The nuclear receptor Nurr1 is a transcription factor essential for the development of midbrain dopaminergic neurons in vertebrates. Recent crystal structures of the Nurr1 ligand binding domain (LBD) and the Drosophila orthologue dHR38 revealed that, although these receptors share the classical LBD architecture, they lack a ligand binding cavity. This volume is instead filled with bulky hydrophobic side chains. Furthermore the “canonical” non-polar co-repressor binding groove is filled with polar side chains; thus, the regulation of transcription by this sub-family of nuclear receptor LBDs may be mediated by some other interaction surface on the LBD. We report here the identification of a novel co-regulator interaction surface on the LBD of Nurr1. We used an NMR footprinting strategy that facilitates the identification of an interaction surface without the need of a full assignment. We found that non-polar peptides derived from the co-repressors SMRT and NCoR bind to a hydrophobic patch on the LBD of Nurr1. This binding surface involoves a groove between helices 11 and 12. Mutations in this site abolish activation by the Nurr1 LBD. These findings give insight into the unique mechanism of action of this class of nuclear receptors.

Nuclear receptors comprise a large and ancient family of intra-cellular transcription factors that regulate many aspects of development and metabolism in metazoans. The activity of many nuclear receptors, especially those in vertebrates, is controlled by the binding of fat-soluble hormones and metabolites within a non-polar cavity in the ligand binding domain (LBD) of the receptor. The mechanisms through which these small molecules switch the activity of the receptor have been established (reviewed in Ref. 1). Ligand binding stabilizes the global structure of the LBD (2, 3) and causes the C-terminal helix (helix 12), which is independently mobile in the absence of a ligand, to adopt a stable conformation packed against the surface of the LBD (4). This results in the displacement of co-repressor proteins and the recruitment of co-activator proteins. Both co-repressor and co-activator proteins bind to the same non-polar groove on the surface of the LBD through related but distinct interaction motifs that adopt an amphipathic helical conformation (5–10). Discrimination between co-activators and co-repressors is achieved because the active position of helix 12 does not allow binding of the longer co-repressor helix but stabilizes the binding of the shorter co-activator helix.

Although this mechanism is relevant for ligand-regulated receptors, it has become clear that in vertebrates a significant number of nuclear receptors are probably not regulated by a ligand (11). This is a reflection of the evolutionarily adaptable nature of the nuclear receptor ligand binding domain that has resulted in many instances of loss (and gain) of ligand regulation (12–14). Such receptors are true orphan receptors that are either active with an unoccupied ligand binding cavity (15), have a constitutive structural co-factor in place of a regulatory ligand (16), or simply lack a ligand binding cavity (17, 18). In vertebrates three closely related receptors, Nurr1 (NR4A2), Nur77 (NGFI-B/NR4A1), and Nor1 (NR4A3), belong to this last group of orphans (19). All these receptors play a role in the central nervous system as well as in other tissues. Much interest has, in particular, focused on Nurr1 because it plays an important role in the development of dopaminergic neurons in the midbrain and may represent a therapeutic target to treat Parkinson’s disease (20).

We know that this group of receptors lacks a ligand binding cavity, because crystal structures of the ligand binding domain from Nurr1 (17) and its orthologue in Drosophila, dHR38 (18), revealed that the ligand binding cavity is almost entirely filled with bulky hydrophobic side chains. This implies that this family of receptors cannot bind and is not regulated by ligands in the conventional fashion. These structures also revealed that the canonical co-regulator binding groove observed in other receptors is filled with polar side chains and is therefore not likely to support conventional non-polar interactions with co-regulators. This would appear to suggest that the LBD of Nurr1 and related receptors might, at first sight, be inert and play no direct role in the regulation of transcription. However, this is clearly not the case, because the Nurr1 LBD exhibits a potent activation function, albeit dependent upon cell type. Thus, the question is how can the Nurr1 LBD activate transcription when the lack of a conventional co-regulator binding groove would appear to preclude interaction with known co-regulator proteins. The simplest answer to this is that there is another, as yet unidentified co-regulator interaction surface.

These issues thus raise two questions: what are the co-regulators that interact with the Nurr1 LBD, and where on the surface of the LBD do they interact? Various interaction studies have shown that the co-repressor proteins SMRT (21) and...
PIASy (22) are able to bind to the Nur77 and Nur1 LBDs, respectively. An interaction was also inferred with an ASC-2 adaptor protein (21). Finally, it was also shown that the N-terminal AF1 domains of Nur77 and Nor1 interact directly with their respective LBDs and that this interaction is stabilized by the co-activator protein SRC-2 (23, 24).

To identify the surface or surfaces on the Nur1 LBD that might mediate interaction with co-activators and the N-terminal AF1 domain, we explored its interactions with peptides from conserved regions of the N-terminal domain of Nur1 and from the co-repressors SMRT and NCoR (a SMRT homologue). None of these peptides have been reported previously to interact with the Nur1 LBD, but such interactions seemed to be a possibility given the homology between Nur1 and both Nur77 and Nor1. The receptor interaction domains from SMRT and NCoR were found to interact with the Nur1 LBD in pull-down, fluorescence quenching, and NMR binding assays. Calculation of the hydrophobic potential of the surface of the Nur1 LBD led to the identification of a highly hydrophobic patch/groove between helices 11 and 12 suggestive of an interaction surface. Mutations in this region confirmed its role in co-repressor interaction using both NMR footprinting and pull-down assays.

MATERIALS AND METHODS

Preparation of Recombinant Nur1—The uniformly 15N-labeled Nur1 LBD (amino acids 353–598 preceded by a non-native glycine; molecular mass of 27.8 kDa) was expressed with a hexahistidin tag and a tobacco etch virus protease cleavage site at the N terminus using the pET-13a expression vector in the Escherichia coli strain BL21 (DE3). Cells were grown in M9 medium supplemented with 1 g/liter 15NH4Cl, 2 g/liter glucose, nitrogen bases, vitamins, and oligoelements as described previously (25). Cells were grown at 37 °C to an A600 of 0.6–0.8 and induced with 0.4 mM isopropyl β-D-thiogalactopyranoside. Three hours after induction, cells were harvested and resuspended in buffer (50 mM sodium phosphate, pH 6.9, 50 mM NaCl, 0.02% NaN3, and protease inhibitors. Nurr1 was further concentrated to 0.3–0.7 mM, and 7% (v/v) D2O was added to the NMR sample.15N labeled peptides were chosen on the basis of minimal regions required for interaction with other nuclear receptors. None of these peptides have been reported previously to interact with other nuclear receptors.

Peptides were coupled chemically to N-hydroxysuccinimide-activated Sepharose and challenged with in vitro translated and [35S]methionine-labeled Nur1 LBD (Fig. 1). The two co-repressor peptides (CR1 and CR2) recruited the Nur1 LBD more strongly than either of the two controls (resin alone and resin coupled to bacterially expressed Nur1 LBD). Furthermore, as has been previously reported for Nur77 (21), mutation of the four conserved hydrophobic residues in the co-repressor peptide (CR1m) abolishes interaction with the Nur1 LBD (Fig. 1). This finding suggests that although Nur1 lacks the canonical non-polar co-repressor interaction groove, the nature of the interaction with the co-repressor peptides might be rather similar to that with other nuclear receptors.

NMR—NMR spectra were recorded on Bruker Avance 800 and DMX 600 spectrometers equipped with 5-mm triple resonance (1H-15N-13C) single-axis gradient probes. Data were processed using the program NMRPipe (26) and analyzed with the program nmrView (27). All NMR data were acquired at 300 K.

Titrations were made by adding successive amounts of peptide (CR1, CR1b, and N1-N5) to a solution containing 0.3 mM protein and acquiring HSQC spectra at peptide to protein ratios of 0.5:1, 1:1, 2:1, 4:1, 6:1, and 8:1. The Nur1 concentration was derived from A280 measurements using an extinction coefficient calibrated by determining the concentration by amino acid analysis on an Amersham Biosciences Biochrom 20-amino acid analyzer after 18 h in 6 M HCl at 110 °C. Peptide concentrations were calculated from dry weight.

Fluorescence Quenching Assays—Fluorescence spectra were recorded in a Luminescence Spectrometer LS 50 B (Perkin Elmer) controlled by a personal computer equipped with FL Winlab software (PerkinElmer Life Sciences). Trypsin-induced quenching was measured at 20 °C. The excitation wavelength was 295 nm, and fluorescence emission scans were recorded between 260 and 450 nm at 200 nm/min. The excitation and emission slit widths were at 5 and 10 nm, respectively.

Titrations were made by adding successive amounts of the CR1 peptide to a 500-μl solution containing 11.3 μM Nur1 in buffer X (the A280 of the solution was between 0.5 and 1 so that the quenching process is linear). The peptide was dissolved in 50 μl of the same protein solution. A three-scan spectrum was recorded after each peptide addition (0–27 μl of the peptide solution). Data were analyzed assuming static quenching using the program Prism® (GraphPad).

RESULTS

Peptide Interaction with the Nur1 LBD—To identify potential interaction surfaces on the Nur1 LBD, we first sought to identify, using a pull-down assay, short peptides that are able to interact with the Nur1 LBD. Candidate peptides of a diverse character were derived from the nuclear receptor co-repressors SMRT and NCoR and the N-terminal AF1 domain of Nur1 (Fig. 1), which have previously been reported to interact with the LBD of this class of receptors (21, 23). A peptide from the co-activator SRC-1, which is not thought to interact with the Nur1 LBD (28), was also included in the assay. The Nur1 AF1 domain peptides were chosen on the basis of a region of high homology with Nur77 and Nor1. The co-repressor peptides were chosen on the basis of minimal regions required for interaction with other nuclear receptors.

Peptides were coupled chemically to N-hydroxysuccinimide-activated Sepharose and challenged with in vitro translated and [35S]methionine-labeled Nur1 LBD (Fig. 1). The two co-repressor peptides (CR1 and CR2) recruited the Nur1 LBD more strongly than either of the two controls (resin alone and resin coupled to bacterially expressed Nur1 LBD). Furthermore, as has been previously reported for Nur77 (21), mutation of the four conserved hydrophobic residues in the co-repressor peptide (CR1m) abolishes interaction with the Nur1 LBD (Fig. 1). This finding suggests that although Nur1 lacks the canonical non-polar co-repressor interaction groove, the nature of the interaction with the co-repressor peptides might be rather similar to that with other nuclear receptors.

As expected, the co-activator peptide CA did not recruit the Nur1 LBD. Of the peptides derived from the N-terminal domain of Nur1, only N1 (comprising the 13 N-terminal residues of the receptor) was observed to recruit the LBD. Significantly, this N-terminal peptide bears no sequence similarity to the co-repressor peptides, suggesting that the interaction between this peptide and the Nur1 LBD might be very different in nature.

To further investigate the interaction between the Nur1 LBD and the various peptides, we examined the effect of adding increasing amounts of a peptide to a 15N-Nur1 LBD sample and recording a 1H-15N HSQC NMR spectrum at each step of the titration. Although we observed an apparently tight interaction between the N1 peptide and the Nur1 LBD in the pull-down assay, titrations with this peptide resulted in no
discernible perturbations in the $^1$H-$^{15}$N HSQC spectra, even at coupled to the candidate peptides and with beads alone (not to the other peptide coupled resins, including the CR1m resin. An asterisk indicates that the peptide is coupled to a C-terminal biotinyl residue. Although the co-repressor peptides CR1 and CR2 both had a C-terminal biotinyl residue, another peptide (NS) also with a biotinyl residue failed to interact with the Nurr1 LBD, confirming that the interaction was not mediated by the biotin moiety. Peptides with and without biotin behaved in the same way in the NMR experiments.

In contrast to the N-terminal peptides, the addition of the CR1 peptide to the $^{15}$N-Nurr1 LBD resulted in numerous changes in the $^1$H-$^{15}$N HSQC spectrum even at a 1:1 ratio. The $^1$H-$^{15}$N HSQC spectra of the Nurr1 LBD contain 191 sharp, well resolved backbone amide resonances as well as a few resolved side chain amide protons. The CR1 peptide caused significant chemical shift perturbations and/or broadening of 56 of these resonances (Fig. 2). The observed changes are reproducible and are essentially identical using peptides derived from either SMRT (CR1) or NCoR (CR1b; inset in Fig. 2).

The addition of more peptide induced further changes in the spectrum, suggesting that the binding is not fully saturated at 1:1 and, therefore, that the binding is rather weak (saturation is reached around 8:1). It is also possible that the peptide binds to multiple sites in fast exchange. However, the number of observed spectral changes and the titration profile suggest that the co-repressor peptide is probably interacting with a single site on the surface of the Nurr1 LBD. Notably, many of the spectral changes on peptide addition involve broadening as well as chemical shift perturbations. This broadening suggests that the exchange may be in the intermediate rate regime (29).

To gain a measure of the binding affinity of the CR1 peptide, we monitored the quenching of tryptophan fluorescence upon the addition of peptide to the Nurr1 LBD in solution (Fig. 3). There are two tryptophan residues in the LBD, residues 420 and 482, both of which are partially exposed to solvent. The titration of bacterially expressed Nurr1 LBD with increasing amounts of the CR1 peptide showed a saturable 30% quench in the total tryptophan fluorescence (Fig. 3a). This finding is most likely due to static (rather than collisional) quenching of one of the tryptophans as a result of peptide binding to the Nurr1 LBD. These data suggest that the peptide is probably interacting with a single site on the surface of the LBD, although it is not possible to rule out binding to multiple sites with similar affinity. The observed $K_q$ (Fig. 3b) indicates a dissociation constant of $\sim 50 \mu M$, assuming static quenching and single site binding. This dissociation constant is apparently two orders of magnitude higher than that of other measured co-repressor-LBD interactions (30). This finding fits with the observed behavior in the NMR experiments but may indicate that this short peptide does not recapitulate full physiological interaction. It is also possible that, as has been suggested for co-activators, the co-repressor interaction could require other stabilizing factors. However it should be noted that the pull-down assays, which include a stringent washing procedure, show a clear recruitment of the Nurr1 LBD by this peptide.

**Prediction of a Peptide Binding Surface**—Given that the Nurr1 LBD is able to interact with the receptor interaction domains from co-repressor proteins but lacks a conventional co-repressor interaction surface, we wished to understand where on the surface of the LBD the co-repressor peptides are binding. Because we and others have found that the interaction requires the conserved hydrophobic residues in the CoR peptide, we might expect this surface to be largely non-polar. Hydrophobic potential mapping has proven to be a useful way of identifying non-polar interaction surfaces on proteins of known structure (for an example, see Ref. 31). For such mapping, the energy of a hydrophobic probe is calculated using the program GRID (32) and displayed as a potential map. Such an analysis of the surface of the Nurr1 LBD compared with that of the TR LBD is quite revealing (Fig. 4a and b). As reported previously (17, 18), the conventional co-repressor interaction surface (labeled Co-reg in Fig. 4a) is quite different between the two receptors; Nurr1 lacks the hydrophobic groove seen in the TR. However, Nurr1 has an alternative groove adjacent to helix 12 that has a strikingly high hydrophobic potential (colored yellow/orange and labeled Nurr1 specific in Fig. 4b). This surface is formed from the side chains of residues Leu-570 and Phe-574 in helix 10/11 and those of residues Phe-592, Leu-593, Leu-596, and Phe-598 in and just C-terminal to helix 12 (Fig. 4,
b and c). For comparison, the largely non-polar region of the LBD surface that mediates heterodimerization with the retinoid X receptor is also indicated (labeled Dim in Fig. 4b). If these residues did contribute to a co-regulator interaction surface, it would explain the finding that mutations in or deletion of helix 12 abolishes the activation activity of the Nurr1 LBD (28). In particular, the mutation of residues Asp-589, Phe-592, and Leu-593, which are largely exposed to solvent on this surface, abolish the activity of the LBD.

Together, these findings suggest that this surface might constitute a co-regulator binding surface on the Nurr1 LBD. We wished to determine whether this might be the site of interaction of the CoR peptide. Because the peptide binding was found to quench tryptophan fluorescence, we asked whether either of the two tryptophans in Nurr1 is exposed near this surface. Significantly, Try-482 is partially exposed on the surface of the LBD and is within 7.2 and 10.5 Å of residues Leu-570 and Phe-592, respectively.

Mapping the Peptide Binding Surface—To test directly whether this highly hydrophobic patch acts as the binding surface for the CoR peptides, we made mutations (F592A, L593A, and F598A) in this region of Nurr1. We then compared the 1H-15N HSQC spectra of each of these mutants with that of the wild-type LBD and finally correlated the observed perturbations due to the site-directed mutations with those resulting from the binding of the CoR peptide.

The HSQC NMR spectra of the 15N-labeled mutants indicate that the mutations perturb the backbone amide signals of between 19 and 48 residues. These perturbations are mostly rather small and are not consistent with any significant disruption of the secondary or tertiary structure of the domain. Thus, we can conclude that the structure and stability of the Nurr1 LBD is unaffected by these predominantly surface mutations. Given the close spatial proximity of the mutated residues to one another, it is not surprising that many signals in the spectra are perturbed by more than one mutation. Furthermore, there is a striking overlap between the signals perturbed by the mutations and those perturbed in the 1:1 complex of the wild-type Nurr1 LBD with the CoR-ID1 peptide. This observation is illustrated for a few specific cases by the expansions of HSQC spectra shown in Fig. 5 and, in a more general way, by the statistics summarized in Fig. 6.
In Fig. 5, the colored arrowheads indicate some of the cross-peaks that are perturbed on binding peptide and by at least two of the mutations. For instance, the cross-peak labeled by the orange arrowhead (Fig. 5) is greatly broadened on the binding peptide. The $^1$H frequency of this cross-peak is shifted upfield in the F592A mutant, slightly downfield in the L593A mutant, and does not move in the F598A mutant. The cross-peak marked by the purple arrowhead (Fig. 5) is shifted and broadened upon peptide binding. The four mutants also induce chemical shift changes in this cross-peak (to various extents and in various directions). The cross-peak marked by the green arrowhead (Fig. 5) is almost certainly the amide signal from Leu-593. This assignment can be made based on the very large chemical shift perturbation (0.7 ppm for $^1$H and 4 ppm for $^{15}$N) of this cross-peak in the spectrum of the L593A mutant, which is much larger than any of the other perturbations due to this mutation. The Leu-593 cross-peak is also perturbed, to a lesser extent, by the other mutations due to their proximity. Upon binding of the CoR peptide, the Leu-593 cross-peak is significantly broadened, allowing a firm assignment of the part of the surface affected by the peptide binding.

We also note that a pair of carboxamide protons (from the side chain of a Gln or Asn residue) are also perturbed, both in the mutants and upon peptide binding. This perturbation is particularly evident in the Phe-598 mutations. Examination of the Nurr1 LBD structure suggests that these cross-peaks are very likely to belong to the side-chain of Glu-571.

More extensive analysis of the HSQC spectra shows that the mutations cause perturbations in 48 (F592A), 19 (L593A), and 20 (F598A) cross-peaks, respectively, which compares with 56 cross-peaks influenced by the CoR peptide binding to the wild-type Nurr1 LBD. Fig. 6 illustrates the overall correlation between these sets of residues. For the Phe-592 mutation, 34 of the signals affected by the mutation are also affected by peptide binding to the wild-type LBD. These signals are represented by the red/blue portion of the pie chart, whereas the blue portion represents the 14 signals affected only by the mutation, and the orange portion represents the 22 signals affected only by peptide binding (in the wild-type LBD). Similarly, for each of the other mutants, a high proportion of the signals affected by the mutation are also affected by peptide binding to the wild-type LBD; in no case is this proportion $< 70\%$. The substantial degree of overlap of these perturbations, along with the specific assignment for Leu-593, provides strong evidence that the CoR peptide binding surface overlaps with the sites perturbed by the mutations. It is particularly notable that the Phe-592 mutation perturbs more signals than the other mutations (presumably because it is partially buried). This means that it was not possible to assign the amide of Phe-592. However it is evident that many of the cross-peaks perturbed by this mutation are also perturbed upon peptide binding. This strong correlation supports the conclusion that Phe-592 plays a central role in the peptide interaction surface. Interestingly, although the Phe-598 mutation perturbs many cross-peaks that are also perturbed on peptide binding, the amide of Phe-598 itself (assigned as for Leu-593) is not perturbed by peptide binding. This finding suggests that Phe-598 lies just outside the peptide binding surface.

Disrupting the Peptide Binding Surface—The comparative NMR analyses of the Nurr1 mutant proteins and of peptide binding to the wild-type Nurr1 LBD clearly confirm the hypothesis that the hydrophobic region adjacent to helix 12 is involved in binding the CoR peptide. To determine whether mutation of residues in this region perturbs binding of the CoR peptide, we used a pull-down assay in which the CoR peptide is covalently coupled to a Sepharose resin and used to recruit radiolabeled Nurr1 LBD. The material remaining bound to the resin after washing was analyzed by electrophoresis and quantified by autoradiography. Wild-type Nurr1 LBD was recruited fairly efficiently to the peptide resin, and the bound material represents 64–66% of the standardized input loading (Fig. 7). The F592A, L593A, and F574A mutant Nurr1 LBDs were less efficiently recruited to the peptide resin, particularly the F574A mutant. The F598A mutation only mildly perturbed peptide binding. These results further support the identification of this region of the protein as being involved in interaction with the CoR peptide and show that peptide binding is reduced in the mutants.

DISCUSSION

Crystal structures of the LBDs of two members of the NR4A family of nuclear receptors revealed that these receptors are true orphans lacking a ligand binding cavity (17, 18). In addition, both structures showed that these receptors lack the canonical co-regulator binding surface seen in other nuclear re-
Receptors. This observation is consistent with the finding that the LBDs of this group of receptors do not interact with known co-activator proteins. However, in apparent contradiction to these observations, the Nurr1 LBD has a potent but cell-type specific transactivation function (28). This finding would suggest that, despite lacking the canonical interaction surface, Nurr1 is nevertheless able to recruit co-activator proteins, although the identity of these proteins has yet to be established.

To address this question, we sought to make use of peptides that interact with the Nurr1 LBD. Pull-down and fluorescence quenching assays confirmed that hydrophobic peptides derived from the co-repressors SMRT and NCoR interact, albeit weakly, with the Nurr1 LBD. Titration of the co-repressor peptide into an NMR sample of the Nurr1 LBD showed perturbation of multiple signals, suggesting specific interaction with one region of the surface of the LBD. However, because no NMR assignment of the Nurr1 LBD is available, it was not known which residues in the LBD correspond to the perturbed signals in the HSQC spectra. Assignment of a protein of 28 kDa is time consuming, requires expensive labeling, and is not always possible; furthermore, the availability of an x-ray structure is usually of relatively little help in making such an
Novel Co-regulator Interface on the Nurr1 LBD

![Diagram showing interaction sites on the Nurr1 LBD](image)

**Fig. 8. Comparison of NR4A family members.** *a*, annotated sequence alignment of Nurr1 and related receptors. Residues that form the surface of high hydrophobic potential are indicated with orange circles. Residues that were shown by NMR to be close to the peptide binding surface are labeled with green circles. Residues that inhibited peptide binding when mutated to alanines are labeled with magenta circles. Residues that were shown previously to abolish the activation activity of the Nurr1 LBD when mutated to alanines are indicated with blue circles (28). Red boxes indicate helical regions. Residues are labeled with single letter amino acid abbreviations coupled with position numbers. *b*, superposition of the structures of the Nurr1 LBD and that of dHR38. Note that helix 12 in Nurr1 (green) adopts a somewhat different position from that of helix 12 in dHR38 (red).

assignment. We sought therefore to implement an NMR footprinting strategy to broadly map the interaction surface with the non-polar co-repressor peptides.

The first step in this strategy is to identify a potential interaction site or sites. In this case, given the hydrophobic character of the co-repressor peptides, we used hydrophobic potential mapping to explore the surface of the Nurr1 LBD. Such an approach has proven informative in several other systems (for an example, see Ref. 31). For the Nurr1 LBD, this mapping revealed a very hydrophobic groove between helices 11 and 12 of the receptor that is formed from a cluster of non-polar side chains on the surfaces of these helices. The second step in the strategy is to make mutations in the candidate binding surfaces and to use NMR spectroscopy to compare the set of signals perturbed by the mutations with those perturbed upon peptide binding. In this case, the perturbations caused by three mutations in the surface highlighted by the hydrophobic potential mapping largely overlap with those perturbations observed upon peptide binding. This observation strongly suggests that the hydrophobic surface near helix 12 serves as part of the binding interface for the co-repressor peptide.

The advantage of this NMR footprinting strategy is that it does not require a full assignment of the protein nor does it require labeled amino acids that are needed in related strategies (33). It also allows exploitation of an existing crystal structure and capitalizes on the advantage of NMR techniques to examine weak interactions (29), thus opening the possibility of probing a structure with peptide libraries to reveal interaction surfaces when partner proteins have not been identified. Finally, the same mutant constructs can be used in functional and biochemical interaction assays but with confirmation from the NMR spectra that the mutations do not grossly perturb the protein architecture.

Using this strategy, we have shown that co-repressor peptides, derived from SMRT and NCoR, interact with the Nurr1 LBD at a site in the vicinity of helix 12. As mentioned above, interaction between the nuclear receptor Nur77 and SMRT has been reported previously (21). It has been proposed that this interaction is biologically relevant and leads to the deactivation of Nur77. Although Nur77 is highly homologous to Nurr1 and many of the residues that contribute to the interaction surface in Nurr1 are the same in the two receptors (Fig. 8), our measurements suggest that the short peptides from SMRT interact rather weakly with the Nurr1 LBD. Indeed the dissociation constant is ~100 times higher than that measured for other receptor/co-regulator interactions (30). This finding suggests that the short peptides may either be insufficient to fully recapitulate the interaction of the full-length co-repressors or that some other factor is needed to form a stable ternary complex analogous to the AF-1/AF-2/SRC2 complex observed previously (23). Alternatively, it is possible that SMRT is not a biologically relevant co-regulator for Nurr1. Because this issue is unresolved, we consider the SMRT peptide to be a probe that allows us to identify a surface on the LBD that is required for interaction with non-polar co-regulator proteins.

The biological relevance of this interaction surface is strongly supported by the results of previous mutagenesis experiments (28). These results showed that mutation of residues Ile-588, Asp-589, Leu-591, Phe-592, and Leu-593 abolishes the transactivation activity of the Nurr1 LBD (summarized in Fig. 8). It was, however, not clear from these studies whether this was the result of a global or a local perturbation of the LBD structure. Our results, though, show that at least two of these mutations do not substantially perturb the structure of the LBD and that they contribute to a hydrophobic peptide binding interface. Because the mutations abolish activity of the Nurr1 LBD, it seems most likely that this peptide binding surface comprises a binding site for an as yet unidentified co-activator protein. The fact that this may coincide with a binding surface for the SMRT co-repressor is strikingly reminiscent of the situation for receptors such as the retinoic acid receptor, where co-activators and co-repressors bind to a single co-regulator interaction surface (34). Interestingly, Sohn et al. showed that SMRT association with Nur77 results in deactivation of the receptor due, in part, to competition between a co-activator protein and the SMRT co-repressor for the same site on the surface of the Nur77 LBD (21).

The finding of a novel co-regulator interaction surface on the Nurr1 LBD raises the question as to why such a situation has evolved. At first sight, one might think that because the LBD is constitutively active and not regulated by ligand binding, a co-regulator interaction site need not be close to the sensor of ligand binding, helix 12. It is notable therefore, that this novel site is adjacent to helix 12. It thus remains possible that helix 12 positioning might still regulate the binding of co-regulator proteins to Nurr1. In this context it is interesting to note that the helix 12 of dHR38 differs in length and positioning with respect to the main body of the LBD (Fig. 8). This observation
may reflect an alternative but fixed position or it could also reflect a means of regulation of the LBD.

In summary, we provide evidence of a novel co-regulator interaction surface on the Nurr1 LBD. The high degree of sequence conservation suggests that this surface may be relevant to other members of the NR4A sub-family of receptors. These findings shed light on the unique mechanism of action of this class of nuclear receptors.

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