Site-Specific Modification of Single Cysteine Pax 3 Mutants Reveals Reciprocal Regulation of DNA Binding Activity of the Paired and Homeo Domain†

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ABSTRACT: The mechanism by which the paired domain (PD) and the homeo domain (HD) act together in the intact Pax3 protein to recognize DNA is unclear and was studied in a Pax3 mutant (Pax3-CL) devoid of cysteines. Pax3-CL binds to PD (P6CON−P3OPT sites) and HD (P2, P1/2 sites) DNA site sequences with near wild-type activity but, contrary to Pax3, in a *N*-ethyl maleimide (NEM) insensitive fashion. The Pax3-CL backbone was used for cysteine scanning mutagenesis and for site-specific NEM modification. Five single cysteine replacements were independently introduced in the PD, while eight were inserted in the HD. NEM sensitivity of PD and HD DNA binding was investigated in DNA-binding competent mutants. In the PD mutant C82, NEM abrogated DNA binding by the PD but also abolished DNA binding by the Cys-less HD. Likewise, in the HD mutant V263C, NEM modification abrogated DNA binding not only by the HD, but also by the Cys-less PD. The transfer of NEM sensitivity to the PD seen in V263C was specific and not due to simple loss of HD DNA binding since alkylation of adjacent V265C and S268C, although impairing HD DNA binding did not affect PD DNA binding. Thus, the PD and HD do not function as independent DNA binding modules in Pax3 but seem functionally interdependent.[1]

Pax-3 is a member of the mammalian Pax family (*1*), a group of nine DNA-binding transcription factors structurally defined by a highly conserved DNA binding domain known as the paired domain (PD[1]), which was initially identified in the paired segmentation gene of *Drosophila* (*2*). Pax proteins play a key role in directing tissue patterning and development of different organs during embryogenesis (*3*), and mutations in Pax genes impair normal development of the skeleton (Pax1), kidney (Pax2), eye (Pax6), pancreas (Pax4), and thyroid (Pax8) (*4*−*12*). Pax3 is expressed in the developing neural tube, in neural crest cell derivatives, and in migrating limb muscle precursor cells (*13*, *14*), and a naturally occurring mutation in mouse *Pax3* (splotch) causes profound defects in neurogenesis (spina bifida and exencephaly) and myogenesis (no limb musculature) (*13*, *15*−*19*). Likewise, mutations in human *PAX3* cause Waardenburg syndrome, a pathology associated with pigmentary disturbances, cranio-facial abnormalities, and sensorineuronal deafness (*20*, *21*). It has been proposed that Pax3 plays a dual role in neurogenesis and myogenesis via transcriptional regulation of specific targets genes and by maintaining replicative potential of specific, Pax3 positive, epithelial, and migratory cell populations (*3*).

Pax proteins show a modular structure. In addition to the PD, certain Pax proteins possess a second DNA binding domain, the paired-type homeo domain (HD) (*1*, *22*). A highly conserved octapeptide motif is also found in certain Pax proteins in the linker separating the PD from the HD. Finally, the C-terminal half of Pax proteins shows a P/S/T domain resembling transactivating domains of other transcription factors. A high-resolution three-dimensional structure has been obtained for the PD−DNA complex of Prd and Pax6 and shows this domain to be bipartite (*23*, *24*) with N-terminal (PAI) and C-terminal subdomains (RED) each folding into three α helices, with the last two forming a helix-turn-helix (HTH) DNA binding motif. In the PAI subdomain, a unique β hairpin structure, consisting of 2 antiparallel β strands joined by a type I β turn, contacts the sugar−phosphate backbone to help anchor the subdomain to DNA. PAI holds a type II β turn lying immediately C-terminal to the hairpin and makes base specific contacts with the minor groove. Helix 3 of PAI fits directly into the major groove and participates in base specific contacts with DNA (*23*, *24*). Both N- and C-terminal subdomains contribute to DNA binding to certain type of sequences (CD19/2, P6CON) (*25*, *26*). Other Pax proteins bind DNA mostly through the PAI domain, while certain isoforms of Pax6 and Pax8 bind DNA exclusively through the RED domain (*27*, *28*). Finally, the linker segment joining the PAI and RED domains also make base-specific contacts in the minor groove (*24*), and alternative splicing of a single glutamine residue in this segment of Pax3 generates proteins with distinct DNA binding properties (*29*, *30*).

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[1] Abbreviations: PD, paired domain; HD, homeodomain; CL, cysteine-less; SCM, single cysteine mutant; NEM, *N*-ethylmaleimide; EMSA, electrophoretic mobility shift assay; EDTA, ethylenediamine tetracetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; kDa, kilodaltons; HA, *Haemophilus influenza* hemagglutinin A.

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Paired-type homeodomains (HD) found in Pax proteins define a specific highly conserved subgroup (*31*). The crystal structure of the DNA bound form of the Paired-type HD has been determined and shows three α helical segments with helices 2 and 3 forming a HTH motif (*32*). Although helix 1 does not make DNA contacts, residues N-terminal to it contact the minor groove. Helix 3 makes extensive DNA contacts in the major groove and is important for sequence specificity (*33*, *34*). A unique characteristic of paired-type HDs is their ability to cooperatively dimerize on palindromic sequences of the type TAAT-(N_{2-3})-ATTA. Position 50 (helix 3) of the HD plays a key role in binding specificity and dimerization potential of HDs; while the HD of Phox (Gln50) can only dimerize on TAAT-(N_3)-ATTA sequences showing a three-nucleotide spacer, the HD of Pax3 (Ser50) can only dimerize (in the context of a PD) on sequences harboring a two-nucleotide spacer (*33*, *35*).

Although both the PD and HD of Prd and Pax3 can bind DNA on their own (*36*), a large body of data suggests that they do not function independently in intact Pax3, perhaps conferring additional sequence binding specificity to the protein. Indeed, both PD and HD are required for function in Pax3 and Pax6 and mutations in either domain show similar loss-of-function phenotypes in vivo (*8*, *21*, *37*). Interestingly, a mutation (G42R) in the PD of Pax3 found in the splotch-delayed mouse mutant (Sp^d) not only shows reduced DNA binding to PD oligos, but also causes reduced DNA binding to HD-specific oligos (*36*). Conversely, a WS mutation in the HD (R53G) also modulates binding of Pax3 to PD-type sequences (*38*). Additional studies in Pax3−Phox chimeras have shown that the Pax3 PD can modulate the DNA binding specificities and dimerization potential of a heterologous HD (*39*). Deletion of helix 2 of the PD in the context of the Sp^d mutation restores DNA binding by the HD, identifying this helix as a key structural element in this regulation (*39*). These results have suggested that the PD and HD are functionally interdependent and interact for final target site selection. However, the mechanistic basis and the protein segments involved in this functional interaction remain poorly understood so far.

Site-specific modification of cysteines with thiol-specific reagents is a versatile tool for structure:function studies (*40*, *41*). This is best accomplished in the Cys-less molecular backbone in which single cysteines are inserted by mutagenesis in predetermined strategic locations (*42*). For example, important information on the chemical environment of a specific residue or protein segment (e.g., transmembrane domains vs solvent-exposed loops) can be obtained by reacting the modified protein with thiol reagents of different chemical and physical properties, such as varying size and degree of hydrophobicity. Residues in substrate-binding pockets can be identified by Cys-scanning mutagenesis as positions that confer NEM sensitivity to substrate binding and, conversely, at which prior reaction with substrate protects against NEM alkylation. Dynamic changes in structure following substrate binding or catalytic activity can be further studied in quenching experiments, using purified protein modified by fluorescent sulfhydryl reagents (*40*). Finally proximity relationships can be studied in mutants containing Cys pairs, by a variety of methods including cross-linking with bifunctional reagents (*40*, *41*), FRET (*43*), excimer fluorescence (*44*−*46*), and several others. In the present study, we have created and functionally characterized a Pax3 mutant in which all Cys residues have been replaced by Ser or Gly. We have used this mutant backbone to reintroduce Cys residues at strategically located positions of the PD and HD in individual mutants. The effect of site-specific modification of these single Cys mutants by sulfhydryl reagents on the DNA binding properties of the PD and HD was characterized.

MATERIALS AND METHODS

Mutagenesis. The construction of the pMT2 expression plasmid encoding a portion of wildtype (wt) *Pax3* cDNA (positions 297−1801) has been previously described (*36*). This pMT2Pax3 construct encodes the full-length 479 amino acid murine Q+ isoform of Pax3. A 1.3 kb *PstI* internal Pax3 fragment from upstream the initiator AUG to nucleotide position 1581 (residues 1−429) that encodes the paired domain (PD) and the homeodomain (HD) was inserted into the corresponding site of plasmid vector pAlter-1™ (ProMega) to create pAlterPax3. The fragment was also inserted into the *Pst I* site of a eukaryotic expression plasmid pMT2 (*38*) to generate pMT2Pax3(B). The pAlterPax3 plasmid encodes the first 429 residues of Pax3 and consequently encodes for six of the seven endogenous cysteines of Pax3. Altered Sites II in vitro mutagenesis systems (Promega) was used with the pAlterPax3 plasmid to mutate all but the most C-terminal cysteine of Pax3, creating pAlterPax3CL/C429. pAlterPax3CL/C429 was used for mutagenesis in the construction of the single cysteine mutants. The mutagenic oligonucleotides used to generate the Cys-less (CL), and the single cysteine mutants are listed in Table 1. The five most N-terminal endogenous cysteines of Pax3 (Cys70, 82, 88, 143, 355) were substituted to serine or glycine. The single Cys mutants CL/C82 andCL/C88 were obtained at intermediate steps of this process.

The pMT2Pax3(B) plasmid was further modified by PCR-mediated mutagenesis in order to insert in-frame at the C-terminus of the protein, both a polyhistidine tail (His_6) and an antigenic hemagglutinin A epitope followed by a new termination codon. This was carried out using sequence-specific oligonucleotide primers (5′)-CAGGTGACAACG-CCTGACGTGGAG-(3′) and (5′)-CCTTTGGAATTCCT-GCAGTCAATGATGATGATGATGATGTCGC-GAAGCGTAGTCTGGCACATCGTATGGGTATACG-TACCTCGAGCTGGCTGACACCGTGGTC-(3′). The mutagenic oligonucleotide also replaces Cys429 by a serine. The resulting 1049 bp PCR product was digested with restriction enzymes *Apa I* and *Eco RI* (sites embedded in the oligo) and ligated into the corresponding sites of pMT2Pax3(B) to produce pMT2Pax3C429S/HA. The 12 single Cys mutants generated in pAlterPax3CL/C429 plasmid (mutant oligonucleotides listed in Table 1) were reconstructed in pMT2C429S/HA using endogeneous Pax3 restriction sites *Sma I* (Pax3; pst 342−672) or *Kpn I* (Pax3; pst 563−1500). This permitted the production of the following expression plasmids: pMT2Pax3CL, pMT2Pax3CL/C82, pMT2Pax3CL/C88, pMT2Pax3CL/S152C, pMT2Pax3CL/S153C, pMT2-Pax3CL/S155C, pMT2Pax3CL/R221C, pMT2Pax3CL/S222C, pMT2Pax3CL/V263C, pMT2Pax3CL/V265C, pMT2Pax3CL/S268C, pMT2Pax3CL/R270C, pMT2Pax3CL/A272C, and pMT2Pax3CL/W274C. Each mutation was verified by

Table 1: Oligonucleotides Used for Pax3 Mutagenesis[a]

substitution	mutagenic primer (5′−3′)
C70S	CATTCGGCCG**A**GCGTCATTTC
C82S	GTCCCATGGATC**C**GTCTCTAAG
C88S	CTAAGATCCTA**G**GCAGGTAC
C143S	GGACGCTGTC**A**GCGATCGGAACACTGTG
C355S	CAGCTCTGCCTAC**A**GTCTTCCCAGCACCAG
S152C	GTGCCCTCAGTG**T**GTTCTATCTCGCGAATCCTGAGGAG
S153C	CACTGTGCCCTCAGTCAGC**T**GTATCAGCCGCATCCTG
S155C	CAGTGAGTTCTATC**T**GCCGCATTCTGAGGAGTAAATTTG
R221C	GAGGAAGCAGCGC**T**G**T**AGCAGAACCACC
S222C	GCAGCGCAGG**T**GCCGTACGACCTTCACGGC
V263C	GCTTACCGAGGCGCGC**TGT**CAGGTCTGGTTTAG
V265C	CGAGTGCAG**TGT**TGGTTTAGCAACCGCCGTGCACGATGGAGGAAAC
S268C	GCAGGTCTGGTTTT**T**GCAACCGGCGCGCCAGATGGAGGAAAC
R270C	GGTTTAGCAAT**T**GCCGTGCAAG
A272C	GCAGGTCTGGTTTTCGAACCGCCGT**TGC**AGATGGAGGAAAC
W274C	CGTGCAAGATG**T**AGGAAACAAGCCGGCGCCAATCAACTG

[a] Nucleotide substitutions leading to amino acid changes are indicated in bold, and those that introduce silent restriction sites are underlined.

nucleotide sequencing, and the accessibility of restriction sites used for cloning was verified by endonuclease fragmentation.

Expression and Detection of Pax3 Mutants. The pMT2Pax3 expression plasmids were introduced into COS7 cells via transient transfection. One million cells were plated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and were transfected by the calcium phosphate coprecipitation method using 15 ug of plasmid DNA doubly purified by ultracentrifugation on cesium chloride density gradients. Cells were exposed to calcium−DNA precipitates for 5 h and then treated with HBS (0.14 M NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 6 mM dextrose, 25 mM HEPES, pH 7.05) containing 15% glycerol for 1 min. Following this treatment the cells were washed once and placed in complete DMEM. Whole cell extracts were prepared 24 h following HBS/15%glycerol treatment by sonication in a buffer containing 20 mM HEPES (pH 7.6), 0.15 M NaCl, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), 0.2 mM EDTA, 0.2 mM EGTA, and a cocktail of protease inhibitors: aprotinin, pepstatin, and leupeptin at 1 μg/mL and phenylmethysulfonyl fluoride at 1 mM. These extracts were stored frozen at −70 °C until use. To assess Pax3 mutant protein expression and stability, aliquots of whole cell extracts were analyzed by electrophoresis on acrylamide-containing SDS gels (SDS−PAGE), followed by electrotransfer onto nitrocellulose membranes and immunoblotting. Immunodetection was performed with mouse monoclonal anti-HA antibody (BabCO, Berkeley, CA) at a dilution of 1:1000 and visualized by enhanced chemiluminescence using a sheep antimouse horseradish peroxidase conjugated secondary antibody (Amersham).

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays were performed as previously described (*36*). Each protein:DNA binding reaction was carried out using approximately 8 μg of total cell extracts from transiently transfected COS-7 monkey cells and 10fmol (0.06 μCi) of radioactively labeled double stranded oligonucleotides containing either PD or HD recognition sites. The final concentration of labeled oligonucleotide in the binding reaction is 0.0005 μM. Whole cell extracts were incubated with ^{32}P-labeled PD specific probes in a volume of 20 μL containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 2 mM spermidine, 2 mg/ml BSA, and 10% glycerol. To reduce nonspecific binding, 1 μg of poly(dI−dC)poly-(dI−dC) was included in binding studies with PD-specific probes, while 2 μg of heat-inactivated salmon sperm DNA was added to binding reactions involving HD specific probes. Following a 30 min incubation at room temperature, samples were electrophoresed at 12V/cm in 6% acrylamide:bis-acrylamide (29:1) gels containing 0.25 or 0.5X TBE (1X TBE is 0.18 M Tris-HCl, 0.18 M boric acid, 4 mM EDTA, pH 8.3). Gels were dried under vacuum and exposed to Kodak BMS film with an intensifying screen. Some films were used to perform densitometry studies to quantitate the amount of radiolabeled probe that was protein-bound using a Fuji LAS-1000. PD-specific sequences P6CON (5′)-TGGAATTCAG-GAAAAATTTTCACGCTTGAGTTCACAGCTCGAGTA-(3′) (*26*) and P3OPT (5′)-TGGTGGTCACGCCTCATTG-AATATTA-(3′) (*47*, *48*) and HD-specific sequences P2 (5′)-′GATCCTGAGTCTAATTGATTACTGTACAGG-(3′) (*33*) and P1/2 (5′)-GATCCTGAGTCTAATTGAGCG-TCTGTAC−(3′) (*33*) were synthesized as complementary oligonucleotide pairs and were designed in order to have recessed 3′ ends for end labeling with [α-^{32}P] dATP (3000 Ci/mmol; NEN) using the Klenow fragment of DNA polymerase.

Thiol-specific reagents *N*-ethylmaleimide (Pierce) and dibromobimane (Molecular Probes) were prepared as 10 mM stocks in water and 100% dimethyl sulfoxide, respectively, and were stored frozen until use. They were added as a 0.5 μL aliquot to a 4 μL volume of whole cell extract, followed by a 30 min incubation at room temperature prior to the addition of the [^{32}P]-labeled probe and EMSA.

RESULTS

Construction and Characterization of a Pax3 Mutant Devoid of Cysteines. The analysis of structure:function relationships by site-specific modification with sulfhydryl reagents is most conveniently carried out in a protein backbone lacking cysteine residues (Cys-less), where single Cys can be introduced at strategic sites by site-directed mutagenesis. Pax3 has 7 Cys residues at positions 70, 82, 88, and 143 in the paired domain (PD) and at positions 355, 429, and 449 downstream the Homeo Domain (HD). A Cys-less Pax3 mutant was created in a Pax3 cDNA fragment (residues 1−429), which lacks the last 50 residues (including Cys449 but which retains wild-type DNA binding activity

Table 2: Conservation of Cysteine Residues in Mouse and Human Pax Proteins

Pax proteins	endogenous Cysteines of Pax3					
	Cys70	Cys82	Cys88	Cys143	Cys355	Cys429
Pax3/PAX3	Cys	Cys	Cys	Cys	Cys	Cys
Pax4/PAX4	Cys	Cys	Gly	Cys	Cys	Pro
Pax6/PAX6	Cys	Cys	Gly	Cys	Met	Ser
Pax7/PAX7	Cys	Cys	Cys	Cys	Ser	Cys
Pax1/PAX1	Cys	Cys	Ala	Cys	Cys	Leu
Pax9/PAX9	Cys	Cys	Ala	Cys	Ser	Leu
Pax2/PAX2	Cys	Cys	Gly	Cys	Thr	Ser
Pax5/PAX5	Cys	Cys	Gly	Cys	Pro	Ser
Pax8/PAX8	Cys	Cys	Gly	Cys	Gly	Tyr
substitution	Ser	Ser	Gly	Ser	Ser	Ser

by the PD and by the HD (*36*). An alignment of human and mouse Pax protein sequences (Table 2) was used to guide the choice of amino acid to be substituted for each Cys residue. Cys70, Cys82, and Cys143 in the PD are invariant among Pax family members and were replaced by Ser. Serine and Cysteine have a very similar structures, differing only by the size of the Oxygen vs Sulfur atom in the side chain, and therefore these substitution are expected to have a minimal structural consequence. Indeed, a C70S mutation in Pax8 has been previously shown to be without consequence on DNA binding (*49*). Five Pax proteins harbor a Gly at position 88 (Table 2); thus, the Cys88 of Pax3 was mutated to Gly. Finally, Cys355 and Cys429 in the P/S/T-rich trans-activation domain are not conserved in other Pax proteins (Table 2) and were substituted to Ser. All mutations were introduced sequentially by site-directed mutagenesis, and the integrity of the final cDNA was verified by nucleotide sequencing.

The Cys-less (CL) Pax3 construct was further modified by the addition of a hemagglutinin (HA) epitope tag at its C-terminus to facilitate protein detection and was introduced in the pMT2 expression vector followed by transient transfection into COS-7 Monkey cells. Immunoblotting of whole cell extracts with an anti-HA monoclonal antibody indicates similar stability and levels of expression of both the wild type (wt) and Cys-less (CL) Pax3 proteins in COS7 cells (Figure 1A). The effect of Cys replacement on DNA binding properties of the PD and HD of Pax3 was examined by electrophoretic mobility shift assays (EMSA). DNA binding by the PD was examined using oligonucleotide probes P3OPT (*26*) and P6CON (*47*, *48*), previously shown to reveal binding determinants present in both the amino (PAI) and carboxy (RED) subdomains of the PD. Results in Figure 1B indicate that the wt and CL Pax3 can both bind these probes, and semiquantitative analysis in the form of dilution series suggests similar affinities of both proteins for P3OPT (Figure 1C) and P6CON (data not shown). The effects of Cys replacement on DNA binding properties of the HD were evaluated using a target sequence (P2) containing the sequence $TAAT(N)_2TAAT$ previously shown to support cooperative dimerization of Pax3 (*33*). In addition, an oligonucleotide containing half of this sequence (half site, P1/2) and revealing monomeric Pax3 binding by the HD was used. Results in Figure 1B,C indicate that CL Pax3 can bind to P1/2 (monomer, empty arrowhead) and can dimerize on P2 (shaded arrowhead), albeit with somewhat reduced proficiency. Similar levels of wt and CL Pax3 proteins were present in all experiments, as revealed by immunoblotting. Taken together, these experiments indicate that removal of all Cys residues from Pax3 does not have a major effect on DNA binding by either the PD or the HD.

Effects of Thiol-Specific Reagents on DNA Binding by WT and Cys-Less Pax3. Sulhydryl or thiol-reactive compounds such as *N*-ethyl maleimide (NEM) or dibromobimane (DBB) can form covalent adducts with Cys residues and can be used efficiently for site-specific modification in structure:function studies. The effect of NEM and DBB on DNA binding by the PD and HD of WT and CL-Pax3 proteins was tested in EMSA. In these experiments, extracts from COS cells expressing each protein were incubated with various amounts of NEM prior to EMSA with PD specific (P3OPT, P6CON) and HD specific (P2, P1/2) oligonucleotides (Figure 2). In WT Pax3, PD-specific DNA binding to P3OPT was completely abrogated by 1 mM NEM. Studies with a narrower NEM concentration range showed that 0.3 mM reduced DNA binding by 50%, while no binding was detectable at 0.5 mM (Figure 2, lower panel, Figure 6B). Identical results were obtained when P6CON was used as the PD target sequence; in some experiments, a Pax3-independent, NEM-sensitive P6CON complex of slower electrophoretic mobility was detected in all samples including the negative control (Figure 2; asterisk). NEM sensitivity of DNA binding by the PD was also seen when other PD probes (Nf3′, CD19−2A) were used in these experiments (data not shown). A similar analysis of the HD revealed that dimerization of the WT Pax3 on P2 sequences is also NEM-sensitive and is abrogated by NEM concentration of ~0.5 mM (Figures 2 and 6B). Likewise, monomeric binding of WT Pax3 to a P1/2 probe is also largely abrogated by NEM concentrations in the range of 0.1−1 mM; however, we note that a small proportion of monomeric binding to P1/2 is NEM-insensitive, and quantitation of the signal indicates that up to 20% of WT Pax3 binding to P1/2 is NEM-insensitive (Figure 2, lower panel and Figure 6B). In contrast, DNA binding by the PD and the HD in the CL Pax3 mutant was completely NEM insensitive in the same assay for all concentrations tested up to 3.6 mM (Figures 2 and 6B). Finally, identical results were obtained when dibromobimane was used in place of NEM in these experiments (data not shown).

Together, results in Figures 1 and 2 indicate that a Pax3 mutant devoid of Cys residues can still bind DNA through its PD and HD. Thus, inactivation of Pax3 DNA binding by NEM (Figure 2) is probably due to introduction of one or more bulky groups into the molecule rather than the removal of a critical sulfydryl group. Importantly, these results show that the CL Pax3 mutant can be used as molecular backbone for site-specific modification in Pax3 mutants containing single cysteines inserted by site-directed mutagenesis.

Construction and Characterization of Single Cysteine Pax3 Mutants. Site-specific modification in single cysteine mutants has been used to study, in a dynamic fashion, structural changes in proteins associated with ligand binding or catalysis (*40*). Our goal was to use such mutants to study possible interactions between the PD and HD of Pax3, including reciprocal regulation of DNA binding properties. Hence, we aimed to study the effect of site-specific modification of single Cys residues in one domain on DNA binding properties of the other domain. Ideal substitutions for this type of analysis would be Cys insertions in one

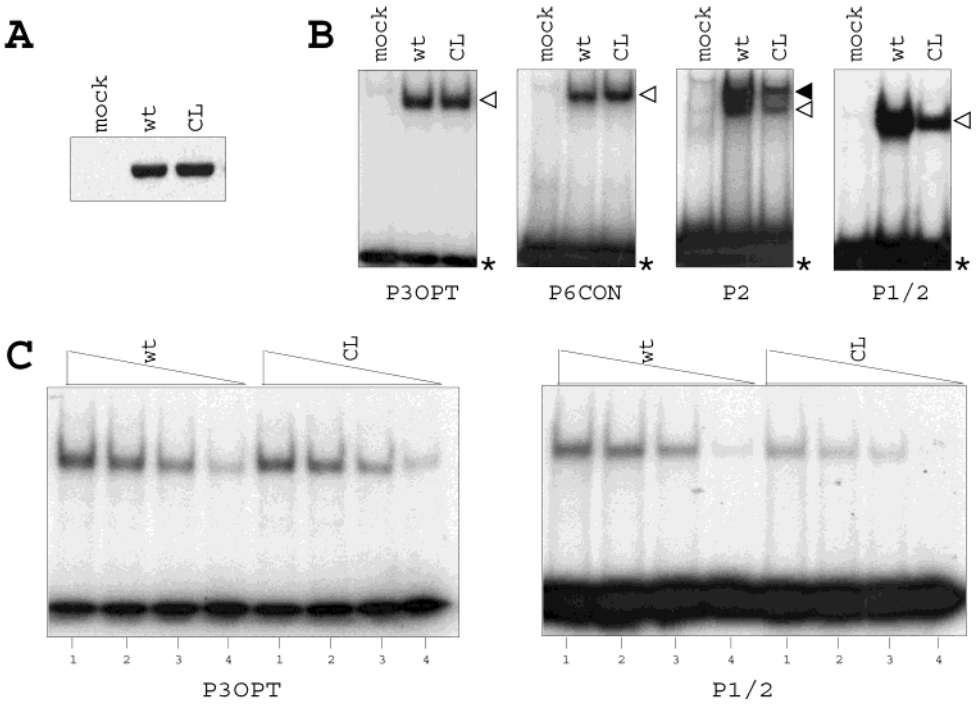

FIGURE 1: Paired domain and homeo domain DNA binding properties of wild-type and of Cys-less Pax3. (A) Immunodetection of wild-type (wt) and Cys-less Pax3 (CL) proteins in whole cell extracts from COS7 monkey cells transfected with corresponding Pax3 cDNAs modified by the in-frame addition of a *Haemophilus influenza* hemagglutinin A (HA) epitope tag at the C-terminus of each protein. Proteins were resolved by SDS-PAGE (10% acrylamide) and transferred to a nitrocellulose membrane. Detection was by enhanced chemiluminescence using a mouse anti-HA monoclonal antibody and a HRP-conjugated secondary antibody. The "mock" labeled lane refers to whole cell extracts from untransfected COS7 cells. (B) Electrophoretic mobility shift assays were used to measure the DNA binding properties of either Pax3 (wt) or CL Pax3 against paired domain (PD) (P3OPT, P6CON) and homeo domain (HD) specific binding sites (P2, P1/2). Protein−DNA complexes were formed using total COS7 cell extracts and were resolved on 6% acrylamide nondenaturing gels, as described in Materials and Methods. Open arrowheads identify monomeric Pax3/DNA complexes, while closed arrowheads identify Pax3 dimers bound to the P2 probe. Free oligonucleotide probe is identified by an asterisk. (C) Dilution series of Pax3 (wt) and CL Pax3 proteins to compare relative affinities for P3OPT (PD) and for P1/2 (HD) probes. For each panel, lanes 2, 3 and 4 correspond to 1.5-, 2-, and 4-fold dilutions of total cell extract in lane 1.

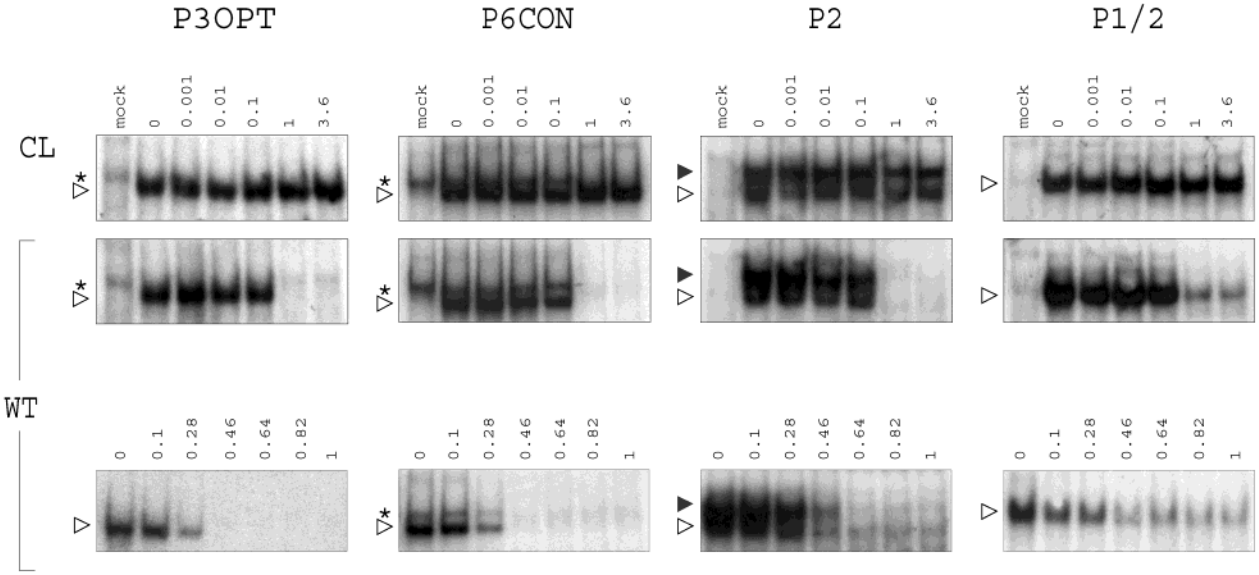

FIGURE 2: Effects of *N*-ethyl maleimide treatment on the paired domain and homeo domain DNA binding properties of WT and CL Pax3. Total cell extracts from COS7 monkey cells (mock) or from cells expressing either wild-type Pax3 (WT) or the Cys-less Pax3 mutant (CL) were incubated in increasing concentrations of *N*-ethyl maleimide (NEM) prior to electrophoretic mobility shift assay. The DNA binding properties of the PD were evaluated with target sites P3OPT and P6CON, while the HD was tested using the P2 and P1/2 sites. Two series of NEM concentrations (in mM) were tested for WT and are indicated on top of each panel. Monomeric Pax3/DNA complexes are indicated by an open arrowhead, while dimers formed on P2 are identified by a shaded arrowhead. The presence of a Pax3-independent band detected in some of the EMSA with P3OPT and P6CON is identified by an asterisk. All gels were exposed for similar time periods.

domain that do not affect DNA binding by this domain but that are strategically positioned close to the DNA molecule so that formation of a bulky adduct at that position upon sulfhydryl modification would hinder DNA binding by this

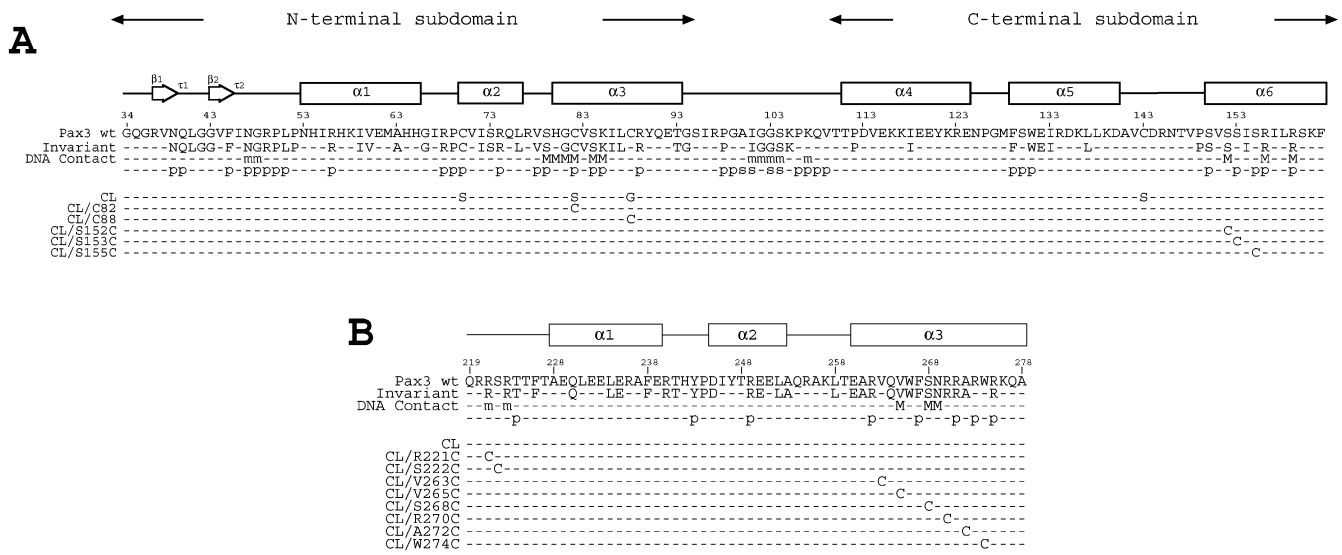

FIGURE 3: Site-directed mutagenesis of the paired domain and homeo domain of Pax3. (A) Schematic representation of the N-terminal and C-terminal subdomains of the paired-domain, together with structural features based on the three-dimensional structure of the paired domain of the *Drosophila* Prd protein (β, β-strand; τ, β-turn; α, α-helix) (*23*, *24*). The amino acid sequence for positions 34−162 of Pax-3 is shown, and invariant residues among all known paired domains are identified below. The type of predicted DNA contacts made by these residues (p, phosphate; m, minor groove; M, major groove) is shown. The position and nature of the mutations introduced in Pax-3 to create the Cys-less (CL) mutant, as well as the positions of single cysteine mutants corresponding either to endogenous Cys positions (CL/C82; CL/C88) or novel insertions, are shown below. (B) Schematic representation of the Pax3 homeodomain, including the presence and position of predicted structural features, invariant amino acid residues, number and types of DNA contacts, and positions of single cysteine insertions (as for panel A).

domain. In addition, these insertions should be accessible to NEM, a parameter that must be determined experimentally. The high-resolution crystal structures of the PD of Pax6 (*24*) and of the HD of Prd (*32*) proteins bound to DNA were used to guide the choice of residues to be mutated to Cys (Figure 3). Five mutations were independently created in the C-terminal helices of each HTH motif of the PD (helices α3 and α6). These two helices make key contacts in the major groove and are essential for DNA binding (*24*). C82 and C88 (α3 helix) are endogenous cysteines highly conserved in Pax proteins; these were re-created as single cys mutants. Likewise serines 152, 153, and 155 in α6 are well conserved in Pax proteins, contact DNA (Figure 3A), and were individually mutated to S152C, S153C, and S155C. In the HD, the N-terminal arm (pst 219−227) is important for regulation of DNA binding (*32*), through DNA contact with the minor groove (Figure 3B); R221 and S222 in this segment were mutated to Cys. Residues of helix 3 of the HTH motif, including four highly conserved arginines, make extensive base-specific and phosphate contacts in the major groove; thus, R221C, S222C, V263C, V265C, S268C, R270C, A272C, and W274C were created in this segment. All mutants were created by site-directed mutagenesis, and the corresponding HA-tagged proteins were expressed by transient transfection in COS-7 cells.

Immunoblotting experiments show that all mutant variants could be expressed to similar levels in COS-7 cells (Figure 4A), indicating that none of the mutations affect protein expression or stability. The effect of single Cys mutations on DNA binding by the PD and HD was analyzed by EMSA, using P6CON−P3OPT and P2−P1/2, respectively (Figure 4B). All the PD SCMs, except C82, have PD and HD DNA binding activities similar to CL Pax3. C82 is the only PD SCM that behaves similarly to WT with respect to DNA binding properties and NEM sensitivity (Figure 4B). Therefore, none of the single Cys mutants inserted in the PD abolished the DNA binding properties of either domain. Most HD SCMs showed near-wild-type activity for PD sequences (P6CON, P3OPT). HD SCMs R221C, R270C, and A272C show a reduction in PD DNA binding activity, though this reduction is small. However, HD mutations had a marked effect on DNA binding by the HD. Although several mutants retained some DNA binding for P1/2, mutants at highly conserved positions R221, R270, and A272 showed severely reduced binding activities. In addition, none except S222C was capable of cooperative dimerization on P2 (Figure 4B). These results confirm the key role of α3 helix in DNA binding by the HD. Reduced P1/2 binding by the mutants is concomitant to an inability to dimerize on P2, showing that integrity of α3 is also essential for cooperative homodimerization of the HD. Of the HD SCMs with binding activity for P1/2, all but S222C have activities less than that of CL Pax3. Due to their severe reduction of HD DNA binding activity, mutants R221C, R270C, and A272C could not be studied further. Nevertheless, several single Cys mutants in the PD and HD did retain robust DNA binding by both domains and were thus suitable for site-specific modification studies.

Effect of Thiol Specific Reagents on DNA Binding by Single Cysteine Pax3 Mutants. Initially, whole cell extracts from COS-7 cells expressing individual mutants were incubated with 1 mM *N*-ethyl maleimide (NEM), and the ability of each mutant to bind to P3OPT and P1/2 was monitored by EMSA. Results were quantitated by densitometry and are expressed as a fraction of protein bound to DNA, expressed as a percentage (Figure 5). For the PD single Cys mutants, only the C82 mutant demonstrated NEM sensitivity to PD binding sites (P3OPT). For the HD, mutants

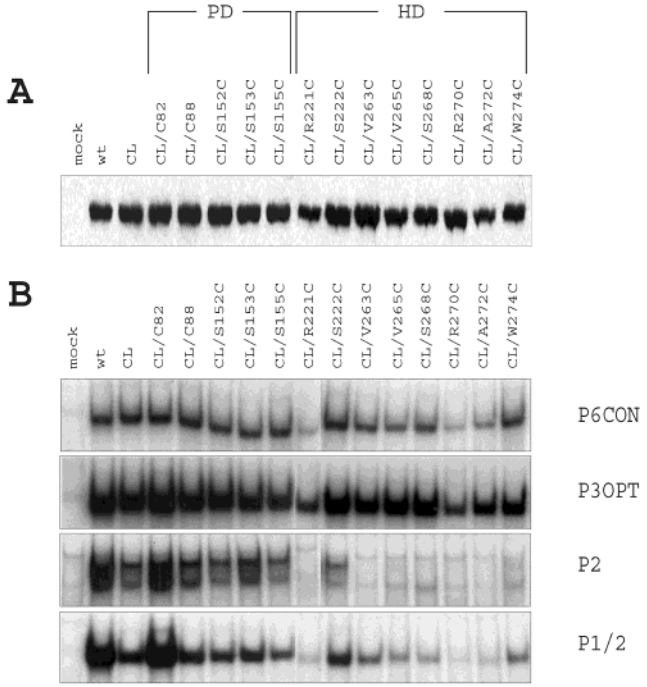

FIGURE 4: Paired domain and homeo domain DNA binding properties of Pax3 mutants bearing single cysteine replacements. (A) *Pax3* mutants bearing single cysteine (single Cys) insertions onto the backbone of a Cys-less (CL) *Pax3* mutant were created by site-directed mutagenesis, cloned into pMT2 expression plasmid, and total cell extracts from transiently transfected COS7 Monkey cells were separated by SDS-PAGE and analyzed by immunoblotting with a mouse anti-HA monoclonal antibody, as described in the legend to Figure 1. (B) EMSA analysis of the DNA binding properties of either Pax3 (wt), CL Pax3 (CL), or single Cys mutants against paired domain (P3OPT, P6CON) and homeo domain specific binding sites (P2, P1/2) was as described in Figure 1.

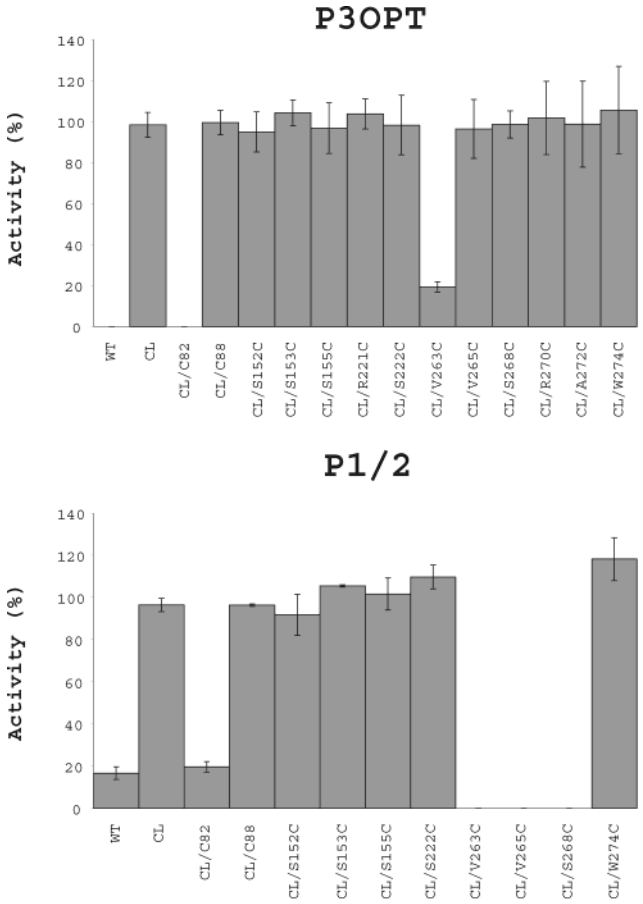

FIGURE 5: Effects of *N*-ethyl maleimide treatment on the paired domain and homeo domain DNA binding properties of Pax3 mutants bearing single cysteine replacements. The effect of NEM treatment of whole cell extracts from COS7 transfected cells on the DNA binding properties of individual mutants was tested for the PD sequence P3OPT and for the HD sequence P1/2, as described in the legend to Figure 2. The signals were quantitated by densitometry from a minimum of three independent experiments, and results are expressed as a ratio (% activity) of the amount of radioactivity present in the DNA/protein complex for each mutant in the absence and presence of prior incubation of the extract with 1 mM NEM.

V263C, V265C, and S268C showed NEM sensitivity for binding to the P1/2 site, while mutants S222C and W274C were NEM insensitive. Importantly, NEM treatment of PD mutant C82 not only abrogated DNA binding to PD sites P3OPT, but also impaired binding of this mutant to the HD sequence P1/2. Conversely, NEM treatment of the HD mutant V263C not only impaired DNA binding to HD site P1/2, but also strongly reduced binding of this mutant to a PD site (P3OPT) (Figure 5). The NEM sensitivity of PD and HD binding seen in V263C was specific and distinct from that of adjacent mutants V265C and S268C, which displayed NEM sensitive binding to HD site but NEM-insensitive binding to PD site. Finally, although the severe reduction of P1/2 binding displayed by mutants R221C, R270C, and A272C (Figure 4B; see above) precluded the testing of NEM sensitivity toward this sequence (data not shown), NEM treatment did not affect DNA binding of these mutants to the PD site P3OPT. For all mutants studied, identical results were obtained when another sulfhydryl reagent, dibromobimane, was used (data not shown).

Additional dose response studies with NEM concentrations ranging from 0 to 3.6 mM were conducted on single Cys mutants showing NEM sensitivity for DNA binding to PD and HD target sites. Primary EMSA results are shown in Figure 6A and are plotted in Figure 6B after quantitation of the radioactivity in the gel retarded complex. In these studies, the effect of NEM on possible dimerization of C82 on a P2 site was also investigated. For the C82 mutant, NEM concentrations >0.1 mM were sufficient to impair binding to P3OPT and P6CON sequences ($IC_{50} \sim 0.35$ mM; Figure 6B), but also to P1/2 ($IC_{50} \sim 0.4$ mM; Figure 6B), with a residual amount of NEM insensitive binding seen for that site (Figure 6A). As expected, NEM also impaired homodimerization of C82 on a P2 HD site (Figure 6A). For the V263C mutant, half-maximal inhibition for binding to PD sites (P3OPT, P6CON) and the HD site (P1/2) were obtained and in both cases were ~0.4 mM NEM (Figure 6A,B). Finally, in both the HD mutants V265C and S268C, although NEM concentrations >0.1 mM were sufficient to abrogate binding to P1/2, binding to PD sites in these mutants was insensitive to [NEM] of up to 3.6 mM (Figure 6A), confirming uniqueness and specificity of NEM sensitivity seen in V263C and C82.

Together, these results demonstrate a reciprocal effect of site-specific modifications of single Cys mutants in the PD and HD of Pax3 on DNA binding by the modified and unmodified site. They support a model in which the PD and HD do not function independently in the mature protein, but rather functionally interact to regulate DNA binding.

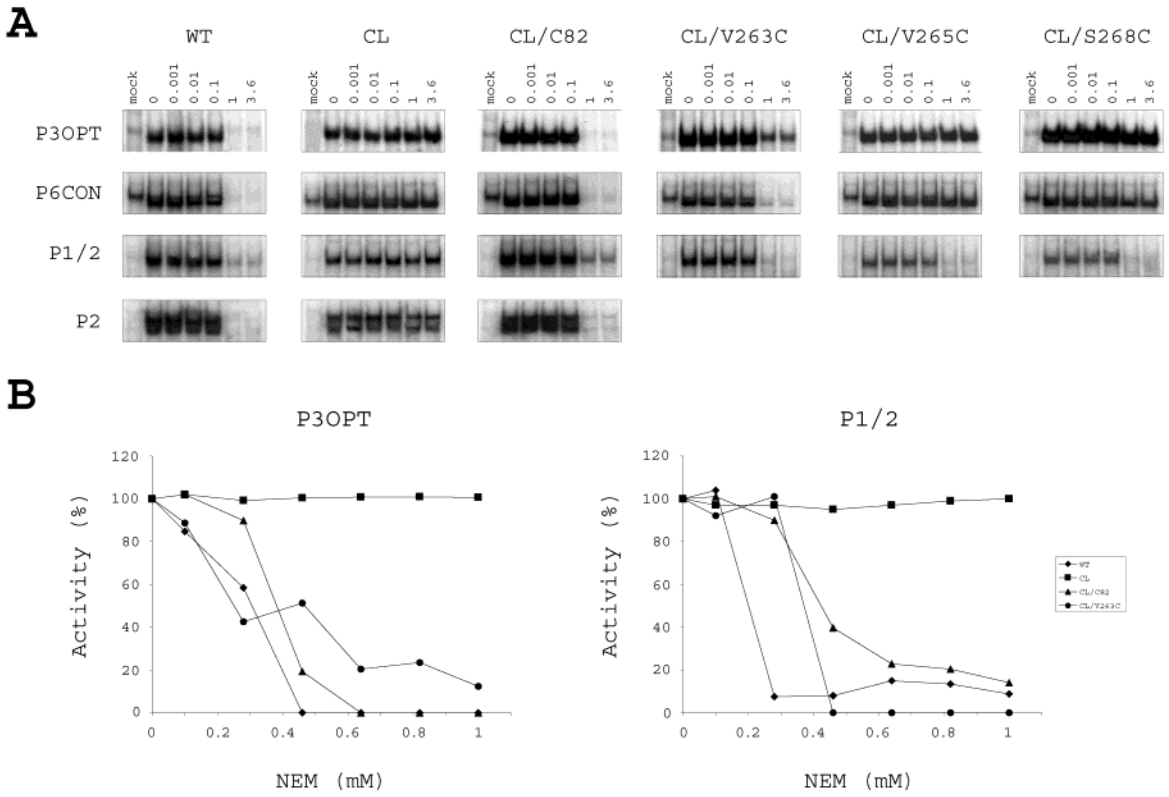

FIGURE 6: Dose-dependent effect of *N*-ethyl maleimide on DNA binding properties of certain single Cys mutants. (A) The NEM-sensitive DNA-binding properties of single Cys mutants in the PD (C82) and in the HD (V263C, V265C, S268C) were investigated in dose-response experiments as described in the legend to Figure 2 and for NEM concentrations ranging between 0.001 and 3.6 mM. NEM effects on DNA binding by the PD were tested using P3OPT and P6CON, while HD DNA binding was measured with P2 (for WT, CL, and C82) and with P1/2 (for all mutants). Gels were exposed for similar periods of time for mutants and WT protein extracts. (B) The signals were quantitated, and results are expressed as a ratio (% activity) of the amount of radioactivity present in the DNA/protein complex for each mutant in the absence and presence of prior incubation of the extract with the indicated concentration of NEM.

DISCUSSION

We have created and functionally characterized a Pax3 mutant devoid of cysteine residues. One of the challenges of using a Cys-less mutant is that ideally, removal of the Cys residues should not affect function, so that reinsertion of single cysteines can be done in the context of an active protein. At the time of initiating these studies, little information was available on the role of cysteines in Pax proteins. In a study of the isolated PD from Pax5 and Pax8, it was observed that the 3 Cys of the PD (also conserved in Pax3) must be reduced for PD binding to DNA (*49*). Trypsin digestion and mass spectroscopy analysis were used to demonstrate that in the oxidized state a reversible disulfide bridge is formed between Cys37 and Cys49 (Cys70, Cys82 in this study) which inhibits DNA binding by the PD (*49*). In normal nuclear extracts, it was shown that the PD of Pax5 and Pax8 are kept in a DNA-binding competent state by the reducing activity of the Ref-1 protein (*49*). These results established that at the very least, eliminating intramolecular disulfide bonds formation in Pax3 should not be detrimental to DNA binding. The CL−Pax3 mutant created herein could bind to PD and HD target site sequences with characteristics similar to that of wild type Pax3, and this binding was resistant to NEM concentrations of up to 3.6 mM. In the wild type Pax3, NEM blocks DNA binding by the PD and HD at concentrations >0.3 mM. These results show that none of the Cys residues in Pax3 are essential for DNA binding and that it is the introduction of a bulky maleimide group as opposed to removal of a functional thiol moiety that accounts for NEM inhibition of wild type Pax3 activity. The differential effect of NEM on WT and CL-Pax3 indicates that over the concentration range tested, NEM appears to specifically modify cysteines and does not seem to alter other residues important for DNA binding. Second, the observation that in wild-type Pax3, HD DNA binding is NEM-sensitive despite the absence of Cys residues in this domain suggests a possible role of the Cys-containing PD on HD activity. Finally, the observations that NEM inhibition of Pax3 DNA binding occurs both over a narrow range and at the same concentration for PD and HD targets suggest that alkylation of one or few critical Cys may be responsible for the effect. In purified PD from Pax5/Pax8, Cys82 appears to be most sensitive to reduction (*49*) and is a good candidate for the NEM effect seen in WT Pax3. The unique sensitivity to NEM of the single cysteine mutant C82 also agrees with results of this study.

Functional studies of Pax3 proteins carrying PD mutations found in certain naturally occurring mutant alleles of the *Splotch* mouse (Sp^d) (*36*) or in certain Waardenburg Syndrome patients (*38*) initially suggested possible regulation of HD DNA binding by the PD. Here we used the CL-Pax3 mutant to further investigate possible functional interdependence of the 2 DNA-binding domains of Pax3. In particular, we wished to determine whether NEM sensitivity of one of the two domains caused by alkylation of a single Cys inserted in this domain would also result in NEM sensitivity in the other, Cys-free domain, and vice-versa. In these studies, we wanted to avoid introducing mutations that would grossly affect the integrity of the DNA binding domains, but rather,

we wanted to insert single Cys at neutral positions in close proximity to the DNA molecule in the DNA-bound complex. Alkylation at such positions could impair DNA binding, thus providing a convenient method of ascertaining accessibility of the inserted Cys to thiol reagents. We focused our Cys insertion sites on the α3 helix of each of the HTH motifs of the PD (*2*) and HD, a helix known to play a key role in DNA binding. In the PD, independent reinsertion of Cys 82 and 88 of PAI was as expected without consequence on PD and HD activity. Likewise, three independent insertions in the RED domain had no effect on DNA binding. In the HD, two insertions were in the N-terminal arm and six were in the α3 helix. Interestingly, 3/8 mutations impaired partly or completely monomeric binding to the P1/2 half site, while 7/8 mutations abolished cooperative dimerization on P2, but none had an effect on PD binding properties. Thus, dimerization on P2 seemed more mutation sensitive than monomeric binding to P1/2 for the mutant set, possibly reflecting either a threshold effect for dimerization, reduced affinity for P2 compared to P1/2 or both. Therefore, subsequent studies were limited to the five mutants having retained significant monomeric binding to P1/2.

The effect of alkylation on the DNA binding properties of each mutant was tested in dose response experiments for both NEM and dibromobimane with identical results. In the experimental scheme used, we did not ascertain the NEM accessibility of each single Cys insertion. NEM accessibility can be inferred only for insertions that imparted NEM sensitivity to DNA binding by the targeted domain, and we will limit our discussion to these positions. NEM-insensitive insertions could reflect lack of effect of alkylation on DNA binding, or inaccessibility of the residue to NEM and will not be discussed further. As described above, WT Pax3 binding by the PD (P3OPT, P6CON) and by the HD (P2) was NEM sensitive. Of note was a small amount of NEM-insensitive binding to P1/2 (10−20%), detected even at the highest NEM concentration tested. Interestingly, this residual binding was not seen in single Cys HD mutants V263C, V265C, or S268C modified by alkylation. This suggests a small amount of NEM-independent monomeric binding to P1/2 by the HD of Pax3 (as expected from its lack of cysteine), which can be abrogated by sulfhydryl modification of a single Cys insertion in helix 3 of this domain.

In the PD, NEM alkylation of Cys82 impaired DNA binding by the PD. As shown in Figure 7, examination of Cys82 in the published structures of DNA-bound Pax6 and in Prd indicates that this residue makes phosphate and base specific contacts in the major groove. Thus, it is not surprising that modification by a bulky maleimide group would affect DNA binding. Surprisingly, the NEM sensitivity of PD DNA binding imparted by Cys82 also caused NEM sensitivity for DNA binding by the otherwise unmodified HD. Thus, a small structural change in the PD abrogates the ability of the HD to bind DNA, again suggesting functional interdependence in the form of regulatory interactions between the two domains in the wild type protein, and possibly interrupted by NEM modification of Cys82. Interestingly, WSI mutations have been identified at the flanking Gly81 (G81A) and Ser84 (S84F)(*21*) positions. These mutations do not affect DNA binding by either the PD or the HD (*38*), suggesting that another molecular aspect of Pax3 function may be impaired in these mutants.

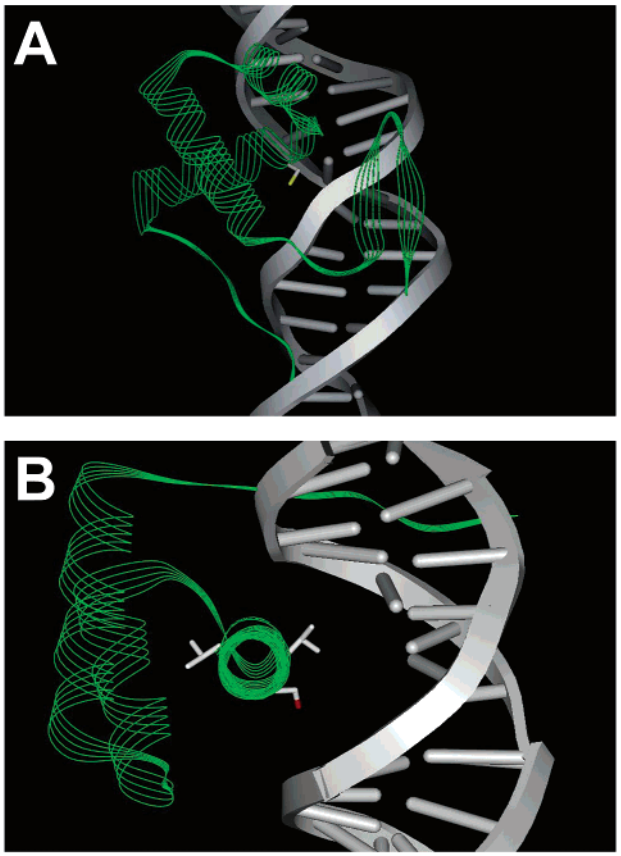

FIGURE 7: DNA-bound structure of the N-terminal subdomain of the paired domain and of the homeo domain. The N-terminal subdomain of the PD (A) and the HD (B) are shown as green ribbons drawn through the α carbon backbone. The DNA strands are shown as gray ribbons through the sugar phosphate backbone, and bases are shown as protrusions from the ribbons. The position of the endogenous PD cysteine C82 is indicated (A) and faces the major groove. (B) The HD is shown bound to DNA as a monomer. The positions of V263, V265, and S268 residues of the HD indicate that V265 and S268 face DNA while V263 is on the opposite face of the helix.

In the HD, alkylation of V263C had similar effects to those seen for Cys82 in the PD, in that it not only impaired DNA binding by the HD but also abrogated DNA-binding by the Cys-less PD present in this mutant. Although Val263 does not make DNA contacts per se, it is tucked between R262 and V265 residues in α3 helix that make phosphate contacts and base-specific contact in the major groove, respectively. Thereby, sulfhydryl modification at V263C may adversely affect important DNA contacts made by neighboring residues, possibly uncoupling DNA binding by the HD. Surprisingly, NEM modification of V263C also impaired DNA binding by the PD, therefore mirroring in a reciprocal fashion the effect seen for Cys82 in the PD. The transfer of NEM sensitivity in DNA binding by the PD seen for V263C is specific for this residue and cannot be explained by either (a) a nonspecific disruption of the HD or (b) a general effect of loss of HD DNA binding on PD binding. Indeed, alkylation of downstream positions in α3 in mutants V265C and S268C although impairing DNA binding by the HD was without consequences on PD DNA binding, demonstrating that it is possible to impair DNA binding by the HD without affecting PD activity in the same molecule. Interestingly, the loss of HD DNA binding while retaining PD binding seen after NEM modification of V265C is identical to the

phenotype of a WS1 mutation (V265F) previously reported at that position (*38*). Close examination of the structures of the HD bound to DNA suggests a possible mechanistic basis for the distinct effect of alkylation of V265C/S268C and V263C on DNA binding by the PD and HD (Figure 7). V265 and S268 make key base-specific contacts in the major groove separated by approximately one turn of the α3 helix (*32*). On the other hand, V263 is solvent-exposed and on the other side of the helix in close proximity (tightly packed) to helices α1 and α2 (Figure 7). Therefore, it is tempting to speculate that introduction of a large maleimide group at V263C may not only affect the binding of α3 to DNA but may also alter the packing of the two other helices (α1, α2) of the HD; this latter disruption may have an allosteric effect on the PD activity, an effect not seen after modification of either V265C or S268C. Studies of the Phox-1 protein have previously shown that its HD is responsible for the recruitment and physical interaction with the nuclear factor SRF (*50*). This property is specific to HDs of the paired class (Pax), is independent of the DNA binding specificity of the HD, and is mediated by pairs of charged residues on the solvent exposed face of helices 1 and 2 of the HD (*51*). Such residues are conserved in Pax3 PD, and they may participate in interactions between the HD and PD which may be disturbed by alkylation of V263C. Additional Cys-scanning mutagenesis in helices of the HTH motif will be required to further characterize this possible allosteric effect.

Together, results of site-specific modification experiments agree with the proposition that the PD and HD do not function as independent DNA binding modules in the full length Pax3 protein. Rather, it appears that both domains may functionally interact, such that one domain can modulate the DNA binding properties of the other domain. These interactions may be critical for target site selection by the Pax3 protein.

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