Characterization of Homoe- and Heterodimerization of Cardiac Csx/Nkx2.5 Homeoprotein*

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Csx/Nkx2.5 is an evolutionarily conserved homeodomain (HD)-containing transcription factor that is essential for early cardiac development. We found that the HD of Csx/Nkx2.5 binds as a monomer as well as a dimer to its DNA binding sites in the promoter of the atrial natriuretic factor (ANF) gene, an in vitro target gene of Csx/Nkx2.5. Csx/Nkx2.5 physically interacts with each other in vitro as well as in cells, and the HD is critical for homodimerization. Lys183 and Arg184, located at the COOH-terminal end of HD, are essential for dimerization. Lys183 is also required for a specific interaction with the zinc finger transcription factor GATA4. Csx/Nkx2.5 can homodimerize with other NK2 homedomain proteins, Nkx2.3 and Nkx2.5/Tix, with different affinities. A single missense mutation, Ile183 to Pro in the HD of Csx/Nkx2.5, preserved homodimerization function, but totally abolished DNA binding. Ile183 → Pro mutant acts in an inhibitory manner on wild type Csx/Nkx2.5 transcriptional activity through the ANF promoter in 10T1/2 cells. However, Ile183 → Pro mutant does not inhibit wild type Csx/Nkx2.5 function on the ANF promoter in cultured neonatal cardiac myocytes, possibly due to failure of dimerization in the presence of the target DNA. These results suggest that complex protein-protein interactions of Csx/Nkx2.5 play a role in its transcriptional regulatory function.

The homeodomain (HD)-containing transcription factors, characterized by their 60-amino acid DNA binding domain, play critical roles in developmental patterning and differentiation. The HD forms three α-helices and contacts the major groove of DNA through the third helix. Contrary to the highly specific biological functions of individual homeobox genes, in vitro DNA binding studies have demonstrated that most HD proteins bind to similar short consensus sequences containing the TAAT motif. This apparent discrepancy may result from target gene’s specificity for each HD protein in vivo being achieved by multiple mechanisms, such as interaction with other factors, small differences in DNA binding affinities to target sites, translational regulation of homeobox gene expression, subcellular localization, or protein phosphorylation.

Homo- or heterodimerization of transcription factors has been proposed to regulate transcriptional activity of many transcription factors. Combinatorial use of a limited number of transcription factors allows the regulation of a larger number of biological processes, increasing both the diversity as well as the specificity of control. However, a very limited number of studies have addressed the homo- and heterodimerization of HD-containing transcription factors among more than 400 members of HD proteins from yeast to human. Homodimerization ability has been demonstrated for Oct1 (10), Paired (11), Cdx2 (12), Even-skipped (13), Mix.1 (14), and Pit1 (15). Heterodimerization was shown for HNF1α-HNF1β (16), Oct1-Oct2 (15), Mix.1-Siamois (14), MCM1-MATα2 (17, 18), and Extra-denticle-Ultrabithorax (19). The monomer of HD proteins is sufficient to interact with DNA, and the DNA-bound monomer recruits other partners to the complex (11, 20). Most likely, the monomer HD proteins regulate through the monomeric DNA binding site, whereas homo- or heterodimerized HD proteins regulate through the dimeric sites. These differential interactions would provide more precise gene regulation at each developmental stage.

The biological significance of dimerization of paired-like class HD proteins has been demonstrated in Xenopus Mix.1, which regulates dorsal-ventral patterning (14). To date, the significance of homodimerization of HD-containing transcription factors has not been well established in mammals. Interestingly, 10 heterozygous mutations of human CSX/NKX2.5 were recently identified in patients with congenital heart disease. These patients show progressive atrioventricular conduction defects, left ventricular dysfunction, atrial septal defect, ventricular septal defect, and tetralogy of Fallot (22, 23). Four

GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MSV, murine sarcoma virus.

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mutations are single missense mutations in the HD that result in markedly reduced DNA binding (24), raising the possibility that if Csx/Nkx2.5 forms homodimers, the mutants with DNA binding defects may dominantly inhibit CSX/NKX2.5 function in human cardiac development and maturation. In Xenopus, injections of mRNA encoding non-DNA binding mutants of Xenopus XNkx2.3 and XNkx2.5 suppressed normal heart formation and resulted in a small heart or no heart formation in the most severe cases (25). This in vivo evidence suggests that a non-DNA binding mutant of Csx/Nkx2.5 may act in a dominant inhibitory manner on wild type Csx/Nkx2.5 through protein-protein interaction. Therefore, it is critical to examine whether Csx/Nkx2.5 proteins homo- or heterodimerize to regulate their transcriptional activity.

The NK2 class of HD proteins, first described in Drosophila (26), is highly conserved from nematode to human and is characterized by a unique try residue at position 54 in the third helix of the HD. The most frequently observed HD binding motif is T/AAAT, but the NK2 class HD binds to the unique motif

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The guanine nucleotide at the fourth position is the most severe cases of this motif. The guanine nucleotide at the fourth position is the seventh position of this motif.

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Recombinant Adenoviruses—FLAG- or HA-tagged wild type or FLAG-tagged Ile183→Pro mutant was inserted into the shuttle vector pADloxp vector (50), creating pADloxp-Csx/Nkx2.5(FLAG-wild) or pADloxp-Csx/Nkx2.5(FLAG-IP). One μg of plasmids was cotransfected with 1 μg of V5 viral DNA into Cref8 cells to obtain adenovirus according to the methods reported previously (50). For control, V5 viral DNA expressing no transgene was infected to 293 cells. The viral particle number was determined by plaque assays, and 5–15 multiplicity of infection was used for infection to neonatal rat cardiac myocytes prepared as described previously (51). The expression of wild type or Ile183→Pro mutant protein was determined by Western blotting and immunostaining using anti-FLAG mAb (Sigma) and anti-HA mAb (Roche Molecular Biochemicals).

Protein-Protein Interaction—Bacterially produced MBP-Csx/Nkx2.5, MBP-HD, MBP, glutathione S-transferase (GST)-GATA4 (provided by B. Markham), and GST protein were made as described previously (47). In vitro translated and transcribed proteins were generated by using TNT-coupled reticulocyte lysate systems (Promega). 1 μl of reticulocyte lysate containing 55S-labeled wild type or mutant Csx/Nkx2.5, Nkx2.5, or Nkx2.6/Ttx protein was mixed with fusion proteins in a 400 μl ml of binding buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, aprotime (2 μg/ml), pepstatin (0.07 μg/ml), 0.1 mM PMSF, 1 mM DTT, and 1% bovine serum albumin) at 4°C for 2 h. Beads were washed with binding buffer (without bovine serum albumin) and subject to SDS-PAGE.

To perform commounoprecipitation assay, 293 cells in 100-mm plates were transacted with 9 μg of pcDNA3-FLAG-Csx/Nkx2.5 and/or 9 μg of pcDNA3-HA-Csx/Nkx2.5 using the calcium phosphate method. Total plasmid amount was adjusted with pcDNA3 empty vector to 18 μg. Cells were lysed in the lysis buffer (20 mM HEPS, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.5% Nonidet P-40, aprotime (2 μg/ml), pepstatin (0.07 μg/ml), 0.1 mM PMSF, 1 mM DTT) and preclared with normal mouse IgG-bound protein G. Approximately 1 mg of protein in 1 ml of lysis buffer was incubated with 3 μg of anti-FLAG mAb affinity gel (Sigma), washed five times with lysis buffer, and resolved on SDS-PAGE and subject to Western blotting using peroxidase-conjugated anti-HA Ab (Roche Molecular Biochemicals).

Reporter Gene Assays—10T1/2 fibroblast cells cultured in six-well plates were cotransfected with 1.0 μg of ANF(-638)-Luc reporter construct (provided by K. R. Chien), 0.4 μg of Rous sarcoma virus β-galactosidase (provided by B. Markham), 0.4 μg of pcDNA3-Csx/Nkx2.5 with or without 0.4 μg or 0.5 μg of pcDNA3-Luc vector plasmid. After glycerol shock using 1 M glycerol, cells were cultured for another 48 h, lysed with 300 μl of reporter lysis buffer (Promega), and assayed for luciferase activity (Promega) and β-galactosidase activity.

To examine whether endogenous Csx/Nkx2.5 homodimerizes in cells, we cotransfected FLAG epitope-tagged Csx/Nkx2.5 expression plasmid with HA epitope-tagged Csx/Nkx2.5 expression plasmid into the human embryonic kidney carcinoma cell line 293 and confirmed that both Csx/Nkx2.5 proteins coimmunoprecipitated with anti-FLAG Ab (Fig. 2B, lane 1). Thus, Csx/Nkx2.5 can homodimerize in solution as well as in cells, and binding to DNA is not required for this interaction.

Homodimerization of Endogenous Csx/Nkx2.5 on the ANF –242 Site—To examine whether endogenous Csx/Nkx2.5 homodimerizes on the ANF –242 site, nuclear extracts prepared from neonatal rat cardiac myocytes were used for EMSA. As shown in Fig. 3A, lane 1, endogenous Csx/Nkx2.5 forms monomers (M) as well as dimers (D). These two bands corresponded to the bands shifted by the nuclear extract from adenovirus infected rat cardiac myocytes that expressed FLAG-tagged Csx/Nkx2.5 (Fig. 3A, lanes 2–4). Because of the high expression levels of Csx/Nkx2.5 in the adenoviral vector-infected cardiac myocytes, 20-fold dilution of the nuclear extracts was required.
to shift the DNA probe to a similar level compared with that of the endogenous Csx/Nkx2.5 (Fig. 3A, lane 1 versus lane 3). When the protein-DNA complex was transferred to the membrane and blotted with anti-Csx/Nkx2.5 Ab, we detected the signal at the monomeric (M) and dimeric (D) protein-DNA complex both in the uninfected and the virus infected nuclear extracts (Fig. 3B).

To ascertain whether Csx/Nkx2.5 protein forms dimers with DNA in cardiac myocytes, adenovirus-encoding FLAG-tagged Csx/Nkx2.5 and/or HA-tagged Csx/Nkx2.5 were coinfected into cardiac myocytes, and the nuclear extracts were mixed with the DNA probe for EMSA analysis (Fig. 3C). The protein-DNA complex was transferred to a membrane followed by Western blot analysis (Fig. 3D). We detected the signal at the monomeric (M) and dimeric (D) protein-DNA complex, similar to Fig. 3B. Additional slow migrating bands observed in these experiments (as in Fig. 3D) corresponded to Csx/Nkx2.5 protein unbound DNA (lanes 4–6 in Fig. 3D). Both FLAG-tagged and HA-tagged Csx/Nkx2.5 were detected at the dimeric protein-DNA complex when both proteins were coexpressed in cardiac myocytes (Fig. 3D, lane 2), suggesting that two Csx/Nkx2.5 molecules homodimerize on DNA.

Lys193-Arg194 within the HD Is Required for Dimerization—To confirm the specificity and to identify the regions that are required for dimerization, we mapped the dimerization domain of Csx/Nkx2.5 using in vitro binding assays. Initially, four [35S]methionine-labeled COOH-terminal deletion mutants were mixed with MBP-HD (Fig. 4A). Two COOH-terminal deletion mutants of Csx/Nkx2.5, 1–230 and 1–199, associated with MBP-HD, whereas the further deletions to 1–159 or 1–149 abolished the association (Fig. 4B, top panel). These results indicate that amino acids between 159 and 199 are necessary for dimerization. Next, 5 amino acid serial deletion mutants from the carboxyl terminus of HD, 1–196, 1–191, 1–186, and 1–181, were examined. The 1–196 protein interacted with the HD, but the 1–191, 1–186, and 1–181 proteins did not (Fig. 4B, middle panel). Further single amino acid deletions revealed that 1–193 dramatically reduced the interaction, and 1–192 completely abolished the association (Fig. 4B, bottom panel). Therefore, two basic amino acids, Lys193 and Arg194, are necessary for the interaction with the HD.

We further mutated Lys193 and Arg194 into neutral or acidic amino acids (Lys193 to Ile, Arg193 to Ile, and Lys193, Arg194 to Ile, Asp194) and examined them for dimerization with the HD as well as with full-length Csx/Nkx2.5 (Fig. 4C). The Lys193 → Ile mutant markedly reduced the interaction with the HD, and an ~50% reduction was observed in Arg194 → Ile mutant. The interaction with the HD was undetectable when both amino acids were mutated.
acids were mutated (Fig. 4C, HD). However, we still detected a weak interaction between Lys193-Arg194 mutant and full-length Csx/Nkx2.5 (Fig. 4C, Full). These findings confirm that two amino acids Lys193 and Arg194, are required for the dimerization of the HD, and additional protein domain(s) outside of HD are also likely to be involved in dimerization.

Involvement of the Region(s) Outside of HD for Dimerization—To identify the domain(s) outside of the HD of Csx/Nkx2.5 that are involved in homodimerization on DNA, we examined DNA binding affinity of HD and full-length protein on the palindromic ANF −242 site (Fig. 5A). The HD protein bound DNA predominantly as a monomer at a low protein concentration (Fig. 5A, panel a, lanes 1–3) and dimerized more at a higher protein concentration (Fig. 5A, panel a, lanes 5 and 6). The monomer to dimer transition was observed between lanes 4 and 5 at a protein concentration of 0.91–2.7 × 10⁻⁶ M in the HD protein (arrow in Fig. 5A, panel a). However, with the full-length Csx/Nkx2.5 (Fig. 5A, panel b), the monomer-dimer transition occurred more abruptly between lane 2 and lane 3 (protein concentration 0.71–2.1 × 10⁻⁷ M). We tested and confirmed that HD and full-length protein bound to the mutated monomeric site with similar affinity ($K_d$ = 1.0–3.0 × 10⁻⁹ M for HD; 0.71–2.1 × 10⁻⁹ M for full-length Csx/Nkx2.5) (Fig. 5A, panels c and d).

To further examine the regions responsible for dimeric DNA binding, we constructed two deletion mutants, a carboxyl terminus deletion mutant (1–250) and an amino-terminal deletion mutant (122–318) and examined their DNA binding on the ANF −242 site (Fig. 5B). In the COOH-terminal deletion mutant (Fig. 5B, panel a), the monomer-dimer transition was observed between lanes 4 and 5 (0.66–2.0 × 10⁻⁷ M), which was similar to that of HD protein. The amino-terminal deletion showed the monomer-dimer transition between lanes 3 and 4 (2.4–7.3 × 10⁻⁹ M) (Fig. 4B, panel b), therefore it required 3-fold lower protein concentration than that of the HD or the carboxyl terminus deletion, but still required 3-fold higher protein concentration than that of the full-length protein. Taken together, although the HD and full-length Csx/Nkx2.5 binds the monomeric DNA binding site with a similar affinity, full-length Csx/Nkx2.5 preferentially forms dimers at 13-fold lower protein concentration than the HD alone. Thus, regions outside of the HD, particularly the COOH-terminal region of Csx/Nkx2.5, seem to facilitate protein-protein interactions involved in the dimerization on DNA.

Lys193 Is Necessary for Association with GATA4—We and others have reported that Csx/Nkx2.5 interacts with the transcription factor GATA4 (42–45). It was demonstrated that the
second zinc finger of GATA4 is involved in the specific interaction with the HD of Csx/Nkx2.5, and amino acids between 182 and 199 are responsible for the direct interaction with GATA4. Our data presented in Fig. 4 revealed that this domain is also responsible for homodimerization. Therefore, we examined whether GATA4 associates with Lys193-Arg194 mutants (Fig. 6A). As shown in Fig. 6A, lane 1, the wild type Csx/Nkx2.5 (1–318) associated with GATA4-GST protein, whereas Lys193Ile (lane 2) and Lys193Ile/Arg194Asp (lane 4) mutants abolished the interaction. Interestingly, the Arg194Ile mutant (lane 3) associated with GATA4 with an apparent higher affinity than wild type Csx/Nkx2.5, in contrast to its lower homodimerization ability (Fig. 4C). These data demonstrate that Lys193 in the HD of Csx/Nkx2.5, which is critical for homodimerization, is also essential for the interaction with GATA4.

Heterodimerization of Csx/Nkx2.5 with Other NK2 Class HD Proteins—Since Xenopus XNkx2.3 and XNkx2.5 are co-expressed in the heart, and Csx/Nkx2.5 and Nkx2.6/Tbx are co-expressed in restricted areas in the mouse heart (31, 32, 42), we examined the potential interaction of Csx/Nkx2.5 with wild type Csx/Nkx2.5 and XNkx2.6/Tbx by using in vitro binding assay. As shown in Fig. 6B, full-length Csx/Nkx2.5 associated with Csx/Nkx2.5 as well as XNkx2.6/Tbx, and weakly with Nkx2.3. Therefore, Csx/Nkx2.5 demonstrates the potential to interact with other NK2 class proteins with varying binding affinity depending on the partner.

Generation of an Inhibitory Mutant—To examine the effects of protein dimerization on transcriptional activity, we attempted to create a mutant protein that does not dimerize, but does bind, DNA. As shown in Fig. 4, we constructed Csx/Nkx2.5 mutants that do not dimerize to the HD. We next examined the DNA binding of these mutants using ANF242 site and found that the Lys193Ile mutant completely abolished DNA binding (Fig. 6A). Lys193-Arg194 mutant bound DNA, but the binding affinity was significantly lower than that of wild type Csx/Nkx2.5 (Fig. 7A). As an alternative to examine the effect of protein dimerization, we generated a converse mutant in which protein dimerization is preserved, but DNA binding is abolished. By mutating Ile183 in the third helix of HD into Pro, DNA binding of Csx/Nkx2.5(Ile183Pro) mutant was completely abolished (Fig. 7B), but this mutant associated with MBP-Csx/Nkx2.5 protein with a similar affinity as that of wild type protein (Fig. 7C, lane 1 versus lane 2). In contrast, this Ile183Pro mutant markedly reduced the interaction with GATA4 (Fig. 7C, lane 6).

We tested the function of the Ile183Pro mutant by transient transfection assays in 10T1/2 fibroblasts using ANF(2638)-luciferase reporter construct (ANF-Luc), which includes the −87 and −242 bp sites shown in Fig. 1A. The Ile183Pro mutant did not bind DNA (Fig. 7B), and the mutant itself did not activate or repress the ANF-Luc (data not shown). When we cotransfected the expression plasmid encoding Ile183Pro—Pro mutant protein with wild type Csx/Nkx2.5 at 1:1 ratio (0.4 μg), the luciferase activity of wild type Csx/Nkx2.5 de-
creased by $\sim$44%. A slight further reduction ($\sim$53%) of ANF-Luc activity was observed when the Ile$^{183}\rightarrow$Pro expression plasmid was increased to 2:1 (0.8 $\mu$g) (Fig. 1D). In the presence of GATA4 expression plasmid, we observed a further increase of ANF-Luc activity from the ANF promoter as reported previously (43). We found that the Ile$^{183}\rightarrow$Pro mutant reduced luciferase activity by $\sim$20% at 1:1 ratio of plasmid amount and by $\sim$44% at 2:1 ratio. These data demonstrate that the non-DNA binding mutant, Ile$^{183}\rightarrow$Pro, acts in an inhibitory manner on wild type Csx/Nkx2.5 in transient transfection assays in 10T1/2 cells.

Ile$^{183}\rightarrow$Pro Mutant Does Not Inhibit the Csx/Nkx2.5-dependent ANF Promoter Activation in Neonatal Cardiac Myocytes—We further examined the inhibitory effect of the Ile$^{183}\rightarrow$Pro mutant on endogenous Csx/Nkx2.5 as well as wild type Csx/Nkx2.5 in cultured neonatal cardiac myocytes. In rat neonatal cardiac myocytes, the base-line ANF-Luc activity was high. When we used the LipofectAMINE transfection method, the base-line ANF-Luc activity was approximately the same as that detected in 10T1/2 cells transfected with the wild type Csx/Nkx2.5 expression plasmid. ANF-Luc activation was suppressed by the cotransfection of Ile$^{183}\rightarrow$Pro expression plasmid by 29%. (Fig. 8A, $^{183}$I-P). When we cotransfected the wild type Csx/Nkx2.5 expression plasmid, ANF-Luc activity was increased by 50% (Fig. 8A, Wild). However, cotransfection of Ile$^{183}\rightarrow$Pro expression plasmid did not inhibit the wild type Csx/Nkx2.5 function (Fig. 8A, $^{183}$I-P+Wild). Similar results were obtained using the calcium phosphate method (data not shown).

Transfection efficiency of primary cardiac myocytes is known to be very low when plasmid vectors are used. Therefore, we infected cardiac myocytes with adenoviral vectors, which exhibit a high efficiency of gene transfer. More than 90% of cardiac myocytes expressed either Ile$^{183}\rightarrow$Pro mutant or wild type Csx/Nkx2.5 (Fig. 8B), and each construct expressed a similar protein amount determined by Western blotting (Fig. 8C). Twenty-four hours after adenovirus infection, ANF-Luc reporter gene was transfected into cardiac myocytes, and the transcriptional activation was measured by luciferase activity. When Ile$^{183}\rightarrow$Pro mutant protein was expressed by the adenoviral vector, ANF-Luc activity was suppressed by 31% (Fig. 8D, $^{183}$I-P). In contrast, wild type Csx/Nkx2.5 activated the ANF-Luc reporter by 3.5-fold (Fig. 8D, Wild), which was not suppressed by coexpression of Ile$^{183}\rightarrow$Pro mutant (Fig. 8D, $^{183}$I-P+Wild). When we examined DNA binding of the nuclear extract from cardiac myocytes expressing wild type alone (Fig. 8E, panel a) or wild type with Ile$^{183}\rightarrow$Pro mutant (Fig. 8E, panel b), there was no significant difference in DNA binding of wild type Csx/Nkx2.5 either as monomers or dimers. These data indicate that the expression of Ile$^{183}\rightarrow$Pro mutant...
positively charged amino acids, Lys193-Arg194 at the COOH-terminal end of the HD of Pit1 are involved in the homodimerization on DNA by forming a protein-protein interface with the POU-specific domain (15). In contrast to Csx/Nkx2.5, Pit1 requires DNA to homodimerize (59). The Lys193-Arg194 mutation, located at the carboxyl end of the HD in Csx/Nkx2.5, markedly reduced DNA binding, which is consistent with the NMR structure of another NK2 class HD protein Drosophila NK-2. The third a-helix (helix III) of NK-2 extends up to amino acid 62 in the presence of DNA (52, 60). We demonstrated that Lys193 is required for the Csx/Nkx2.5 and GATA4 interaction as well as for homodimerization of Csx/Nkx2.5.

Regions Outside the HD Facilitate Dimerization on DNA—Cooperative dimerization of HD proteins has been characterized in paired and paired-like HD proteins (11). Paired HD proteins cooperatively bind DNA with a palindromic TAAT sequence separated by 3 bp. The presence of Arg28 or Arg43 prevents cooperative dimerization, and paired class HD proteins do not have Arg residues at the 28 and 43 positions (11). In contrast, 50% of HD proteins have conserved Arg28 or Arg43 residues among ~350 HD proteins (53). NK class HD proteins, as well as engrailed, bcd, POU, and msh class proteins do not have Arg28 or Arg43, suggesting the possibilities for cooperative dimerization in these classes of HD proteins.

In Csx/Nkx2.5, regions outside of the HD, particularly the region carboxyl terminus to the HD (aa 251–318), appear to facilitate cooperative dimerization on DNA. Compared with the DNA binding of HD of Csx/Nkx2.5 or the COOH-terminal deletion mutant, we found that the full-length protein facilitated dimerization, and the monomer-dimer transition occurred at ~13-fold lower protein concentrations than that of the HD or the carboxyl terminus deletion mutant. Our observation that Csx/Nkx2.5 homodimerizes through the HD as well as outside of the HD supports the hypothesis that protein-protein interactions play important roles in cooperative dimerization. Alternatively, a full-length Csx/Nkx2.5 molecule may “bend” DNA to facilitate the binding of a second Csx/Nkx2.5 molecule to DNA, or full-length Csx/Nkx2.5 proteins may be more stable than HD proteins in a dimerized form on DNA. It is also possible that these effects may function cooperatively to regulate the transcriptional activation of target genes.

Palindromic NK2-specific binding sites are also identified in the enhancer of the Drosophila ind gene, which is the target of...
Another NK2 class HD protein Vnd (61). Also, Tinman, the *Drosophila* homologue of *Cxs/Nkx2.5*, binds the sequence located at -5.4 kilobases of the *Dmef2* gene, which contains two NK2 binding sites separated by 165 bp (62). Mutations that disrupt either one of two Tinman binding sites caused loss of binding to the *Dmef2* gene, leading to the hypothesis that the physical interaction of Tinman molecules occurs by looping of the 165-bp intervening segment (62). Although it has yet to be shown that Tinman protein homodimerizes, our data are consistent with this hypothesis.

**Heterodimerization with Other NK2 Class Proteins**—Several NK2 class HD proteins are coexpressed both temporally and spatially, suggesting that NK2 class HD proteins may heterodimerize. As shown in Fig. 2A, *Cxs/Nkx2.5* and *Nkx2.6/Tix* associated with each other, but the association of *Cxs/Nkx2.5* and *Nkx2.3* was significantly weaker. Although further quantitative analyses are necessary, these results suggest that NK2 class HD proteins potentially interact with each other, and the affinity of the interaction is different depending on heterodimer pairs. In this study, we did not examine the possible heterodimerization of *Cxs/Nkx2.5* with other classes of HD proteins. Of note, mouse *Nkx2.3* is not expressed in the heart, and *Nkx2.6/Tix* expression is restricted to the sinus venosa and outflow tract of the mouse heart. Other NK2 class HD proteins coexpressed temporally and spatially similar to *Cxs/Nkx2.5* in the heart have not been described in mouse and human (29, 41, 63).

The Effect of Non-DNA Binding Mutant on Wild Type *Cxs/Nkx2.5*—Based on the studies of phenotypes caused by the non-DNA binding mutants of *Cxs/Nkx2.5* in patients and *Xenopus* (22–25), and on the evidence for the formation of homodimers of *Cxs/Nkx2.5* in our study, non-DNA binding mutants might act in a dominant inhibitory manner. We generated a single missense mutation in the third helix of the HD Csx/Nkx2.5(Ile183->Pro), which abolishes DNA binding, but preserves dimerization ability. The interaction between GATA4 and Csx/Nkx2.5(Ile183->Pro) was significantly weaker than that of wild type Csx/Nkx2.5; therefore, it is likely that Csx/Nkx2.5(Ile183->Pro) will not sequester GATA4 from wild type Csx/Nkx2.5 when it is overexpressed. The transcriptional activation of wild type Csx/Nkx2.5 on ANF(-638) promoter was indeed suppressed by the coexpressed Csx/Nkx2.5(Ile183->Pro) in a dose-dependent manner in 10T1/2 cells.

ANF(-638) promoter activity was slightly suppressed by

**Fig. 7.** A non-DNA binding mutant with preserved homodimerization acts in an inhibitory manner in vitro. A, two mutants with protein dimerization defects were examined for DNA binding. EMSA shows Lys183->Ile completely abolished DNA binding (middle panel), and Lys183->His/Arg184->Asp significantly decreased DNA binding (right panel) compared with wild type (left). B, an Ile183->Pro mutant was created by mutating Ile183 into Pro in the third helix of HD (see Fig. 3A). EMSA shows Ile183->Pro completely abolished DNA binding (right panel). C, protein dimerization of wild type (W) and Ile183->Pro mutant (IP). -35-Labeled wild type (W) (lanes 1, 3, 5, 7) or Ile183->Pro mutant (IP) protein (lanes 2, 4, 6, 8) were mixed with MBP-wild type Csx/Nkx2.5 (lanes 1 and 2), MBP (lanes 3 and 4), GATA4-GST (lanes 5 and 6), or GST fusion protein (lanes 7 and 8). Complexes were resolved by SDS-PAGE and autoradiographed. The Ile183->Pro mutant associated with MBP-Csx/Nkx2.5 protein (lane 2) similar to wild type Csx/Nkx2.5 (lane 1). The association between Ile183->Pro mutant and GATA4 (lane 6) was markedly reduced compared with wild type (lane 5). D, inhibition of Csx/Nkx2.5-dependent transactivation in the presence of increasing amount of Ile183->Pro expression plasmid. 10T1/2 cells were transiently transfected with 0.4 µg of pcDNA3-wild type Csx/Nkx2.5 expression plasmid, ANF-Luc, and RSV-β-GAL plasmid and the indicated amount of pcDNA3-Csx/Nkx2.5(Ile183->Pro) expression plasmid. Approximately 44% reduction in transactivation was seen upon transfection with 0.4 µg, and -53% reduction with 0.8 µg of pcDNA3-Csx/Nkx2.5(Ile183->Pro) (left panel). The right panel shows the inhibitory effect of Ile183->Pro mutant and GATA4 expression vector. Cotransfection with GATA4 plasmid increased ANF-Luc activity as reported previously (43). The Ile183->Pro mutant reduced ANF-Luc by ~20% at a 1:1 ratio (0.4 µg) and by 44% at a 2:1 ratio (0.8 µg). Values are means ± S.E.
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Csx/Nkx2.5(Ile^{183}→Pro) in neonatal cardiac myocytes where endogenous Csx/Nkx2.5 is expressed. However, when both wild type and Csx/Nkx2.5(Ile^{183}→Pro) mutants were overexpressed in cardiac myocytes, transcriptional activation by wild type was not suppressed by the Csx/Nkx2.5(Ile^{183}→Pro) mutant. Thus, unlike in 10T1/2 cells, Csx/Nkx2.5(Ile^{183}→Pro) does not seem to act as a typical dominant inhibitor mutant on the ANF(−638) promoter in cultured cardiac myocytes. The EMSA using cell lysates prepared from adenovirus-infected cardiac myocytes revealed that coexpression of Csx/Nkx2.5(Ile^{183}→Pro) mutant does not inhibit the specific binding of wild type Csx/Nkx2.5 to the ANF −242 site (Fig. 8E). Since the Csx/Nkx2.5(Ile^{183}→Pro) mutant expressed in cardiac myocytes did not bind to the ANF −242 site (data not shown), it is possible that Csx/Nkx2.5(Ile^{183}→Pro) mutant loses the ability to form dimers with wild type Csx/Nkx2.5 on DNA and, therefore, does not inhibit the function of wild type Csx/Nkx2.5 on the ANF promoter in cardiac myocytes. It is also possible that the inhibitory effect of Csx/Nkx2.5(Ile^{183}→Pro) observed in 10T1/2 cells may occur through a mechanism independent of wild type Csx/Nkx2.5. Mutant Csx/Nkx2.5 protein may quench a transcription factor that is critical for the ANF(−638) promoter activity in 10T1/2 cells.

Protein homodimerization of Csx/Nkx2.5 yields the potential for it to precisely regulate a number of genes by utilizing monomeric and dimeric binding. It is possible that the genetically dominant effect of the human CSX/NKX2.5 missense mutation (22–24) may in part be due to an inhibitory effect of the mutant protein over the wild type protein on target genes that require dimeric binding.

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