Communication between the ERRα Homodimer Interface and the PGC-1α Binding Surface via the Helix 8–9 Loop*

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Although structural studies on the ligand-binding domain (LBD) have established the general mode of nuclear receptor (NR)/coactivator interaction, determinants of binding specificity are only partially understood. The LBD of estrogen receptor-α (ERα), for example, interacts only with a region of peroxisome proliferator-activated receptor coactivator (PGC)-1α, which contains the canonical LXXLL motif (NR box2), whereas the LBD of estrogen-related receptor-α (ERRα) also binds efficiently an atypical, LXXYL-containing region (NR box3) of PGC-1α. Surprisingly, in a previous structural study, the ERα LBD has been observed to bind NR box3 of transcriptional intermediary factor (TIF)-2 untypically via LXXYL, whereas the ERRα LBD binds this region of TIF-2 only poorly. Here we present a new crystal structure of the ERRα LBD in complex with a PGC-1α NR box3 peptide. In this structure, residues N-terminal of the PGC-1α LXXYL motif formed contacts with helix 4, the loop connecting helices 8 and 9, and with the C terminus of the ERRα LBD. Interaction studies using wild-type and mutant PGC-1α and ERRα showed that these contacts are functionally relevant and are required for efficient ERRα/PGC-1α interaction. Furthermore, a structure comparison between ERRα and ERα and mutation analyses provided evidence that the helix 8–9 loop, which differs significantly in both nuclear receptors, is a major determinant of coactivator binding specificity. Finally, our results revealed that in ERRα the helix 8–9 loop allosterically links the LBD homodimer interface with the coactivator cleft, thus providing a plausible explanation for distinct PGC-1α binding to ERRα monomers and homodimers.

The ligand-binding domain (LBD) of nuclear receptors (NRs) acts as a ligand-dependent molecular switch recruiting diverse cofactor complexes (1–3). It exhibits a canonical three-layered α-helical sandwich fold generally composed of 12 α-helices (H1–H12) and a β-sheet. Binding of activating ligand (agonist) to the LBD triggers conformational changes that result in coactivator (CoA) recruitment. CoAs typically interact with the LBD via a short α-helix containing a LXXLL sequence motif, also termed the NR box (4). The leucine residues of this motif form hydrophobic contacts with the so-called CoA cleft of the LBD, which is constituted by H3, H4, and H12. Binding of the LXXLL helix is further stabilized by a “charge clamp” interaction with two conserved, charged residues of the LBD (lysine or arginine in H3 and glutamate in H12). Numerous proteins are recruited to the agonist-bound NR LBD including CBP/p300, the p160 CoAs (steroid receptor coactivator-1, transcriptional intermediary factor (TIF)-2, activator of thyroid and retinoid receptor), the p220 subunit of the DRIP-TRAP-Mediator complex (5, 6), and members of the family of peroxisome proliferator-activated receptor (PPAR) CoAs (PGC-1α, PGC-1β, and PRC) (7, 8).

PGC-1α was initially characterized as a CoA of PPARγ and thyroid hormone receptor-β and a key regulator of adaptive thermogenesis in brown adipose tissue and skeletal muscle (9). Meanwhile, evidence has been provided that PGC-1α also coactivates several other NRs including PPARα (10), hepatocyte nuclear factor-4α (HNF-4α) (11), retinoid X receptor-α (RXRα) (12), estrogen receptor-α (ERα), (13), and estrogen-related receptor (ERR) α and γ (14–17). PGC-1α thereby plays a role in diverse processes including energy metabolism, mitochondrial biogenesis, and resistance to insulin in type 2 diabetes (7, 8).

The large number of potentially interacting NRs and CoAs, and the relatively small interaction surface constituted by the CoA cleft and the LXXLL helix, have raised the question how selective binding is achieved (18). Several examples of preferential or selective NR/CoA interaction have been documented.

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‡The atomic coordinates and structure factors (code 3D24) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4The abbreviations used are: LBD, ligand-binding domain; BHK, baby hamster kidney; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; CoA, coactivator; DOR, direct repeat with 0 bp spacing; EMSA, electrophoretic mobility shift assay; ER, estrogen receptor; ERE, estrogen response element; ERR, estrogen-related receptor; GST, glutathione S-transferase; H, helix; HNF, hepatocyte nuclear factor; ID, interaction domain; NR, nuclear receptor; PDB, Protein Data Bank; PPAR, peroxisome proliferator-activated receptor; PGC, PPAR coactivator; RXR, retinoid X receptor; TIF, transcriptional intermediary factor.
Early work showed that residues flanking the LXXLL motif can contribute to binding specificity (19, 20). Furthermore, the binding preference of the LBD of the glucocorticoid receptor or constitutive androstane receptor for the third LXXLL motif (NR box3) of TIF-2 appears to result from a second charge clamp interaction between CoA cleft residues and charged residues at positions + 2 and + 6 (relative to the first leucine residue) of the box3 motif (21, 22). In comparison, multiple features account for selective repression of liver receptor homolog-1 activity by a small heterodimer partner; key residues in the CoA cleft, orientation of the main chain C-terminal of H12, and the precise positioning of H12 affecting the shape of the CoA cleft (23). The understanding of CoA binding selectivity is further complicated by the observation that some NRs, such as ERRα or androgen receptor, can interact with the untypical motifs LLKYL or FXXLF, respectively (17, 24). Although the binding preference of FXXLF peptides for androgen receptor was reported to be determined solely by CoA cleft residues that differ between steroid hormone receptors (25), the determinants of specific ERRα interaction with the LLKYL region of PGC-1α have not been addressed in detail.

PGC-1α harbors a putative (box1) and two confirmed (box2 and box3) NR interaction motifs (17). Several NR LBDs, including that of PPARα (10), HNF-4α (11), RXRα (12), and ERα (13), interact only with the canonical box2 region (spanning LKKLL148), whereas ERRα and ERRγ can also bind the untypical box3 region (spanning LLKYL214) (17). Importantly, PGC-1α interacts only with ERRα homodimers but not with monomers (26). A previously reported crystal structure of the ERRα LBD in complex with a PGC-1α box3 peptide shows that the untypical motif binds in an α-helical conformation (like LXXLL peptides), with the exception that Leu(+4) is replaced by tyrosine (27). Such a LXXYL binding mode has already been observed earlier in the crystal structure of the ERα LBD complexed with a TIF-2 box3 peptide (28). Because of a particular amino acid composition (LLRYLL), the TIF-2 box3 region has the potential to interact with NRs in either a canonical (LXXLL) or an untypical (LXXYL) manner. However, based on additional functional studies, it was concluded that untypical binding to ERRα may be favored by the crystal packing and that in solution TIF-2 box3 likely interacts via LXXYL (28). Together, these observations raise the question as to what determines efficient binding of the LXXYL region of PGC-1α box3 (but not of TIF-2 box3) to ERRα (but not to ERα). ERs are known to interfere with ER signaling in tissues where the receptors are coexpressed (29–31). Thus, the understanding of specific CoA recruitment to ERRα may, as an alternative to the modulation of the receptor with ligands (32), help to design specific peptide antagonists of potential therapeutic interest.

Here we present a structural and functional analysis of PGC-1α binding to the ERRα LBD, which is based on a comparison between our new crystal structure of the ERRα LBD complexed with a PGC-1α box3 peptide at 2.1 Å resolution and previously presented ERRα/PGC-1α box3 and ERRα/TIF-2 box3 structures (27, 28). We co-crystallized the ERRα LBD with a “wild-type” PGC-1α box3 peptide (198QQQKPQRRPCSELLKYLITNDD219), whereas in the case of the reported structure (27) a shorter, mutant peptide (205RPASELLKYLTT216; C207A) was used (probably to avoid potential oxidation problems during crystallization). In our crystal structure, residues N-terminal of the LXXYL motif form additional contacts with the ERRα surface, notably with H4, the H8-H9 loop, and the C terminus of the LBD. Through extensive functional interaction studies using wild-type and mutant forms of PGC-1α, ERRα, and ERα, we provide evidence that the contacts between N-terminal flanking residues of PGC-1α and the H8-H9 loop of ERRα are a major determinant of coactivator binding specificity. Furthermore, our results show that the H8-H9 loop allosterically links the homodimer interface with the CoA cleft, providing a plausible explanation for distinct PGC-1α binding of ERRα homomers and homodimers.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Plasmids**—cDNA fragments encoding wild-type or mutant human ERRα LBD (residues 189–423) (GenBank™ accession number NP_004442) and human ERRγ LBD (residues 307–548) were generated by PCR. The wild-type ERRα cDNA fragment was cloned at the BamHI/HindIII sites of a modified pET-24d expression plasmid (Novagen) in which the T7 tag had been replaced by a hexahistidine (His) tag (MRSHHHHHGPPGVPGRS). Wild-type and mutant cDNA fragments of ERRα and ERRγ were also cloned at the EcoRI/BamHI sites of the eukaryotic expression plasmid pCMX-VP16, resulting in fusion with the VP16 transactivation domain. Similarly, cDNA fragments encoding wild-type or mutant NR interacting regions of human PGC-1α or human TIF-2 were generated by PCR and cloned at the BamHI or EcoRI/BamHI sites of pCMX-Gal4, resulting in fusion with the Gal4 DNA-binding domain (amino acids 1–147). The coding region of full-length human ERRα was cloned by PCR at the EcoRI/BamHI sites of pCMX-K-ATG containing a Kozak sequence 5’ to an ATG start codon and the multiple cloning site. ERRα mutants were subcloned into pCMX-K-ATG from the corresponding pCMX-VP16 plasmids using an internal SpeI site and a 3’ BamHI site present in both pCMX vectors. The coding sequence for PGC-1α interaction domain (ID) and PGC-1α ID(L144A/L210A) was subcloned from pCMX-Gal4 at the BamHI site of a modified pET15b plasmid (Novagen) containing the coding sequence for glutathione S-transferase (GST) 5’ of the hexahistidine tag sequence. A detailed description of the cloning of all plasmids is available upon request.

**Protein Production and Purification**—The His-tagged ERRα LBD (residues 189–423) was produced in *Escherichia coli* BL21(DE3). Cultures were grown in Terrific Broth medium at 37 °C until an A600 of 0.4 was reached. The temperature was then decreased to 18 °C, and cultures were induced at an A600 of 0.8 overnight with 0.1 mM isopropyl 1-thio-β-d-galactopyranoside. Harvested bacterial pellets were resuspended in buffer containing 20 mM Tris/HCl (pH 8.0) and 200 mM NaCl. After sonication of the bacterial cells and ultracentrifugation the tagged ERRα LBD was purified by affinity chromatography using a Ni2+-loaded HiTrap chelating column (GE Healthcare) and by gel filtration using a HiLoad 16/60 Superdex 200 column (GE Healthcare). The purified protein (estimated purity, >95%) was concentrated to about 10 mg/ml in gel filtration buffer containing 20 mM Tris/HCl (pH 8.0) and 150 mM...
NaCl. GST-PGC-1α ID and GST-PGC-1α ID(L144A/L210A) fusion proteins were produced at 37 °C in E. coli BL21(DE3) using a PET15b-GST expression plasmid. The GST fusion proteins were purified using glutathione-Sepharose (GE Healthcare) according to the manufacturer’s instructions and dialyzed against buffer containing 20 mM Tris (pH 8.0) 100 mM NaCl.

Protein Complex Crystallization, Data Collection, and Processing—Crystallization trials were carried out in the presence of a 3-fold molar excess of PGC-1α box3 peptide ([QQQ-KPQRRPCELLKYLTTNDDD])219). Crystals of the ERRα LBD/GST-PGC-1α box3 complex were obtained at 17 °C by vapor diffusion in sitting drops. Reservoir solutions contained 0.1 M bis-Tris (pH 5.5), 15% polyethylene glycol 3350, and 0.2 M Mg(NO3)2. Crystals were cryoprotected in reservoir solution supplemented with 20% ethylene glycol and flash-frozen in liquid nitrogen. Data collection was performed at 100 K at the European Synchrotron Radiation Facility (beamline ID 14-2) in Grenoble. Crystals belong to the monoclinic space group C2 with one homodimer per asymmetric unit. Data were integrated and scaled using HKL2000 (33).

Structure Determination, Refinement, and Comparison—The structure was solved by molecular replacement with AMoRe (34) using the published ERRα LBD crystal structure (27) as a model probe. Refinement involved cycles of manual building and refinement calculations. The programs CNS-SOLVE (35), COOT (36), and QUANTA (37) were used throughout the structure determination and refinement. Anisotropic scaling and a bulk solvent correction were applied. Data collection and refinement statistics are summarized in Table 1.

Structure Comparison of ERRα/PGC-1α Box3 and ERRα/TIF-2 Box3 Complexes—We solved a new crystal structure of the ERRα LBD in complex with a PGC-1α box3 peptide ([QQQ-KPQRRPCELLKYLTTNDDD])219). In a previously reported structure of the ERRα-PGC-1α complex (PDB ID: 1XB7) (27), a shorter mutant peptide ([RPASSEELLKYLTT])216; C207A) was used. The C207A mutation was probably introduced to avoid potential oxidation problems during crystallization.) Our crystals of the ERRα-PGC-1α complex belong to space group C2 and contain one LBD homodimer (subunits A and C in the PDB file) per asymmetric unit. The structure was solved by molecular replacement and refined to 2.1 Å resolution. Data collection and refinement statistics are summarized in Table 1.

Although the overall interaction of the LXXYL core region of PGC-1α with the ERRα LBD is as described (27), we observed additional electron density for amino acids Arg-205, Pro-206, and Cys-207 (Fig. 1, A and B), which were not included in the final model of the reported structure (Fig. 1, C). These residues are in contact with H4, the H8-H9 loop, and H12. Notably, Arg-205 of PGC-1α forms a hydrogen bond with Gln-262 (H4), and water-mediated hydrogen bonds with Ser-337 (H8-H9 loop) and the main chain carbonyl oxygen of Ala-420 (H12). Furthermore, we could trace the C-terminal residue of the LBD (Asp-423) that interacts with His-341 (H8-H9 loop) and with Arg-205 of PGC-1α.

These observations suggested that a network of interacting amino acids from H4, the H8-H9 loop, and H12 of ERRα and N-terminal flanking residues of PGC-1α (comprising Arg-205,
Pro-206, and Cys-207) contribute to specific ERRα/PGC-1α interaction. A potential contribution of residues N-terminal of the LXXYL region has not been addressed by Kallen et al. (27), who explained efficient PGC-1α binding by favorable van der Waals contacts of Leu-211 and Tyr-213 with the ERRα CoA cleft and by intramolecular interactions involving Glu-209, Lys-212, Thr-215, and Thr-216, which stabilize the CoA helix.

A favorable contribution of Arg-205, Pro-206, and Cys-207 to the ERRα/PGC-1α interaction is also interesting with respect to the crystal structure of the ERα LBD bound to a TIF-2 box3 peptide (740KENALLKYLLDK751; PDB ID: 1GWR) (28). In the crystal, the TIF-2 box3 region interacts with the receptor via LXXYL (Fig. 1D). As in the case of the reported ERRα/PGC-1α structure (27), the TIF-2 helix is short and N-terminal flanking residues have not been included in the model. The residue of TIF-2 that corresponds to Cys-207 of PGC-1α is Glu-741 (not present in the model).

This structure comparison indicated that a cysteine at position 207 of PGC-1α may play a role in efficient ERRα binding and that other residues, such as alanine, serine, or glutamic acid, would negatively affect ERRα/PGC-1α interaction. We further hypothesized that Cys-207, for example through a defined main chain conformation, helps to correctly position Pro-206 and Arg-205. Consequently, we addressed the importance of individual residues of ERRα and PGC-1α for efficient interaction in subsequent functional assays.

It must be noted that in the second molecule (subunit A) of the ERRα homodimer Arg-205 of PGC-1α does not contact the receptor surface (supplemental Fig. S1). Furthermore, there is no clear electron density for the C terminus of the ERRα LBD in this subunit. To date, the reason for this "asymmetry" within the ERRα homodimer is unclear. We speculate that distinct coactivator binding to the ERRα subunits is either due to the crystallization conditions or the result of an intramolecular communication of the subunits via the homodimer interface.

Interaction of Wild-type or Mutant PGC-1α Fragments with the ERRα LBD—To initiate functional studies that address the relative importance of PGC-1α residues, we performed mammalian two-hybrid interaction studies in transiently transfected eukaryotic cell lines. Wild-type and mutant NR interacting regions of PGC-1α were expressed in fusion with the Gal4

### TABLE 1

| Data collection and refinement statistics |
|------------------------------------------|
| **Data processing***                     |
| Resolution (Å)                           | 50.2-2.1 (2.19-2.11) |
| Crystal space group                      | C2                  |
| Cell parameters (Å or °)                 | a = 119.4; b = 56.0; c = 96.2; β = 106.4 |
| Unique reflections                       | 31,886 (2,740)      |
| Mean redundancy                          | 3.5                 |
| R<sub>free</sub> (%)                     | 3.1 (23.8)          |
| Completeness (%)                        | 88.9 (76.9)         |
| Mean I/σ (%)                            | 26.2                |

*Values in parentheses correspond to the highest resolution shell.
*Root-mean-squared deviations (r.m.s.d.) are given from ideal values.

**Refinement**

- No. protein atoms: 3,532
- No. water molecules: 272
- r.m.s.d. bond length (Å): 1.054
- r.m.s.d. bond angles (°): 0.005
- R<sub>cryst</sub>, (%)<sup>a</sup>: 21.2
- R<sub>free</sub>, (%): 25.5
- Ramachandran plot (%): 91.8
- Core: 82.0
- Allowed: 91.8

![Interaction of CoA peptides with the ERRα and the ERRα LBD](image)

**FIGURE 1. Interaction of CoA peptides with the ERRα and the ERRα LBD.** Representations complexed with a PGC-1α box3 peptide (198QQQKPQRRPCSELLKYLTTNDD219) (A), the ERRα LBD (subunit C)-PGC-1α box3 complex contoured in electron density at 1σ (B), the published ERRα LBD (PDB ID: 1XB7) in complex with a mutant PGC-1α box3 peptide (200RPASELKLTTNDD11; 207A) (C), and the published ERRα LBD (PDB ID: 1GWR) co-crystallized with a TIF-2 box3 peptide (204KENALLKYLLDK751) (D). In all three cases the CoA peptide binds in the untypical LXXYL mode (probably representing a crystal artifact in the case of the ERRαTIF-2 complex). In A and B, residues of PGC-1α that N-terminally flank the LXXYL helix (Arg-205, Pro-206, and Cys-207) interact with the ERRα surface, notably with H4, the H8–H9 loop, and the C terminus. In comparison, N-terminal flanking residues are not observed in C and D. In all structures, the length of H12 and the conformation of the C-terminal residues differ.
DNA-binding domain (Fig. 2, left panel) and the ERRα LBD in fusion with the VP16 activation domain. A construct spanning the NR ID of PGC-1α, including the box2 and box3 motifs, strongly interacted with the ERRα LBD, thus resulting in a dramatic increase in luciferase expression in COS-1 cells (Fig. 2, right panel). Relative to the ID, the interaction of constructs containing a single point mutation in the box2 or box3 motif (ID(L144A) or ID(L210A), respectively) was reduced but still robustly above base levels. In contrast, the ERRα LBD no longer interacted with the double point mutant ID(L144A/L210A). In agreement with these observations, the interaction of the smaller constructs, PGC-1α box2 or PGC-1α box3, with ERRα was comparable to the corresponding ID single point mutants and depended on an intact LXXLL or LXXYL motif (see mutants box2(L144A) and box3(L210A)).

Although mutation of the first leucine residue (Leu-210) of the LXXYL motif abolished interaction of PGC-1α box3 with the ERRα LBD, the single point mutations L211A or Y213A had a less dramatic effect on ERRα binding. In comparison, a C207S or a C207E mutation in PGC-1α box3 affected the interaction more strongly. In agreement with a previous report (17), these observations showed that PGC-1α efficiently interacts with ERRα via a canonical LXXLL (box2) and an untypical LXXYL (box3) motif. Although Leu-144 of box2 and Leu-210 of box3 are essential for the binding of PGC-1α to ERRα, Leu-211 or Tyr-213 do not play a crucial role in the interaction of the box3 region. Furthermore, Cys-207 at position −3 relative to LXXYL contributes importantly to efficient binding.

Flanking Residues at Positions −6 to −3 of the PGC-1α LXXYL Motif Determine Efficient Binding to ERRα—Next we asked whether mutation of residues N-terminal of Cys-207 of PGC-1α also affects ERRα interactions. Deletion of 13 N-terminal amino acids from PGC-1α box3 (resulting in the mutant PGC-1α box3206) reduced the interaction with the ERRα LBD by only ~2-fold (Fig. 3A). More importantly, deletion of four more residues (C202PQR) dramatically reduced binding of the resulting mutant PGC-1α box3206 to almost base-line levels. Thus PGC-1α box3206 defines a minimal high affinity box3 interacting region. Point mutation of Pro-202 and Gln-203 to

FIGURE 2. Interaction of wild-type or mutant PGC-1α fragments with the ERRα LBD in mammalian two-hybrid assays. Left panel, schematic representation of wild-type and mutant PGC-1α fragments fused to the Gal4 DNA-binding domain (amino acids 1–147). The major NR ID of PGC-1α contains a canonical (LXXLL) and an untypical (LXXYL) NR binding motif (dark shading). An asterisk indicates a point mutation. In PGC-1α box3(C207E), Cys-207 of PGC-1α has been mutated to the corresponding residue of TIF-2 box3 binding to ERα in the LXXYL mode. Right panel, mammalian two-hybrid interaction assay in COS-1 cells using Gal4(3)-TK-LUC, pCMX-Gal4-PGC-1α constructs, and pCMX-VP16-ERRα LBD. Bars represent the mean ± S.D. (n = 10). p values were calculated for the PGC-1α constructs relative to Gal4-PGC-1α ID (*, p < 0.0001) or relative to Gal4-PGC-1α box3 (#, p < 0.0001).

FIGURE 3. Residues N-terminal of LXXYL determine binding of PGC-1α to ERRα. A, left panel, schematic representation of PGC-1α box3 deletion and point mutants. The amino acid sequence of box3 is 189WSNKAK-210. Truncation mutants PGC-1α box3206 and box3208 start at residues Pro-202 and Pro-206, respectively. PGC-1α box3208(PQ) and box3208(RR) contain the double point mutation P202A/L210A. Although Leu-144 of box2 and Leu-210 of box3 are essential for the binding of PGC-1α to ERRα, Leu-211 or Tyr-213 do not play a crucial role in the interaction of the box3 region. Furthermore, Cys-207 at position −3 relative to LXXYL contributes importantly to efficient binding.
Alanine resulting in PGC-1α box3202(PQ) did not influence interaction with ERRα, whereas replacement of Arg-204 and Arg-205 with alanine (mutant PGC-1α box3202(RR)) reduced binding to minimal levels. Together with the observations from the crystal structure (Fig. 1A), these results revealed that amino acids at positions −6 to −3 (Arg-204/Arg-205 and Cys-207) determine high affinity binding of the PGC-1α box3 region to the ERRα LBD.

Next, we asked whether the TIF-2 box3 could interact with ERRα via LXXYL or whether swapping of the N-terminal flanking residues of PGC-1α box3 into TIF-2 box3 could enable or enhance such an interaction. As explained, the TIF-2 box3 region (containing 744LLRYLL749) has the potential to interact with ERRα either in the canonical manner via LXXYL or untypically via LXYL. In mammalian two-hybrid assays, Gal4-TIF-2 box3 interacted only weakly with VP16-ERRα LBD, resulting in a 2-fold stimulation of luciferase activity (Fig. 3B). This interaction probably occurs via the canonical LXXYL motif, because mutation of Leu-745 (the first leucine residue of this motif) to alanine abolished the weak binding. Importantly, swapping of residues 202–209 of PGC-1α into Gal4-TIF-2 box3(L745A) resulted in a strong increase in ERRα binding via LXXYL. Our experiments thus show that N-terminal flanking residues determine whether the LXXYL region of PGC-1α or TIF-2 interacts efficiently with ERRα.

**Residues Outside of the ERRα CoA Cleft Contribute to Selective PGC-1α Binding**—In the next set of experiments we addressed the question as to which parts of the ERRα surface are involved in specific binding of PGC-1α, in particular of the box3 region. We first considered that the distinct CoA binding properties of ERRα and ERRβ may result from amino acid exchanges in the CoA cleft, where ERRα differs from ERRβ by two residues, Met-258 and Gln-262 of ERRα, corresponding to Val-376 and Glu-380 of ERRβ, respectively (see Fig. 1). Exchange of these residues strongly reduced the interaction of the resulting mutant ERRα(M258V/Q262E) with PGC-1α ID, box2, and box3 (Fig. 4A). The dramatic loss of box2 binding was unexpected, as the ERRα LBD has been reported to interact efficiently with the canonical LXXYL region of PGC-1α (13), and ERRα(M258V/Q262E) was therefore expected to gain ERRα-like properties. In the case of box3, we note that Arg-205/204 and Cys-207 of PGC-1α are located in the vicinity of Gln-262 of ERRα and that Arg-205 forms a hydrogen bond with Gln-262 (Fig. 4B). Mutation of Gln-262 to glutamate changes the electrostatic potential and solvation properties of the ERRα surface and may thereby perturb binding of the N-terminal flanking residues of PGC-1α. However, because the interaction with

**FIGURE 4.** The H8–H9 loop region of ERRα is required for efficient PGC-1α interaction. A, mammalian two-hybrid interaction assay of PGC-1α ID, box2, and box3 with ERRα mutants. Residues that differ between the CoA clefts of ERRβ and ERRα have been exchanged in the mutant ERRα(M258V/Q262E). In the mutant ERRα(Δ3C), the three C-terminal residues (Met-421 to Asp-423) have been deleted. The single point mutants ERRα(Δ3C) and ERRβ(D423A) probe the relevance of a hydrogen bond between the side chains of these residues observed in subunit C of the asymmetric unit. Transient transfection assays in COS-1 cells were performed as described for Fig. 2. Bars represent the mean ± S.D. (n = 6); p values were calculated for ERRα mutants relative to the corresponding values of wild-type ERRα**, p ≤ 0.0001; *, p ≤ 0.001. **

B, schematic representation of selected parts of the ERRα CoA cleft and the homodimer interface. The H8–H9 loop lies within the proximity of the CoA cleft and is directly contacted by N-terminal flanking residues of PGC-1α. The H8–H9 loop links via multiple hydrogen bonds the homodimer interface with the CoA cleft. C, ERRα mutants probing the contribution of the H8–H9 loop to PGC-1α binding. In ERRα(SwapH6/H9) the H8–H9 loop region (amino acids 338–341) has been replaced with the corresponding region of ERRβ (amino acids 457–468). In ERRα(S259H) the serine residue has been mutated to the corresponding residue of ERRβ. Transient transfection assays in COS-1 cells were performed as described for Fig. 2. Bars represent the mean ± S.D. (n = 8), p values were calculated for ERRα mutants relative to the corresponding values of wild-type ERRα**, p ≤ 0.0001. **
both PGC-1α box2 and box3 was strongly reduced, our observations suggest that in the context of ERα, Met-258 and Gln-262 account for overall binding affinity but do not determine CoA binding selectivity.

Next, we investigated the functional relevance of contacts between N-terminal flanking residues of PGC-1α and H12 or the H8–H9 loop of ERα. The ERRα C terminus (Met-421, Met-422, Asp-423) differs from the corresponding region of ERα (see Fig. 5A), and in the published ERα crystal structure (27) it has neither been modeled nor has it its contribution to PGC-1α binding been evaluated. Deletion of the three C-terminal amino acids of ERRα (Met-421 to Asp-423) significantly reduced the interaction of the resulting mutant ERRα Δ3C with PGC-1α box3, whereas binding to the box2 region was less strongly affected (Fig. 4A). Thus, the box3 region of PGC-1α requires the ERRα C terminus for maximal interaction. In comparison, point mutation of His-341 (H8–H9 loop) or of Asp-423 (C terminus), which form a hydrogen bond in one subunit of the asymmetric unit (Fig. 4B), affected PGC-1α box3 binding only moderately.

Finally, we addressed the role of the H8–H9 loop in CoA interaction. The loop is part of a hydrogen bonding network that centers on Ser-337 and Asp-338 and links the ERα homodimer interface with the CoA cleft (Fig. 4B). This observation is intriguing because it indicates possible allosteric interactions between both surface regions. Furthermore, the H8–H9 loop differs significantly in ERRα and ERα, and Asp-338 is not conserved in ERα (see Fig. 5, A–C). Because this region might account for distinct CoA binding, we evaluated the effect of mutations aimed at perturbing the overall conformation of the H8–H9 loop in mammalian two-hybrid assays.

A complete exchange of the H8–H9 loop of ERαs (amino acids 338–341) for that of ERα (amino acids 457–468) abolished interaction of the resulting mutant ERα(SwapH8–H9) with PGC-1α (Fig. 4C) providing evidence for the involvement of the loop in CoA binding. Mutation of Ser-259 (H4) to histidine (the corresponding residue in ERα) had a small effect on PGC-1α or box2 but a larger effect on box3 binding. In comparison, PGC-1α interaction of ERα(E343A) remained almost unchanged. Importantly, however, mutation of Asp-338 (H8–H9 loop) or Arg-315 (H7) in the homodimer interface to alanine strongly reduced PGC-1α binding. Together, these observations suggested that changes that significantly perturb the conformation of the H8–H9 loop influence CoA interaction.

The H8–H9 Loop Plays a Role in Efficient and Selective PGC-1α Box3 Binding—As mentioned, the H8–H9 loop region differs significantly in ERα and ERRα (Fig. 5A). Interestingly, this region has only been modeled in some ERα crystal structures, possibly explaining why potential functions have not been addressed to date. Superimposition of an ERα LBD crystal structure in which the H8–H9 loop has been modeled (PDB ID: 1QKU) with the ERα LBD showed that in ERα H9 is longer and the H8–H9 loop adopts a distinct conformation (Fig. 5B). The superimposition also suggested that in ERα(SwapH8–H9) the hydrogen bonding network connecting the homodimer interface with the CoA cleft is particularly perturbed by the bulky side chain of Tyr-459. On the other hand, some amino acids that contribute to the hydrogen bonding network...
centered around Ser-337 and Asp-338 in ERRα are conserved in ERα, e.g. Arg-315 (H7) and Arg-389 (H10) of ERRα corresponding to Arg-434 and Arg-515 of ERα, respectively (see Fig. 4B and data not shown). Amino acids of potential functional importance that are not conserved comprise Ser-263 (H4) and Glu-311 (H7) of ERRα corresponding to Cys-381 and Ala-430 of ERα, respectively. In the ERα homodimeric interface, Arg-434 and Arg-515 adopt distinct side chain conformations to accommodate the bulky side chain of Tyr-459 (data not shown). However, upon swapping of the H8–H9 loop of ERRα into ERα, the arginine side chains might contribute to the allosteric control of CoA binding as suggested for ERRα.

Consequently, to provide further evidence for an allosteric role of the H8–H9 loop in PGC-1α binding, we asked whether replacement of the H8–H9 loop alone or in concert with other mutations would enable more efficient interaction of ERα with PGC-1α, in particular with the box3 region. In mammalian two-hybrid assays, the wild-type ERα LBD interacted in an estradiol (E2)-dependent manner, detectable only with the PGC-1α ID and box2 but not with box3 (Fig. 5D). The overall response was only about 10% of that obtained with the ERRα LBD. Relative to wild-type ERα, mutation of CoA cleft residues to the corresponding ones found in ERRα (resulting in mutant ERα(V376M/E380Q)) enhanced the interaction with PGC-1α ID and box2. In contrast, no binding to box3 was detected. PGC-1α binding was similarly enhanced in the case of the mutant ERα(SwapH8–H9) containing the H8–H9 loop of ERRα. Importantly, this mutant gained significant interaction with PGC-1α box3. PGC-1α interaction was even further enhanced in ERα(3mut), comprising the double point mutation in the CoA cleft, the swapped H8–H9 loop, and H373S/H377S to avoid potentially reduced CoA binding as observed for ERRα(S259H) (see Fig. 4C). Finally, however, when we additionally swapped the three C-terminal residues of ERRα into ERα (resulting in mutant ERα(4mut)), PGC-1α interaction was reduced. We hypothesize that in the context of ERα the bulky side chains of Met-421 and/or Met-422 cannot be accurately accommodated, such that the dynamic behavior of H12 is perturbed. In summary, our data show that residues outside the CoA cleft such as the H8–H9 loop and, to a lesser extent, C-terminal residues critically contribute to efficient and selective PGC-1α binding, in particular to binding of the box3 region.

Mutations in the H8–H9 Loop Affect DNA and PGC-1α Binding of ERRα—In the final set of experiments, we asked whether mutations in the H8–H9 loop region influence DNA or CoA binding of full-length ERRα. To address this question, we assayed the binding of in vitro translated wild-type and mutant receptors (supplemental Fig. S2A) to double-stranded oligonucleotides containing either two direct AGGTCA repeats without spacing (DR0) or two inverted AGGTCA repeats with three-base pair spacing (ERE) in EMSAs (Fig. 6). In agreement with previous studies (26, 40), we observed efficient homodimeric binding of ERRα to both elements (Fig. 6A). Importantly, mutation of residues in the H8–H9 loop that contribute to the homodimeric interface (mutants R315A and D338A; see Fig. 4B), or exchange of the H8–H9 loop for that of ERα, resulted in partial monomer binding to the DR0 element. Monomer binding was observed only on the DR0 but not on the ERR element, as only the DR0 encompasses an extended half-site sequence (TCAAGGTCA), which allows stabilizing interactions of the T/A box (adjacent to the DNA-binding domain) of ERRα with the minor groove of the TCA extension (41). Compared with R315A, D338A, and SwapH8–H9, the E343A mutation, which is located outside the homodimeric interface, did not significantly alter DNA binding. Furthermore, homodimeric DNA binding of the mutant SwapH8–H9 to both the DR0 and the ERR element was drastically reduced. These observations showed that an intact H8–H9 loop is required for optimal DNA binding and that single point mutations such as R315A and D338A can affect homodimerization of full-length ERRα on DNA.

Next, we tested whether the interaction of PGC-1α with DNA-bound ERRα was also affected by exchange or mutation of the H8–H9 loop. On the DR0 and the ERR, the addition of small amounts of E. coli-expressed, partially purified GST-PGC-1α ID fusion protein resulted in an upshift of DNA-bound ERRα (Fig. 6, B and C). This upshift was not observed when GST-PGC-1α ID(L144A/L210A) was added. Compared with the wild-type receptor, all DNA-bound mutants were less efficiently shifted by GST-PGC-1α ID. Furthermore, DNA-bound ERRα(SwapH8–H9) did not interact with GST-PGC-1α ID, even at the highest CoA concentration tested (supplemental Fig. S2B and data not shown). Thus, mutations in the H8–H9 loop such as R315A or D338A influence not only homodimerization but also CoA binding of DNA-bound, full-length ERRα. Together, these data provide further evidence for the role of H8–H9 loop in ERRα function and show that it is an important discriminatory feature between ERRα and ERα.

DISCUSSION

Previous work has established that binding and coactivation of several NRs such as PPARα, HNF-4α, RXRα, and ERRs by PGC-1α depends on the canonical LXXLL motif (NR box2) (10–13). In comparison, an untypical LXXYL-containing region (NR box3) contributes to the interaction of PGC-1α with ERRα or ERRγ (17). In agreement with a previous structural study (27), we observed that PGC-1α box3 binding to the ERRα LBD resembles that of canonical peptides, i.e. Tyr(+)4 occupies the position of Leu(+)4 of LXXLL peptides (Fig. 1). Interestingly, LXXYL binding has also been observed earlier in the crystal structure of the ERRα LBD complexed with a TIF-2 box3 peptide (28). TIF-2 box3 contains the sequence LLRYLL, which potentially allows canonical LXXLL as well as untypical LXXYL binding. However, despite untypical LXXYL interactions in the crystal, it was concluded from functional studies that, in solution, binding was probably canonical. Our new ERRα/PGC-1α box3 crystal structure revealed contacts between N-terminal flanking residues of PGC-1α and the ERRα surface (Fig. 1, A and B). This observation suggested that selective ERRα/PGC-1α box3 interactions are not an inherent characteristic of the LXXYL core region but depend rather on N-terminal flanking residues of the CoA.

In functional studies we found, in accordance with other reports (17, 27), that binding of PGC-1α box2 and box3 to ERRα critically depends on the +1 leucine residue (Leu-144 and Leu-210, respectively), as it is abolished by leucine-to-alanine mutations (Fig. 2). In comparison, Leu-211 and Tyr-213
play a less crucial role and stabilize rather than determine the interaction. Importantly, in accordance with our structural data, we observed that residues at positions 6 to 3 of the LLKYL motif contribute to efficient ERRα binding, as the interaction is strongly reduced in the case of the PGC-1α mutants C207S, C207E, and R204A/R205A (Figs. 2 and 3A). The role of these N-terminal flanking residues is further underlined by TIF-2 box3(L745A-Swap), which gains interaction with ERRα via LXXYL (Fig. 3B). Because of the comparable size of cysteine and serine, we did not anticipate that the C207S mutation would severely reduce interaction with ERRα. We speculate that this effect is due to the distinct solvation properties of these residues, which in the case of cysteine results in a distinct conformation of the PGC-1α main chain allowing proper positioning and optimal binding of Arg-205.

In a recent report, high-affinity interacting ERRα peptides with N-terminal flanking regions that differ from that of PGC-1α have been identified (42). It will be interesting to analyze the exact binding mode of these peptides in future studies.

To investigate the contribution of the LBD to CoA binding selectivity, we first considered amino acid differences between the CoA clefts of ERRα and ERα. This approach was chosen because few amino acids in the CoA cleft have previously been implicated in the specific CoA binding properties of androgen receptor (25), although partially conflicting data are presented in other reports (43, 44). The exchange of residues that differ in the CoA cleft of ERRα and ERα had significant effects on PGC-1α binding, resulting in strongly decreased interaction with ERRα(M258V/Q262E) and slightly increased interaction with ERα(V376M/E380Q) (Figs. 4A and 5D). However, these effects do not explain CoA binding selectivity, as ERRα(M258V/Q262E) unexpectedly lost almost all binding to PGC-1α box2, whereas ERα(V376M/E380Q) did not gain interaction with PGC-1α box3. Therefore we examined, in mammalian two-hybrid assays, whether the interaction between N-terminal flanking residues of PGC-1α and the H8–H9 loop of ERRα observed in the crystal structure was functionally rel-
evant. It is important to note that the H8–H9 loop links the homodimer interface with the CoA cleft via multiple main chain and side chain contacts (Fig. 4B). The marked structural difference between the H8–H9 loop of ERα and ERβ (Fig. 5) further supported the idea that it could be involved in selective PGC-1α binding. In accordance with its anticipated role, swapping of the H8–H9 loop of ERα into ERRα abolished interaction with PGC-1α. This may be explained by the disruption of the hydrogen bonding network that in wild-type ERRα centers around Ser-337 and Asp-338 or by steric effects due to the presence of the bulky Tyr-459 in the swapped H8–H9 loop (ERα numbering) (Fig. 5C). Importantly, the reverse swap mutant ERα(SwapH8–H9) gains significant interaction with PGC-1α box3. Furthermore, participation of Asp-338 in the homodimer interface suggested that the H8–H9 loop allosterically links this functional surface with the CoA cleft. In support of this idea, single point mutations in the dimer interface (D338A and R315A) strongly perturbed interaction of the ERRα LBD with PGC-1α (Fig. 4C). Notably, perturbed homodimerization and CoA binding of the mutants R315A and D338A was also observed in vitro in the context of DNA-bound, full-length ERRα (Fig. 6).

Although the above mentioned mutants show the strongest effects, two other mutations affected PGC-1α binding to a lesser but still significant extent. Deletion of the three C-terminal residues or replacement of Ser-259 (H4) with the corresponding residue of ERβ (resulting in the mutants ERRα(D3C) and ERRα(S259H), respectively) strongly reduced interaction with PGC-1α box3, whereas box2 binding was only modestly affected (Fig. 4, A and C). The C-terminal deletion may result in fewer contacts with the PGC-1α N-terminal flanking region or influence the dynamic behavior of H12, whereas a histidine at position 259 in H4 possibly perturbs the conformation of the H8–H9 loop. The three remaining single point mutants, H341A, E343A, and D423A, showed only small effects.

Despite extensive efforts, we did not succeed to convert ERα into a receptor with full ERRα-like CoA binding specificity (Fig. 5D). The mutant ERRα(3mut) (containing V376M/E380Q/H373S/H377S/SwapH8–H9) interacts slightly better with PGC-1α than ERα(SwapH8–H9). However, swapping the three C-terminal residues of ERRα into ERRα(3mut) decreases PGC-1α binding of the resulting mutant ERRα(4mut), which may be the consequence of a perturbed dynamic behavior of H12. These results reflect the mechanistic complexity of CoA binding to NR LBDs. Accordingly, in previous studies several distinct determinants of CoA binding selectivity have been documented. These include specific contacts within or immediately adjacent to the CoA cleft, the exact positioning of H12 and the CoA helix, and the location of residues C-terminal of H12 (19–23, 25, 44). We provide, for the first time, evidence for an allosteric role of the H8–H9 loop in PGC-1α binding to ERRα. In a recent study, Molnar et al. (45) found contacts between residues in H4, the H8–H9 loop, and H12 to be important for agonist-independent CoA association of PPARγ. However, the conclusions of that study do not apply to our data, because in the case of ERRα, (i) single point mutations aimed at disrupting H4, H8–H9 loop, or H12 contacts (H341A, E343A, D423A) show only small effects on PGC-1α binding, and (ii) none of the ERRα mutants gains ligand-independent activity.

Previous studies have addressed the topic of allosteric communication within the NR LBD (46–48). As a unifying scheme, these studies describe networks of interacting amino acids that couple the functional surfaces of the LBD: the homo- or heterodimer interface, the ligand-binding pocket, and the CoA cleft. Brelivet et al. (47) identified two sets of differentially conserved residues, which partition the NR superfamily according to oligomeric behavior, whereas Shulman et al. (48) characterized the role of allosterically coupled amino acids in ligand activation of RXR heterodimers, and Nettles et al. (46) provided evidence for a role of H11 as a conduit of structural information between ligand and H12 in ER homodimers. Interestingly, Asp-338 in the H8–H9 loop of ERRα corresponding to Asp-379 of RXRα is part of the network of allosterically coupled amino acids described by Shulman et al. (48). However, this residue is not conserved in any other steroid hormone receptor. Furthermore, Arg-315 in H7 of ERRα (the other important residue in the ERRα homodimer interface identified in this study) is conserved neither in RXRα nor in other steroid receptors except ERs. These observations argue for an allosteric control mechanism in homodimeric ERRs that shares some features with heterodimeric NRs but is still distinct from that of homodimeric steroid receptors and RXR heterodimers.

In which scenarios might the specific allosteric role of the H8–H9 loop of ERs be important? It has been documented that DNA binding and dimerization influence the transcriptional properties of ERs (26, 40, 49, 50). PGC-1α, for example, has been reported to bind only to ERRα homodimers, the formation of which depends on the exact sequence of the DNA-binding site and phosphorylation of the DNA-binding domain (26, 40, 49, 50). The allosteric connection of the homodimer interface with the CoA cleft via the H8–H9 loop provides a mechanistic explanation for distinct CoA binding of ERR monomers and homodimer. It also plausibly explains why small conformational changes in the H8–H9 loop, which may be provoked by distinct relative orientations of the LBDs within the homodimer due to distinct DNA-binding sites or induced by flanking residues of CoAs, can result in distinct transcriptional activities. In support of these ideas, we observe in EMSAs that mutation of Arg-315 and Asp-338 in the H8–H9 loop affects homodimeric DNA binding and CoA interaction (Fig. 6). Given the important role of the H8–H9 loop, it is tempting to speculate that it can serve as a docking site for as yet unidentified cofactors, the binding of which may result in altered DNA binding or cofactor recruitment. A precedent for cofactor binding to the H8–H9 loop of a NR has recently been presented for cyclin H and RARα. In that case, direct binding of cyclin H to the H8–H9 loop of the RARα LBD, which is allosterically controlled by a distant phosphorylation event, directs phosphorylation of the RARα activation function 1 (51). Finally, the H8–H9 loop might, as an alternative to the ERRα ligand-binding pocket (32), serve as a target for small molecule drugs.

In summary, our structural and functional analysis of the ERRα/PGC-1α box3 interaction identifies the H8–H9 loop as a surface region that contributes to selective CoA binding by allo-
sterically linking the LBD homodimer interface with the CoA cleft.

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