Hormone-Dependent Coactivator Binding to a Hydrophobic Cleft on Nuclear Receptors

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The ligand-binding domain of nuclear receptors contains a transcriptional activation function (AF-2) that mediates hormone-dependent binding of coactivator proteins. Scanning surface mutagenesis on the human thyroid hormone receptor was performed to define the site that binds the coactivators, glucocorticoid receptor–interacting protein 1 (GRIP1) and steroid receptor coactivator 1 (SRC-1). The residues involved encircle a small surface that contains a hydrophobic cleft. Ligand activation of transcription involves formation of this surface by folding the carboxyl-terminal α helix against a scaffold of three other helices. These features may represent general ones for nuclear receptors.

Nuclear receptors stimulate transcription in response to hormone binding by interacting with coactivator proteins in a hormone-dependent manner (*1*). The nature of the nuclear receptor–coactivator interaction site is not known. The site could be a large surface, as is usual with peptide hormone–receptor interactions (*2*), or a small surface with complementarity between the coactivator and nuclear receptor (*3*). Alternatively, the interaction could involve the folding of one protein into the partner (*4*). Coactivator binding is impaired by mutations in the COOH-terminal α helix of the receptor ligand-binding domain (LBD) (*5*–*7*), but it is not known how this helix contributes to coactivator binding. An autonomous COOH-terminal helix function might be suggested, since the isolated helix confers activation when linked to a heterologous DNA-binding domain (*8*). However, in the liganded thyroid hormone receptor (TR), this helix packs against the LBD and thus the COOH-terminal helix might be part of a larger surface. A conserved Lys outside of the COOH-terminal helix has been implicated in coactivator binding to the estrogen receptor (ER) (*9*), but it is difficult to interpret effects of this single mutation, which might disrupt overall receptor folding (*10*) or affect TR heterodimerization with the retinoid-X receptor (RXR) (*11*, *12*).

We mapped the surface of the TR LBD for its interaction with coactivator. We used the TR LBD x-ray crystallographic structure (*13*) to guide placement of 37 mutations in the human (h) TRβ1 LBD surface. Reasoning that bulky, surface-charged residues might disrupt coactivator binding, yet preserve global structure and solubility, we mutated selected residues preferentially to charged residues (Arg, Lys, or Glu) (*14*).

Wild-type (WT) TR and most of the TR mutants liganded to 3,5,3′-triiodo-L-thyronine (T_3) bound equally well to the coactivator glucocorticoid receptor-interacting protein 1 (GRIP1) (Fig. 1A, upper panel) (*15*). In all cases, GRIP1 binding was hormone dependent (*16*). As expected (*5*, *6*), mutations $Lys^{454} \rightarrow Arg^{454}$ (L454R) (*17*) and E457K in surface residues of helix 12 abolished GRIP1 binding. Mutations in two residues of helix 3, V284R and K288A, and two residues of helix 5, I302R and K306A, also impaired binding. Five of the mutations with diminished GRIP1 binding (V284R, K288A, I302R, L454R, and E457K) also showed decreased binding to another coactivator, steroid receptor coactivator 1a (SRC-1a) (Fig. 1B) (*18*). Thus, two differ-

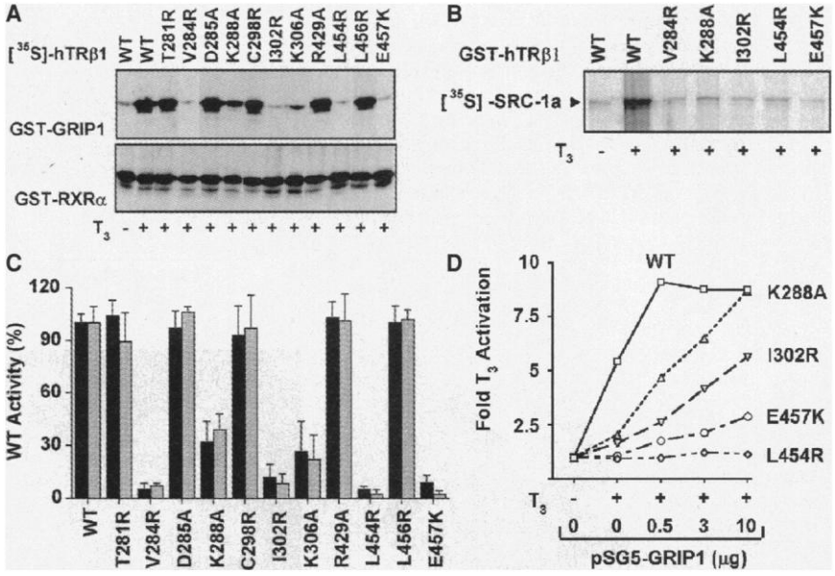

Fig. 1. Binding of GRIP1, SRC-1, and RXRα to hTRβ1 mutants and transcriptional activation by hTRβ1 mutants. (**A**) In vitro binding of ^{35}S-labeled full-length WT and representative mutants of hTRβ1 to GST-GRIP1 (upper panel) and to GST-hRXRα (lower panel). The GST-GRIP1 used included GRIP1 amino acids 721 to 1121; the same results were obtained with a GST-GRIP1 construct that included GRIP1 amino acids 563 to 1121 (*16*). The binding assay was analyzed by autoradiography after separation with 10% SDS–polyacrylamide gel electrophoresis (PAGE) (*25*). The mutations are indicated above the gels. The absence or presence of 1 μM T_3 is indicated below the gels as − or +, respectively. (**B**) In vitro binding of ^{35}S-labeled full-length SRC-1a to WT and mutant GST-hTRβ1. Analysis and display as for Fig. 1A. The same results were obtained when a GST-SRC1 construct including SRC-1a amino acids 381 to 882 was tested for binding of [^{35}S]Met-labeled full-length hTRβ1 WT and mutants (*16*). (**C**) Specific effects of mutations on hTRβ1 transcriptional activation in HeLa cells and correlation with effects on binding to GST-GRIP1. The multiple increases (fold) of T_3 activation (black bar) and of the phosphorimager quantitation of the GST-GRIP1 binding from Fig. 1A (gray bar) are both expressed as percentages of the WT values and graphed for each representative mutant (*25*, *26*). Not all 37 mutants are shown. Error bars indicate the SD of three experiments. (**D**) Overexpression of full-length GRIP1 rescued the loss of transcriptional activation by hTRβ1 mutants. Indicated are amounts of the expression vector for full-length GRIP1, pSG5-GRIP1, that were included in the cotransfections, which were otherwise performed as for Fig. 1C (*26*). The WT and representative hTRβ1 mutants are indicated. The data represent the averages of three independent experiments, with SD <10%.

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ent coactivators recognize the same TR surface residues.

To determine which residues are involved in ligand-mediated transcription activation, we tested the TR mutants in HeLa cells. T_3 increased reporter gene activity fivefold in cells expressing either WT TR or mutated TRs that showed normal GRIP1 binding (representative mutants are shown in Fig. 1C). By contrast, TR mutants with diminished or absent GRIP1 binding (V284R, K288A, I302R, K306A, L454R, and E457K) showed a diminished or absent response to T_3 that correlated with the GRIP1 binding defect. Overexpression of GRIP1 increased activation by the WT TR and rescued activation by TR mutants roughly in proportion to the severity of the defect of GRIP1 binding and activation (Fig. 1D). Thus, the same residues may be required for coactivator binding, functioning of one or more endogenous coactivators in HeLa cells, and responsiveness of TRs to GRIP1; these residues also probably define the TR transcriptional activation function (AF-2).

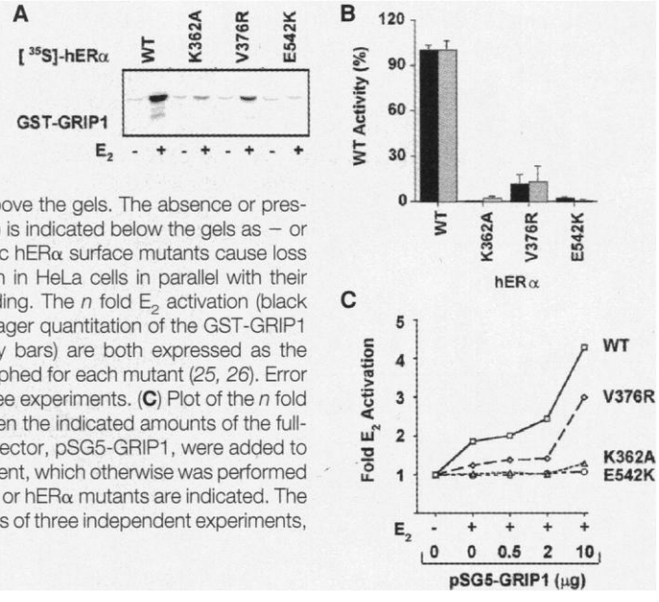

Fig. 2. GRIP1 and binding and transcriptional activation by hERα mutants. (**A**) In vitro binding of WT and mutant ^{35}S-labeled hERα to GST-GRIP1 (GRIP1 amino acids 721 to 1121) (*25*). The mutations are indicated above the gels. The absence or presence of 1 μM estradiol (E_2) is indicated below the gels as − or +, respectively. (**B**) Specific hERα surface mutants cause loss of transcriptional activation in HeLa cells in parallel with their loss of in vitro GRIP1 binding. The *n* fold E_2 activation (black bars) and the phosphorimager quantitation of the GST-GRIP1 binding from Fig. 2A (gray bars) are both expressed as the percentage of WT and graphed for each mutant (*25, 26*). Error bars indicate the SD of three experiments. (**C**) Plot of the *n* fold E_2 activation observed when the indicated amounts of the full-length GRIP1 expression vector, pSG5-GRIP1, were added to the cotransfection experiment, which otherwise was performed as for Fig. 2B (*26*). The WT or hERα mutants are indicated. The data represent the averages of three independent experiments, with SD <10%.

The effects of the mutations on other receptor functions were also examined. All of the mutants bound radiolabeled thyroid hormone (K_d values, 6 to 234% that for native receptor) (*14*). Occasional lower values were expected because some residues have partially buried side chains; at the high T_3 concentrations used, saturated hormone binding for all mutants was achieved in the assays used. None of the residues that decreased GRIP1 binding affected TR binding to a glutathione S-transferase (GST)–RXR fusion protein (Fig. 1A, lower panel), or to DNA, as determined with three different DNA half-site arrangements testing with or without added RXR (*16*). Some mutations that affect GRIP1 binding occur in a region spanning helices 3 to 5, which has been suggested as important for TR–RXR heterodimerization (*11, 19*); our results suggest that these AF-2 residues do not contribute to this heterodimerization. Further, mutation of the AF-2 residues does not affect the T_3-dependent inhibition of the Jun and Fos transcription factor activities at an AP-1 site (*5*). These unaffected activities also serve as controls, showing that the amounts of labeled mutant TRs put into the binding reactions and the expression levels of the mutant TRs in the cell culture assays are comparable with those of WT receptors.

The identified TR AF-2 residues lie within portions of the TR sequence that are conserved between nuclear receptors (*10, 11, 20*), suggesting that the coactivator-binding surfaces for other nuclear receptors

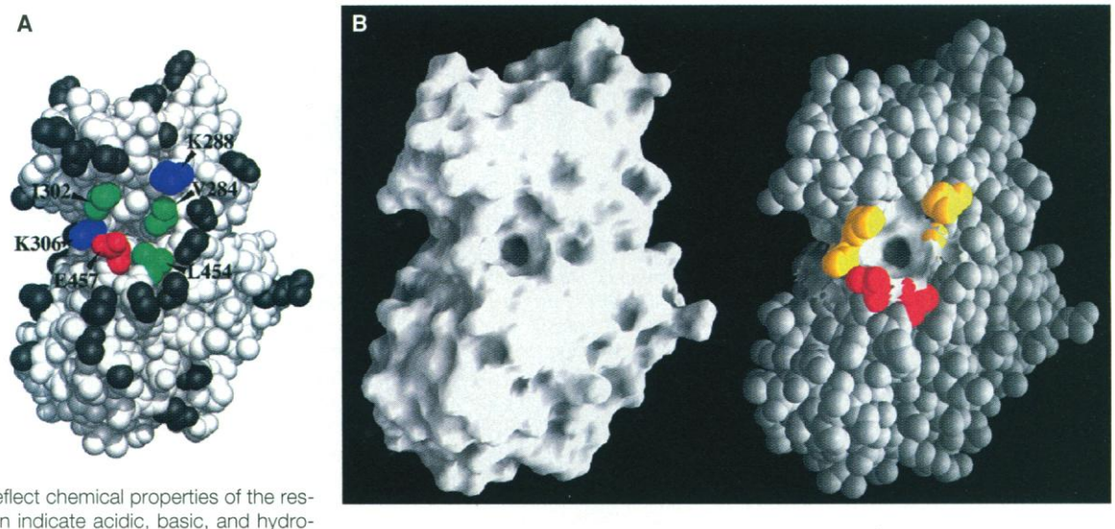

Fig. 3. Scanning surface mutagenesis defines a small cluster of effective mutations that surround a surface cleft containing central hydrophobic residues. (**A**) A space-filling model of the TRα LBD shows the LBD surface locations of mutations made in the full-length hTRβ1. Mutated residues that have no effect on GRIP1 binding or on activation in HeLa cells are shaded dark gray. Mutated residues with diminished GRIP1 and SRC-1a binding and diminished activation in HeLa cells are colored to reflect chemical properties of the residues: Red, blue, and green indicate acidic, basic, and hydrophobic residues, respectively. The main-chain structures of the TRα LBD and TRβ LBDs are the same (*16*). Computer graphic prepared with MidasPlus (*27*). (**B**) The AF-2 surface contains a cleft, one side of which is formed by conformationally hormone-responsive residues. Left, a view of the TR LBD molecular surface, showing the concave surfaces in gray; note the cavity at the center of the figure. Right, a space-filling model of the TR LBD, overlayed with a molecular surface view restricted to a 12-Å radius of the hydrophobic cavity. The hormone-insensitive residues of mutated AF-2 (V284, K288, I302, and K306) are located on one side of the cleft and are colored yellow. The mutated AF-2 residues that likely undergo a conformational change upon hormone binding (L454 and E457) are located on the opposite side of the cleft and are colored red. Computer graphics were prepared with GRASP (*28*).

are similar to that for TR. TR residues K288 and E457 are completely conserved between different nuclear receptors (*10*), and other residues show conservation of residue type—hydrophobic for TR residues V284, I302, and L454, polar or charged for TR residue K306. We created three separate mutations (K362A, V376R, and E542K) in human estrogen receptor-α (hERα), which align to three of the effective positions in the hTRβ1 (K288A, I302R, and E457K). All three mutations diminished GRIP1 binding (Fig. 2A) and abolished transcriptional activation (Fig. 2B); mutant V376R, with 10% residual GRIP1 binding, was rescued partially by overexpression of GRIP1 (Fig. 2C). As a control, the ER mutants demonstrated a normal hormone-dependent ability to activate a vitellogenin-LUC hybrid reporter gene, GL45, which responds to the ER NH_2-terminal activation function (*16*, *21*). Thus, the AF-2 surface residues appear to be similar in different nuclear receptors.

The residues that define AF-2 (Fig. 3A) cluster within a small area with well-defined borders. The surface contains charged and hydrophobic residues at its periphery but only hydrophobic residues at its center. Amino acids that form the surface cleft include residues I280, V284, and K288 from helix 3; I302, L305, and K306 from helix 5; C309 from helix 6; and L454, E457, and V458 from helix 12. This small interaction surface may match a complementary surface on the coactivator with the hydrophobic cleft at the center of the surface driving the coactivator binding reaction. The volume of the cleft, although small (~300 Å^3), can accommodate, for example, a Leu (124 Å^3) or Phe (135 Å^3) residue. A short conserved motif that can mediate recognition of nuclear receptors by coactivators of several types, including GRIP1 and SRC-1 (*22*), contains leucines (LXXLL) that could fit into the target surface hydrophobic cleft. The features of the hydrophobic cleft make it a suitable target for the rational design of pharmaceuticals to block the receptor-coactivator interaction.

The inferred AF-2 surface appears to comprise two parts (Fig. 3B, right). Residues contained in helices 3, 5, and 6 (Fig. 3B, yellow residues) probably form a constitutive part (*10*), since their positions are identical in all nuclear receptor structures reported, including the liganded, activated states of the TR, retinoid-A receptor, and ER, the unliganded RXR, and the inhibitor-liganded ER (*13*, *23*, *24*). By contrast, the residues of helix 12 (Fig. 3B, red residues) are positioned differently in the active and inactive states reported. Thus, the coactivator binding surface is formed in response to an active hormone by positioning helix 12 against a scaffold formed by helices 3 to 6. Because the binding surface is so small, changes in the position of helix 12, such as that induced by the ER antagonist raloxifene (*23*), could impair coactivator binding and thus receptor activation also.

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14. TR mutants were created by ligating double-stranded oligonucleotides to encode the mutant sequence into the pCMX vector that encodes the full-length 461-amino-acid hTRβ1 sequence. Some of the mutations of hTRβ1 in the CMX vector and all three mutations of hERα in the pSG5-ER-HEGO vector [L. Tora *et al.*, *EMBO J.* **8**, 1981 (1989)] were created with the use of Quick Change Site-Directed Mutagenesis Kits (Stratagene). The mutated sequences were verified by DNA sequencing with Sequenase Kits (Stratagene). The T_3 binding affinity constants (K_d) for in vitro–translated WT and mutant TRs were measured by using [^{125}I]T_3 in gel-filtration binding assays as described [J. W. Apriletti, J. D. Baxter, K. H. Lau, B. L. West, *Protein Expr. Purif.* **6**, 363 (1995)]. Both the K_d and standard error were calculated with the Prism computer program (GraphPad Software, San Diego, CA). Mutations are indicated by the single-letter amino acid abbreviations (*17*), with the native residue name being followed by the primary sequence position number and then by the mutated residue name. The affinity of the WT TR is 81 ± 12 pM. The relative affinity was determined by dividing the WT K_d by the K_d for each mutant. The 37 mutants tested, and their relative affinities (%) were: E217R (123%), E227R (109%), K242E (92%), E267R (117%), H271R (123%), T277R (7%), T281R (145%), V284R (105%), D285A (89%), K288A (98%), C294K (94%), E295R (118%), C298A (87%), C298R (141%), E299A (171%), I302A (86%), I302R (99%), K306A (6%), K306E (6%), P384R (164%), A387R (107%), E390R (151%), E393R (146%), L400R (95%), H413R (109%), H416R (153%), M423R (156%), R429A (48%), S437R (170%), L440R (174%), V444R (89%), T448R (234%), E449R (36%), P453E (32%), L454R (26%), L456R (46%), and E457K (71%).
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17. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y. Tyr.
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25. The WT or mutant pCMV-hTRβ1 vector and the pSG5-GRIP1 and pCMX–SRC-1a vectors were used to produce radiolabeled full-length receptors with the TNT-coupled Reticulocyte Lysate System (Promega) and [^{35}S]Met (DuPont). GST-GRIP1 (amino acids 721 to 1221, from M. Stallcup), GST-GRIP1 (amino acids 563 to 1121, from M. Stallcup), GST–SRC-1a (amino acids 381 to 882), GST-hTRβ1 (full-length, WT or mutants, WT from C. Costa), and the GST-hRXRα (full-length, from C. Costa) fusion proteins were produced in *Escherichia coli* strain HB101 according to the manufacturer's protocol (Pharmacia Biotech). The binding experiments were performed by mixing glutathione beads containing 10 μg of GST fusion proteins (Coomassie Plus Protein Assay Reagent, Pierce) with 1 to 2 μl of the ^{35}S-labeled WT or mutant hTRβ1 (25 fmol, 4000 cpm of receptor), or 1 to 2 μl of coactivators, in 150 μl of binding buffer (20 mM HEPES, 150 mM KCl, 25 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) containing bovine serum albumin (2 μg/ml) for 1.5 hours in the presence or absence of 1 μM T_3. After the beads were washed three times with 1 ml of binding buffer, the bound proteins were separated with use of 10% SDS–PAGE and visualized by autoradiography. Binding was quantitated by phosphorimaging with ImageQuant (Molecular Dynamics).
26. HeLa cell transfection and assay conditions are described [P. Webb, G. N. Lopez, R. M. Uht, P. J. Kushner, *Mol. Endocrinol.* **9**, 443 (1995)]. For TR assays, we used 5 μg of the reporter p(DR-4)$_2$–TK-LUC consisting of two copies of the DR-4 element [a direct repeat of the consensus TR response element (TRE) spaced by 4 bp] placed upstream of a minimal (−32/+45) thymidine kinase gene promoter linked to luciferase coding sequences. A reporter containing palindromic TREs gave the same results (*16*). We used 2 μg of the TR expression vector, pCMX-TR (WT or mutant), and 0.5 μg of the control vector, pJ3LacZ, which contains the SV40 promoter linked to the β-galactosidase (β-Gal) gene. For the hERα assays, we used 5 μg of estrogen-responsive reporter plasmid encoding chloramphenicol acetyltransferase (CAT), pERE-ColITATA [Y. Sadovsky *et al.*, *Mol. Cell. Biol.* **15**, 1554 (1995)], 0.5 μg of expression vector encoding full-length hERα, pSG5-ER HEGO (WT or mutants), and 2 μg of pJ3LacZ. For the experiments of Figs. 1D and 2C, 0.5 μg of a full-length GRIP1 expression vector, pSG5-GRIP1, was also included in the transfection. Transfected cells were treated with or without 1 μM T_3 or E_2, as indicated. After cell culture for 24 hours, the LUC or CAT activities were assayed; β-Gal activities were also assayed to correct for differences in transfection efficiencies.
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29. We thank M. Stallcup and C. Costa for providing the GST-GRIP1, GST-RXRα, and GST-TRβ expression vectors; P. Webb and M. Tsai for providing the pSG5-GRIP1 and pCMX–SRC-1a expression vectors; K. Yamamoto, P. Webb, M. G. Rosenfeld, and T. Scanlan for suggestions; and T. Chen and K. Nakamura for technical assistance. W.F. is a recipient of a postdoctoral fellowship from the National Institutes of Health (DK09516). Supported by NIH grants to J.D.B. (DK51281) and to the Computer Graphics Laboratory at the University of California, San Francisco (P41-RR01081).

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