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Genetics

LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones

(glucocorticoid receptor/yeast/*PDR5*/*STS1*)

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ABSTRACT **The rat glucocorticoid receptor confers hormone-dependent transcriptional enhancement when expressed in yeast, thereby enabling the genetic identification of nonreceptor proteins that function in the hormone signal-transduction pathway. We isolated a yeast mutant, *lem1*, with increased sensitivity to dexamethasone and triamcinolone acetonide; responsiveness to a third agonist, deoxycorticosterone, is unaffected. Cloning of wild-type *LEM1* revealed a putative transport protein of the ATP-binding cassette family. Dexamethasone accumulation is increased in *lem1* cells, suggesting that wild-type LEM1 decreases dexamethasone potency by exporting this ligand. LEM1 appears to affect certain steroids and not others. We propose that transporters like LEM1 can selectively modulate the intracellular levels of steroid hormones. Differential activities of such transporters in mammalian cells might regulate hormone availability and thereby hormone signaling in a cell-type specific manner.**

Glucocorticoids function by binding to the glucocorticoid receptor (GR), an intracellular protein that, once activated by hormone, binds to specific DNA sequences and regulates the transcription of adjacent genes. The protein machinery with which the receptor interacts in the course of signaling and transcriptional regulation has been evolutionarily conserved, as the rat GR can confer hormone-dependent transcriptional activation when expressed in cells from heterologous species as diverse as fungi, insects, and plants (1–4).

Interestingly, the biological efficacies and potencies of certain steroid ligands differ when mammalian GR is expressed in different species. Two strong agonists in mammalian cells, dexamethasone (dex) and triamcinolone acetonide (TA), are very weak agonists in *Saccharomyces cerevisiae* (5); in contrast, agonists like deacylcortivazol and RU28362 efficiently activate GR in all species tested. Thus, dex and TA are more potent GR agonists than deoxycorticosterone (DOC) in mammalian cells, whereas DOC is more potent than dex and TA in yeast. Similarly, dex-mesylate, an antagonist in mammalian cells, is a strong agonist in cultured *Drosophila melanogaster* cells (3). Clearly, cellular factors other than the receptor must serve as determinants of ligand responsiveness by GR. By this view, species- or cell-type-specific differences in these factors would produce the distinct ligand response characteristics of mammalian, *Drosophila*, and *S. cerevisiae* cells.

Factors that modulate ligand effects might interact either with GR or with the ligand itself. GR-interacting factors could affect ligand binding or alter the activity of the hormone-bound receptor; factors that interact with the ligand could modify it, sequester it, or affect its intracellular accumulation. Ligand accumulation could be affected by proteins that alter its flux across the cell membrane. Steroids are small hydrophobic molecules commonly assumed to diffuse through the membrane. However, steroid transport may be an active process. Corticosterone uptake by isolated rat liver membrane vesicles is saturable and produces elevated intravesicular hormone levels relative to the medium (6). In mouse L929 fibroblasts, cortisol and dex are exported in a saturable, energy-dependent, temperature-sensitive process (7, 8). Thus, in a given cell type, accumulation of a specific ligand may depend on the nature and activities of selective transporters.

The ability of GR to function in yeast provides an opportunity to genetically identify nonreceptor factors in the glucocorticoid signal-transduction pathway. To search for proteins that selectively modulate the effect of particular steroid ligands (here termed LEM for ligand effect modulator), we screened for yeast mutants that increase GR responsiveness to TA and dex without altering responsiveness to DOC.

MATERIALS AND METHODS

Plasmids. Plasmids pG1N795, pG1F620S, pTCA/N795, and pLCA/N795 express wild-type (N795) or a point mutant (F620S) GR, from the constitutive yeast *GPD* promoter (1, 5). Plasmids pH2/ER (9), pH2/MR (9), and YEphPR-B (10) express respectively, the estrogen receptor (ER), the mineralocorticoid receptor (MR), and the progesterone receptor (PR). Reporter plasmids pΔs26x (1) and pUCΔsERE (11) contain three glucocorticoid/progesterone response elements and one estrogen response element, respectively, upstream of a minimal yeast *CYC1* promoter driving the *Escherichia coli lacZ* gene. Plasmid pTCA/LEM1 carries the *LEM1* gene with 1200 bp of 5′ and 400 bp of 3′ sequences inserted into the polylinker of pRS314 (12). In pTCA/*lem1*::*LEU2*, the *LEM1* sequences from −360 bp to +3950 bp relative to the translation initiation site were replaced by the *LEU2* gene.

Yeast Strains and Genetic Methods. We used the following yeast strains: BJG26.1 contains a GR-responsive β-galactosidase (β-gal) reporter gene (5); YPH252 (**a**, *LEM1*) (12); YNK100 (**a**, *lem1-1*), derived by crossing the original m17 isolate twice to YPH252. The *LEM1* locus was disrupted in the haploid YPH252 and YPH250 and the diploid YPH274 (12) strains by the one-step replacement method (13) and by using the DNA insert of the pTCA/*lem1*::*LEU2* plasmid.

Mutagenesis and Genetic Screen. BJG26.1 cells containing pTCA/N795 were grown in 5 ml of selective medium at 30°C to OD_{600} = 0.7, harvested by centrifugation (5000 × *g* for 5 min), washed twice with phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4), resuspended in 1 ml of PBS, and incubated with 50 μl of ethyl methanesulfonate (EMS; Sigma) for 1 h at 25°C. The EMS was neutralized with five volumes of 5% sodium thiosulfate, and

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Abbrevations: GR, glucocorticoid receptor; ER, estrogen receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; dex, dexamethasone; DOC, deoxycorticosterone; TA, triamcinolone acetonide; LEM, ligand effect modulator; ABC, ATP-binding cassette; Mdr, multidrug resistance; β-gal, β-galactosidase.

cells were centrifuged (5000 × *g* for 5 min), washed with H_2O, and resuspended in 1 ml of H_2O. Cell viability after mutagenesis was 30–50%. Mutagenized cells (8000 colonies) were grown at 30°C on plates containing 20 nM TA and analyzed by β-gal plate assays. Blue colonies were tested by quantitative β-gal assays. The m17 isolate was cured of pTCA/N795, retransformed with pLCA/N795, and crossed twice to YPH252; sporulation and tetrad analysis of the resulting diploids indicated that the mutant phenotype was not linked to the GR expression vector and was due to a single genomic mutation. Responsiveness of GR to dex in *lem1-1/LEM1* heterozygotes was intermediate to responsiveness in homozygous wild type and homozygous *lem1-1*, suggesting that the mutation was semi-recessive. Further characterization of *lem1-1* was carried out with a GR mutant (F620S) that, in yeast, displays higher affinity than wild-type GR (N795) for TA and dex (5) and is more amenable to biochemical studies; *lem1-1* had similar effects on wild-type and F620S GR.

Cloning and Sequencing of *LEM1*. YNK100 cells containing pG1F620S and pΔs26x were transformed with a *S. cerevisiae* genomic library cloned in the low copy number plasmid pRS200 (a gift from C. Connelly and P. Hieter, Johns Hopkins School of Medicine, Baltimore), grown on selective plates containing 0.5 μM dex and tested by β-gal plate assays. A 6-kb *Cla* I–*Bam*HI DNA fragment that complemented the *lem1-1* phenotype was subcloned into pRS314 (12), subjected to unidirectional *Exo* III deletion, and sequenced on both strands. To demonstrate linkage of the cloned DNA to the *LEM1* locus, we subcloned the 6-kb *Cla* I–*Bam*HI fragment into pRS303 (12) and transformed *lem1-1* cells with a linearized form of this construct. Lem^+ His^+ transformants were subjected to Southern analysis to confirm that the plasmid had integrated at the locus of the cloned DNA and crossed to YPH250. Resulting diploids were sporulated and subjected to tetrad analysis. Twelve tetrads and 44 random spores were Lem^+, suggesting that the site of plasmid integration—i.e., the cloned DNA locus—was tightly linked to the *lem1-1* mutation.

β-gal Assays. β-gal plate and liquid assays were performed as described (9).

Hormone-Binding Assays. *In intact cells.* [^{3}H]dex (42 Ci/mmol; 1 Ci = 37 GBq; Amersham) or [^{3}H]estradiol (94.8 Ci/mmol, ICN) were incubated with yeast cells expressing GR or ER at 30°C for 2 h (dex) or 1 h (estradiol) in the absence or presence of a 300-fold excess of unlabeled ligand. Cells were then harvested by centrifugation (12,000 × *g* for 5 min at 4°C), washed three times with cold PBS containing 2% (wt/vol) glucose, recentrifuged, and suspended in 50 μl of PBS, and the amount of bound hormone was quantitated by liquid scintillation. Specific binding was determined as the counts bound in the absence of excess unlabeled ligand minus the counts bound in its presence.

In cell extracts. Cells from 300-ml cultures of yeast (OD_{600} = 0.8–1.0) containing pG1F620S were washed twice with PBS, centrifuged, resuspended in 1 ml of lysis buffer [10 mM Tris·HCl, pH 7.5/20% (vol/vol) glycerol/150 mM KCl/20 mM sodium molybdate/15 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/1 μg each of leupeptin, 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 centrifugation (12,000 × *g* for 10 min, followed by 350,000 × *g* for 30 min, at 4°C) and incubated with 1–300 nM of [^{3}H]dex in the absence or presence of a 250-fold excess of unlabeled dex, in a final volume of 75 μl, and at a protein concentration of 6–7 mg/ml at 4°C for 9 h. Subsequent steps were as described (5). Binding data were analyzed using a nonlinear least-squares curve fitting program (14) with a three-parameter model (one specific- and one nonspecific-binding site). Approximately 2% of the GRs present in the extracts binds dex, as estimated by immunoblotting.

RESULTS

Isolation of the *lem1* Mutant. We mutagenized yeast cells containing a GR expression vector and a GR-responsive *lacZ* reporter gene and screened for mutants with an elevated response to TA and unaltered response to DOC. Wild-type yeast colonies are blue in the presence of 20 nM DOC and the indicator 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and white in the presence of 20 nM TA and X-Gal. Three yeast colonies that were blue in the presence of 20 nM TA were tested in quantitative β-gal assays for responses to TA and DOC. One displayed increased response to TA and wild-type response to DOC; genetic characterization revealed a single, semi-recessive genomic mutation. GR response to TA, dex, and DOC, in wild-type yeast and the isolated mutant *lem1-1* is shown in Fig. 1. TA and dex were significantly stronger agonists in *lem1-1* than in wild-type yeast, whereas the activity of DOC was similar in the two strains. In the case of dex, approximately 1/10th the concentration of hormone required in wild-type cells for half-maximal induction of β-gal was required in *lem1-1* (Fig. 1*B*). Maximal GR activity at high concentrations of dex and TA was similar in wild-type and mutant cells. Thus, the *lem1-1* mutation renders GR responsive to lower concentrations of dex and TA but alters neither the response to DOC nor the maximal activation seen with these hormones.

Cloning and Sequencing of the *LEM1* Gene. We cloned wild-type *LEM1* by complementation of the mutant phenotype. *lem1-1* cells bearing a GR expression plasmid and a β-gal-reporter plasmid were transformed with a genomic library from wild-type yeast and grown on plates containing 0.5 μM dex, conditions under which *lem1-1* but not wild-type colonies are blue in X-Gal. White transformants were tested for retention of the wild-type DOC response. Plasmids from these transformants were isolated and reintroduced into *lem1-1* yeast to confirm that they selectively suppressed responsiveness to dex. Of 15,000 transformants screened, one plasmid efficiently complemented the mutant phenotype. Deletion analysis of the plasmid insert defined a 6-kb genomic DNA fragment with complementing activity. A plasmid bearing this insert rendered the GR response to dex in *lem1-1* similar to that in wild type (Fig. 2*A*), decreased only modestly the response of GR to dex in wild-type cells (Fig. 2*A*), and had no significant effect on the GR response to DOC in either strain (Fig. 2*B*). Furthermore, the 6-kb insert directed integration of the plasmid DNA to the *LEM1* locus (see *Materials and Methods*), confirming that the cloned DNA fragment encoded the *LEM1* gene. Finally, disruption of *LEM1* showed that it is not essential, and that the phenotypes of *lem1-1* and the null *lem1* are similar (data not shown).

Sequencing of *LEM1* revealed a single open reading frame of 4533 nucleotides, predicting a polypeptide of 1511 amino acids. The deduced amino acid sequence showed significant similarity to members of the ATP-binding cassette (ABC) superfamily of putative membrane-spanning transport proteins (15). Comparisons with ABC transporters from bacteria, yeast, insects, and mammals suggested that LEM1 contains two ABC domains (Fig. 3). Hydropathy plots (22) predicted two hydrophobic domains containing potential α-helices, TM_1 and TM_2, that displayed significant identity to the predicted TM domains of the ABC transporters SNQ2 (16), ADP1 (17), and White (18) (Fig. 3). Recently, *LEM1* was cloned by others as *PDR5* (23) and *STS1* (24), a gene that confers resistance to cycloheximide and sporidesmin, respectively, when overexpressed. The predicted LEM1 amino acid sequence is analyzed in those reports (23, 24).

LEM1 Modulates Intracellular dex Accumulation. The sequence of LEM1, the increased dex response in *lem1*, and the decreased dex sensitivity in $Lem1^+$ cells, suggested that the wild-type LEM1 protein may selectively export dex, reducing

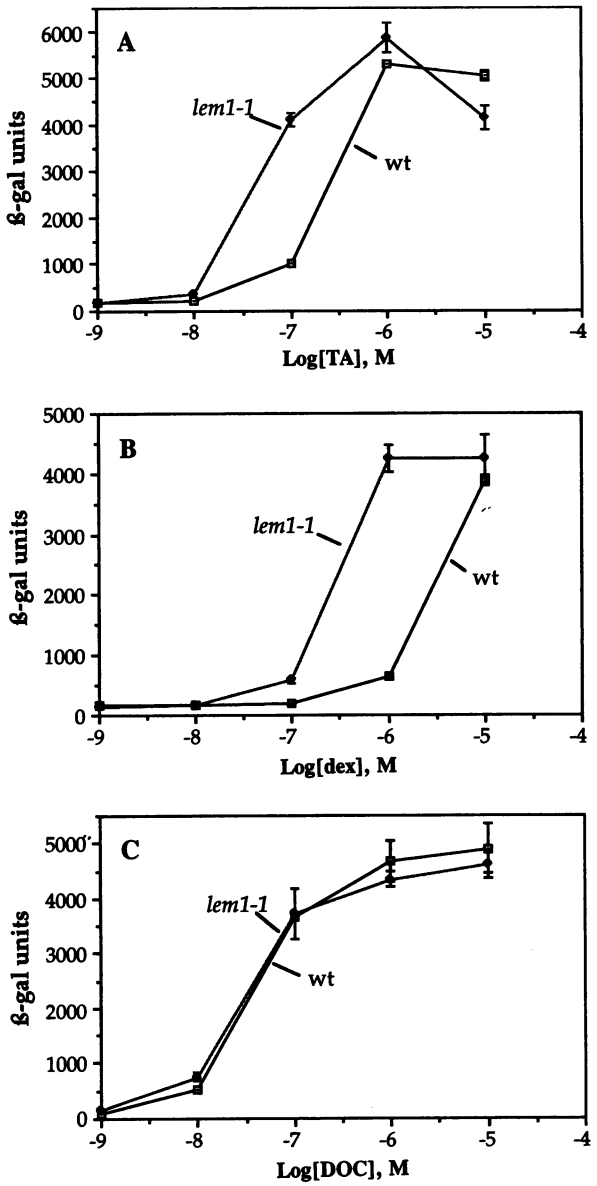

FIG. 1. Ligand response of GR in wild-type (wt) and *lem1-1* yeast. Cells carrying the GR expression vector pG1F620S and the reporter plasmid pΔs26x were treated with the indicated concentrations of TA (*A*), dex (*B*), or DOC (*C*) and assayed for β-gal activity. Data are the mean and range of results from two independent transformants from one experiment and are representative of three or more experiments.

its availability to GR. This predicts that LEM1 should decrease dex binding to GR *in vivo* without altering the affinity of GR for dex or the level of GR protein. We first measured binding of dex in intact yeast expressing GR (Fig. 4*A*). As predicted, we found significantly higher levels of bound dex in *lem1-1* than in wild-type cells: at 0.1 μM dex (a concentration ≈6-fold higher than the K_d of GR for dex), no detectable binding was seen in wild-type cells; at 1 μM dex, binding of dex in wild-type cells was one-fifth that observed in *lem1-1* cells and similar to the binding seen at 0.1 μM in *lem1-1* cells. Specific binding of dex was not detected in wild-type or mutant yeast cells lacking GR (data not shown). Next, we prepared yeast lysates and showed that receptor–ligand affinity was unaffected by the mutation (K_d = 16.6 ± 3.1 nM in wild type and 16.3 ± 1.5 nM in *lem1-1*). Comparable levels of GR protein were present in the lysates from the two strains, measured by immunoblotting (data not shown) and by dex binding (359 ± 162 fmol of GR per mg of protein in wild type and 524 ± 131 fmol of GR per

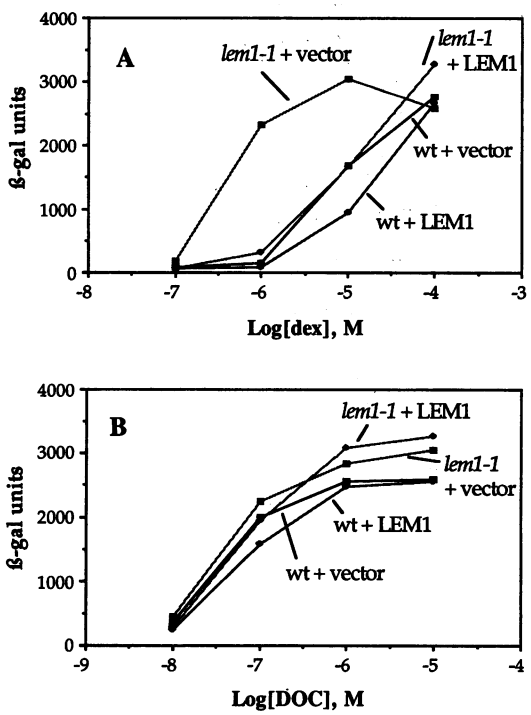

FIG. 2. Complementation by LEM1. Cells carrying the GR expression vector pG1F620S, the reporter plasmid pΔs26x, and either the vector (pRS314) or the LEM1 expression plasmid (pTCA/LEM1) were treated with dex (*A*) or DOC (*B*) and assayed for β-gal activity. Data are from one experiment and are representative of three or more experiments. wt, Wild type.

mg of protein in *lem1-1*). We conclude that the *lem1-1* mutation results in increased intracellular accumulation of dex, apparently without altering the receptor itself.

To assess the ligand specificity of the *lem1-1* effect, we measured the binding of estradiol to wild-type and *lem1-1* cells expressing ER. We observed only a modest increase (<50%) in estradiol binding in *lem1-1* cells compared with the 5-fold effect on dex binding (Fig. 4*B*). We conclude that LEM1 preferentially affects intracellular accumulation of dex.

Effects of LEM1 on the Potency of Other Steroid Ligands. To examine the effect of LEM1 on the responses of other steroid receptors to their respective ligands, we constructed wild-type and *lem1-1* strains expressing MR, ER, or PR and assayed their response to corticosterone, estradiol, and progesterone, respectively. Corticosterone was a more potent agonist of MR in the mutant *lem1-1* than in wild-type cells (Fig. 5*A*); as expected, *lem1-1* had no effect on the responsiveness of MR to DOC (data not shown). The *lem1-1* mutation did not significantly affect the responsiveness of ER to estradiol or of PR to progesterone (Fig. 5 *B* and *C*).

DISCUSSION

The *lem1-1* mutation potentiates the responsiveness of GR to dex and TA without affecting responsiveness to DOC. It acts by increasing dex accumulation in intact yeast without altering the affinity of GR for dex or the level of GR protein. The wild-type *LEM1* gene encodes a putative ABC transporter. We propose that LEM1 specifically exports a subset of steroids, thereby limiting the availability of these ligands to intracellular receptors.

LEM1 has been cloned by others as *PDR5* (23) and *STS1* (24), a gene whose overexpression can confer resistance to cycloheximide and sporidesmin, respectively; disruption of *PDR5* causes hypersensitivity to cycloheximide, chloramphenicol, lincomycin, erythromycin, and antimycin (25, 26). What

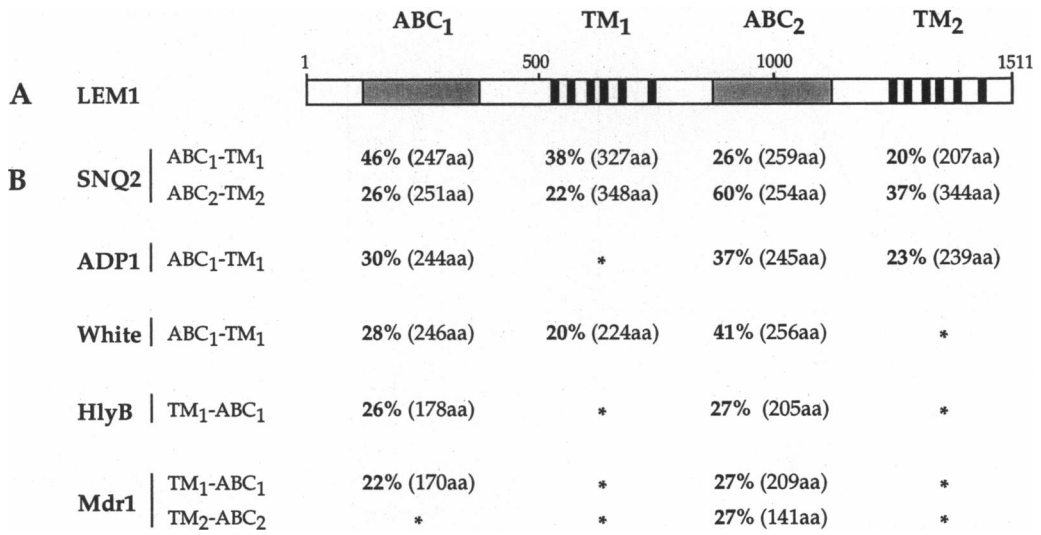

FIG. 3. (*A*) Structural organization of the LEM1 protein. Grey boxes represent the two ABC's. The two blocks of vertical bars represent the putative membrane-spanning helices of the transmembrane domains (TM) 1 and 2. (*B*) Comparison of LEM1 to other members of the ABC-transporter superfamily. Shown is the percent identity of each ABC [ABC_1, amino acid (aa) 171–420; ABC_2, aa 881–1135] or TM (TM_1, aa 486–835; TM_2, aa 1162–1511) of LEM1 to each of the ABC or TM, respectively, of the following proteins: yeast SNQ2 (16), yeast ADP1 (17), *Drosophila* White (18), *E. coli* HlyB (19), and human Mdr1 (20). Numbers in parentheses indicate length, in amino acids, of the region of identity. At left, the number and arrangement of the ABCs and TMs are given for each protein. Sequence comparisons were from FASTA (21). *, Domains with <20% sequence identity and FASTA scores of <70.

is the function of LEM1 in yeast? Although yeast lack classical steroid hormone receptors, they contain steroid-binding proteins (27, 28) and their membranes include ergosterol. Conceivably, LEM1 might be involved in ergosterol homeostasis or in the putative function of the intracellular yeast steroid-binding proteins. Alternatively, given its broad substrate specificity, the physiological substrates of LEM1 may be unrelated to steroids.

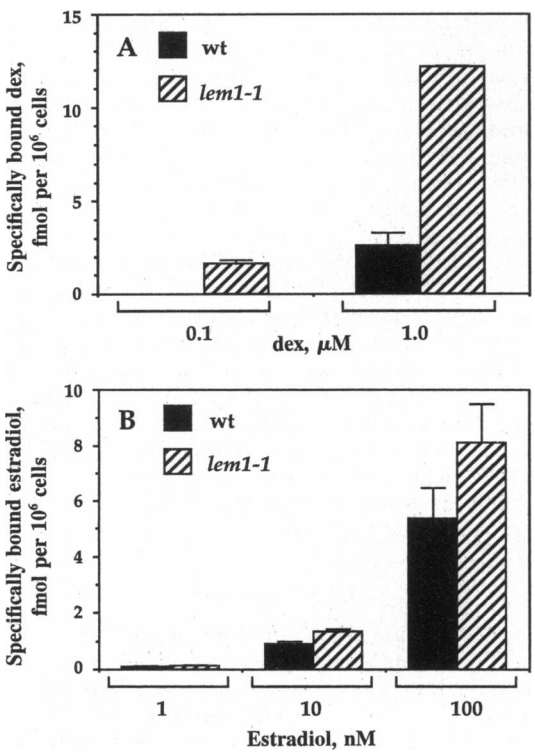

FIG. 4. Hormone binding to wild type and *lem1-1* yeast. (*A*) Specific binding of dex to intact wild-type (wt; black bars) and *lem1-1* (hatched bars) cells carrying the GR expression vector pG1F620S. (*B*) Specific binding of estradiol to intact wild-type (black bars) and *lem1-1* (hatched bars) cells carrying the ER expression vector pH2/ER. Data indicate mean and range of results from two independent transformants from one experiment and are representative of three or more experiments.

Typical ABC transporters include two nucleotide-binding domains (ABC) and two hydrophobic domains containing the putative transmembrane helices (TM); these domains can reside in one to four proteins (15). LEM1 includes all four domains in a single protein; its strongest similarity to ABC transporters resides in the nucleotide-binding domains (Fig. 3). Its putative transmembrane regions are most similar to SNQ2, a yeast transporter conferring resistance to a number of drugs (16), ADP1, a yeast transporter of unknown function (17), and White, a *Drosophila* half-size ABC transporter that imports an eye pigment precursor (18). Interestingly, *SNQ2* has also been isolated as a gene whose overexpression decreases hormone responsiveness of GR (J. A. Lefstin and K.R.Y., unpublished data), suggesting that it may export certain steroids upon overexpression. LEM1 and SNQ2 are readily distinguished, however, as they effect resistance to different drugs when overexpressed in yeast (24).

The active and selective export of steroids from yeast raises the possibility that similar mechanisms operate in mammalian cells. The energy-dependent, temperature-sensitive export of steroid ligands from L929 cells (7, 8) supports this view. On the basis of sequence similarity, none of the known mammalian ABC transporters is an obvious LEM1 homologue. However, Mdr1, whose overexpression confers multiple drug resistance to mammalian cells (20, 30–32), displays functional similarities to LEM1. Overexpressed Mdr1 appears to export dex, corticosterone, and aldosterone but not progesterone (33–35). Moreover, a dex-resistant cell line, S7CD-5, derived by double selection in dex and colchicine, expresses elevated levels of Mdr1 (36). Notably, whether wild-type Mdr1 expressed at normal levels can affect GR responsiveness is unknown. Overexpression of LEM1 in yeast can decrease the response to steroids that are not affected by normal levels of LEM1—e.g., estradiol and DOC (data not shown)—showing that substrate selectivity can be abrogated under these conditions. Conceivably, a yet unidentified mammalian ABC transporter, perhaps

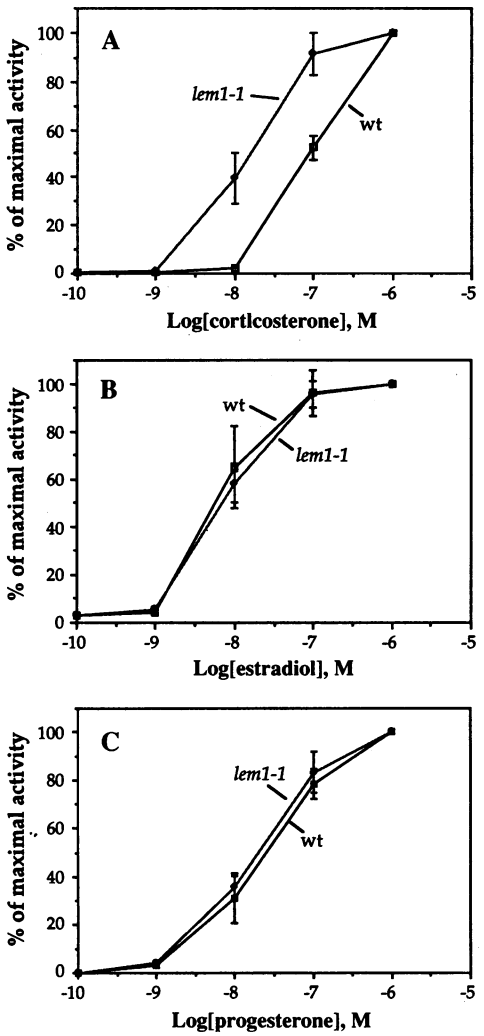

FIG. 5. Ligand responses of MR (*A*), ER (*B*), and PR (*C*) in wild-type (wt) and *lem1-1* yeast. Cells carrying expression plasmids pH2/MR (*A*), pH2/ER (*B*) or YEphPR-B (*C*) were incubated with corticosterone, estradiol, or progesterone, respectively. Data are expressed as percent of maximal activity and are the mean ± SD of results from five or more assays (from three independent transformants assayed in duplicates). Maximal β-gal activities, obtained at 1 μM ligand, were as follows (expressed in β-gal units ± SD): MR in wild types, 1190 ± 684; MR in *lem1-1*, 637 ± 169; ER in wild type, 519 ± 150; ER in *lem1-1*, 503 ± 109; PR in wild type, 1618 ± 216; PR in *lem1-1*, 1851 ± 181.

related to Mdr1 or LEM1, may export steroid ligands as part of its normal function.

Putative mammalian steroid exporters may be regulable or cell-specific, providing a mechanism for selective response to a given level of circulating hormone. Thus, even though GR is virtually ubiquitously expressed, this mechanism could operate in combination with others, such as enzymatic modification of ligands (37, 38) and regulatory factor interactions (29), to impose selectivity on glucocorticoid activity. Our findings establish that an export mechanism can influence the availability of hormone to its intracellular receptor. Together with evidence supporting the existence of membrane-bound steroid importers (6), we suggest that cellular response to steroid hormones can be actively modulated at the cell membrane.

The distinct pattern of ligand response of GR in yeast and mammalian cells led to the isolation and characterization of *LEM1*. Importantly, LEM1 is not the only factor that contributes to the distinct GR-ligand response in yeast. The potency of dex is still low in a *lem1* null strain compared with the potency of other ligands in yeast or the potency of dex in mammalian cells. By using a strategy similar to that described here, it may be possible to identify and characterize additional factors that modulate the response of GR to dex.

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