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REVIEW ARTICLE

Hirotoshi Tanaka · Noritada Yoshikawa · Noriaki Shimizu
Chikao Morimoto

Selective modulation of glucocorticoid receptor function toward development of novel antiinflammation: lessons from a phenylpyrazolosteroid cortivazol

Abstract A Frequent association of side effects has been a long-standing dilemma in clinical glucocorticoid therapy. Recent progress in molecular biology of glucocorticoid hormone action, however, has prompted researchers to tackle the dissociation of side effects and therapeutic effects based on the assumption that selective modulation of its receptor function could be achieved by as yet unknown compounds. Already a number of selective modulators of the glucocorticoid receptor (SGRMs) have been reported, and certain compounds have dissociating characteristics in vivo. We have addressed ligand-dependent modular recruitment of AF-1 function using a phenylpyrazologlucocorticoid cortivazol, suggesting the possibility of developing tissue-specific SGRMs. It should also be emphasized that SGRMs do not always have a steroid structure.

Key words Co-activator · Conformation · Glucocorticoid receptor (GR) · Ligand binding domain (LBD) · Transcription

Introduction

Glucocorticoids (GCs) are produced in the adrenal cortex under the strict control of the hypothalamus-pituitary-adrenal axis, and they have a variety of biological actions: regulation of glucose metabolism, lipolysis, and participation in the immune system, cardiovascular system, electrolyte metabolism, and central nervous system.[1,2] Moreover, GCs have been widely and successfully used in the treatment of acute and chronic inflammatory diseases for more than 50 years. They exert their effects by distinct mechanisms that interfere with many inflammatory pathways.

Unfortunately, the desired antiinflammatory and immunosuppressant effects are often accompanied by severe side effects (e.g., diabetes mellitus, peptic ulcer, osteoporosis, psychosis, glaucoma). The use of GCs, therefore, is limited by these side effects; and there is hence an urgent clinical need for the development of compounds with the antiinflammatory potency of classical GCs but with reduced side effects.[3,4] On the other hand, new insights into the molecular mechanisms of GC-mediated actions have provided opportunities for identification of substances with a better therapeutic index.[3–5] Given this background, this review focuses on recent advances in the molecular biology of GC action and pharmacological development of novel GCs and GC-related compounds.

Molecular biology of GC action

After entry of GCs into target cells, GC actions are believed to be mediated by the binding to their cognate receptor, the glucocorticoid receptor (GR), which belongs to the nuclear receptor superfamily and includes receptors for the mineralocorticoids (MRs), estrogens (ERs), progestins (PRs), and androgens (ARs), as well as those for peroxisome proliferators, vitamin D, and thyroid hormones. Phylogenetic analysis and sequence alignments show that the GRs, MRs, PRs, and ARs form a subfamily of oxosteroid receptors.[6]

Like most nuclear receptors, the GR is a modular protein that is mainly organized into three major domains: an N-terminal activation function-1 domain (AF-1), a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The C-terminal part of the LBD is termed the AF-2 helix, and it plays essential roles in transactivation with co-activators. Among nuclear receptors, AF-2 helix is extremely conserved, whereas the molecular size and amino acid composition of AF-1 is diverse.

H. Tanaka (✉) · N. Yoshikawa · N. Shimizu · C. Morimoto
Division of Clinical Immunology, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
Tel. +81-3-5449-5547; Fax +81-3-5449-5547
e-mail: hirotnk@ims.u-tokyo.ac.jp

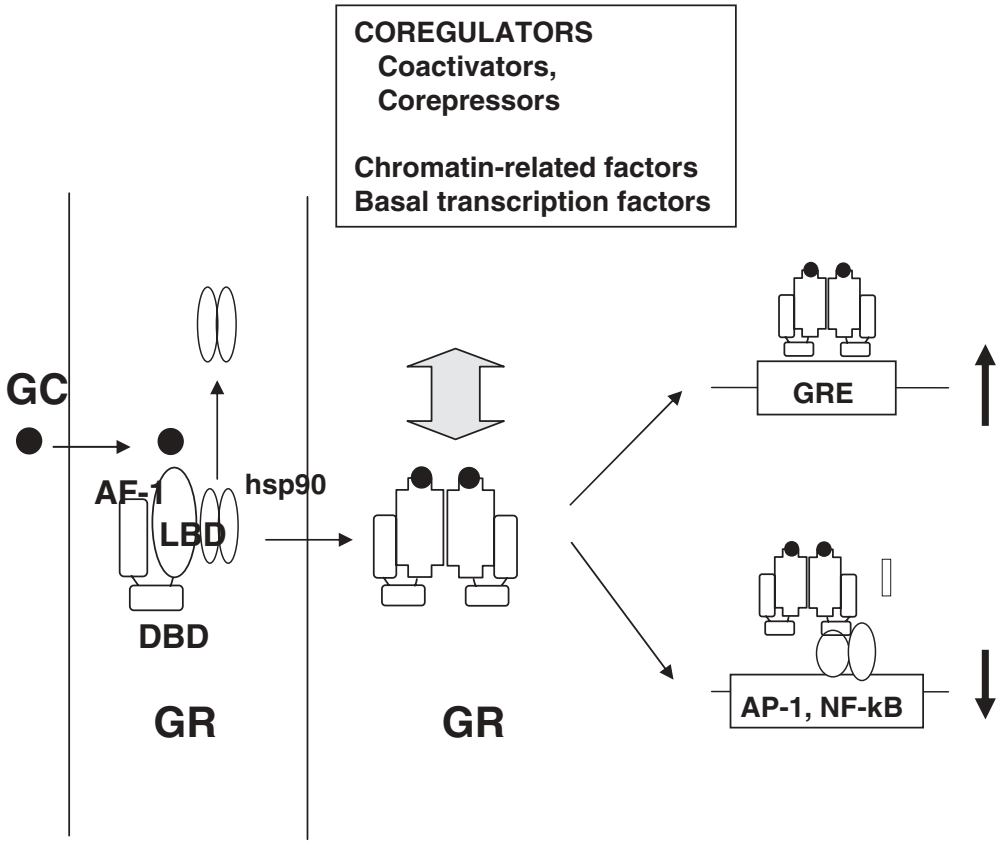

Fig. 1. Hypothetical model for glucocorticoid-mediated gene regulation. *GC*, glucocorticoid; *GR*, glucocorticoid receptor; *GRE*, glucocorticoid response element; *AF-1*, activation function-1; *DBD*, DNA binding domain; *LBD*, ligand binding domain

In the absence of ligand, the GR is retained in the cytoplasm by association with chaperone proteins, such as heat shock protein 90 (hsp90). Hormone binding initiates release of the chaperone proteins from the GR, allowing dimerization and translocation of the receptor into the nucleus. In the nucleus, the GR binds to a DNA promoter element termed the GC response element (GRE) and can either activate or repress transcription depending on the context of the target promoters. In addition, the GR cross-talks with other transcriptional factors, such as nuclear factor-κB (NF-κB) and AP-1, to repress their gene activation activities, at least in part, via protein–protein interaction.[7–10] This GR-mediated repression has been postulated to be one of the molecular bases for the antiinflammatory and immunosuppressive activities of glucocorticoids. Moreover, gene expression of a large number of proinflammatory cytokines and key inflammatory mediators such as tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-12, and prostaglandin E_2 is suppressed by GCs[3,9,10] (Fig. 1).

Indeed, this assumption has been confirmed by investigations with transgenic mice expressing a dimerization-deficient GR. Although transactivation is suppressed in these mice, the transrepression function is intact, and GCs inhibit edema in a croton oil-induced ear inflammation test as effectively as in wild-type animals.[11] It should be noted, however, that GCs also activate a perhaps smaller number of antiinflammatory genes such as IL-10, IL-4, and transforming growth factor-β (TGFβ), eliciting antiinflammation.[5,9]

The side effects of GCs are also associated with both repression and activation of specific genes. Such activities include transcriptional activation of enzymes involved in gluconeogenesis, lipid metabolism, and muscle metabolism, such as glutamine synthetase and gelsolin. For example, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, the two most important enzymes of gluconeogenesis (an essential pathway in the development of diabetes), are both induced by GCs. Moreover, tissue-selective involvement of co-activators may also play a role in undesirable side effects. For example, PGC-1 co-activator is reported to be related to hepatic glucocorticoid-induced gluconeogenesis.[5] In contrast, a key mechanism for suppression of the hypothalamo-pituitary-adrenal axis, the decreased release of adrenocorticotrophic hormone (ACTH) by corticotrophin-releasing hormone, is mediated by the GR via a transrepression mechanism.[2] Other GC-mediated side effects (e.g., osteoporosis, skin atrophy, growth retardation, Cushing's syndrome) are subject to complex regulation involving a variety of mechanisms. However, a transactivation mechanism seems to be at least partially involved in the regulation of these side effects as well. These complexities suggest that it may be necessary to find a gene- or tissue-specific ligand to achieve the desired therapeutic profile.[4,5]

Role of ligand for determination of three-dimensional structure of the GR

Although GR shows a variety of biological actions, it should be noted that the ligand gives a critical cue for each of them. That is, conformational alteration of the receptor after ligand binding is a key step for diversity of subsequent GR-mediated signals. Recent crystallographic analyses of the nuclear receptors have established a paradigm of receptor activation in which agonist binding induces AF-2 helix to form a charge clamp for co-activator recruitment. The human GR LBD was crystallized in a ternary complex with dexamethasone (DEX) and LXXLL co-activator peptides. The GR LBD is similar to other nuclear receptor LBDs and composed of α-helices and β-strands folded into a three-layer helical sandwich. The ligand-binding pocket is composed of residues from helices 3, 4, 5, 6, 7, and 10 and the AF-2 helix, as well as residues from β-strands between helices 5 and 6. Following the AF-2 helix is an extended strand that forms a conserved β-sheet with a β-strand between helices 8 and 9. This C-terminal γβ-strand also appears to play an important role in receptor activation by stabilizing the AF-2 helix in an active conformation. In the GR LBD structure, the LLRYLL sequence in the TIF2 motif forms a two-turn α-helix that orients the hydrophobic leucine side chains into a groove formed in part by the AF-2 helix and residues from helices 3, 3′, 4, and 5. The N- and C-terminal ends of the co-activator helix are clamped by Glu-755 from AF-2 helix and Lys-579 in helix 3, respectively.

Mutation either in the first charge clamp (Glu-755) or the second charge clamp (residues Arg-585 and Asp-590) dramatically reduced the activation mediated by the GR LBD, demonstrating that both charge clamps are critical for the transactivation function of the GR.[12,13] In contrast, little information is available on the conformation and function of the AF-1 regions, and only a few AF-1 regions have been studied in detail. Although AF-1 has been considered to be ligand-independent and regulated by cell- and promoter-context and phosphorylation, recent evidence strongly suggests that AF-1 communicates with the LBD/AF-2, the receptor specific-AF-1 cofactors, or both.[14,15]

Role of co-regulators in GR-mediated gene expression

A large number of transcriptional co-regulators have been identified and characterized. Many but not all of these co-activators and co-repressors are recruited to the DNA template via interactions with transcription factors including nuclear receptors. Some co-regulators are direct intermediaries between transcription factors and the general/basal transcriptional machinery. On the other hand, many co-activator complexes (which include TRAP, SMCC, mediator, SRB complex, CRSP, DRIP, NAT, p300/CBP, and others) can serve as a bridge between transcription factors and the general/basal transcriptional machinery. Another distinct class of cofactors are chromatin-related co-regulators, which are also thought to be recruited by the nuclear receptors. The chromatin-related co-regulators affect transcription indirectly by remodeling nucleosomes or by covalent modification of histones (e.g., by acetylation, methylation, phosphorylation, ubiquitylation, and ADP-ribosylation) or the DNA template. Other co-regulators are not recruited by the nuclear receptors but, instead, interact directly with RNA polymerase II and modulate the efficiency of transcriptional elongation. These phenomena reveal the diversity and complexity of transcriptional regulation.

In eukaryotes, there are also other modes of regulation, such as the covalent modification of histones, nucleosome remodeling, and the formation of higher-order chromatin structures. It is important to understand the contexts in which each of the co-regulators is required for transcriptional control and how these factors work in concert to potentiate the transcriptional signals that emanate from the nuclear receptors.[16,17]

In terms of the GR, many AF-2 co-activators for the GR have been identified to date, including SRC-1, TIF2/GRIP-1, and CBP/p300. On the other hand, AF-1 co-activators have only recently been described. For example, basal transcription factors including TBP and TFIID have been shown to be associated with autonomous transactivational function of GR AF-1. TSG101 and DRIP150 have been reported to interact with GR AF-1 and regulate GR function in a reciprocal manner; GR transcriptional activities are repressed by TSG101 but enhanced by DRIP150. These cofactors have been shown to interact with distinct regions of AF-1.[16,18–23] On the other hand, we have recently identified a novel repressor for the GR, termed HEXIM1. HEXIM1 suppresses GR function via an entrapment mechanism in the nucleus, again indicating the complexity of GR-mediated gene regulation (N. Shimizu et al., in preparation).

Taking the modular nature and the presence of many accessory proteins of the nuclear receptors into consideration, functional interplays, either direct or indirect, between each domain would determine how full-length receptor works in a ligand-dependent manner.[22] In the PR B form and AR, for example, direct interaction between AF-1 and LBD of the receptor has been reported. Such interaction takes place only when an agonist is bound and antagonist binding prevents it, suggesting a conformational change taking place in the agonist-bound LBD, which is well suited for the interaction between AF-1 and LBD.[24,25] It has also been reported that AF-1 and AF-2 interact via mutual binding of other proteins, such as co-activators. The synergy between the ER AF-1 and AF-2 is reported to be due to cooperative recruitment of members of p160 co-activators.[26] This type of bridge protein interaction has been observed between AF-1 and AF-2 regions for PPARγ, SF-1, AR, and RARα.[27,28] In the GR, DRIP205, which has been shown to associate with AF-2, could modulate GR function in concert with AF-1 cofactor DRIP150.[29] It is poorly understood, however, how ligand, most possibly via conformational change of the LBD, modulates communication between cofactors and two AFs of the GR. This issue

Table 1. Selective glucocorticoid receptor modulators (SGRM) in the literature

Compounds	Binding					Transcriptions		References
	GR	MR	PR	AR	ER	Activation	Repression	
RU24858	+	n.d.	n.d.	n.d.	n.d.	−	+	48
Ursodeoxycholic acid	−	−	−	−	−	−	+	49
A276575	++	+	+	n.d.	n.d.	−	++	50
AL 438	++	+	+/−	+/−	−	+	+	51
5-allyl-2,5-dihydro-2,2,4- trimethyl-1H-[1]benzopyrazolo[3,4-f]quinidine-based-ligand	++	+	+/−	+/−	−	+/−	++	52
ZK216348	++	+	+	−	−	+	+	53
10-methoxy-2,2,4-trimethylbenzopyrazopyrazol [3,4-f]quinoline-based-ligands	++	n.d.	+/−	n.d.	n.d.	+/−	+	54

GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; ER, estrogen receptor

appears to be extremely important for understanding the mechanism for tissue-specific regulation of the GR function, as AF-1 activity is believed to be tissue-specific.

Selective modulators of GR

Although the molecular mechanisms of GC-induced side effects are complex and often not yet well understood, it appears justified to assume that some of these undesired effects, such as steroid diabetes, require GR–DNA interaction and transactivation. Therefore, ligands that preferentially induce the transrepression and not the transactivation function of the GR should be as effective as classical GCs but with fewer undesirable effects[1] and can be considered to be a prototype for selective modulators of GR (SGRMs). In this line, a number of compounds have been documented (summarized in Table 1). Of note, some of them do not contain steroid backbone but bind the GR with high affinity.

As described previously, GR-mediated gene regulation is a complex process, and dissociation of transactivation and transrepression is not a unique criteria for SGRM. Indeed, bone-related side effects are associated with transcriptional repression of genes involved in osteoblast function and bone formation, such as osteocalcin and osteoprotegerin in vitro[5] and clinically. These complexities suggest that it may be necessary to find a gene- or tissue-specific ligand to achieve the desired therapeutic profile. A number of selective ER modulators that can control ER-mediated cellular responses in tissue-specific fashion have been reported. The molecular basis for activities of these selective ER modulators, at least in part, is considered to be variable regulation of not only AF-2 but also AF-1 activity.[30] Similar situations are also known in the AR. In this context, future strategies for the development of SGRM should include, for example, the screening of effects on AF-1 activity in various tissues.

Cortivazol, a unique GR agonist

The phenylpyrazologlucocorticoid cortivazol (CVZ) (Fig. 2A) is a unique synthetic glucocorticoid agonist that has been reported to have two dissociation constants for the GR and be more potent than DEX.[31] We previously demonstrated that CVZ specifically binds to the GR but not to the MR; and that based on two criteria, functional interaction of CVZ with the GR LBD is different from that of DEX. First, not DEX but CVZ can bind to GR-(1–765), in which 12 amino acids from the C-terminal end of the GR are deleted. Second, mutant GR GRL753F, in which the Leu-753 in AF-2 is substituted to Phe, can efficiently recruit TIF2 to the LBD on binding with CVZ but not with DEX.[32] Indeed, several derivatives of CVZ, including deacylated CVZ, have been shown to be as potent as CVZ.[33,34] These results prompted us to speculate that CVZ-bound GR LBD might have unique effects on the structure and function of the whole GR molecule.

Putative docking of CVZ into GR LBD in silico

The calculated volumes of DEX and CVZ are 386 Å and 541 Å,[3] respectively, reflecting the difference in attachment to the A-ring of the steroid backbone. The estimated volume of ligand-binding pocket of the DEX–GR LBD complex is 600 Å.[3] and DEX occupies 65% of the pocket.[13] CVZ thus seemed to be too large to be docked into the pocket observed in the crystal structure of DEX-bound GR LBD without modifying the pocket structure. However, it has recently been reported, that, in liver X receptor b, the structure of the LBD is flexible to adopt a different size of ligand; the larger agonist GW3965 shifts many side chains and enlarges the volume of the ligand-binding pocket, resulting in induction of differential conformation in a part of the LBD when compared with the smaller agonist T0901317, keeping the interaction between the ligand and

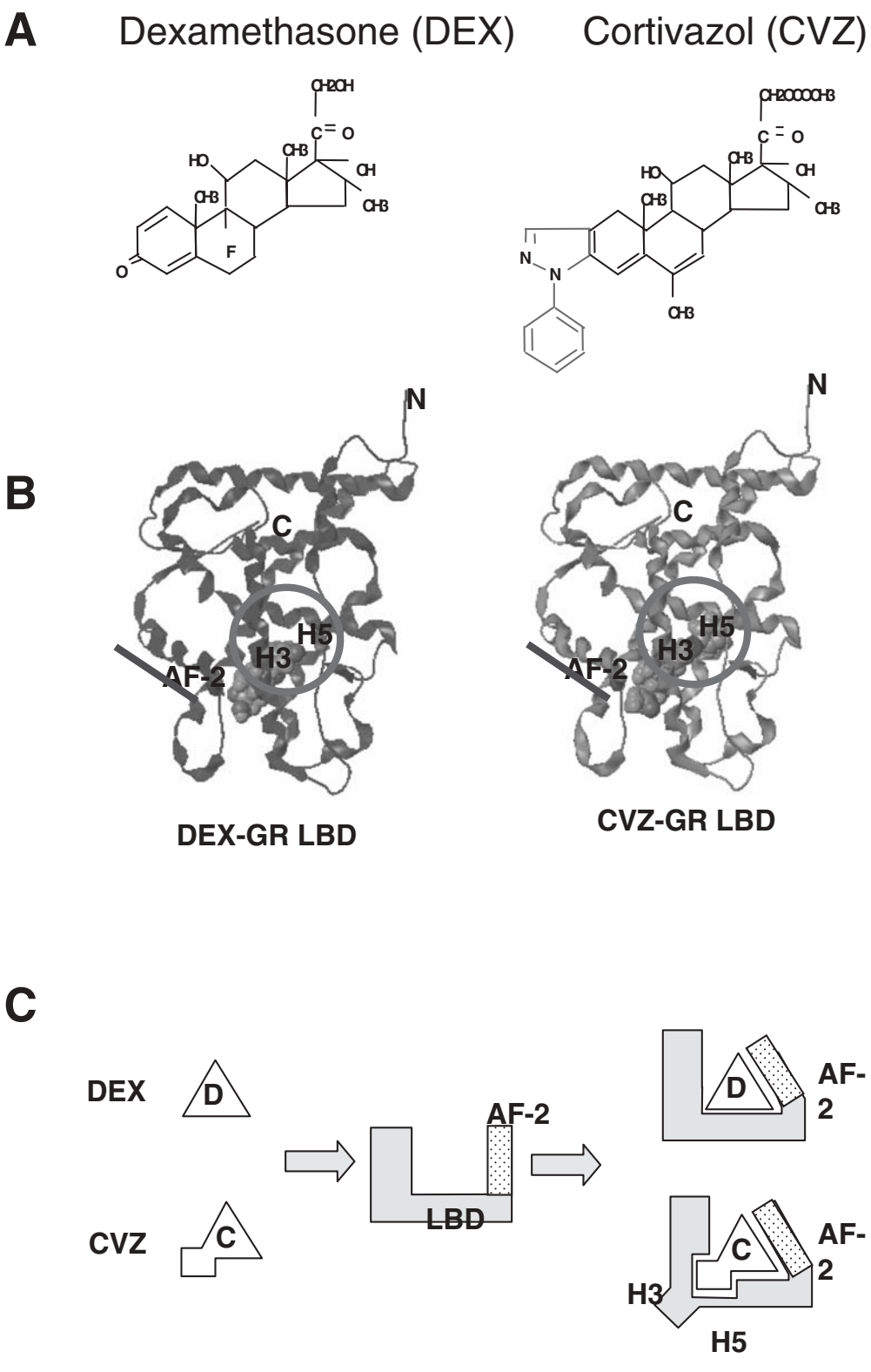

Fig. 2. Putative three-dimensional structure of the GR LBD docked with dexamethasone (*DEX*) and cortivazol (*CVZ*). **A** Structure of dexamethasone and cortivazol. **B** Overall arrangement of DEX- and CVZ-bound GR LBD. Ribbon represents the α-helix and β-sheet, and the tube represents loop of protein. The GR LBD docked with DEX or CVZ is depicted with space fill model. **C** Hypothetical model for the interaction between either DEX or CVZ and GR LBD

helix 12 intact.[35] Along this line, although it is obvious that crystallographic analysis for CVZ-bound LBD should be done for precise determination of the CVZ-bound LBD structure, this assumption might be the case.

The fact that CVZ worked as an agonist for the GR in various experiments strongly indicated that the docking manner of CVZ is similar to that of DEX. When we applied the structural model derived from recent X-ray crystallographic analyses for the GR LBD, in which substitution of Phe-602 to Ser (F602S) was introduced, CVZ was manually docked into the ligand-binding pocket of the GR LBD by superimposing its steroid backbone with that of DEX in silico. Energy minimization of the CVZ/GR LBD complex suggested that CVZ could be accommodated in the GR

LBD by the induced fit mechanism. The resultant model for CVZ-GR LBD is shown in Fig. 2B; CVZ, as well as DEX, is completely enclosed in the bottom half of the GR LBD, and the spatial position of the β-helices and β-strands of the CVZ-bound GR LBD is almost identical to that of the DEX-bound one, including the orientation of helix 12. In contrast, when CVZ is applied, the position of the side chain of Arg-611 should be shifted outward of the ligand binding pocket, and the side-chain conformations of Asn-564, Gln-570, Met-604, Leu-608, Met-646, and Phe-749 are somewhat changed, resulting in a distinct hydrogen bond network. These alterations could be due to space-occupying effects of the bulky phenylpyrazole ring and the 21-acetoxyl group of CVZ. It is of note that all of these amino acids except Phe-749 are involved in helices 3 and 5, suggesting that, if our prediction could be applicable, CVZ might elicit distinct effects via regional alteration in conformation of these helices. These results, at least in part, may support such assumption that helices 3 and 5 are important for ligand recognition of the GR, and a bulky substituent at A-ring might influence not only their conformation but also receptor function (Fig. 2C). In this regard, the three-dimensional structural model of a mutant MR, based on the crystal structure of the PR, also clearly addressed both structural and functional importance of helices 3 and 5. Substitution of Ser-810 to Leu in helix 5 of the MR alters receptor specificity, and the transactivation function is induced not only by aldosterone but also by the antagonist spironolactone or progesterone via de novo creation of interaction between these ligands and helices 3 and 5.[36]

Distinct effects of DEX and CVZ on the function of C-terminal truncated GR

It has been reported that in several amino acids deletion of the C-terminal end of the LBD abolishes not only its ligand-binding ability but also co-activator recruitment to AF-2 and AF-2-dependent transactivation function.[13,37,38] Because the GR LBD for crystallization involved substitution of Phe-602 to Ser, we also introduced such a mutation in the GR-(1–765) to make the resultant mutant able to bind DEX. [The resultant mutant is GR-(1–765)/F602S).] Surprisingly, although GR-(1–765)/F602S was capable of nuclear entry as a DNA-binding species with DEX and CVZ, DEX scarcely induced its transactivation even at high concentrations. CVZ elicited concentration-dependent transactivation in not only GR-(1–765) but GR-(1–765)/F602S as well. Because these C-terminal truncated GR mutants could not recruit TIF2 to AF-2 irrespective of ligand binding, CVZ binding is likely to recruit TIF2, either directly or indirectly, to the other portion of the receptor (i.e., AF-1).

Ligand-dependent scenario for communication between AF-1 and the LBD

In the case of the GR, CBP has been known to interact directly with AF-1 or AF-2, or both.[27] Given the previous reports showing that TIF2 does not directly bind to GR AF-1,[20,39] it appears likely that in the C-terminal truncated GR binding of not DEX but CVZ enables TIF2 to influence AF-1 function as a component of a co-activator complex with CBP. On the other hand, it is also possible that TIF2 forms a complex with known AF-1 co-activators DRIP150, Ada2, TFIID, and TBP.

Reporter gene assays using the chimeric GR, which consists of foreign AF-1 and DBD and LBD of the GR, revealed that induction of transactivation is not determined merely by co-activators and ligand-bound LBD but also by AF-1. Indeed, both DEX and CVZ efficiently induced the transactivation function of AF-1 of the GR and PR in contrast to that of ER, MR, and AR. Although the relevance of alignment or structural similarity of AF-1 among steroid receptors remains controversial, this result may be deemed useful for analyzing the common functional features of AF-1. For instance, we may raise the possibility that each receptor has some kind of relation in the class of AF-1 co-activators because, for example, Jun dimerization protein 2 has been reported to act as co-activator for both GR and PR but not for ER.[40] Furthermore, both glucocorticoid and aldosterone are known to bind to the MR and activate its transcriptional function.[41–43] However, it has been reported that MR-specific co-activator RNA helicase A is recruited to AF-1 of the MR by aldosterone but not by hydrocortisone.[44] Again, together with our result that AF-1 of the MR could not mimic GR AF-1 in either DEX- or CVZ-bound chimeric GR, we may speculate that the ligand employs differential recruitment of AF-1 cofactor species, and that this stringent function of ligand may be due to conformational change of the LBD specific to each ligand. We therefore suggest that ligand, via formation of distinct conformation of the LBD, could modulate the interaction between LBD and AF-1 in a variable fashion, and that cellular cofactors also take part in such interdomain communication (Fig. 3). It is of note that in the AR disturbance of such communication between AF-1 and LBD causes androgen insensitivity syndrome,[45] suggesting the physiological importance of interdomain synergism. In any case, thorough identification of AF-1 cofactors in a variety of tissues definitely contribute to understanding the precise role of ligand for communication between AF-1 and the LBD and the tissue-specific regulation of GR function.

Concluding remarks: lessons from CVZ and future direction for SGRM

It has been reported that a synthetic glucocorticoid agonist RU27144, which has a phenylpyrazole A-ring, does not bind to the MR.[46] Together with the present results for CVZ, it is conceivable that such a steroid ligand with a bulky phenylpyrazole substituent at the A-ring could be a prototype for a distinct class of SGRMs with both GR selectivity and AF-1 modulation, as well as anti-NF-κB activity. Surprisingly, some nonsteroidal phenylpyrazole analogues

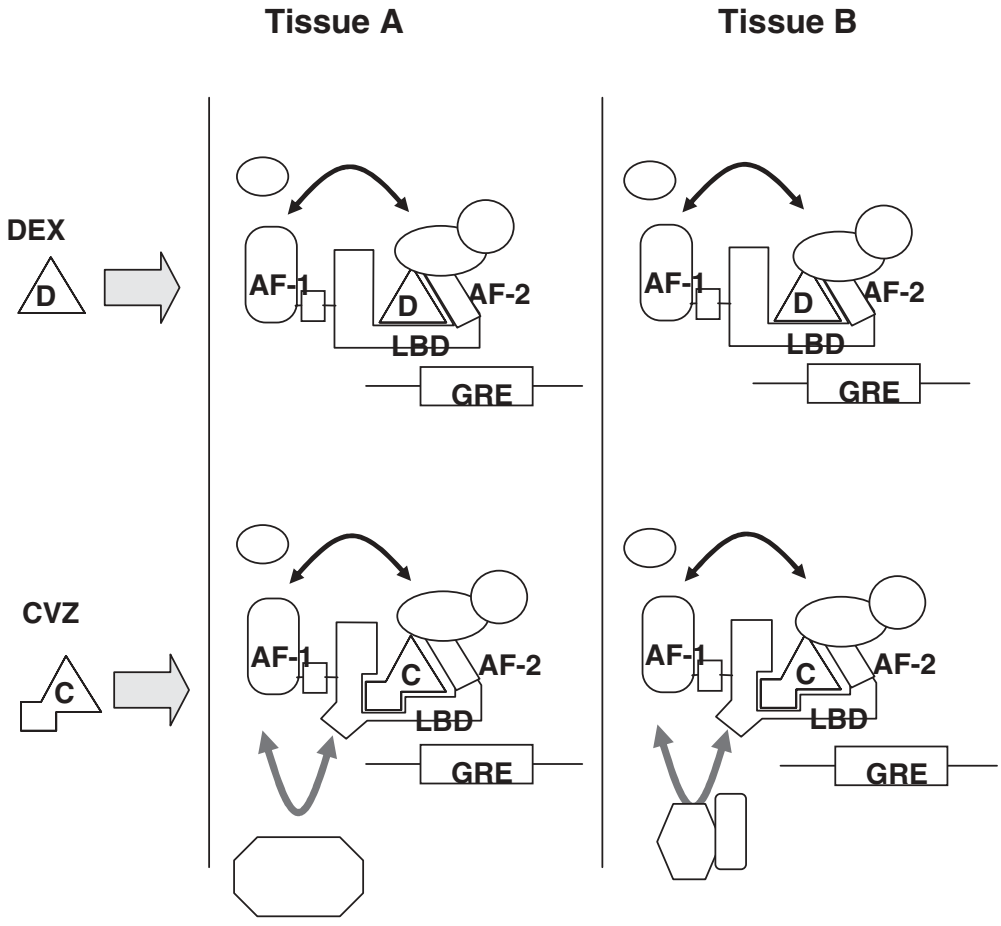

Fig. 3. Hypothetical model for multiple regulation of AF-1 function by dexamethasone (*DEX*) and cortivazol (*CVZ*)

display higher repression activity for interleukin-6 than activation activity for tyrosine aminotransferase promoter.[47] Further structural analysis of the complex between phenylpyrazole ligands and the LBD, therefore, would definitely contribute to the development of a distinct category of SGRMs.

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