Steroid hormones bind and activate intracellular receptors that are ligand-regulated transcription factors. Mammalian steroid receptors can confer hormone-dependent transcriptional enhancement when expressed in yeast, thereby enabling the genetic identification of nonreceptor proteins that function in the hormone signal transduction pathway. Pdr5p (Lem1/Sts1/Ydr1p), a yeast ATP-binding cassette transporter, selectively decreases the intracellular levels of particular steroid hormones, indicating that active processes can affect the passage of steroids across biological membranes. In yeast, the immunosuppressive drug FK506 inhibited Pdr5p, thereby potentiating activation of the glucocorticoid receptor by dexamethasone, a ligand that is exported by Pdr5p. In mammalian L929 cells but not in HeLa cells, FK506 potentiated dexamethasone responsiveness and increased dexamethasone accumulation, without altering the hormone-binding properties of the glucocorticoid receptor. We suggest that an FK506-sensitive transporter in L929 cells selectively decreases intracellular hormone levels and, consequently, the potency of particular steroids. Thus, steroid transporters may modulate, in a cell-specific manner, an initial step in signaling, the availability of hormone to the receptor.

Cells use intracellular receptors to sense and respond to extracellular signals such as steroid, thyroid, retinoic, and vitamin D hormones (1). Responses to these hormones can be modulated by other signals and by cellular context. For example, activators of kinases can potentiate or abrogate glucocorticoid action in a cell-type specific manner (2, 3). Resistance to glucocorticoids or mineralocorticoids in patients with wild-type receptors for these steroids further demonstrates that factors in addition to the receptors can determine hormone response (4–7). One step at which response could be modulated is the availability of hormone to the intracellular receptors. The levels of hormone that can interact with and activate the receptors could be determined by proteins affecting hormone influx, efflux, or metabolism.

The existence of proteins that actively affect the influx or efflux of steroid ligands has been controversial. Although steroids are small lipophilic molecules that can diffuse freely in lipid bilayers, corticosterone uptake into liver membrane vesicles is saturable and produces elevated hormone concentration inside the vesicles (8). Studies in yeast, where expression of the mammalian glucocorticoid receptor (GR)1 supports hormone-dependent transcriptional regulation of appropriate reporter constructs (9, 10), have revealed a yeast ATP-binding cassette (ABC) transporter, Pdr5p (Lem1/Sts1/Ydr1p), that decreases intracellular accumulation of particular steroids (11), and of other non-steroid drugs (12–14). The identification of a steroid exporter in yeast raised the possibility that similar transporters might modulate steroid potencies in mammalian cells. In support of this notion, L929 mouse fibroblasts have been reported to export cortisol and dexamethasone (Dex) by a saturable, energy-dependent, temperature-sensitive process (15). Furthermore, several studies in mammalian cells suggest that overexpressed Mdr1, a mammalian ABC-transporter that can confer multidrug resistance (reviewed in Ref. 16), can also transport particular steroids (17–20). The nature of the activity that exports steroids from L929 cells and the role of putative mammalian steroid transporters, including Mdr1, in hormonal regulation have not been yet determined. Indeed, control of steroid transport across the cell membrane has not been considered as a regulatory step that, together with other mechanisms (e.g. ligand metabolism and transcription factor interactions), may contribute to the cell-type specificity of steroid hormone action.

In yeast, the ABC transporter Pdr5p selectively decreases the responsiveness of the mammalian GR to specific ligands (11). Do mammalian cells express Pdr5-like proteins that can modulate cellular responses to glucocorticoids? The distinct substrate selectivity of ABC transporters that display sequence similarities in their transmembrane regions (21, 22) discouraged us from probing for Pdr5-like proteins on the basis of sequence homology. We therefore took a pharmacological approach and sought drugs that might affect the function of a mammalian steroid exporter. Given the precedent of drugs that inhibit a given process in both mammalian and yeast cells (e.g. brefeldin A (23)), we identified an inhibitor of the yeast Pdr5p, and then examined its effects on steroid accumulation and response in mammalian cells.

The macrocyclic lactone FK506 is used clinically as a potent immunosuppressant. It interacts with a family of cellular proteins, termed FK506-binding proteins (FKBP), and interferes with T-cell activation by inhibiting the signaling phosphatase calcineurin (reviewed in Ref. 24). Interestingly, FK506 has also been shown to interact with Mdr1 (25), to be a substrate of Mdr1 (26), and to inhibit Mdr-mediated transport, thereby reversing multidrug resistance (27, 28). We therefore tested whether FK506 might inhibit Pdr5p in yeast, and then determined the effect of FK506 on hormone responsiveness in mammalian cells.

1 The abbreviations used are: GR, glucocorticoid receptor; ABC, ATP-binding cassette; Mdr, multidrug resistance; Dex, dexamethasone; TA, triamcinolone acetonide; PBS, phosphate-buffered saline; β-Gal, β-galactosidase; FKBP, FK506-binding protein.

* This work was supported in part by a grant from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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EXPERIMENTAL PROCEDURES

Plasmids

Yeast plasmids pG1F620S and pTCA/PDR5 (previously denoted as pTCA/LEM1) express, respectively, a point mutant (F620S) rat GR from the constitutive yeast GDP promoter (11, 29) and Pdr5p from its own promoter (11). Reporter plasmid pAS266 (29) contains three glucocorticoid response elements upstream of a minimal yeast CYC1 promoter driving the Escherichia coli iacZ gene. The mammalian reporter TAT3-Luc contains three glucocorticoid response elements upstream of a minimal alcohol dehydrogenase promoter driving the luciferase gene (30). Plasmid 6RZ contains the E. coli iacZ gene under the control of the Rous sarcoma virusLTR.

Yeast Strains and Methods

The following strains were used: YPH252 (α, PDR5) (31), YNK100 (α, pdr5–101) (previously referred to as lem1–1) (11), and YNK102 (α, pdr5::LEU2) (11). Cells were transformed by a standard lithium acetate protocol (32).

β-Gal Assays

Liquid β-gal assays were performed as described (29). Briefly, yeast carrying the plasmids pG1F620S and pAS266 were grown to saturation in selective media, diluted 1:10 in fresh media containing hormone, FK506 (kindly provided by Fujisawa U. S. A.) or carrier ethanol, and grown for an additional 12 h at 30 °C. Enzyme activity was determined as described (29). FK506 was not toxic to cells under any of the experimental conditions tested.

Hormone Binding

In Whole Cells—Yeast cells expressing GR (1.5 ml of a culture at A600

= 0.8) were incubated with [3H]Dex (1.05 Ci/mmol) at 30 °C for 2 h, in the absence or presence of 300-fold excess unlabeled Dex, and in the presence of 0.1% carrier ethanol or 10 μM FK506. Cells were harvested by centrifugation (16,000 × g, 5 min at 4 °C), washed 3 times with cold PBS containing 2% glucose, resuspended in 50 μl of PBS, and counted by liquid scintillation. Mammalian cells (1–4 × 106 cells/60-mm dish) were incubated for 2 h with labeled [3H]Dex (1.05 Ci/mmol; [3H]TA, 11 Ci/mmol), in the absence or presence of 150-fold excess unlabeled ligand, and in the presence of 0.1% carrier ethanol or 10 μM FK506. Cells were washed once with PBS, treated with 0.3 ml of trypsin followed by 0.5 ml of charcoal-stripped serum, transferred to Eppendorf tubes, washed twice with PBS, harvested by centrifugation, suspended in 50 μl of PBS, and counted by liquid scintillation. Specific binding was determined as the counts bound in the absence of unlabeled ligand minus the counts bound in its presence.

In Extracts—Extracts were prepared from L929 cells or YPH252 yeast expressing the rat P202S GR as described (11, 29) except that: (a) cells were treated with either 0.1% carrier ethanol or 10 μM FK506 for 2 h prior to lysis, and (b) lysis and incubation with [3H]Dex were in the presence of 0.1% carrier ethanol or 10 μM FK506. Binding in yeast extracts was as described (11). L929 cell extracts were incubated with 0.2–18 nm [3H]Dex for 6 h, in a final volume of 75 μl and at a protein concentration of 2 mg/ml. Binding data were analyzed using a nonlinear least-squares curve-fitting program (33) with a three-parameter model (one specific and one nonspecific binding site) for binding in yeast extracts and a two-parameter model (one specific binding site) for binding in L929 extracts.

Immunoblots

The yeast cell extracts that were used for hormone binding were also used to assay GR protein levels. Pdr5p, a membrane protein, was not detectable in these soluble extracts. Thus, crude membranes were prepared from YPH252 cells that had been treated for 2 h with either 0.1% carrier ethanol or 10 μM FK506. Cells (15 ml at A600

= 0.8) were washed with cold PBS, resuspended in 50 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM mannitol, 1.5 mM MgCl2, 2.5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM aprotinin, and pepstatin A per ml), and lysed by vortex mixing with glass beads at 4 °C for 30 min. The lysates were cleared by low speed centrifugation (3,000 × g for 10 min), crude membranes were pelleted by high speed centrifugation (150,000 × g for 30 min at 4 °C) and resuspended in 50 μl of lysis buffer. Thirty μg of soluble cell extract (for GR) or crude membranes (for Pdr5p) were separated on 7% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). GR was detected with the monoclonal antibody BUGR2 (34); Pdr5p was detected with a rabbit polyclonal antibody raised against Sts1p (Pdr5p) (35) and kindly provided by K. Kuchler. Blots were developed with alkaline phosphatase-conjugated secondary antibodies (Bio-Rad).

Cell Culture and Transient Transfections

L929 (mouse fibroblasts) or HeLa (human cervical carcinoma) cells grown in Dulbecco’s modified Eagle’s medium (DME-H21; Life Technologies, Inc.) supplemented with 8% charcoal-stripped fetal calf serum were plated at ~1.5 × 106 cells/35-mm dish, 12–18 h before they were transfected by the calcium phosphate precipitation method. Briefly, 1 μg of TAT3-luc, 1 μg of 6RZ, and 3 μg of pBlueScript (Stratagene) were mixed with 20 μl of 2.5 mM CaCl2, 200 μl of 2 × HBs (50 mM Hepes, pH 6.95, 1.5 mM Na2HPO4, 280 mM NaCl), and H2O to a total volume of 400 μl, and added to the cells. Twelve hours later, the transfected HeLa cells were shocked by exposure to 15% glycerol in DME-H21 for 2 min, washed twice with PBS lacking calcium and magnesium (L929 cells were not glycerol shocked, and were washed three times with PBS lacking calcium and magnesium), and incubated for an additional 24 h with fresh DME-H21 media supplemented with 5% charcoal-stripped serum and containing varying concentrations of hormone (Dex or TA), and 0.1% carrier ethanol or 10 μM FK506. Cells were then harvested, and luciferase and β-Gal activity were assayed as described (30).

RESULTS AND DISCUSSION

To determine whether FK506 can inhibit the yeast Pdr5p, we tested the effect of FK506 on GR responsiveness to Dex, an agonist that is exported by Pdr5p, and to deoxycorticosterone, an agonist that is unaffected by Pdr5p (11), in yeast that do or do not have functional Pdr5p. Wild-type yeast (Pdr5p

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) or yeast lacking calcium and magnesium (L929 cells were not glycerol shocked, and were washed three times with PBS lacking calcium and magnesium), and incubated for an additional 24 h with fresh DME-H21 media supplemented with 5% charcoal-stripped serum and containing varying concentrations of hormone (Dex or TA), and 0.1% carrier ethanol or 10 μM FK506. Cells were then harvested, and luciferase and β-Gal activity were assayed as described (30).

Fig. 1. Effect of FK506 on GR function in yeast. A and B, effect of 10 μM FK506 on GR responsiveness to hormone in wild-type and pdr5–101 yeast. YPH252 (wild-type) and YNK100 (pdr5–101) cells carrying the GR expression plasmid pG1F620S and the reporter plasmid pAS266 were treated for 12 h with the indicated concentrations of Dex (A) or deoxycorticosterone (DOC) (B) in the presence of 0.1% carrier ethanol (open symbols) or 10 μM FK506 (closed symbols), and assayed for β-Gal activity, as described under “Experimental Procedures.” Data are the mean and range of results from two independent transformants from a given experiment and are representative of three or more experiments. Squares, wild-type circles, pdr5–101 yeast. C, effects of various doses of FK506 on Dex responsiveness. YPH252 (wild type) and YNK100 (pdr5–101) cells carrying pG1F620S, pAS266, and a Pdr5p expression plasmid (pdr5–101 + Pdr5p) were treated for 12 h with the indicated concentrations of FK506 and 1 μM Dex, and assayed for β-Gal activity, as described under “Experimental Procedures.”
cells (Fig. 1B), indicating that FK506 altered neither the transcriptional regulatory properties of GR nor the general permeability properties of yeast membranes. Dosage studies revealed that 1 μM FK506 increased Dex responsiveness to ~50% of that seen in cells lacking Pdr5p, and that 10 μM FK506 produced almost maximal induction by 1 μM Dex (Fig. 1C). At all concentrations, FK506 had no effect on the Dex response in pdr5–101 cells. Importantly, introduction of a plasmid expressing Pdr5p in pdr5–101 cells restored both reduced responsiveness to Dex in the absence of FK506 and potentiation of the Dex response by FK506 (Fig. 1C). We conclude from these results that FK506 acts by counteracting the function of Pdr5p, i.e. export of Dex.

A prediction of Pdr5 inhibition by FK506 is that FK506 would increase the intracellular accumulation of Dex in cells that express Pdr5p, without altering the Dex-binding properties of GR. We therefore assayed the effect of 10 μM FK506 on Dex binding in whole cells and in extracts of yeast that express GR. FK506 increased the levels of Dex binding in wild-type cells, rendering them similar to the levels seen in cells that lack Pdr5p (Fig. 2A). The increased levels of Dex binding could not be attributed to altered GR properties, as FK506 did not increase the levels of GR protein (Fig. 2B) or the affinity of GR for Dex (Table I). Moreover, FK506 treatment had no effect on Dex accumulation in pdr5–101 cells that express GR (Fig. 2A). In summary, FK506 treatment of wild-type yeast rendered the cells phenotypically pdr5–, suggesting that FK506 inhibits export of Dex by Pdr5p. Interestingly, FK506 modestly increased Pdr5p protein levels, suggesting that it inhibits the activity and not the expression of Pdr5p (Fig. 2B).

![Image]

**Fig. 2. Effect of FK506 on Dex binding in vivo, and on protein levels of GR and Pdr5p.** A, FK506 effect on Dex accumulation in wild-type and pdr5–101 yeast. Specific binding of Dex in whole cells was measured, as described under "Experimental Procedures," in yeast that carried the GR expression plasmid pG1F6205 and that had been preincubated for 2 h with either 0.1% carrier ethanol (no FK506, black bars) or 10 μM FK506 (10 μM FK506, hatched bars). No specific binding was seen in cells that do not express GR. B, protein levels of GR and Pdr5p. GR (indicated by the arrow) and Pdr5p (indicated by the bracket) were detected in 30 μg of total protein of either soluble extracts (GR) or crude membranes (Pdr5p) from yeast that expressed GR and that were treated for 2 h with either 0.1% carrier ethanol (lanes 1, 3, and 5) or 10 μM FK506 (lanes 2 and 4). Lanes 1, 2, 4, and 5, YPH252 (wild-type) yeast; lane 3, YNK102 (Pdr5–) yeast.
ABC transporters comprise a superfamily of prokaryotic and eukaryotic proteins that can import or export a wide range of substrates, from small molecules like vitamins, nutrients, or drugstopolypeptidessuch as bacterial toxins (37). What transportermight operateonDexinL929cells? Potential candidates include various well characterized mammalian ABC transporters (e.g. Mdr1 (also called Mdr1b P-glycoprotein), Mdr3 (also called Mdr1a P-glycoprotein), and MRP (38)), less well characterized members such as Spgp (39) or an unknown novel transporter. Among the characterized candidates, Mdr1 and Mdr3 have been shown to transport specific steroids (17–20) and overexpressed Mdr1 can export Dex efficiently enough to affect GR response (19). We thus probed L929 membranes with the anti-Mdr1 antibody C219 (Signet Laboratories, MA) that recognizes Mdr1, Mdr3, and Spgp (16, 39). We failed to detect a cross-reacting protein (data not shown), suggesting that if one or more of the above transporters are expressed, they are at levels lower than those in adrenal Y1 cells, which we used as a positive control. If indeed low levels of Mdr1, Mdr3, or Spgp cause the dramatic decrease in intracellular Dex accumulation in L929 cells, then they must export Dex very efficiently. Given that there are several candidate transporters and many steroids, it is also possible that multiple transporters act on steroids, each with a different steroid selectivity. Interestingly, the yeast Pdr5p and the putative transporter in L929 cells show overlapping but distinct substrate selectivity, as Pdr5p, but not the L929 activity, can reduce the cellular levels of TA (11).

As mammalian ABC transporters can function in yeast (40, 41), it may be possible to clone the L929 Dex exporter by complementation of the Pdr5− phenotype. We have expressed the human Mdr1 in yeast and shown that it confers resistance to valinomycin as previously reported (40). However, Mdr1 does not affect GR response to Dex in Pdr5− or Pdr5− cells, suggesting that Mdr1 may not export Dex efficiently, at least in yeast.

The stimulation of Dex activity in L929 cells by FK506 has been reported previously (36, 42) and interpreted as a direct effect on GR, as unliganded GR resides in a hetero-oligomeric aporeceptor complex that includes FKBP-59, an FK506-binding immunophilin and peptidylprolyl isomerase (43, 44). However, FK506 has little or no effect on the affinity of GR for Dex or TA (42, 45), and the effects of FK506 analogs on Dex responsiveness do not correlate with their inhibition of peptidylprolyl isomerase (FKBP-59) or calcineurin activity (42). FK506 also potentiates the response of progesterone receptor in yeast to the steroid R5020, an effect that was attributed to enhanced steroid-induced receptor phosphorylation by sub-saturating levels of R5020 (46). If, however, FK506 inhibits R5020 export, the increase in phosphorylation might instead reflect elevated intracellular R5020 levels. It will be interesting to test whether R5020 is transported by Pdr5p or by another yeast ABC transporter. In summary, our experiments support strongly the idea that Pdr5p acts as a membrane transporter for steroids.

2 A. Kralli and K. R. Yamamoto, unpublished results.
that FK506 alters the availability of intracellular steroids without affecting the GR per se, and that it acts in a ligand- and cell type-specific manner.

By extension, our findings imply that responses to steroids and signals for other intracellular receptors may similarly be modulated at the level of plasma membrane transport. In an accompanying study, Ribeiro et al. (48) suggest that intracellular thyroid hormone levels and response are subject to similar controls; they find that verapamil, another inhibitor of Mdr proteins, potentiates thyroid hormone responsiveness in cells that express proteins of the Mdr family, and decreases thyroid hormone efflux from Mdr-expressing cells, primary hepatocytes, and cardiocytes (48).

In principle, hormone transporters could protect specific cells in particular developmental or physiological states from the effects of high hormone concentrations (e.g., during stress or pregnancy). If the transporter itself were under hormonal regulation, it could be used to shape the duration and magnitude of a hormone response. Deregulation of the levels or activity of such a transporter might lead to steroid hormone resistance and disease. In addition, steroid transporters may render hormone responses sensitive to non-steroid molecules, such as FK506, that can affect the activity of the transporters. These findings suggest approaches to cell- and/or ligand-selective modulation of hormone effects that could prove useful in the design of drug therapies. Notably, FK506 is commonly used in conjunction with glucocorticoids for immune suppression following organ transplants. Although high levels of FK506 are required to potentiate Dex effects in L929 or yeast cells relative to those administered to patients (47), it is conceivable that some drug interactions may occur in certain cells in this regimen. In any case, isolation and characterization of mammalian steroid transporters will allow an assessment of their role in ligand- or cell type-specificity of steroid action.

Acknowledgments—We thank members of the Yamamoto laboratory and U. Muller for helpful discussions; K. Kuchler for sharing reagents prior to publication; I. Bekersky of Fugisawa U.S.A. for their gift of FK506; J. D. Buxter, L. Z. Benet, S. Bohem, R. Cavaleri, R. Grosschedl, I. Herskowitz, J. LesFtin, N. Lonri, D. Pearce, R. Ribeiro, B. F. Scharschmidt, and D. B. Starr for their comments on the manuscript; and R. Ribeiro for communication of results prior to publication.

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