Structural Studies of Mutant Glucocorticoid Receptor Transactivation Domains Establish a Link between Transactivation Activity *in Vivo* and α-Helix-Forming Potential *in Vitro*†

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ABSTRACT: We have previously shown, using circular dichroism spectroscopy, that the τ1 core peptide has α-helix-forming potential *in vitro* [Dahlman-Wright et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1699−1703]. The τ1 core peptide is a 58-amino acid peptide, constituting the core of the transactivation activity of the τ1 major transactivation domain of the human glucocorticoid receptor [Dahlman-Wright et al. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1619−1623]. Further structural studies of the peptide, using NMR spectroscopy, identified three segments with α-helical character. In this report we show that reduced protein expression or stability is not responsible for the reduced *in vivo* transactivation potential of τ1 core peptides with proline substitutions in proposed α-helical regions. Rather, the reduced α-helix propensity of the corresponding purified peptides *in vitro* suggests that α-helices are involved in the molecular mechanism of glucocorticoid receptor mediated changes in gene activity.

The glucocorticoid receptor (GR)[1] belongs to the family of nuclear receptors (Evans, 1988). Binding of ligand transforms the receptor into an active species able to interact with specific DNA sequences termed glucocorticoid response elements or GREs (Wright et al., 1993, and references therein). Binding of the receptor to these DNA sequences often results in an increase in the rate of transcriptional initiation of associated genes (Wright et al., 1993, and references therein). The GR, like most other gene regulatory proteins contains discrete domains responsible for DNA-binding and transactivation activity (Giguère et al., 1986). The major transactivation activity of the human GR (hGR) was initially shown to reside between amino acids 77−262 of the 777-amino acid receptor protein and was termed τ1 (Hollenberg & Evans, 1988). We have extended these studies and shown that amino acids 187−244 of the hGR constitute the core of the transactivation activity, retaining 60%−70% of the transactivation activity of the intact τ1 fragment, and we have called this segment of the protein the τ1 core (Dahlman-Wright et al., 1994).

The molecular mechanism by which DNA-bound transcription factors enhance the rate of transcriptional initiation remains to be established. It is thought that transactivation domains enhance the rate of one or more rate-limiting steps in the assembly of the initiation complex by interacting, directly or indirectly, with one or more of the basal transcription factors (Ptashne & Gann, 1990; Roeder, 1991; Zawel & Reinberg, 1995, and references therein). Interaction of a transactivation domain with components of the general transcription machinery could stabilize interactions within the initiation complex, leading to an increase in the rate of transcriptional initiation. Recently, interactions between transactivation domains and several of the basal transcription factors have been described (Zawel & Reinberg, 1995, and references therein). Several nuclear receptors have been shown to interact with the basal transcription factor TFIIB *in vitro* (Ing et al., 1992; Baniahmad et al., 1993; Fondell et al., 1993; McDonald et al., 1995; Blanco et al., 1995). The viral transcription factor VP16 has been demonstrated to interact with the basal transcription factors TBP and TFIIB and the TBP-associated factor TAF40 *in vitro* (Stringer et al., 1990; Lin et al., 1991; Goodrich et al., 1993). Studies of mutant proteins show a correlation between loss of function *in vivo* and reduced protein−protein interactions *in vitro* (Ingles et al., 1991; Lin et al., 1991). The human transcription factor Sp1 has been demonstrated to interact with the TBP-associated factor TAF 110 *in vitro*, and mutants which interfere with the transactivation potential *in vivo* reduce this interaction (Gill et al., 1994).

Structural studies, using circular dichroism (CD) and NMR spectroscopy, of transactivation domains from several transcriptional activator proteins have shown that these are largely unstructured in aqueous solution at neutral pH (Donaldson & Capone, 1992; O'Hare & Williams, 1992; Van Hoy et al., 1993; Dahlman-Wright et al., 1995). This is in marked contrast to the DNA-binding domains of transcription factors which appear to have well-defined three-dimensional structures (Härd et al., 1990; Harrison, 1991). However, two transcriptional activation domains from the yeast transcription factors GAL4 and GCN4 were shown to acquire a significant β-sheet structure at low pH (Van Hoy et al., 1993). The transactivation domains from the VP16 viral transcription factor and a transactivation domain from the p65 subunit of the NFkB transcription factor were shown to acquire significant α-helical structure in trifluoroethanol (TFE) but

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[1] Abbreviations: BV, bed volume; CD, circular dichroism; DTT, dithiothreitol; GR, glucocorticoid receptor; hGR, human glucocorticoid receptor; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol.

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adopt random coil conformations in aqueous solution (Donaldson & Capone, 1992; Schmitz et al., 1994). TFE is believed to induce α-helical structure in peptides which have a propensity for α-helix formation (Lehrman et al., 1990; Dyson et al., 1988, 1992). However, it is still unclear whether the α-helix-inducing potential of TFE is restricted to peptides which naturally form α-helices or whether it can also induce α-helical structures in peptides which do not naturally adopt this conformation. It has been speculated that, in the cell, transactivation domains are unstructured until they touch their putative target factors and that the interaction of the transactivation domain with its target factor leads to a structural change in the transactivation domain by an induced fit mechanism (Tjian & Maniatis, 1994). The above conditions, inducing secondary structure, might mimic conditions experienced by transactivation domains when involved in interactions with target factors.

We have recently shown that the τ1 core peptide is largely unstructured in aqueous solution at a variety of pH (Dahlman-Wright et al., 1995). However, the peptide acquires significant α-helical structure in the presence of TFE including three segments with clear α-helical character (Dahlman-Wright et al., 1995). The identification of the position of α-helical segments in a transactivation domain allows us to address the importance of α-helices for transactivation *in vivo*. In this study we demonstrate that the reduced transactivation potential of τ1 core peptides with proline substitutions in proposed α-helical segments is not due to reduced expression or stability of mutant proteins. The corresponding purified τ1 core peptides show reduced α-helix-forming potential *in vitro*, suggesting that α-helix potential is important for GR-mediated transactivation *in vivo*.

MATERIALS AND METHODS

Generation of Expression Plasmids, Protein Expression, and Protein Purification. The construction of vectors expressing mutant τ1 core−LexA DBD fusion proteins in yeast cells has been described previously (Dahlman-Wright et al., 1995). Vectors expressing mutant proteins in *Escherichia coli* were constructed as follows. DNA coding for wild type and mutant τ1 core peptides was amplified from the corresponding yeast expression vectors using the polymerase chain reaction (PCR) with primers including *Bam*HI and *Bgl*II sites, respectively. The PCR products were cleaved with *Bam*HI and *Bgl*II and cloned into the *E. coli* expression vector pQE12 (Qiagen) which had been cut with *Bam*HI and *Bgl*II. This vector expresses inserted sequences fused to six C-terminal histidine residues. The correct sequence of the resulting clones was confirmed by DNA sequencing. The *E. coli* strain M15 pREP4 (Villarejio & Zabin, 1974) was used for protein expression. Cells were inoculated into 10 mL of LB medium supplemented with 0.5% glucose, 0.5% casamino acids, 100 μg of ampicillin/mL, and 25 μg of kanamycin/mL and were grown overnight at 37 °C. This culture was used to inoculate 500 mL of the same medium. Cells were grown at 37 °C to $OD_{600} = 0.6$. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and growth was continued for 2 h. Cells were harvested by centrifugation and stored at −70 °C. Cells were resuspended in 5 mL 50 mM Tris-HCl, pH 8.0, 20 mM β-mercaptoethanol and freeze−thawed three times. Lysozyme was added to a final concentration of 1 mg/mL, and the lysate was incubated on ice for 15 min. DNase I and $MgCl_2$ were added to final concentrations of 100 μg/mL and 1 mM, respectively, and incubation was continued for 15 min. NP40 was added to a final concentration of 0.05%, and incubation was continued for 15 min. NaCl was added to a final concentration of 500 mM, and the soluble fraction was prepared by centrifugation at 150 000*g* for 30 min. The lysate was passed over a 1 mL Ni-NTA column equilibrated in buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM β-mercaptoethanol). The column was washed with 20 BV of buffer A and eluted with buffer A + 250 mM imidazole. Protein-containing fractions were dialyzed against 20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1 mM DTT. The protein was applied to a Superdex 75 gel filtration column (Pharmacia) eluted in the same buffer.

Protein concentrations were determined by measuring OD_{280} using the extinction coefficient $\epsilon = 6000$ (calculated from tryptophan and tyrosine absorptions). Protein concentrations were also determined using the Bradford protein assay reagent (Pierce), which gave very similar results.

Transactivation Assays in Yeast Cells and Western Immunoblotting. Yeast cells were inoculated into SGal medium lacking uracil and leucine and grown overnight at 30 °C. The cells were diluted to $OD_{600} = 0.2$, grown at 30 °C for 8 h, and harvested by centrifugation. Extracts were prepared as described and assayed for β-galactosidase activity as described previously (Wright et al., 1990). For Western immunoblot analysis equal numbers of cells were directly resuspended in SDS sample buffer. SDS−PAGE was performed as described (Laemmli, 1970). Western blot analysis was performed as described (Burnette, 1981) using rabbit polyclonal antibodies raised against LexA DBD. The antibodies were a kind gift from Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA). Development of blots was performed using an chemiluminescence system (Amersham).

Circular Dichroism Spectroscopy. Samples for circular dichroism spectroscopy contained 100 μg of protein/mL in 10 mM phosphate buffer, 1 mM DTT, and indicated concentrations of TFE. Spectra were recorded on an AVIV 60DS CD instrument with a bandwidth of 1.5 nm and scan steps of 0.5 nm. The temperature was kept constant at 25 °C. Experiments were repeated twice with different batches of protein.

RESULTS

Proline-Substituted τ1 Core−LexA DBD Fusion Proteins Are Stably Expressed at Levels Similar to the Wild Type Protein. We have previously described that introduction of proline substitutions into the first two of three proposed α-helical regions interferes with the transactivation potential of the τ1 core *in vivo* (Dahlman-Wright et al., 1995). The mutational analysis focused on the first two helices since we had shown that the region including these two α-helices is most important for transactivation *in vivo* (Dahlman-Wright et al., 1994). In these experiments various τ1 core derivatives were fused to the LexA DNA-binding domain. Such proteins, in which residues within putative helical regions were replaced with prolines, showed reduced transactivation activity when expressed in yeast. Although consistent with an important role of α-helical conformation

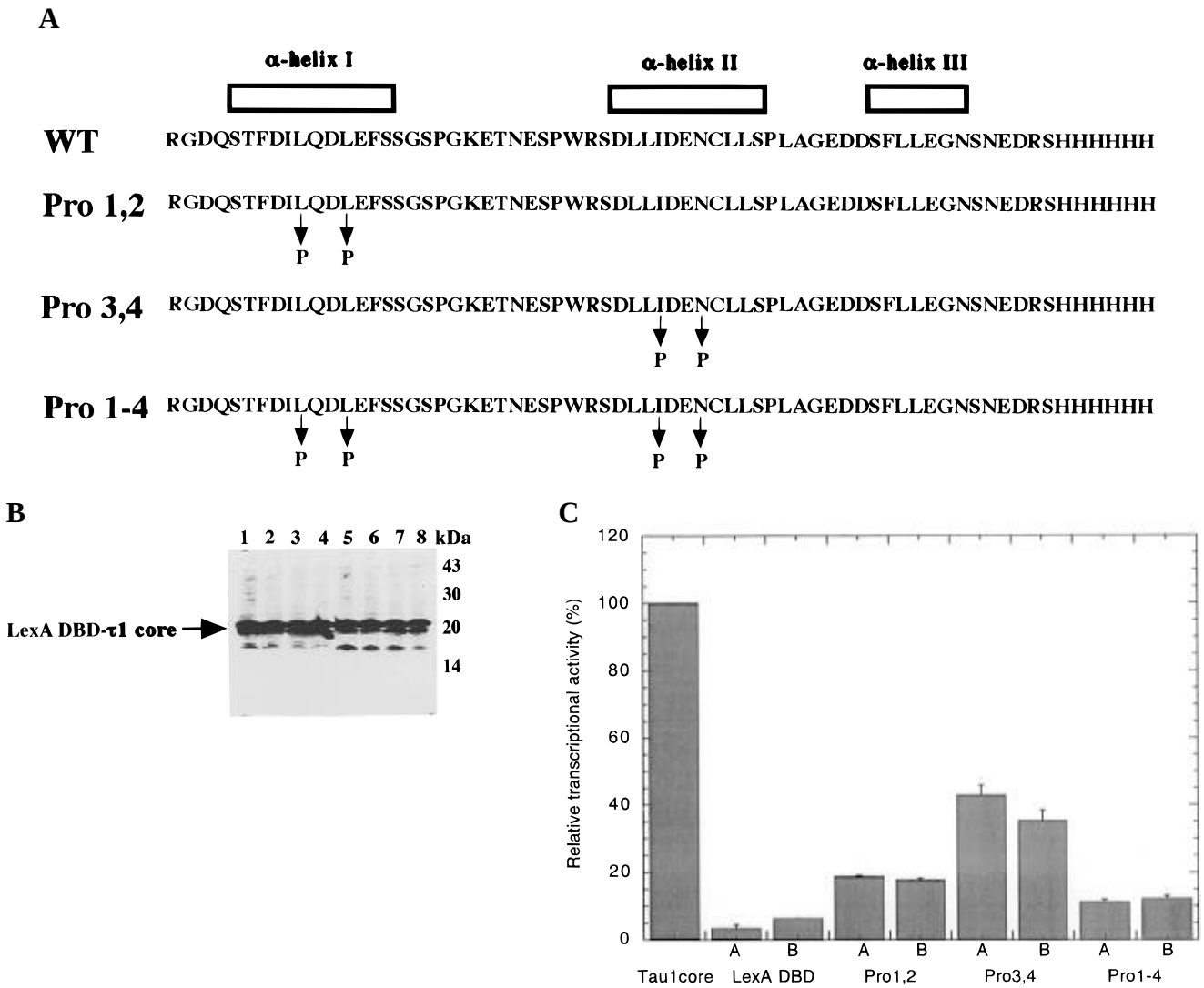

FIGURE 1: (A) The sequence encoding the τ1 core peptide is shown. The locations of α-helices identified by NMR spectroscopy (Dahlman-Wright et al., 1995) have been indicated as have the position of the various proline substitution mutations investigated here. (B) Western blot showing the expression and stability of various τ1 core−LexA DBD peptides in yeast cells; lanes 1 and 2, WT−LexA DBD; lanes 3 and 4, Pro 1,2−LexA DBD; lanes 5 and 6, Pro 3,4−LexA DBD; lanes 7 and 8, Pro 1−4−LexA DBD. In each case two independent transformants were analyzed. Blots were probed with anti-LexA DBD antibodies. The position of expressed fusion proteins is indicated by an arrow. (C) Results from transactivation assays of various τ1 core−LexA DBD fusion proteins and the isolated LexA DBD are shown relative to that of the fusion protein including the wild type τ1 core protein. In each case, the same two transformants as in B were analyzed. The mean and standard deviation ($n = 3$) are shown.

in transactivation activity it was also possible that the reduced activity resulted from destabilization of the protein leading to more rapid turnover and consequently lower expression levels. Figure 1B shows that wild type and proline-substituted τ1 core−LexA DBD fusion proteins are expressed at equal levels in yeast cells, and thus they appear to be equally stable. Figure 1C shows that the activity of the expressed proteins in the cells used for the immunoblots is indeed reduced as expected from previous results. Therefore we conclude that the proline substitutions primarily affect the specific transactivation activity of the mutant proteins.

Purification of Wild Type and Mutant Transactivation Domains. Since reduced transactivation activity results from reduced specific activity of the fusion proteins, we wanted to test directly whether the reduced transactivation potential *in vivo* correlated with reduced α-helix-forming potential of the corresponding peptides *in vitro*, thus establishing a link between transactivation capacity and α-helix-forming potential. In order to achieve this, wild type and mutant τ1 core proteins were expressed in *E. coli* fused to six C-terminal histidine residues. Recombinant proteins were purified by nickel affinity chromatography and gel filtration. The purified proteins were estimated to be at least 95% pure as estimated from Coomassie staining of SDS−polyacrylamide gels (Figure 2).

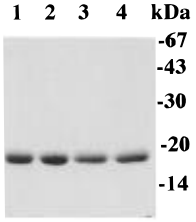

FIGURE 2: Coomassie staining of an SDS−PAGE gel showing the purified τ1 core peptides analyzed in this report: lane 1, the wild type peptide; lane 2, Pro 1,2; lane 3, Pro 3,4; lane 4, Pro 1−4.

Proline Substitutions Severely Reduce the α-Helix-Forming Potential of the τ1 Core. CD spectroscopy can be used to determine the overall content of secondary structure in

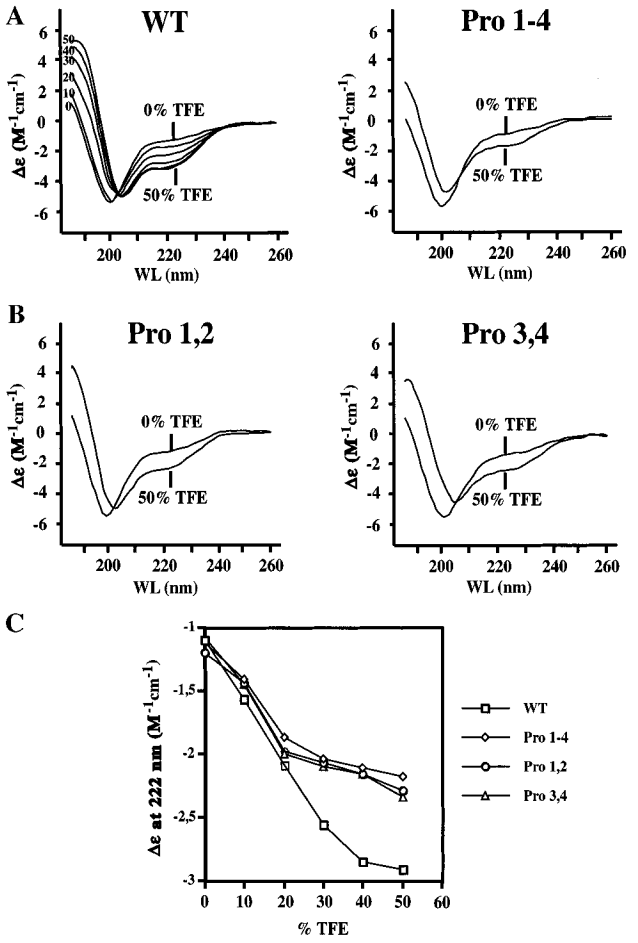

FIGURE 3: (A) CD spectra of the wild type τ1 core peptide and the Pro 1−4 peptide at various TFE concentrations. For clarity, only the spectra in 0% and 50% TFE are shown for Pro 1−4. (B) CD spectra of the Pro 1,2 and Pro 3,4 peptides at various TFE concentrations. For clarity, only the spectra in 0% and 50% TFE are shown. (C) The CD signal at 222 nm is given as a function of the TFE concentration.

peptides (Johnson, 1990). We have previously used this technique to show that the τ1 core peptide is largely unstructured in aqueous solution but that it acquires significant α-helical structure in the α-helix-promoting solvent TFE (35% α-helix at 50% TFE; Dahlman-Wright et al., 1995). There was no evidence of any other stable secondary structures (Dahlman-Wright et al., 1995). Figure 3A shows CD spectra of the wild type τ1 core protein and the Pro 1−4 mutant protein, with four proline substitutions, at various TFE concentrations. As previously described, the spectra of the wild type τ1 core peptide in aqueous solution displays the features of a random coil configuration with a minimum at 197 nm (Dahlman-Wright et al., 1995). Increasing TFE concentrations induce α-helical structures as evident from the maximum at 190 nm and the minima at 208 and 220 nm, characteristic of α-helical structures (Dahlman-Wright et al., 1995). As expected, the CD spectra of the Pro 1−4 mutant τ1 core peptide, with proline substitutions in both of the first two α-helices, shows that the peptide is also largely unstructured in aqueous solution. However, this peptide remains relatively unstructured even at high TFE concentrations (Figure 3A). Thus introduction of proline residues into the regions with propensity to form α-helices strongly reduces secondary structure formation in the presence of TFE.

Loss of Transactivation Activity Correlates with Reduced Secondary Structure Formation in Proline-Substituted Mutants. To determine further whether there is a correlation between loss of transactivation activity and reduced secondary structure formation potential, the intermediate mutants, Pro 1,2 and Pro 3,4, were studied. These proteins also acquire a low level of α-helical structure in TFE consistent with them carrying proline substitutions in only one α-helix (Figure 3B). The CD signal at 222 nm can be used as a measure of the α-helical content of a peptide, particularly if its secondary structure is a mixture of random coil and α-helix, since in this region of the spectrum the signal derived from random coil conformations is negligible. This applies to the τ1 core peptide (see above). Figure 3C shows a clear correlation between the number of proline substitutions and the α-helix-forming potential of the various τ1 core peptides in this study. A comparison of Figures 1C and 3C shows a clear correlation between transactivation activity *in vivo* and the α-helix-forming potential *in vitro* for various derivatives of the τ1 core transactivation domain.

DISCUSSION

This study has addressed two important points: First, how selective is the capacity of TFE as an α-helix-inducing solvent? Second, is there a correlation between the transactivation activities of eukaryotic transactivation domains *in vivo* and the ability for secondary structure formation *in vitro*? Figure 3A shows that introduction of four proline

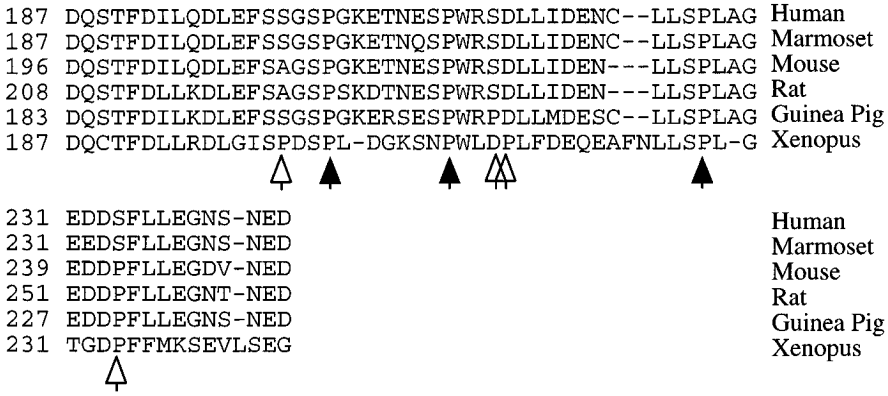

FIGURE 4: Shown is a comparison of the amino acid sequence corresponding to the hGR τ1 core from human, marmoset, mouse, rat, guinea pig, and xenopus GR. Human GR (Hollenberg et al., 1985); marmoset GR (Brandon et al., 1991); mouse GR (Danielsen et al., 1986); rat GR (Miesfeld et al., 1986); guinea pig GR (Keigthley & Fuller, 1995); xenopus GR (Gao et al., 1994). Black arrows indicate proline residues in the human GR. White arrows indicate additional proline residues in other GRs.

substitutions into a 70-amino acid fragment including the τ1 core transactivation domain from the hGR almost abolishes the ability of the peptide to acquire α-helical structure in TFE, demonstrating a considerable specificity in the α-helix-inducing capacity of TFE. Comparison of Figures 1C and 3C shows that the reduced activation potential of proline-substituted τ1 core transactivation domains correlates with the potential of these peptides for α-helical formation *in vitro*, indicating that α-helical structures are very likely to be involved in the mechanism of τ1 core mediated transactivation.

A comparison of the hGR τ1 core amino acid sequence with the corresponding sequence for five other species reveals that proline residues are present at four additional positions. Interestingly, these proline residues are always outside or at the borders of identified α-helices (Figure 4), consistent with selection against α-helix-breaking proline residues in the three identified α-helical segments.

Our knowledge about the structure of transactivation domains when not involved in the process of transcriptional activation as well as possible structural changes associated with this process remains limited. The lack of transactivation domain structure when not involved in transcriptional activation would present some potential advantages. (i) Nonproductive interactions of transactivation domains with target factors would be minimized if transactivation domains were only to be structured upon interactions with target factors in functional initiation complexes. (ii) An unstructured transactivation domain could provide more flexibility. Several laboratories, including our own, have presented evidence that isolated transactivation domains are unstructured in aqueous solution but that they have a potential for structure formation. To our knowledge this is the first study addressing the *in vivo* significance of structural elements for the activity of transactivation domains. However, further studies of transactivation domain target factor complexes will clearly be required to firmly establish that the various structures acquired by transactivation domains in various solvents or at nonphysiological pH reflect structures involved in the mechanism of transcriptional activation. For the GR τ1 core domain, as for other transactivation domains, the search for putative target factors is in progress.

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