Helix 2 of the paired domain plays a key role in the regulation of DNA-binding by the Pax-3 homeodomain

Anouk S. Fortin, D. Alan Underhill and Philippe Gros*

Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

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ABSTRACT

Pax3 contains two structurally independent DNA-binding domains, a paired domain (PD) and a homeodomain (HD). Biochemical and mutagenesis studies have shown that both domains are functionally interdependent. In particular, it has been shown that the PD can regulate the DNA-binding specificity and dimerization potential of the HD. To delineate Pax3 protein segments that are involved in the regulation of HD DNA-binding, a series of chimeric proteins were created in which the HD and linker region were gradually replaced with corresponding sequences from a heterologous HD protein, Phox. Characterization of chimeric proteins by electrophoretic mobility shift analysis (EMSA) suggests that a portion of the linker region contributes to the functional interaction between the PD and HD. In addition, stepwise removal of sequences from the Pax3 PD was used to define regions within this domain that are involved in the regulation of HD DNA-binding. EMSA of these proteins in the context of the chimeric Pax3/Phox backbone provided two key findings: (i) the C-terminal subdomain of the PD does not play a major role in the regulation of HD DNA-binding and (ii) the N-terminal subdomain and, in particular, the second α-helix are essential for modulation of HD DNA-binding. Significantly, deletion of helix 2 was found to be sufficient to uncouple regulation of HD DNA-binding by the PD.

INTRODUCTION

Pax3 belongs to the mammalian Pax family of transcription factors, which is composed of nine members that are structurally defined by the presence of a unique DNA-binding domain known as the paired domain (PD) (1). Members of the Pax family are expressed throughout embryogenesis and play key regulatory roles in a number of developmental processes during mammalian development (1). Specifically, expression of Pax3 in the neural tube, neural crest and in migrating myoblasts had suggested an important role for this transcription factor in neurogenesis and myogenesis (1). This has been confirmed with the discovery that the Pax3 gene is mutated in independent alleles of the mouse Splotch mutant (2–4). Homozygous Splotch mice suffer from a complete absence of limb musculature, and show severe neural tube defects and neural crest cell deficiencies that together lead to embryonic lethality by midgestation (5–8). Mutations in the human PAX3 homologue are associated with Waardenburg syndrome (9), a condition characterized by pigmented disturbances, dystopia canthorum and sensorineural deafness (10).

In addition to the PD, the Pax3, 4, 6 and 7 proteins also possess a second DNA-binding domain, the paired-type homeodomain (HD) (1,11). The two DNA-binding domains are separated by a linker segment that contains a highly conserved octapeptide motif of unknown function. Finally, transcriptional regulatory domains are located at the C-terminus of the protein (12). The high resolution crystal structures of the PD and HD have been solved for the closely related Drosophila Paired protein (13,14). The PD is a bipartite DNA-binding domain that contains two structurally independent subdomains (PAI and RED), each comprising three α-helices that can form a helix–turn–helix motif and can act either independently or cooperatively in the recognition of DNA. The paired-type HD also comprises three α-helices that form a helix–turn–helix motif which is characterized by a serine residue at position 50 (14). Interaction of the HD with DNA occurs principally through the N-terminal arm and the third helix which makes base-specific contacts in the major groove (14). The paired-type HDs are characterized by their ability to cooperatively dimerize on palindromic sites of the type 5′-TAAT(N)₂-3ATT₃-3′ (15).

Although the PD and HD can bind to their target DNA sequences in isolation, both domains appear functionally interdependent when present in the same molecule and can interact cooperatively in DNA binding. Indeed, composite binding sites for the PD and HD of Drosophila Paired have been identified by oligonucleotide selection procedures (16) and also through the analysis of target promoters in vivo (17). In the case of Pax3, characterization of the Splotch delayed (Sp³) mutation (G42R) revealed that a mutation within the PD can abrogate DNA-binding by the HD (18), suggesting that the domains may functionally interact. This notion was supported by the systematic analysis of naturally occurring Waardenburg syndrome mutations that are distributed throughout the PD where a strong correlation was seen between PD and HD loss of DNA-binding (19). Furthermore, this effect was shown to be reciprocal with the demonstration that a WS mutation in the HD could reduce DNA-binding by the PD (19).

Lastly, we have previously shown that the PD can modulate the sequence specificity of the Pax3 and Phox HDs by limiting their ability to dimerize on palindromic recognition motifs with a 3 bp spacer [TAAT(N)₂ATTA] (20).

In the present study, the ability to transfer this functional interdependence to the Phox HD has been exploited to identify protein segments within Pax3 that sustain this interaction. The PD and HD DNA-binding properties of chimeric proteins were assessed by EMSA either in the presence of an intact PD or in a

*To whom correspondence should be addressed. Tel: +1 514 398 7291; Fax: +1 514 398 2603; Email: gros@med.mcgill.ca

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series of deletion constructs which removed progressively larger portions of the PD. Our results establish a critical role for helix 2 of the PD in the regulation of HD DNA-binding.

MATERIALS AND METHODS

Plasmid construction

The construction of the pMT2 expression plasmids encoding wild type (wt) Pax3 and Pax3ΔPD has been previously described (18). The wt Pax3 construct encodes the full length 479 amino acid Pax3 polypeptide while the Pax3ΔPD construct lacks an Xmal restriction fragment (positions 342–674 in the Pax3 cDNA) which encodes amino acids 17–126 of the Pax3 protein and includes the first 92 residues of the PD. The pCGNPhox expression plasmid (21) contains a cDNA fragment which encodes the 217 amino acid Phox polypeptide (22) and a 21 amino acid N-terminal HA epitope (MASSYPYDVPDY-ASLGPPRSM) (23). Chimeric constructs (χ) that contain the Pax3 PD fused to Phox were made by inserting Pax3 cDNA sequences in pCGNPhox. χ1 (20) codes for a polypeptide where residues 1–87 of Phox are replaced by amino acids 18–230 of Pax3. χ2 was produced by recombinant PCR amplification, as follows: the Pax3 segment (nucleotides 539–947) was amplified using the Pax3 oligonucleotide P3I (positions 539–558, 5′-GGTGT-CAGGCCTCTG-3′) and the chimeric oligonucleotide Pax3χ2 (5′-CTGTTTTCTCTTCAGGCCG-3′) which contains sequences derived from Pax3 (positions 937–947) and Phox. The Pax3 portion was amplified involved the introduction of a novel ClaI restriction site (bold) and Phox (underlined, positions 222–228). The Phox segment (nucleotides 217–426) was amplified using oligonucleotides Phox 885–908, 5′-GAGAGAAAGAGCCCG-3′ and PhoxNhel (positions 410–426, 5′-TTATTTGGCGTAGCATGG-3′) which spans an Nhel restriction site (bold) in the Phox cDNA. The amplification products were gel purified, annealed through their complementary ends, and used in PCR with oligonucleotides P3I and PhoxNhel. The resulting product was gel purified, digested with KpnI (Pax3 position 563) and Nhel (Phox position 414) and used to replace the corresponding restriction fragment in χ1. χ3 was the product of a 3-way ligation that made use of the existing Pax3 KpnI and Phox Nhel restriction sites in χ1 and involved the introduction of a novel BspEI site in both Pax3 and Phox by PCR mutagenesis (24). The Pax3 portion was amplified using oligonucleotides P3I and Pax3BspEI (Pax3 positions 885–908, 5′-CCTGGTCCTCCCGAGTCAGATGAGG-3′) which introduces a BspEI restriction site (bold). Similarly, the Phox segment (positions 172–426) was PCR amplified using oligonucleotides PhoxBspEI (positions 172–186, 5′-CCTCTGCCGGAGTCAGATGAGG-3′) to create the BspEI site (bold), and the PhoxNhel oligonucleotide. For χ4, the Pax3 segment (positions 138–426) was amplified with oligonucleotides PhoxClaI (positions 138–156, 5′-GGATGCTACGAGATGCTCCGGAGA-CTC-3′), to introduce a ClaI site (bold), and PhoxNhel. The amplification product was purified and digested with ClaI and Nhel and ligated to replace the equivalent fragment in χ1 (ClaI site at position 859 in Pax3). χ5 was produced by PCR amplification of a Pax3 segment (positions 296–748) using the oligonucleotide Pax3ΔβI (positions 296–320, 5′-GGATGACCACCGCTGGCGCTGTG-3′) and the Pax3BspI oligonucleotide (5′-AACTG-CAGGGTCTGCTGCAGACCAGTCGTTCC-3′) which is complementary to positions 732–748 in the Pax3 cDNA and introduces a BspI restriction site. The amplification product was digested with Xmal (position 342) and PstI and ligated to replace the Xmal–PstI fragment in pCGNPhox (positions 9–28). A Phox PstI–PstI fragment (positions 28–262) was subsequently reintroduced to restore the complete open reading frame. χ6 was produced by PCR amplification of a Pax3 segment (positions 942–1138) using oligonucleotides Pax3BspEI (5′-AAATCGAGACCGAGAGCCGCG-CAGGCAATGACCAGGTGAACTCAGAAGAA-AAAAGAAGAGGAAGGCGCGACGCGAGG-3′), containing both Pax3 (positions 942–960) and Phox sequences (underlined, positions 171–216), and which creates a BspEI site (bold). The second oligonucleotide was Pax3Nhel which contains Pax3 sequences (positions 1129–1138, 5′-TTATTTGCGTAGC-A-CTGCCCAGCTGGGTGTTTTCCT-3′) and Phox sequences (underlined, positions 409–422), and includes a Nhel site (bold). The resulting PCR product was gel purified, digested with BspEI (introduced at Phox position 175) and Nhel (Phox position 414) and ligated to replace the equivalent fragment in χ3. For χ7, the Pax3 segment (positions 985–1138) was amplified with oligonucleotides Pax3χ7 (5′-TACGGCCACGCTGGGAGCTCGG-3′) containing Pax3 (positions 985–999) and Phox sequences (underlined, positions 252–257) and Pax3Nhel. The chimeric segment was amplified from χ7 with oligonucleotides P3I and Phoxχ7 (Phox positions 246–267, 5′-CTTCCAGTCGCTATGGAAG-3′). These PCR products were gel purified and annealed, followed by amplification of the resulting chimeric segment with oligonucleotides Pax3I and Pax3Nhel. The resulting product was restriction digested with KpnI (Pax3 position 563) and Nhel (site introduced in oligonucleotide Pax3XmaI and ligated to replace the corresponding fragment in χ1.

N-terminal deletions in the PD (constructs χ1 or χ3ΔA18-48, Δ18-66, Δ18-77, Δ18-95 and Δ18-112) were made by replacing the Xmal fragment in Pax3 (positions 342–672) by PCR amplification products in which a 5′ Xmal site has been introduced at nucleotide 439, 492, 525, 578 or 631. PCR amplifications were carried out using the 5′Xmal oligonucleotide (positions 667–686, 5′-CTAAACATGCCCGGCTCTTC-3′) and one of the following oligonucleotides in which the Xmal site is underlined:

\[\Delta\beta\] (positions 439–457, 5′-TAATACCGGGGACGCGCTCTGTG-3′),

\[\Delta\beta\] (positions 492–506, 5′-TAATACCGGGGACGCTGTCACGG-3′),

\[\Delta\beta\] (positions 525–539, 5′-TAATACCCGGGACGCTGTTCTGTG-3′),

\[\Delta\beta\] (positions 578–593, 5′-TAATACCCGGGACTCTGACCCGAC-3′),

\[\Delta\beta\] (positions 631–645, 5′-TAATACCCGGGACTCTGACCCGAC-3′). Internal deletions in the PD (constructs χ1 and χ3ΔA13-125, Δ15-125, Δ66-125 and Δ48-125) were made by replacing the Xmal fragment in Pax3 (positions 342–672) by PCR amplification products in which a 3′ Xmal site has been introduced at nucleotide 636, 580, 493 or 440. PCR amplifications were carried out using the 5′Xmal oligonucleotide (positions 340–357, 5′-GACGACGGCGGACGAGATAC-3′) and one of the following oligonucleotides in which the Xmal site is underlined:

\[\Delta\beta\] (positions 622–636, 5′-TAATACCGGGGACGCTGTTCTCG-3′),

\[\Delta\beta\] (positions 568–580, 5′-TAATACCCGGGACTCTGACCCGAC-3′).
Δ23L4 (positions 480–493, 5′-TATATACCGGCCCATGGTG- GGCCATC-3′),
Δ123L4 (positions 427–440, 5′-TATATACCGGGCCGCTTAGA- TAAATAC-3′).

The Pax3G42RA65-79 construct was generated by deleting a NcoI fragment (positions 489–534) from the pMT2Pax3 ΔP (20).

Expression and detection of the Pax3 proteins in COS-7 cells

Transient transfection of individual pMT2 constructs into COS-7 monkey cells was carried out as previously described (18,25).

Whole cells extracts were prepared 24 h after transfection by sonication in an ice-cold buffer (250 µl/10^6 cells) containing 20 mM HEPES (pH 7.6), 0.15 M NaCl, 0.5 mM dithiothreitol (DTT), 0.2 mM EDTA, 0.2 mM EGTA and protease inhibitors (aprotinin, pepstatin and leupeptin at 1 µg/ml and phenylmethylsulfonyl fluoride at 1 µM), and were stored at –80°C until use. Protein expression was monitored by western blotting, using a monoclonal anti-HA antibody (BabCO) at a 1:5000 dilution and visualized by enhanced chemiluminescence using a sheep anti-mouse horseradish peroxidase conjugated secondary antibody (Amersham).

Electrophoretic mobility shift assays (EMSA)

Double stranded oligonucleotides Nf3′ (5′-CTAGTGTTGTCATG- ACGTATTAAAAATTGATTAGTAGTTGAG-3′) (26), P2′ (5′-GAT- CCTGAGTATTGATTGACTGAGG-3′), P1/2′ (5′-GAT- TCCTGAAGTCAATTGAGCGTGCTGTA-3′) (15) and P3′ [5′-(N11)TAAATTGAGT(N8)N3′]-TAGTAATGTGTGTC-3′ (15) were end labeled with [α-^32P]dATP (3000 Ci/mmol, New England Nuclear) using the Klenow fragment of DNA polymerase I. Binding reactions were carried out for 30 min at 20°C, in a 20 µl volume containing 5 fmol of radiolabeled probe, 10 mM Tris pH 7.5, 2 mM MgCl2, 50 mM NaCl, 1 mM EDTA; 1 mM DTT; 5% glycerol, whole cell extract and a non-specific competitor: 1 μg of poly[(dI-dC):(dI-dC)], 2 μg of salmon sperm DNA for HD specific probes (P2, P1/2, P3). Samples were electrophoresed at 12 V/cm in 6% polyacrylamide gels containing 0.5× TBE (1× TBE is 0.18 M Tris–HCl, 0.18 M boric acid and 2 mM EDTA). Gels were dried and exposed to Kodak XAR films or to a phosphorimaging plate for quantitation with a Fuji BAS 2000 phosphorimaging station.

RESULTS

The major structural features of Pax3 (Fig. 1A) include two DNA-binding domains, the PD and the paired type HD which are separated by a 56 amino acid linker segment. Although the PD and HD are highly conserved amongst Pax proteins, the linker domain joining the two is poorly conserved, with the exception of an octapeptide motif. We have reported that the PD and HD of Pax3 are functionally interdependent and showed that point mutations in each domain have reciprocal effects on DNA binding by the other domain (18–20). Moreover, the PD of Pax3 can modulate in cis the DNA-binding activity of a heterologous HD such as that of Phox (20).

The HDS of Phox and Pax3 share 67% sequence identity but a glutamine (Phox) and a serine (Pax3) at position 50 impart different DNA-binding specificities on these HDS. Pax3 cooperatively dimerizes on palindromic recognition sequences of the PD co-type [TAAT(N2)ATTA] but binds as a monomer to recognition sequences of the P3 type [TAAT(N3)ATTA] (Fig. 1C, lanes 1 and 3). In contrast, Phox binds to P2 probes as a monomer but dimerizes on P3 sequences (Fig. 1C, lanes 5 and 9). Two experiments indicate that the PD can modulate the P3-binding properties of the Pax3 and Phox HDs. Firstly, deletion of 92 amino acids in the N-terminal portion of the PD is sufficient to restore dimerization on P3 sequences (Fig. 1C; lane 4). Secondly, when the PD and linker sequence of Pax3 up to the first helix of the HD are fused to the HD of Phox, the resulting chimera (χ1) no longer dimerizes on P3 probes (Fig. 1C; lane 6) (20). Again, deletion of the PD restores the P3 dimerization potential of the heterologous HD (Fig. 1C; lanes 4 and 7). Finally, a mutation in the PD (ΔP′ G42R) which abrogates DNA-binding by the PD and HD in Pax3 (18) also causes loss of binding of the Phox HD in χ1 (Fig. 1C; lanes 8 and 12). Thus, the PD of Pax3 can modulate DNA-binding of homologous and heterologous HDs, when fused in the same molecule.

DNA-binding studies in vitro using the isolated PD and HD from Drosophila Prd (16) and in vivo transcomplementation studies (27) indicate that cooperative DNA-binding and functional complementation requires linkage of the PD and HD in the same molecule. To investigate a possible role of the linker segment (L) in HD/PD cooperative interactions, five chimeras were constructed in which the PD and portions of the linker domain of Pax3 were fused to the HD of Phox (Fig. 2A). The effect of these replacements on PD/HD interactions was monitored by measuring the HD DNA-binding properties of Phox. In chimera χ1, (positions 22–107 of Phox replaced by positions 18–230 of Pax3), the N-terminal arm of the HD from Pax3 is fused to the first α-helix of Phox HD. In chimera χ2, the PD and complete linker sequence of Pax3 are fused to the complete HD of Phox (positions 22–95 of Phox replaced by positions 18–218 of Pax3). In chimera χ3, an additional 18 residues N-terminal of the HD, up to but not including the conserved octapeptide, are from Phox (positions 22–77 of Phox replaced by positions 18–200 of Pax3). In chimera χ4, the conserved octapeptide motif is disrupted by replacement with Phox sequences (positions 22–65 of Phox replaced by positions 18–188 of Pax3). Finally, in chimera χ5, the entire linker is derived from Phox (positions 22–77 of Phox replaced by positions 18–150 of Pax3). In all chimeras, the spacing between the PD and HD was preserved at 56 residues. The chimeras were expressed in COS-7 cells by transient transfection and protein expression was monitored by western blotting (Fig. 2C). Similar amounts of protein were used in EMSA to monitor DNA-binding properties of the Pax3 PD and Phox HD, using Nf3′ and P3 oligonucleotides, respectively (Fig. 2B).

Chimeras χ1 and χ2, in which most or all of the HD is derived from Phox, both bound to P3 as monomers (Fig. 2B). Surprisingly, replacing the Pax3-derived 18 amino acid segment that immediately precedes the HD by its Phox counterparts in χ3 abolished DNA-binding by the HD. Replacement of additional Pax3 linker sequences by their counterpart in Phox (χ4 and χ5) did not restore DNA-binding by the HD (Fig. 2B). DNA-binding by the PD (Fig. 2B, Nf3′) was unaffected in all chimeras indicating that the inserted Phox sequences do not interfere with PD function, and suggesting that gross misfolding of the chimeric proteins is not responsible for the observed modulation of HD DNA-binding (Fig. 2B). Furthermore, changes in the composition of the linker sequence per se do not seem to affect HD DNA-binding properties as deletion of the PD in chimeras χ1 to χ5 (in constructs χ1 to χ5ΔPD, Fig. 2D) restores DNA-binding and dimerization of the Phox HD on P3 (Fig. 2E; lanes 4–9). Finally, a splotch mutation (G42R, ΔP′) that abrogates DNA-binding by the PD and HD in Pax3 also eliminates PD and HD DNA-binding in chimeras χ1, χ3 and χ4 (Fig. 2D and E). These results suggest that the Pax3 linker...
Figure 1. PD modulation of HD DNA-binding. (A) Schematic representation of the structural features of Pax3, based on the three dimensional structure of the PD and HD of the *Drosophila* Prd protein (13,14). The PD contains two subdomains (PAI and RED) each composed of three α-helices (numbered black boxes) and an N-terminal β-hairpin structure (β). The linker joining the PD and HD contains the conserved octapeptide (black box, OP). The HD contains an N-terminal arm (N) and three α-helices (numbered black boxes). (B) Schematic representation of Pax3 deletion and chimeric proteins analyzed for DNA binding. The numbers in plain text represent the amino acid position in Pax3, according to the sequence published by Goulding et al. (32). Italicized numbers represent amino acid positions in Phox (shaded area), according to the sequence published by Graeber et al. (21). (C) HD DNA-binding properties of Pax3, PD deletion of Pax3 (left panel), Phox, and Phox-Pax3 chimaera (right panel) by EMSA using the HD specific P2 and P3 oligonucleotides. The protein extracts used are indicated above the autoradiogram and the probe used is identified below. The location of monomeric (M) and dimeric complexes (D) as well as the free probe (F) are indicated by arrowheads.

**Figure 1.**

**A**

**PAIRED DOMAIN**

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\[\beta 1 3 2 4 5 6\]
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**HOMEODOMAIN**

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\[\text{OP} \text{N} 1 2 3\]
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**B**

<table>
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**C**

![Diagram showing EMSA results for Pax3, PD deletion of Pax3, Phox, and Phox-Pax3 chimaera](image)

(Please note that the actual image should be included here for a complete understanding.)

A segment is important to mediate the regulatory effect of the PD on HD DNA-binding. In the context of the Pax3/Phox chimeras tested here, its replacement by a heterologous sequence leads to loss of HD function.

To determine if the effect of the linker domain on HD binding detected in the Pax3/Phox chimeras (transition of χ2 to χ3) could be recapitulated in the otherwise intact Pax3 protein, two additional chimeras were constructed. A sequence alignment of the 18 amino acid segment of Pax3 and Phox located N-terminal to the HD (Fig. 3A) reveals that this region is not conserved, although its highly charged nature is preserved, a typical structural feature of flexible solvent exposed linker domains. The adjacent N-terminal arm and first few residues of the first α-helix of Phox and Pax3 HD are identical at 12/16 positions. In chimera χ6, the divergent 18 residue segment N-terminal to the HD is derived from Phox (Pax3 residues 201–219 replaced by Phox residues 77–95), while in chimera χ7, both the 18 residue segment and the conserved N-terminal arm of the HD are from Phox (Pax3 residues 201–231 replaced by Phox residues 77–107). These constructs were expressed in COS-7 cells (Fig. 3B) and 2-fold protein dilution series were used in EMSA to monitor DNA-binding by the PD (Nf3′) and HD (P2 and P3) (Fig. 3D). χ6 and χ7 were found capable of binding both P2 and P3 oligonucleotides as dimers and monomers, respectively. However, compared to wild type Pax3, χ6 and χ7 showed reduced affinity for both P2 and P3 by a factor of 2–4× as determined in competition assay and Scatchard analyses (Fig. 3E), indicating that this portion of the linker region does influence the functional interaction between the PD and HD. As seen with the other chimeras, χ6 and χ7 continue to bind the PD specific probe Nf3′ with characteristics similar to wild type Pax3 (data not shown). The lack of dimerization of χ6 and χ7 on P3 oligos (Fig. 3C) also suggests that the 18 residue linker and N-terminal HD segment of Phox are not sufficient to uncouple the PD regulation of HD DNA-binding. In addition, comparing the reduction in HD binding of χ6 and χ7 to the loss of Phox HD binding detected in χ3, χ4 and χ5 would suggest that additional sequences in the HD are important for regulation of HD DNA-binding by the PD.

Although mutagenesis studies have clearly established that functional interactions between the PD and the HD take place in the intact Pax3 protein (18–20), the discrete structural domains of the PD responsible for modulation of HD DNA-binding have not been identified. To identify such segments, we created two series of bidirectional deletions in the PD, in the context of Pax3/Phox chimera χ1 and χ3. We monitored the effect of such deletions on two distinct DNA-binding properties of the fused Phox HD (Fig. 4A) by using P3 and P1/2 target DNA sequences. P1/2 is a HD half site that contains only one TAAT recognition sequence and does not allow HD dimerization (15). In a series of N-terminal deletions, segments including the β-hairpin structure (χ1Δ18–48), the first α-helix (χ1Δ18–66), the second α-helix (χ1Δ18–77), the third α-helix (χ1Δ18–95) and the inter-subdomain linker (χ1Δ118–112) of the PD were deleted. In a second set of internal deletions of χ1, segments including helix 4 (χ1Δ113–125), the inter-subdomain linker and helix 4 (χ1Δ95–125), helix 2 to helix 4 (χ1Δ66–125), and helix 1 to helix 4 (χ1Δ48–125) were deleted (Fig. 4A). Transfected COS-7 cell extracts expressing similar amounts of recombinant protein (Fig. 4B) were used for EMSA analysis. Using the PD specific probe Nf3′, it was observed that all PD deletions resulted in loss of PD DNA-binding, with the exception of the construct in which the fourth helix alone had been internally deleted (χ1Δ113–125) (Fig. 4B; second panel). When the N-terminal
deletions (Δ18-48 to Δ18-112) were assayed for HD DNA-binding with the P3 probe (Fig. 4B; lower left panel), we observed that deletion of the β-hairpin structure, up to Gly 48 (χ1Δ18-48), or the β-hairpin structure and helix 1 (χ1Δ18-66) restored some of the Phox HD dimerization potential on P3 by the χ1 chimera. However, the most striking finding was that deletion of helix 2 (χ1Δ18-77) restored dimerization of the Phox HD on P3 to levels observed in the control APD construct (Fig. 4B). The quantitative effects of these N-terminal deletions on Phox HD DNA-binding were easily seen when binding was assessed with the P1/2 probe (Fig. 4, third left panel). Indeed, deletion of the β-hairpin structure and helix 1 of the PD had little effect on DNA-binding to P1/2, while deletion of helix 2 in χ1Δ18-77 caused a dramatic increase in the monomeric binding to this probe. Further deletion of helix 3 and the linker between the PAI domain and the specific requirement for helix 2 in the modulation of HD DNA-binding (Fig. 4A and C). Analyses of the HD DNA-binding activity of such internal deletions (Fig. 4B and D; right panels) also pointed at the critical role of the PAI domain. Deletion of helix 4, or helix 4 and the linker (Δ113-125 and Δ95-125), produced proteins that bound P3 with characteristics similar to the full length parental chimeras. In the case of chimera χ1, monomeric binding to P3 (albeit at a reduced level) was retained for χ1Δ113-125 and χ1Δ95-125 (Fig. 4B; lower right panel, compare lanes 9 and 10 to lane 8), while in the case of chimera χ3, no significant binding to P3 was observed for χ3Δ113-125 and χ3Δ95-125 (Fig. 4D; lower right panel, lanes 9 and 10). Further deletion of helix 2 and 3 in the background of χ1 and χ3Δ66-125 restored DNA-binding and dimerization on P3 to levels seen in the control APD constructs (Fig. 4B and D; compare lanes 11 and 12 to lane 13). Similar results were obtained when the χ1 and χ3 deletion constructs were assayed for binding to the P1/2 half site HD probe: deletion of helices 2 and 3 was sufficient to restore DNA-binding to P1/2 (Fig. 4B and D). These results suggest that helix 2 and 3 of the PAI subdomain are essential for PD mediated modulation of HD DNA-binding.

Finally, the critical role of helix 2 in the PD-mediated regulation of HD DNA-binding was further tested in the following manner.
A mutant Pax3 protein which bears the G42R substitution abrogates DNA-binding of the PD and HD to target sequences this mutant (Pax3Δ encoding helix 2). Analysis of the DNA-binding characteristics of was further altered by internal deletion of a small segment that deletion of helix 2 restored DNA binding by the HD which results further sustain the contention that helix 2 plays a key role in the PD mediated regulation of DNA-binding of homologous (Pax3) and heterologous (Phox) HDs.

DISCUSSION

The Pax family of transcription factors is defined by the presence of a highly conserved DNA-binding domain, the PD (1). The mammalian Pax3, 4, 6 and 7 proteins, like Drosophila prd and gsb, contain a second DNA-binding domain, the paired-type HD (1). The presence of either DNA-binding domain in a number of polypeptides and the characterization of their individual DNA-binding properties (15,28) and structure (13,14) have clearly established the ability of these two domains to bind DNA in isolation. In contrast, their presence together in many proteins suggests that the PD and HD may cooperate in the recognition of DNA sequences. Furthermore, it was shown that a heterologous HD can be rendered dependent on the PD (20 and Fig. 1) through the modulation of its substrate specificity and dimerization potentials. In this study, we have attempted to identify regions of Pax3 polypeptide that contribute to the functional interaction between the PD and HD. For this, we have created a series of chimeras and deletion mutants in which various portions of the Pax3 PD were fused to the heterologous HD of Phox. The effect of such alterations on the DNA-binding properties of the PD and HD were monitored using oligonucleotides specific for each domain.

We first assessed the role of the linker region in PD/HD interactions. This rationale derives from the study of POU proteins, which contain a POU-specific domain and a POU HD, and where the sequence separating these two DNA-binding domains was found to influence DNA-binding specificity (29). In the case of Oct-1, both the length and composition of its interdomain linker have been shown to affect DNA-binding affinity and specificity, in addition to contributing to cooperation between the two domains by tethering (30). Therefore, to determine if the composition of the linker is important for the modulation of HD DNA-binding activity by the Pax3 PD, we created chimeras in which the HD and various portions of the linker region were derived from the Phox protein (Fig. 2A). In the full length constructs, any changes in the linker sequence resulted in loss of HD binding while replacing the Pax3 HD N-terminal arm sequences by the homologous Phox sequences had no effect (Fig. 2B). Changes in linker sequences had no effect on DNA-binding by the PD (Fig. 2B; left column) nor on the activity of the Phox HD in the absence of the PD (Fig. 2E). This loss of HD DNA-binding suggests that the linker composition is important for the PD modulation of HD DNA-binding. Significantly, when the chimeric linker is present in the wt Pax3 background, DNA-binding by the Pax3 HD was also affected, as evidenced by a 2- and 4-fold decrease in the level of binding to the P2 and P3 oligonucleotides, respectively (Fig. 3E). Together, these results demonstrate that linker region plays an important role in the functional interaction between the PD and HD.

The fact that the Pax3 HD continues to bind the P3 probe as a monomer (Fig. 3C) indicates that this change in linker sequence is not sufficient to uncouple the regulation of HD DNA-binding by the PD. This is consistent with observations made for the Drosophila Prd protein where it was shown that the PD and HD retain a portion of their cooperative binding to composite sites that contain recognition motifs for both domains, even when expressed as separate polypeptides. Consequently, tethering is only partly responsible for the cooperative interactions between these two domains (16). These data would therefore suggest that
Figure 4. Effects of PD deletions on HD DNA-binding properties. (A) Schematic representation of the different PD deletion constructs made in the χ1 chimera backbone. Shaded areas correspond to Phox derived protein segment, and Pax3 amino acid residues forming the boundaries of the deleted segment are identified. (B) Immunodetection and DNA-binding properties of the χ1 deletion constructs. (Top panel) immunodetection in whole cell extracts of the different constructs with a monoclonal anti-HA antibody. (Second panel) EMSA with the PD specific Nf3′ probe. (Third panel) EMSA with the HD specific half site probe P1/2. (Bottom panel) EMSA with the HD specific probe P3. Location of the monomeric (M) and dimeric (D) complexes as well as the free probe (F) is indicated by arrowheads. Asterisk indicates partial proteolysis degradation complex.

other determinants are present within the PD and HD themselves that support cooperative interactions.

Independent studies have underlined the crucial importance of the PAI subdomain of the PD in DNA-binding properties of Pax proteins and suggest that it is responsible for the modulation of HD DNA-binding. For example, mutagenesis studies of Pax3 have suggested that a clustered series of phosphate and non-base specific contacts made by the PAI subdomain are essential for both PD and HD DNA-binding (19). In addition, in vivo rescue and transcomplementation experiments (15,31) have shown that both the PAI subdomain and the HD are absolutely required for Prd function, while the RED subdomain was shown to be dispensable. The present deletion studies extends these observations and further defines the PAI subregion as an important regulator of HD DNA-binding. Although internal deletions may affect non-specifically the DNA-binding properties and three dimensional structure of the Pax3 PD, independent deletion series in this study point at a critical role of helix 2 of the PD in regulating HD DNA-binding. Deletions were made from the N-terminus of the PD to probe the importance of the β-hairpin, each of the PAI subdomain helices and the linker between the two subdomains in modulating HD DNA-binding (Fig. 4A). To confirm the results obtained with these constructs and determine if the spacing between the different structures is important, we characterized the effect of internally deleting the same PD structures. Both sets of deletions were made in the chimeric backgrounds of χ1 and χ3. The χ1 construct has lost the ability to dimerize on P3 while the χ3 construct no longer binds DNA through its HD. Removal of the β-hairpin structure and of helix 1 of the PD did not appear to have a major effect on HD DNA-binding (Fig. 4B and D; lanes 2, 3, 11 and 12). Remarkably, the deletion of helix 2 in both the χ1 (Fig. 4B; lane 4) and χ3 (Fig. 4D; lane 4) backgrounds restores dimerization of the Phox HD to the P3 oligonucleotide. Additional deletion of helix 3, the subdomain linker and helix 4
Figure 5. The second α-helix of the PD modulates HD DNA-binding. (A) Schematic representation of wild type Pax3. The G42R Pax3 mutant (Pax3G42R; Spd) and the G42R Pax3 mutant bearing a deletion of the second α-helix of the PD. Arrow indicates location of the G42R mutation. (B) EMSA of the three proteins with the HD specific probe P2. Location of the dimeric complex (D) and free probe (F) is indicated with arrowheads. The asterisk indicates degradation product.

had no further effect on HD binding properties (Fig. 4B and D; lanes 5, 6, 7, 11, 12 and 13). Consistently, the modulation of HD DNA-binding by the PD is seen for every construct in which helix 2 is present in both chimeric backgrounds (Fig. 4B and D; lanes 1, 2, 3, 8, 9 and 10), indicating that helix 2 plays a key role in the modulation of HD DNA-binding. Moreover, its relative position with respect to the HD can be altered, at least by 30 residues, without affecting cooperative interactions between the two DNA-binding domains (internal deletion constructs, Fig. 4B and D; lanes 9 and 10), and suggesting that the RED subdomain (helices 4 and 5) is dispensable for the modulation HD DNA binding (Fig. 4B and D; lanes 6 and 9).

Mutations located throughout the PAI subdomain of Pax3 have been shown to result in loss of PD and HD DNA binding (18–20). These mutations likely disrupt critical DNA contacts made by the PD (13) but the mechanism by which these mutations affect HD DNA-binding remains to be elucidated. The deletion series described in this study suggest that the PD helix 2 plays a key role in the modulation of HD DNA-binding. We thus wanted to investigate if this structure is involved in transmitting to the HD the effect of the PD mutations. To do so, we generated a construct harboring the Spd mutation (G42R) (4) in which helix 2 has been deleted (Fig. 5A). Although the Spd mutation normally abrogates HD DNA-binding, deletion of helix 2 within the PD is sufficient to restore dimerization of the Pax3 HD on the P2 oligonucleotide (Fig. 5B). These results substantiate the notion that helix 2 is a key structure responsible for the modulation of HD DNA-binding by the PD and that it mediates the negative effect of PD mutations on HD DNA-binding.

Characterization of Prd binding to the composite recognition sequence PHO revealed that the PD and HD bind to their respective half site only when these are arranged in a specific orientation (everted) and with maximum cooperation when these half sites are juxtaposed (0 bp spacing) (16). These observations have suggested a structural model in which the PAI subdomain is adjacent to the HD, with helix 2 of the PD in close proximity to the N-terminal arm of the HD or of a region N-terminal to the HD. Furthermore, according to this model, these two structures would be the only one capable of mediating a direct interaction between the PD and HD. In accordance with this model, our study has identified that helix 2 of the PD and the region N-terminal to the HD play a key role in the PD modulation of HD DNA-binding.

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