxidase-conjugated rabbit anti-goat IgG, RPMI-1640 powdered medium and iron-supplemented newborn calf serum were obtained from Sigma Chemical Company.

Cell culture, cytosol preparation and immunoadsorption

S49 cells were cultured at 37°C/5% CO_2 in RPMI 1640 medium supplemented with 10% newborn calf serum. Unless otherwise noted, cytosols were prepared by washing the S49 cells with ice-cold Hank's buffered saline solution, followed by Dounce homogenization in hypotonic HE buffer (10 mM Hepes, 1 mM EDTA, pH 7.4) and centrifugation at 8225 *g* for 5 min. Immunoadsorption of GR and associated proteins (Fig. 3) from cytosols was achieved by dilution of the cytosols with TEG buffer (10 mM TES, 5 mM EDTA, 10% v/v glycerol and 50 mM NaCl, pH 7.6) and simultaneous addition of BuGR2 monoclonal antibody against the GR (hybridoma fluid) to a final concentration of 10% (v/v) and 50 μl of protein A-Sepharose resin. After mixing on ice for 16–20 h, the protein A resins were washed with 5 × 1 ml changes of TEG buffer and the bound proteins were eluted with SDS sample buffer and analyzed by Western-blotting.

FK506-affigel affinity chromatography

In the experiments of Fig. 1, aliquots of S49 cytosols were incubated in batch with 50 μl of FK506-affigel pre-equilibrated in HE buffer containing 1 mM sodium molybdate. After mixing on ice for 16–20 h, the resins were washed with 3 × 1 ml changes of HE buffer containing 10 mM molybdate. The bound proteins were then eluted with SDS sample buffer and analyzed by Western-blotting.

DNA- and steroid-binding assays

In the experiments of Fig. 2, aliquots of S49 cytosol were incubated in batch with 150 μl of a DNA–cellulose suspension (12.5% v/v) preequilibrated in HE buffer containing 10 mM sodium molybdate. After mixing on ice for 1 h, the DNA resins were washed with 5 × 1 ml changes of HE buffer, and the DNA-bound receptors were eluted with SDS sample buffer and analyzed by Western-blotting.

In the experiments of Fig. 4, aliquots (45 μl) of cytosol treated as described were incubated on ice for 4 h with increasing concentrations (0.5, 1.0, 2.5,

5.0, 10.0, 25.0, 50.0, 75.0 and 100.0 nM) of [^{3}H]triamcinolone acetonide (TA) in the presence or absence of 50 μM radionert Dex. The bound radioactive ligand was separated from free ligand by application of a 150 μl suspension of dextran-coated charcoal (1%) in HE buffer, followed by centrifugation and measurement of the radioactivity remaining in the supernatant. Specific-binding was calculated by subtracting the bound radioligand values obtained in the presence of Dex from those obtained in its absence and normalizing to protein content.

Gel electrophoresis and quantitative Western-blotting

Samples were resolved by electrophoresis in 7% polyacrylamide SDS gels as described by Laemmli [25]. The relative amounts of GR or GR-associated hsp90 were determined using a quantitative Western-blotting technique previously described [26]. The probe antibodies employed were the BuGR2 anti-GR monoclonal antibody [23] or a polyclonal serum against hsp70 (åhsp70) that also recognizes hsp90 [27]. The blots were then sequentially reacted with peroxidase- and ^{125}I-conjugated counter antibodies. After color development and autoradiography, the peroxidase-stained GR or hsp90 bands were excised and ^{125}I cpm were determined via liquid scintillation spectrophotometry. GR- or hsp90-specific cpm values were derived by subtracting the cpm value measured in a background slice of comparable area.

RESULTS

FK506 affinity chromatography of the untransformed S49 cell GR

As an initial step in the characterization of FK506 effects on the GR of S49 lymphocytes, we assayed for the presence of hsp56 in the untransformed GR complex. In the experiment of Fig. 1(A), S49 cytosols made from cells grown in the absence of hormone and maintained at 0°C were immunoadsorbed with the BuGR2 antibody against GR. Western-blotting of these samples using the UPJ56 rabbit serum [24] revealed the presence of GR-associated hsp56 whether or not the cytosols were prepared in the presence of sodium molybdate. These results indicate that hsp56 is indeed a subunit of untransformed GR in S49 lymphocytes and that molybdate is not needed in order to stabilize the interaction of hsp56 with the GR complex. For this reason, all subsequent experiments, unless otherwise noted, employed cytosols made in the absence of molybdate.

In order to determine if the S49 cell GR contains hsp56 capable of binding FK506, we subjected S49 cytosols to affinity chromatography using FK506-

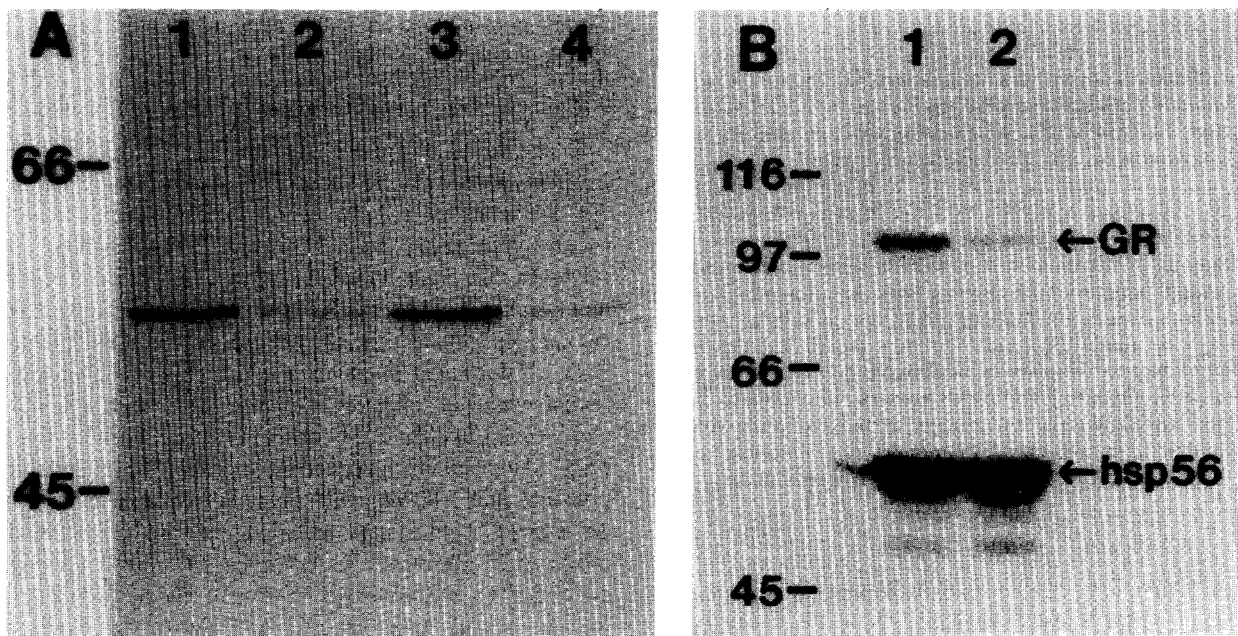

Fig. 1. The untransformed GR complex of S49 cells contains hsp56 and is retained by FK506 affinity chromatography. Panel A: aliquots of S49 cell cytosol made in hypotonic buffer with (lanes 1 and 2) or without 1 mM sodium molybdate (lanes 3 and 4) were immunoadsorbed to protein A-Sepharose with BuGR2 anti-GR monoclonal antibody (lanes 1 and 3) or with non-immune mouse IgG (lanes 2 and 4). After washing of the protein A resins with TEG buffer ± molybdate, the samples were resolved by Western-blotting using the UPJ56 rabbit serum against hsp56. Panel B: Dex (0.1 μM final) was added to equal aliquots of S49 cytosol. After incubation on ice for 3 h, one aliquot was maintained on ice (lane 1), while one was warmed at 25°C for 45 min to transform the GR complex (lane 2). Each aliquots was then incubated with FK506-affigel, and the bound proteins were resolved by Western-blotting using the BuGR2 antibody (blot above 66 kDa marker) or the UPJ56 serum (blot below 66 kDa marker).

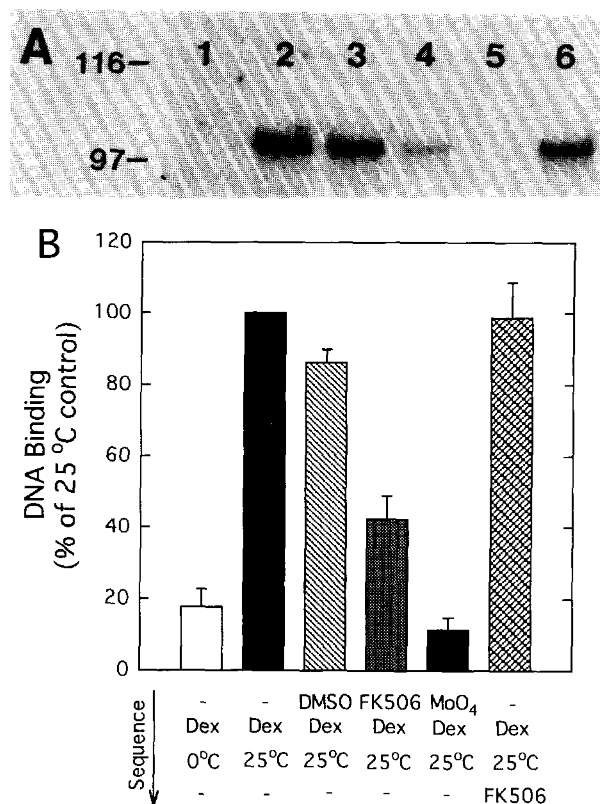

Fig. 2. FK506 inhibits transformation of the S49 cell GR to the DNA-binding state. Following a 1 h preincubation on ice with buffer, 10 μM FK506, DMSO vehicle or 1 mM sodium molybdate, aliquots of S49 cell cytosols were incubated on ice with 0.1 μM Dex for an additional 2 h. All aliquots except one (lane 1) were then warmed to 25°C for 1 h. After warming, one untreated sample was then made 10 μM for FK506 and incubated for an additional h on ice (lane 6). DNA-binding was then assayed at 0°C by rotation of each sample with a 12.5% suspension of DNA–cellulose. After washing, the DNA–cellulose binding proteins were eluted with SDS sample buffer and analyzed by a quantitative Western blotting procedure employing the BuGR2 anti-GR monoclonal antibody as probe and both peroxidase-conjugated and ^{125}I-conjugated counter antibodies. Panel A: autoradiogram of typical blot. Lanes 1–6 correspond to the conditions noted in bars 1–6 of panel B. Panel B: peroxidase-labeled bands corresponding to GR protein were excised from the blots and subjected to liquid scintillation spectrophotometry. Values depicted are expressed as percent of the hormone-treated, temperature transformed control and represent the mean $\pm$ SEM of three independent experiments.

affigel [Fig. 1(B)]. Prior to incubation of the cytosols with the FK506 resin, one aliquot of cytosol was bound with Dex and kept on ice, while the second aliquot was bound with Dex and warmed at 25°C to transform the GR. As can be seen from the results, there was significantly less GR retained by the FK506 resin following warming of the cytosol, while large amounts of "free" hsp56 were retained under both conditions. These results suggest the following. First, that the hsp56 component of the untransformed GR complex can indeed bind FK506. Second, that binding of FK506 to GR-associated hsp56 does not disrupt the GR complex.

FK506 inhibits transformation of the GR to the DNA-binding state

As the binding of FK506 did not appear to cause or facilitate dissociation of the untransformed GR even when bound with hormone [Fig. 1(B)], we wondered if FK506 instead had a stabilizing effect on the GR complex. We tested this notion by measuring the effects of FK506 on hormone-mediated transformation of the GR to the DNA-binding state using a DNA–cellulose binding assay (Fig. 2). In these experiments, aliquots of S49 cytosol were preincubated on ice with FK506 or control reagents before addition of 0.1 μM Dex. After hormone-binding, all samples but one were warmed at 25°C for 1 h in order to transform the GR to the DNA-binding state. As can be seen from the results, acquisition of DNA-binding function was dependent on warming of the cytosols and this process was completely inhibited by sodium molybdate, a transition metal oxyanion known to stabilize steroid receptors against hormone- and temperature-mediated transformation [28]. Interestingly, FK506 also had an inhibitory effect on GR transformation, allowing only 40% of the GR to bind DNA. This inhibitory effect by FK506 was not at the level of a post-transformation step, such as a direct blockade of the GR–DNA interaction, because treatment with FK506 after transformation (lane 6) had no effect on GR DNA-binding.

FK506 inhibits hormone-induced GR/hsp90 dissociation

The results of Fig. 2 suggest that FK506 somehow inhibits hormone-mediated transformation of the GR. As the work of several laboratories have shown that transformation of the GR to the DNA-binding stage requires dissociation of hsp90 [6, 7, 29], one possible mechanism by which FK506 inhibits transformation is by simply preventing this dissociation. On the other hand, it has also been shown that hormone-mediated transformation of progesterone receptors results in acquisition of a distinct conformational state by the PR once it has dissociated from hsp90 [30]. Thus, it is possible that FK506 inhibits transformation by causing an altered conformational state of the GR to form, while still allowing dissociation of hsp90 to occur. In order to discriminate between these possibilities, we measured the effects of FK506 on hormone-induced GR/hsp90 dissociation (Fig. 3). The results demonstrate a concentration-dependent inhibition of GR/hsp90 dissociation by FK506, with $\approx$48% of the GR–hsp90 complexes stabilized against hormone-induced dissociation at 10 μM drug. Although FK506 was not as efficient as molybdate (100%) in stabilizing the GR complex, the level of GR stabilization observed was approximately the same as the level of FK506 inhibition of GR DNA-binding (Fig. 2), suggesting

that FK506 inhibits GR transformation by preventing hsp90 dissociation.

Effects of FK506 on GR hormone-binding function

Although the data presented to this point are consistent with a model in which FK506 through hsp56 stabilizes the GR–hsp90 interaction against hormone-induced dissociation, it is also possible that FK506 acts by actually reducing the ability of the GR to bind hormone. We have tested this possibility by measuring the effects of FK506 on GR hormone-binding function. In the experiments of Fig. 4(A), aliquots of S49 cytosol were preincubated for 2 h on ice with 10 μM FK506 before addition of increasing concentrations of [^{3}H]triamcinolone acetonide. The results show an FK506-dependent increase in specifically-bound ligand at low concentrations of hormone (0.5–10 nM) followed by a decrease in ligand-binding at higher concentrations of hormone (25–100 nM). When these data are subjected to Scatchard analysis [Fig. 4(B)], it was found that FK506 can cause a small but reproducible decrease in the dissociation constant of the S49 cell GR (K_d = 3.36 nM) as compared to the vehicle and no-addition controls (K_d = 8.42 and 9.82 nM, respectively). Interestingly, a decrease in maximal specifically-bound ligand (B_{max}) was also observed in response to FK506 (102.1 fmol/mg) as compared to the vehicle and no-addition controls (120.1 and 128.5 fmol/mg,

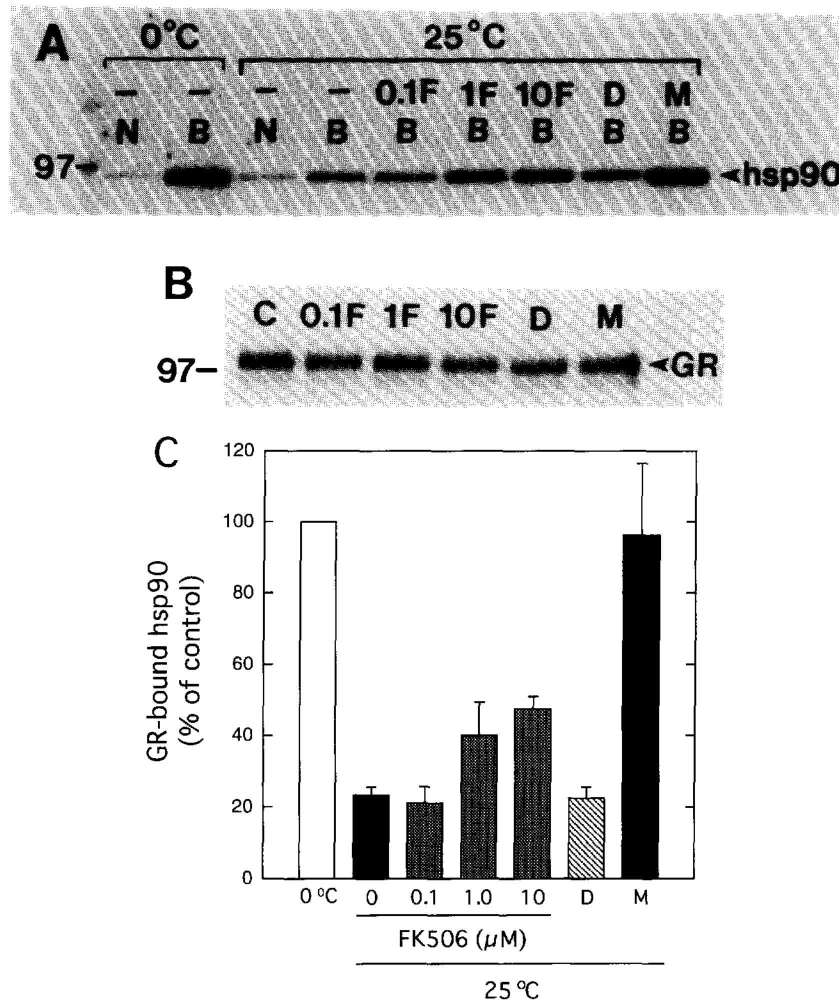

Fig. 3. FK506 inhibits hormone-mediated dissociation of the S49 cell GR/hsp90 complex. Panel A: aliquots of S49 cell cytosols were preincubated with no additions (–), 0.1, 1.0 or 10 μM FK506 (F), DMSO vehicle (D) or 1 mM sodium molybdate (M) for 1 h on ice. Each aliquot was then made 1 μM for Dex and incubated an additional 2 h on ice. All aliquots except one were warmed at 25°C for 1 h and this temperature-mediated transformation was stopped by the addition of sodium molybdate (1 mM final concentration) and re-chilling on ice for 30 min. Two aliquots (0 and 25°C) were then split in half and immunoadsorbed to protein-A Sepharose with either non-immune mouse IgG (N) or BuGR2 anti-GR monoclonal antibody (B), while all other conditions were immunoadsorbed solely with BuGR2. After washing, the immunoadsorbed complexes were eluted with SDS sample buffer and analyzed by Western-blotting, using the åhsp70 polyclonal serum against hsp90 as probe. Panel B: aliquots of S49 cytosol warmed at 25°C in the presence of FK506 (F), DMSO (D), molybdate (M) or buffer (C) were bound with Dex and the GR protein levels were resolved by immunoadsorption and Western-blotting with BuGR2. Panel C: quantitation of GR-associated hsp90. The values depicted represent the mean ± SEM of three independent experiments.

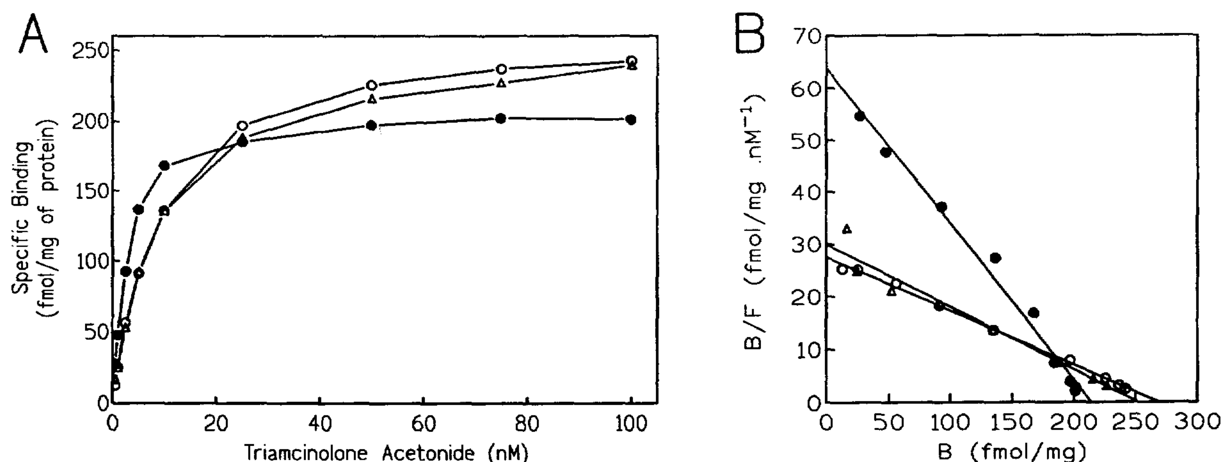

Fig. 4. Effect of FK506 on GR steroid-binding affinity in S49 cytosol. Aliquots of S49 cytosol were untreated (○) or treated with DMSO (△) or 10 μM FK506 (●) on ice for 1 h. Following exposure to 25°C for 1 h, the samples were returned on ice for 30 min and assay for steroid binding was performed as described under "Experimental". Panel A: a representative saturation curve of [^{3}H]TA-specific binding. Panel B: Scatchard analysis of data from panel A. B, bound; F, free.

respectively), suggesting that FK506 can both increase the hormone-binding affinity of the S49 GR while decreasing the number of binding sites.

Given the above results, we considered the possibility that the reduction in hormone-reduced transformation observed in the experiments of Figs 2 and 3 is simply the result of an FK506-induced loss of GR protein and, as a consequence, a loss of hormone-binding sites. However, several aspects of our data argue against this possibility. First, the reduction in maximal binding sites from 128.5 to 102.1 in response to FK506 corresponds to a decrease of only $\approx 21\%$, while the FK506 inhibitory effect on DNA-binding (Fig. 2) was considerably larger ($\approx 60\%$). Second, treatment with FK506 resulted in greater amounts of GR-associated hsp90 [Fig. 3(A)], suggesting that the loss of binding sites in response to FK506 is not due to a loss of GR. And indeed no reduction in GR protein in response to 0.1, 1.0 or 10 μM FK506 was observed [Fig. 3(B)].

Taken together, the results presented thus far provide evidence that FK506 treatment of the S49 cell GR can cause an inactivation of binding function for a small subset of GR molecules, while at the same time increasing the hormone-binding affinity for the majority of the GR population. In theory, this overall increase in steroid-binding affinity by FK506 should have resulted in a stimulation of hormone-induced transformation. Yet the results of Figs 2 and 3 document an inhibitory effect by this drug on transformation. We have attempted to clarify this issue by measuring the effect of FK506 on GR DNA-binding function at low concentrations of hormone (0.5–25 nM TA), conditions under which we observed an increase in hormone-binding function [Fig. 4(A)]. However, even at these low concentrations of TA, FK506 continued to have an inhibitory effect on GR transformation to the DNA-binding state (data not shown).

DISCUSSION

The data presented herein provides evidence that the immunosuppressive ligand FK506 can both increase the hormone-binding affinity of the untransformed GR of S49 lymphocytes and stabilize the GR against hormone-induced transformation to the DNA-binding state. In addition, we showed that the inhibition of transformation by FK506 occurs through an inhibition of GR/hsp90 dissociation, an effect that we have also observed in cytosols derived from mouse L929 cells (data not shown). As a series of papers by Pratt and co-workers have conclusively shown that hsp90 association with the GR is required for the GR to exhibit hormone-binding function [31–33], and as hsp56 has been shown to bind untransformed steroid receptor complexes through the hsp90 component [34], the simplest interpretation of our results is that FK506 acting through hsp56 in the GR complex can somehow stabilize or tighten the binding of hsp90 to the GR, which in turn results in an increase in hormone-binding affinity by the GR. How then could this stabilization of the GR–hsp90 interaction by FK506 occur? One possibility is that stabilization of the GR–hsp90 complex results from an inhibition of hsp56 rotamase activity by FK506. As a class, immunophilins exhibit peptidyl-prolyl *cis-trans* isomerase (rotamase) activity that is inhibited by the binding of FK506 and other immunosuppressive macrolides [35, 36], and indeed the human form of hsp56 has been shown to have rotamase activity that can be inhibited *in vitro* by the binding of FK506 [14]. In addition, rotamase activity by immunophilins has also been implicated in

protein folding processes [37]. Thus, the proposal that FK506 stabilizes the untransformed GR complex by inhibiting the rotamase function of GR-associated hsp56 is plausible.

However, there are other possibilities that need to be considered. First, it is possible that the effects of FK506 on GR hormone-binding and stabilization may be independent events. For example, FK506 may increase GR steroid-binding affinity by binding hsp56 in the receptor complex, but the stabilization against hormone-induced transformation may be mediated by "free" hsp56 [38] or other FK506-binding proteins, such as FKBP-12 [39]. As the principal target of FK506-bound FKBP-12 has been shown to be calcineurin [39, 40], a Ca^{2+} calmodulin-dependent phosphatase which is inactivated by the FK506–FKBP-12 complex, it is possible that inhibition of calcineurin by FK506 may be a factor in controlling GR transformation under our conditions. Of course, calcineurin involvement, if any, may not be restricted to stabilization of the GR complex, but may also be responsible for the observed effects on hormone-binding function. In either case, it will be interesting to see if there is an increase in phosphorylation levels for the GR or GR-associated hsp90 following FK506 treatment. Yet it should be emphasized that hsp56 and FKBP-12 are but a few of the potential targets for FK506 in our system. For example, a variety of less well characterized FK506-binding proteins have been identified by several laboratories [41, 42], and a novel PR-associated protein, p54, has recently been shown to share sequence homology with hsp56 [16, 17].

The results reported here are in good agreement with a recent report by Renoir *et al.* [22], in which the authors tested the effects of FK506 on PR present in rabbit uterus cytosol. In this work, it was similarly found that FK506 could cause a small increase in hormone-binding affinity by the PR, but no decrease in hormone-binding sites was observed. It was also shown that FK506 could partially stabilize the untransformed PR complex against salt-induced dissociation. Taken together then, the results of our two laboratories provide evidence that FK506 can effect both the macromolecular structure and hormone-binding functions of steroid receptors in a variety of *in vitro* systems. In addition, our results and those of Renoir *et al.* [22] demonstrating an FK506-induced increase in steroid-binding affinity provide a plausible explanation for our prior report documenting a potentiation of GR-mediated gene expression following FK506 treatment of intact cells [21]. In contrast, the FK506 stabilization effect against hormone-induced transformation observed here under *in vitro* conditions is not consistent with the FK506-mediated increase in hormone-induced GR nuclear translocation and transactivation observed *in vivo* [21]. Although it is possible, as proposed by Renoir *et al.* [22], that a combination of stabilization of steroid receptor complexes and an increase in hormone-binding affinity can lead to an overall "increase in hormonal response", it is also possible that differences in responses by steroid receptors to FK506 and other immunosuppressive ligands will exist between *in vitro* and *in vivo* systems. One dramatic distinction, of course, between these systems is the complete absence under *in vitro* conditions of the translocation mechanisms that must exist in the intact cell. With this in mind, it is likely that the number of convergence points between the immunophilin and steroid receptor signal pathways will be greater in the intact cell than in cytosols, further adding to the complexities of deciphering immunophilin effects on steroid receptors.

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