Glucocorticoid-specific Gene Activation by the Intact Human Glucocorticoid Receptor Expressed in Yeast

GLUCOCORTICOID SPECIFICITY DEPENDS ON LOW LEVEL RECEPTOR EXPRESSION

Anthony P. H. Wright‡ and Jan-Åke Gustafsson
From the Centre for Biotechnology and Department of Medical Nutrition, Karolinska Institute, NOVUM, Huddinge University Hospital, Huddinge S-141 57, Sweden

In the presence of appropriate reporter genes mammalian nuclear receptors are competent to transactivate gene expression when expressed in yeast cells. Thus yeast genetics could be used to identify determinants of steroid specificity for these mammalian proteins. However, unlike the estrogen, progesterone, vitamin D₃, and thyroid hormone receptors, the glucocorticoid receptor shows an apparently abnormal steroid specificity in yeast (1). The glucocorticoid receptor in yeast appears to result from an artifact of the assay system and is not due to an abnormal receptor structure. This mechanism could account for all our data and so could provide the sole explanation of the abnormal specificity observed. However, it is also possible that part of the abnormal specificity could result from structural or other changes in receptor function, which occur when the receptor expression level is increased.

The glucocorticoid receptor (GR) is a member of a family of nuclear receptor proteins that includes receptors for steroid hormones, thyroid hormones, retinoic acid, and vitamin D₃. All members of the family show structural similarity, and all function as conditional regulators of gene expression, requiring the presence of their cognate ligands for activity. The glucocorticoid (2, 3), estrogen (4), progesterone (5), vitamin D₃ (6), and thyroid hormone (7) receptors have been shown to function as ligand-dependent regulators of appropriate reporter genes when expressed in yeast cells. This provides the possibility of using yeast genetic techniques to identify determinants of ligand binding specificity for these mammalian receptors. However, unlike the other receptors which appear to show normal ligand specificity when expressed in yeast, the rat GR has been reported to show an abnormal specificity. Thus dexamethasone, normally regarded as a potent glucocorticoid, did not result in receptor-dependent transactivation of a reporter gene whereas several other corticosteroids did (2).

The reason for aberrant steroid specificity of the glucocorticoid receptor in yeast is not clear. The GR differs from some other receptors because it only binds hormone with high affinity when complexed with the heat shock protein, hsp 90 (8, 9). hsp 90 is also required for steroid-dependent transactivation of gene expression by the GR in yeast (10). Thus one possibility is that interaction with the yeast homologue of hsp 90 leads to a slightly altered steroid binding surface. However, this is unlikely since we have previously shown that a truncated derivative of the human GR, lacking the N-terminal half of the protein, appears to be associated with hsp 90 and transactivates with a near normal steroid specificity (3). Furthermore, the steroid-binding domain of the GR expressed in Escherichia coli, in the absence of hsp 90, binds steroid with normal specificity, albeit with reduced affinity (11). Thus the steroid specificity determinants appear to be an exclusive property of the receptor protein per se.

Before using yeast genetics in attempts to isolate receptor mutants altered in steroid specificity we wished to determine whether the abnormal specificity reported for the intact GR in yeast was due to a defect in folding of the expressed protein or to some other aspect of the assay system. We have shown, at least under conditions of low level expression, that the GR shows essentially normal steroid specificity and is presumably folded correctly in yeast. The reason for the abnormal steroid specificity seen at higher expression levels of receptor protein is also investigated.

MATERIALS AND METHODS

Strains—Transcriptional transactivation assays were performed using yeast strain, W303-1A/K396-11B, described previously (3). Yeast strains were grown transformed with plasmid DNA, and used for transcriptional transactivation assays as described previously (3).

Plasmid Constructions—Plasmids were constructed and amplified using standard techniques. pKV-NX (12) and the deletion derivatives, pKV-NX-Δ51, and pKV-Δ1-AX (13), have been described in detail previously. The intact GR and its truncated derivatives (above) were expressed at similarly high levels from the pKV plasmids. By contrast, 50-100-fold lower levels of GR expression were achieved using the pKV-NX-AT and pKV-AX (13), have been described in detail previously. The intact GR and its truncated derivatives (above) were expressed at similarly high levels from the pKV plasmids. By contrast, 50-100-fold lower levels of GR expression were achieved using the pKV-NX-AT and pKV-AX (13), have been described in detail previously. The intact GR and its truncated derivatives (above) were expressed at similarly high levels from the pKV plasmids. By contrast, 50-100-fold lower levels of GR expression were achieved using the pKV-NX-AT and pKV-AX (13), have been described in detail previously.
pLGZ-TAT has been described previously (3), and pLGZ-2TAT is identical except that it contains an additional glucocorticoid response element, identical to the first, with a spacing of 32 base pairs between the centers of the elements.

RESULTS

Steroid Specificity of the Intact Human GR Expressed in Yeast—To determine whether the intact human GR shows abnormal steroid specificity of gene activation in yeast cells similar to that previously reported for the rat GR (2), yeast cells containing the pKV-NX expression plasmid and the pLGZ-TAT reporter plasmid (Fig. 1) were used. Expression of the GR protein was induced by growth on medium containing galactose as carbon source prior to division of the culture into smaller cultures containing different levels of a variety of steroid hormones. After 7 h the cells were harvested, protein extracts prepared, and the activity of the β-galactosidase reporter gene assayed. The dose-response curves are shown in Fig. 2A, and induction levels, at a sub saturating hormone concentration, are tabulated in Table I (column 1). The specificity of the GR for the different steroid hormones is clearly abnormal with the mineralocorticoid, deoxycorticosterone, showing the highest level of activation at low concentrations and with progesterone functioning equally well as corticosterone. Some specificity exists to the extent that testosterone and estradiol activate poorly, if at all, and only at the highest concentrations. The curves do not look like typical dose-response curves because they fail to lower activity levels at higher concentrations of hormone instead of forming the expected plateau. This probably results from the phenomenon of transcriptional squelching (see "Discussion"). This experiment shows that the intact human GR shows an abnormal steroid specificity when expressed in yeast, and therefore species differences or differences in yeast strains do not account for the different steroid specificity properties reported for the full-length rat GR (2) and a truncated human GR (3), from which the N-terminal half (residues 1–414) had been deleted. Therefore, the difference must be due to the different proteins per se, even though the N terminus of the receptor has not previously been shown to influence hormone binding (14, 15).

Abnormal Steroid Specificity Is Caused by the \( \tau_1 \) Transcriptional Transactivation Domain—To determine whether the activity that causes abnormal steroid specificity could be localized to a smaller part of the GR N-terminal domain, steroid-dependent transactivation by a GR protein deleted for the \( \tau_1 \) transactivation domain (expressed from pKV-NX-Δ\( \tau_1 \), Fig. 1B) was tested. Fig. 2B and Table I (column 2) show that this gave a more normal specificity, similar to that previously reported for the N-terminal deleted derivative of the GR (3). Although deoxycorticosterone remains the most potent steroid at relatively low hormone concentrations, it is superseded by the glucocorticoids triamcinolone acetonide, dexamethasone, and corticosterone at higher concentrations. Progesterone, which for the intact GR was equally effective as corticosterone, produces almost no activation. It is notable that the hormone concentrations required to see activation with this protein are much greater than for the intact protein (Fig. 2A) (see "Discussion"). To show whether the \( \tau_1 \) domain is sufficient to cause the abnormal steroid specificity it was fused to the N-terminally deleted GR derivative which has a near normal steroid specificity (3). This protein (expressed from pKV-\( \tau_1 \)-AX, Fig. 1B) showed an abnormal specificity resembling that of the intact receptor (compare Fig. 2C with Fig. 2A, and Table I, column 3, with Table I, column 1). Therefore, the \( \tau_1 \) transactivation domain of the GR is sufficient to impose abnormal steroid specificity on the N-terminally truncated GR.

Intact GR Shows Glucocorticoid-specific Transactivation When Expressed at a Low Level—The abnormal steroid specificity profile caused by the \( \tau_1 \) domain of the GR could be the result of an altered folding of the yeast-expressed protein or of the \( \tau_1 \)-mediated transactivation properties of the expressed GR per se. The latter is not unlikely since the \( \tau_1 \) domain accounts for most of the transactivation potential of the GR (16). To determine whether the amount of GR-derived transactivation potential in the cells, as distinct from the constitution of the receptor protein, could influence the specificity profile, the expression level of the GR was reduced. This was done by cloning the expression cassette from pKV-NX (Fig. 1B) into a single copy number vector to produce the new plasmid, pJV-NX (Fig. 1A). Based on the reduction in copy number the expression level from pJV-NX was expected to be about 1–2% of that from pKV-NX, and Western blot and gel mobility shift experiments indicated a large reduction in the level of the GR (data not shown). As shown in Fig. 2D and Table II (columns 1–3), the specificity in this case is near normal, albeit after addition of high amounts of hormone. Thus, the GR appears to be folded correctly in yeast cells, at least when it is expressed at low levels. The nature of the specificity profile appears to reflect the level of GR-dependent transactivation potential in the cells such that a high level of intact GR or of derivatives potent in transcriptional activation is correlated with the abnormal specificity phenotype.

Varying Reporter Gene Sensitivity Influences Steroid Specificity—Another way to change the transactivation potential of the expressed GR with respect to the reporter gene, without changing the level or the structure of the expressed receptor
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Fig. 2. Response to a variety of steroid hormones of a reporter gene (β-galactosidase activity) in yeast cells containing different combinations of expression and reporter plasmids. A, cells containing pKV-NX and pLGZ-TAT; B, cells containing pKV-NX-Δr and pLGZ-TAT; C, cells containing pKV-TA; AX and pLGZ-TAT; D, cells containing pJV-NX and pLGZ-TAT; E, cells containing pJV-NX and pLGZ-TAT. Each experiment was repeated two or more times giving similar relative activities for the different steroids, albeit with some variation (±2-fold) in the absolute values measured. A representative example is shown by each graph (see Tables I and II for comparison of relative values ± S.D.). Steroid hormones are abbreviated as follows: triamcinolone acetonide (TA), corticosterone (C), dexamethasone (DEX), cortisol (H), deoxycorticosterone (DOC), progesterone (PROG), testosterone (TEST), and estradiol (E2).

DISCUSSION

The GR expressed in yeast, irrespective of the derivative expressed and the expression level, consistently shows ligand specificity for transcriptional transactivation to the extent that testosterone and estradiol do not activate under any conditions tested. Presumably, their structures are not sufficiently related to those of glucocorticoids. However, within a group of more closely related steroid hormones, including various glucocorticoids, deoxycorticosterone (a mineralocorticoid), and progesterone, the ligand specificity profile for activation varies considerably depending on the composition of the expressed receptor derivative, its expression level, and the sensitivity of the reporter gene. The intact GR shows near normal steroid specificity when expressed at a low level and assayed using a relatively insensitive reporter gene. Thus, an important conclusion of the study is that, using these conditions, it should be possible to use yeast genetics to isolate receptor mutants which are altered in their steroid specificity.

The reason for the abnormal steroid specificity reported for the rat GR in yeast (2) has been of interest to us since we showed that an N-terminally truncated version of the human GR showed normal specificity (3). Since we now know that the intact human GR in our system also shows abnormal specificity under some conditions, we can exclude that the apparent discrepancy between these previous results was primarily due to differences between the rat and human receptors or to differences in the yeast strains used.

Several putative explanations for the abnormal steroid specificity sometimes seen for the intact GR are possible. First, the intact GR may be folded incorrectly when translated in yeast cells, thus creating an altered ligand binding surface. In yeast yielding up to 10–20-fold higher expression of the reporter gene from a double GRE (13). When a reporter plasmid containing a double GRE was used combined with the low expression plasmid (pJV-NX) the results shown in Fig. 2E and Table II (columns 4–6) were obtained. In addition to the increased reporter gene activity observed, there are clear changes in the relative potency of the different ligands compared with the data obtained using a single GRE reporter (Fig. 2D and Table II, columns 1–3). At a concentration of 2 μM, deoxycorticosterone was the best ligand and gave a 5.1-fold induction of the reporter gene containing a double GRE, compared with a 2.5-fold induction by corticosterone at the same concentration. Using the reporter gene with a single GRE, deoxycorticosterone showed almost no induction (1.4-fold) and was similar in potency to corticosterone (1.2-fold).

Progesterone caused a dose-responsive increase in induction from 1.8-fold at 2 μM to 10.2-fold at 50 μM with the double GRE-containing reporter gene, whereas the induction level was almost insignificant (1.2-fold) at both concentrations with the single GRE reporter gene. We conclude that steroid specificity varies according to the sensitivity of the reporter system used, in conditions where the level and structure of the GR are the same.

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**Table I**

Quantitation of steroid hormone-induced transactivation by different GR derivatives at subsaturating hormone levels

Values indicate the level of induction by steroids (at the concentration shown) relative to controls to which no hormone was added. The highest hormone concentration that does not saturate the response of any of the steroids was chosen in each case according to data in Fig. 2. Standard deviations are shown (n = 2 for column 1, n = 3 for columns 2 and 3). Numbers in parentheses show ranking of the different steroid hormones according to their ability to activate the different GR derivatives.

| Hormone                        | Relative level of induction |
|--------------------------------|-----------------------------|
|                                | pKV-NX (0.4 μM)             | pKV-NX-A71 (50 μM) | pKV-A1-AX (2 μM) |
| Triamcinolone acetonide        | 3.2 ± 0.03 (4)              | 47.0 ± 12.5 (1)    | 4.9 ± 0.61 (3)   |
| Dexamethasone                  | 1.3 ± 0.30 (5)              | 45.9 ± 17.1 (2)    | 2.2 ± 0.26 (5)   |
| Corticosterone                 | 5.9 ± 1.30 (5)              | 33.0 ± 14.9 (3)    | 0.2 ± 0.50 (1)   |
| Cortisol                       | 1.3 ± 0.27 (4)              | 27.5 ± 1.5 (4)     | 1.8 ± 0.30 (6)   |
| Deoxytocorticosterone          | 8.1 ± 1.14 (1)              | 22.1 ± 4.4 (5)     | 5.0 ± 1.31 (2)   |
| Progesterone                   | 6.1 ± 0.74 (2)              | 7.0 ± 0.2 (6)      | 4.3 ± 0.51 (4)   |
| Testosterone                    | 0.9 ± 0.01 (7)              | 1.7 ± 0.3 (7)      | 1.5 ± 0.25 (7)   |
| Estradiol                      | 0.9 ± 0.07 (8)              | 1.1 ± 0.1 (8)      | 1.3 ± 0.15 (8)   |

According to our data, the incorrect folding would occur only at high expression levels, perhaps by an aggregation-type mechanism, and would be mediated exclusively by the τ₃ transactivation domain since τ₃ is both necessary and sufficient for imposition of the abnormal phenotype.

Second, it is possible that interactions between the expressed GR and yeast proteins that are needed for receptor function are not exactly the same as in mammalian cells such that steroid specificity is effected. This might result in an altered steroid binding specificity since it is known that the expressed receptor is contained in a large protein complex (3), one constituent of which is yeast hsp 90 (10). Alternatively, GR complexed with different ligands might interact with yeast proteins involved in transcription in different ways giving rise to an abnormal steroid specificity. The latter possibility would be consistent with the involvement of τ₃ in the abnormal specificity. The dependence of the abnormal specificity phenotype on high level expression of the GR in these cases could result from formation of abnormal interactions with host proteins only under conditions in which the levels of one or more yeast proteins become limiting.

Third, abnormal steroid specificity could result from changes in the transactivation assay which occur as a consequence of expressing different receptor derivatives at different levels. One consequence both of removing the τ₃ transactivation domain and reducing the receptor expression level is that the transactivation assay becomes less sensitive. These sensitivity changes could cause apparent specificity changes in receptor function if the different steroid hormones were differentially available in yeast. When the transactivation assay is sensitive, steroids which are readily available but have poor affinity for the receptor could give a response at concentrations at which high affinity ligands are not available for binding. Under less sensitive assay conditions poor affinity interactions would still take place but would not be detected. Factors that could cause variable availability of ligands include uptake by yeast cells, metabolism within cells, and sequestration of steroids either inside or outside the cells.

To distinguish the first two models from the third we changed the sensitivity of the transactivation assay, without changing the level or constitution of the GR derivative expressed, by using more or less sensitive reporter genes. Using the least sensitive reporter gene, the low level expressed GR had a near normal specificity. However, under the same expression conditions using the more sensitive reporter gene, both deoxytocorticosterone and progesterone showed increased activity relative to glucocorticoids. These data suggest that activation by deoxytocorticosterone and progesterone is seen at relatively low concentrations only in combination with the more sensitive reporter gene because the signal to noise ratio in this assay is about 10-fold that in the less sensitive assay. Thus the same interactions occur in the less sensitive assay, but the transactivation signals from the reporter gene cannot be detected above background.

At least some of the specificity changes seen when the level or constitution of GR derivatives is altered are likely to result from the same mechanism, since it is presumably the change in the assay sensitivity per se, rather than the method by which it is changed, that affects the steroid specificity. Several observations support this view. First, as expected in this model, abnormal steroid specificity is correlated with transactivation of the reporter gene at relatively low steroid concentrations, while the reverse correlation can be made for normal specificity.

Second, there is a tendency for dose-response curves to cross so that abnormal steroids that are most potent at low
concentrations are superseded in potency by normal steroids at higher concentrations. This is more easily accounted for by the variable steroid availability model than by the receptor-based models which would infer a complicated hormone concentration-dependent change in the receptor's steroid specificity.

Third, the saturated response levels in Fig. 2, A and C, are probably due to squelching of transcription by the overexpressed receptor, similar to that reported previously (17), rather than saturation of available receptor binding sites. Thus the highest transactivation levels achieved by the GR at both high and low expression levels are very similar (compare Fig. 2, A and D). This means that the hormone concentrations giving half the maximal transactivation activity in Fig. 2, A and B, cannot be used to compare the binding affinities of different steroids.

Fourth, the variable steroid availability model requires that deoxycorticosterone and progesterone should be more readily available for interaction with the GR than dexamethasone, for example. Little is known in this respect except that progesterone is very readily available in yeast since it stimulates the expressed progesterone receptor at low concentrations, saturating at a concentration of about 10 nM (5).

The specificity changes that we have observed for the GR expressed in yeast can be accounted for by the model based on variable steroid availability and assay sensitivity. However, we do not have direct evidence to exclude that high expression levels combined with the presence of the \( \gamma \), transactivation domain affect receptor structure or function in some way which contributes to some of the specificity changes that we have observed. It is interesting to speculate that there might be physiological systems in which varying ligand availability could modulate the apparent specificity of steroid hormone action. Such a system has been reported in which the specificity of the mineralocorticoid receptor in the kidney is due to a reduction in the availability of cross-reacting glucocorticoids as a result of their rapid inactivation by 11\( \beta \)-hydroxysteroid dehydrogenase (18).

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