A SOX9 Defect of Calmodulin-dependent Nuclear Import in
Campomelic Dysplasia/ Autosomal Sex Reversal

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Running Title: Calmodulin regulates the nuclear localisation of SOX9.

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Summary

During mammalian sex determination, SOX9 is translocated into the nuclei of Sertoli cells within the developing XY gonad. The N-terminal nuclear localization signal (NLS) is contained within a SOX consensus calmodulin (CaM) binding region, thereby implicating CaM in nuclear import of SOX9. By fluorescence spectroscopy and glutaraldehyde cross-linking, we show that the SOX9 HMG domain and CaM interact in-vitro. The formation of a SOX9/CaM binary complex is calcium-dependent and is accompanied by a conformational change in SOX9. A CaM antagonist, calmidazolium chloride (CDZ), was observed to block CaM recognition of SOX9 in-vitro and inhibit both nuclear import and consequent transcriptional activity of SOX9 in treated cells. The significance of the SOX9-CaM interaction was highlighted by analysis of a missense SOX9 mutation, A158T, identified from a XY female with campomelic dysplasia/autosomal sex reversal (CD/SRA). This mutant binds importin β normally despite defective nuclear import. Fluorescence and quenching studies indicate that in the unbound state, the A158T mutant shows a similar conformation to that of the WT SOX9, but in the presence of CaM, the mutant undergoes unusual conformational changes. Furthermore, SOX9-mediated transcriptional activation by cells expressing the A158T mutant is more sensitive to CDZ than cells expressing WT SOX9. These results suggest firstly that CaM is involved in the nuclear transport of SOX9 in a process likely to involve direct interaction and secondly, that CD/SRA can arise, at least in part, from a defect in CaM recognition, ultimately leading to reduced ability of SOX9 to activate transcription of cartilage and testes-forming genes.
Introduction

SOX (Sry-related HMG\textsuperscript{1} box) proteins are a large family of transcription factors that play critical roles in cell-fate, differentiation and development and show diverse, overlapping expression profiles (1, 2). SOX proteins activate the transcription of target genes by binding to DNA in a sequence-specific manner through their HMG box and by interacting with specific partner-proteins (1, 2).

SOX9, an early embryonically expressed gene, has a role in binding to and regulating a number of genes in the chondrogenesis pathway (3, 4, 5) and testis formation pathway (6). The influence of SOX9 upon these pathways is evident where mutations in SOX9 result in the disease campomelic dysplasia/autosomal sex reversal (CD/SRA), a severe bone malformation syndrome in which most XY individuals show male-to-female sex reversal (7, 8). Unlike frameshift and nonsense mutations which occur throughout the open reading frame of human SOX9, all known missense point mutations in SOX9 that cause CD have been localized at the HMG box (9).

During early human and mouse embryogenesis, SOX9 is expressed in the cytoplasm of Sertoli cells in both sexes, but by gestational week 7, at the onset of SRY expression in male embryos, SOX9 moves into the nucleus (10, 11). Here, SOX9 activates the gene for mullerian inhibitory substance (MIS), which is required for the regression of the female reproductive system (10). SOX9 also activates the testis determining gene, SF1 (10). Thus, the regulation of nuclear import of SOX9 in particular and SOX transcription factors generally is likely to play a critical role in their regulation of gene expression given their shared HMG box. SOX proteins contain two nuclear localisation signals (NLSs) located at the N- and C-termini of the HMG box (Fig. 1). The N-terminal NLS resembles the bipartite NLS identified in nucleoplasmin, a small nuclear
protein that consists of two highly basic regions separated by 9-12 residues (KRPAATKKAGQAKKKK) (12). The C-terminal NLS of SOX proteins resembles the NLS of the SV40 large-T antigen, which consists of a short stretch of basic residues (PKKKRK) (13). The fact that both the N- and C-terminal NLS are highly conserved throughout the whole SOX family suggests that the mechanism of nuclear import of SOX proteins is likely to be conserved.

Nuclear import of NLS-containing proteins occurs in two steps. The first step requires recognition of the NLS-containing protein by the transport receptor and docking to the nuclear pore complex (NPC). The second step is an energy-dependent translocation of the NLS-containing protein through the NPC, which requires GTP (14, 15). In conventional nuclear import pathways, the transport receptor importin α binds to the NLS in the protein. Docking of the protein to the cytoplasmic side of the NPC requires importin β, which binds to the importin α-NLS-protein complex. Importin β interacts with multiple nuclear pore proteins (known as nucleoporins) at the NPC, which then facilitates translocation of the importin/NLS-containing protein complex through the NPC (14, 15). Translocation to the nucleoplasmic side of the NPC requires multiple docking and undocking reactions involving importin β and nucleoporins, the guanine nucleotide-binding protein, Ran, and several modifying proteins (16). Once the complex is translocated to the nuclear side of the NPC, binding of RanGTP to importin β causes dissociation and release of the importin α/β-NLS-protein complex. While most protein NLSs bind to importin α, several bind directly to importin β (17, 18), including the C-terminal NLS of the SRY HMG box and the SOX9 HMG box (19, 20). This indicates that the importin/Ran GTP-dependent pathway may mediate the nuclear import of SOX proteins by direct binding of the transport receptor, importin β, to the C-terminal NLS.
located in the HMG box. The N-terminal NLS is also a functional NLS, but this NLS is not recognized by importins $\alpha$ or $\beta$ (20, 21).

Other mechanisms of nuclear protein import have been described (14, 15). Intracellular calcium plays an important role in protein nuclear import. When cytosolic calcium is depleted from the lumen of the endoplasmic reticulum (by treatment of cells with thapsigargin or ionophores), both signal-mediated and passive diffusion of proteins across the nuclear pore complex is inhibited. Sweitzer and Hanover, using a digitonin-permeabilised HeLa cell assay system observed a similar effect on nuclear accumulation of the intrinsically fluorescent algal protein, B-phycoerythrin (to which the NLS of the Simian Virus (SV) 40 large T antigen is linked) (22, 23). After treatment with thapsigargin, nuclear accumulation of proteins occurred only in the presence of GTP, indicative of a Ran/GTP–dependent nuclear import pathway (22, 23). However, at high cytosolic calcium levels, nuclear import occurs in the absence of GTP, suggesting an alternative Ran/GTP–independent nuclear import pathway (23).

One of the major cytosolic $\text{Ca}^{2+}$-binding proteins is calmodulin (CaM), an intracellular calcium receptor, which is located in both the nucleus and cytoplasm of cells (24). CaM antagonists, such as calmidizolium (CDZ), W7 and W12, block GTP-independent nuclear import of the SV40 large-T antigen NLS at elevated cytoplasmic calcium levels in permeabilised HeLa cells (23). The addition of recombinant CaM and ATP to cells restored nuclear import (23). Based on these observations, Sweitzer and Hanover proposed a model where, in the presence of cytoplasmic calcium, CaM stimulates nuclear import of proteins in a GTP-independent manner (23). CaM itself has unusual nuclear import properties (25). CaM import is inhibited by the addition of wheat germ agglutinin and chilling (which inhibits nucleopore complex-mediated transport and
passive diffusion respectively) in a permeabilised assay system suggesting that CaM import is facilitated. However ATP depletion was found to not affect CaM import indicating that it is not an active process (25). Hence the actual mechanism of CaM nuclear import is not clearly understood but may involve associations with CaM binding proteins.

Studies have shown that CaM interacts directly with the C-terminal domain of p21<sup>Clp1</sup>, an inhibitor of the cyclin-dependent kinase, Cdk4 (26, 27). This domain of interaction, has a highly basic amphiphilic region, which is characteristic of CaM binding domains, and also contains several putative NLSs (27). Treatment of normal rat kidney (NRK) cells with the CaM antagonist, W7, resulted in cytoplasmic accumulation of p21<sup>Clp1</sup> (26, 27), indicating that CaM may be able to mediate nuclear import of proteins that contain classical NLS signals.

The SRY HMG box interacts with CaM in a calcium-dependent manner in-vitro (28). The interaction of SRY with CaM was mapped to a 25-amino acid sequence, located in helix 1 of the SRY HMG box (Fig. 1) (28). The residues within this domain consist mainly of hydrophobic and basic amino acids that overlap the bipartite N-terminal NLS region, which along with the SV40-like NLS is required for maximal nuclear import of SRY and SOX9 (29). This domain is highly conserved in the SOX family at both the amino acid sequence and structural levels forming a basic amphiphilic α-helix (Fig. 1). In this present study, we report that SOX9 can directly bind Ca<sup>2+</sup>-CaM in a 1:1 complex and that this interaction results in a conformational change in the structure of the SOX9 HMG box. Treatment of cultured cells that express SOX9 with CaM antagonists reveal that CaM is involved in mediating SOX9 nuclear import and consequent transcriptional activation activity. This suggests that CaM, along with the
transport receptor, importin β, regulate the nuclear localisation of SOX9 and other SRY/SOX proteins.
Experimental Procedures

**SOX9 HMG protein production and purification.** Recombinant SOX9 HMG protein (amino acids 101-184) was expressed and purified from *Escherichia coli* as described (30) with a modification to the protocol as follows. SOX9 HMG protein was extracted from cell pellets which were washed in phosphate buffered saline, followed by sonication in 100 ml HEDA buffer (50 mM HEPES, pH 7.9; 1 mM EDTA; 1 mM DTT; 50 µM AEBSF). The sample was then brought to a final concentration of 450 mM NaCl and mixed with a 0.1 volume of 50% DEAE-Sephadex equilibrated in HEDA/450 mM NaCl for 10 min at 4°C. Following centrifugation (Sorvall RC5B GS3 rotor; 6000 rpm, 10 min, 4°C) the salt concentration of the supernatant was reduced to < 0.2 M by diluting the sample volume 2.5-fold with HEDA buffer and the supernatant was filtered through a 0.45 µM membrane. The sample was injected onto a 10 ml SP-Sepharose FPLC column pre-equilibrated in HEDA buffer containing 200 mM NaCl. Bound proteins were eluted over 40 min with a salt gradient of HEDA buffer containing 200 mM to 1000 mM NaCl and 5 ml fractions collected. SOX9 HMG protein elutes in fractions at about 0.7 M NaCl. SOX9 HMG protein was desalted and concentrated using Amicon Centricon 3 columns. Protein concentrations were determined using Bradford assay reagent kit (BIORAD) and bovine serum albumin as a standard.

**Native polyacrylamide gel electrophoresis.** Gel and buffer composition were essentially as for SDS-PAGE in standard Tris-glycine buffers but lacking SDS, and consisting of a 12.5% separating gel and a 5% stacking gel (28). Reaction mixtures were made to a final volume of 20 µl, typically containing 1 µg (3 µM) CaM (Calbiochem) and 3-4 µg (43 µM) purified SOX9 HMG box protein or 1 µg Protein Kinase II (PKII) peptide (Calbiochem). Reactions were incubated for 1 h on ice prior to
electrophoresis. After electrophoresis, gels were stained overnight with Neuhoff stain (31).

**Glutaraldehyde cross-linking of CaM and full-length SOX9.** SOX9 was synthesised in-vitro with incorporation of $[^{35}\text{S}]$-methionine ($^{35}\text{S}$-Met) using a TNT T7/T3 transcription and translation kit (Promega). Cross-linking reagent, 0.01% glutaraldehyde (Sigma Aldrich) was added to 0.025 µg of SOX9 protein translate in the presence of 5 mM CaCl$_2$ and increasing amounts of CaM (0.025 – 2.5 µM). The reactions were incubated for 20 min at room temperature followed by inactivation with SDS sample buffer at 95°C. Proteins were resolved by SDS-PAGE (12.5%) and detected by autoradiography.

For experiments analyzing the amount of CaCl$_2$ required for CaM-SOX9 interaction, calcium was removed from SOX9 translate by passing the protein through a CentriSep gel filtration column (Princeton Separations). To reactions containing 1 nM SOX9, CaM was added to 5 µM, and increasing amounts of CaCl$_2$ (2 – 50 µM) were added to replica tubes and the mixture incubated on ice for 20 minutes. Cross-linking reagent, glutaraldehyde (Sigma Aldrich) was then added to a final concentration of 0.01% and incubated for 10 minutes at room temperature. The reaction was inactivated by addition of SDS-PAGE sample buffer and heating at 95°C for 2 minutes. Proteins were resolved by SDS-PAGE (10%) and detected by autoradiography.

**Measurement of Fluorescence spectra.** Fluorescence spectra were recorded at 20°C on a SPEX Fluorolog-τ2 frequency domain spectrofluorometer using 0.5 ml cells. The solutions contained 0.58 µM of purified SOX9 HMG protein, 0.94 µM annealed oligonucleotides or 0.7 µM CaM. Double-stranded wildtype DNA (S9CONwtF [GGGTTAACAGAACAATGGAATCTGGTGA] and its complement, S9CONwtR) used
contained the high-affinity SOX9 DNA binding site (underlined) (29). Double-stranded mutant DNA consisted of AllMuTF (GGGGTTAACGTCCCGGTAAATCTGGTAGA) and its complement, AllMuTR. SOX9 was incubated with CaM on ice for 30 minutes in 25 mM Tris, pH 7.0/0.1 M KCl/50 mM CaCl$_2$ and fluorescence measurements were taken. DNA was added, and the mixture was incubated on ice for a further 30 minutes before measurements were taken.

Excitation of tryptophan residues was accomplished using an excitation wavelength of 295 nm from a 450 W Xenon lamp. Emission spectra were measured in the wavelength range of 300 nm to 500 nm, and through a polarizer oriented at the magic angle (54.7°). The spectral band pass of excitation and emission was 5 nm. Spectra were fully corrected for the wavelength response of the detection system. The cell block was maintained at 20°C with a circulating water bath.

Acrylamide quenching was performed using an excitation wavelength of 295 nm to avoid tyrosine excitation and distortion by acrylamide. 10 µl aliquots of 4M acrylamide were added successively to 400 µl of a 2 µM protein sample, and the fluorescence intensity recorded at 350 nm with a Perkin Elmer LS-5 spectrofluorometer. The fluorescence was corrected for dilution and inner-filtering by acrylamide. The quenching profiles were analyzed in terms of a two-site quenching model using the following equation; $I/I_0 = \alpha + (1-\alpha)/(1+K[Q])$ where $I$= fluorescence in the presence of quencher, $I_0$= fluorescence in the absence of quencher, $[Q]$=concentration of quencher, $\alpha$=fraction of fluorescence not accessible to quenching and $K$=Stern-Volmer accessibility to quencher.

**Transient Transfection.** COS7 cells were transfected by FuGene 6 as recommended by the manufacturers (Roche Diagnostics). The antagonists W7, W12,
cyclopiazonic acid (CPA) and CDZ (Sigma Aldrich) were added 24 h after transfection and incubated for a further 24 h prior to cell harvesting. To measure SOX9 transcriptional activity, CAT-ELISA assays were performed as described previously (32). Linear regression (Microsoft Excel) was used to calculate the slope of transcriptional activation and its projection was used to determine the 50% inhibition constant (IC$_{50}$) of the drug on transfected cells.

**Immunohistochemistry.** COS7 cells were transiently transfected with 500 ng pcDNA3-SOX9 using lipofectamine (GIBCO-BRL) according to manufacturer instructions. The antagonists W7, W12, CPA and CDZ at concentrations ranging from 3 µM to 300 µM were added 24 h after transfection and the transfected COS7 cells incubated for a further 24 h prior to protein detection. SOX9 protein was detected as described previously (32). Similarly, EGTA was added 24 h after transfection, and the transfected cells were incubated for a further 20 min before immunohistochemistry. Confocal laser scanning microscopy (CLSM) (BIORAD MRC-500) was used for quantitation of nuclear accumulation of SOX9 protein and was performed as described (33). Measurements were taken of the density of fluorescence from the cytoplasm and the nucleus with the background fluorescence subtracted from the equation: \[ \frac{F_n}{c} = \frac{n - bkgdn}{cp - bkgdncp} \] where \( n \) = nucleus and \( bkgdn \) = background in the nucleus, \( cp \) = cytoplasm and \( bkgdncp \) = background in the cytoplasm.
Results

We showed previously that the SRY HMG box can interact with CaM in a calcium-dependent manner through a 25-amino acid sequence, located in helix 1 of the SRY HMG box which includes the N-terminal NLS (Fig. 1) (28). This domain is highly conserved among members of the SOX family suggesting that CaM is likely to bind to other SOX proteins (Fig. 1). E.coli recombinant SOX9 HMG box was purified to about 95% homogeneity as observed by Coomassie stained SDS-PAGE gel (Fig. 2A, Lane 2). Next, SOX9 HMG box protein was electrophoresed on a native PAGE gel in the presence or absence of CaM, and the calcium dependence of the interaction between SOX9 and CaM was assessed. In this gel system SOX9 HMG box alone is too basic to migrate into the gel (Fig. 2B, Lane 1). For mixtures of SOX9 and CaM in the presence of the chelating agent, EGTA, only CaM was observed (Fig. 2B, Lane 3), In the presence of calcium, migration of CaM was retarded relative to that in the presence of EGTA (Fig. 2B, Lane 2 and 5). In the presence of calcium and SOX9, the migration of CaM was further retarded due to the formation of a Ca$^{2+}$-CaM-SOX9 complex (Fig. 2B, Lane 6). A CaM-binding peptide of PKII, which binds with high affinity to CaM, was added to the assay to assess the specificity and strength of interaction between CaM and SOX9. At equimolar concentrations of PKII and CaM, the Ca$^{2+}$-CaM-SOX9 complex was abolished (Fig. 2B, Lane 7). Hence, the HMG box of SOX9, like SRY, binds to CaM, in a calcium-dependent manner. Binding is competed by PKII peptide, which suggests that SOX9, like PKII, binds to CaM in a conventional way, with the two globular domains of CaM engulfing the α helix of the target.

To determine the stoichiometry of the binding, CaM and radiolabelled SOX9 were incubated with a cross-linking agent. $^{35}$S-Met labelled SOX9 migrates as a single band
at about 65 kDa, slightly higher than its predicted molecular weight of 61 kDa (Fig. 2C, Lane 6). SOX9, in the presence of glutaraldehyde, still migrated as a 65 kDa band suggesting that free SOX9 is a monomer and does not homodimerise (Fig. 2C, Lane 1). In the presence of calcium and CaM, a complex migrating at about 85 kDa was observed (Fig. 2C, Lane 3), consistent with a 1:1 SOX9-CaM complex, the molecular weight of bovine brain CaM used being 17 kDa. No higher order complexes were evident when ten-fold more CaM was added because the 1:1 complex did not increase in size (Fig. 2C, Lane 4). In the presence of EGTA, no SOX9-CaM complex forms (Fig. 2C, Lane 5). To determine the limiting calcium concentration, SOX9 and CaM were crosslinked in CaCl$_2$ at concentrations between 2 and 50 µM. The SOX9-CaM complex was detected at CaCl$_2$ concentrations at 3 µM CaCl$_2$ or greater (Fig. 2D). Since reported intracellular levels of CaCl$_2$ are between 1 – 10 µM (34, 35), our result suggests that CaM-SOX9 interaction occurs within the physiological range of intracellular calcium.

We tested, by fluorescence spectroscopy, if binding of CaM to SOX9 was accompanied by conformational changes in SOX9. We have shown previously that SRY fluorescence emission undergoes characteristic shifts in wavelength maximum upon DNA or CaM binding when compared to free SRY HMG box (28). Here we measured the fluorescence properties of SOX9 in free, CaM-bound and DNA-bound states. The excitation wavelength used was 295 nm, which excites tryptophan residues, and their emission was measured from 300 nm to 500 nm.

The addition of CaM to SOX9 caused a blue shift in emission maximum from 342 nm in the free SOX9 HMG protein (Figure 3A, curve 1) to 341 nm in the complex with a concomitant increase in fluorescence intensity (Figure 3A, curve 2). This shift was
calcium-dependent firstly because no change was evident with apo-CaM and secondly because the addition of EGTA reversed the effect (data not shown). Upon addition of wildtype double stranded DNA bearing the high affinity binding site of SOX9 (30), the fluorescence of the preformed SOX9 HMG-CaM complex is quenched, with the emission maximum shifted to 339 nm (Figure 3A, curve 3). The addition of a mutated DNA binding site to the SOX9-CaM complex caused no change in fluorescence emission and intensity (Figure 3A, curve 4). The intensity of the fluorescence emission in curve 3 is lower and differs from that for SOX9 HMG box alone. In order to characterize the complex in curve 3 further, a second experiment was carried out. The addition to free SOX9 HMG protein of wildtype DNA binding site (Figure 3B, curve 6) but not mutant DNA (Figure 3B, curve 7) caused a blue shift in emission maximum from 342 nm in the free SOX9 HMG to 338 nm in the SOX9-DNA complex (Figure 3B, curve 5) with a concomitant decrease in fluorescence intensity. Curve 3 is characteristic of curve 6 which represents DNA-bound SOX9, suggesting (i) that SOX9 forms binary complexes with CaM or with DNA and (ii) that DNA has an ability to compete with CaM in a sequence-dependent manner. SOX9 shows the same trend as SRY (28), adopting unique conformations in the presence of CaM or DNA. The absolute wavelength values differ for SOX9 compared to those of SRY suggesting that the tertiary structures of these molecules may be different.

To further investigate the SOX9-CaM interaction, a CaM antagonist, calmidazolium chloride (CDZ), was used to directly test its effect on SOX9-CaM complex formation on native gels. CDZ is an imidazole compound that binds very strongly to CaM (36, 37). CDZ varying from 0.3 to 30 μM was incubated with SOX9 and CaM (Fig. 4). Densitometric analysis of Coomassie blue-stained bands revealed that 0.3 μM CDZ reduced SOX9-CaM complex by about 2-fold and 3 μM CDZ by a further 2.5-
fold whereas 30 µM CDZ reduced complex formation by a further 20-fold. Thus the CDZ inhibition of SOX9-CaM complex had the largest effect between 3 and 30 µM. We conclude that CDZ can directly inhibit the interaction between SOX9 and CaM in-vitro in a dose-dependent manner.

To study the effects of CaM antagonists on nuclear accumulation of SOX9, COS7 cells were transiently transfected with pcDNA3-SOX9 and treated with 3 µM of CDZ, W7, W12 or CPA. SOX9 protein was detected by indirect immunofluorescence and CLSM (Fig. 5A). CaM antagonist W7, a naphthalene sulfonamide derivative binds directly to and inhibits CaM at the dosage used in this study (38, 39, 40). W12 is a compound structurally related to W7 but with much lower affinity for CaM (38, 39, 40, 41). All three compounds bind to the exposed hydrophobic surface of CaM preventing the binding of Ca²⁺ to CaM. This prevents CaM from adopting the conformation required for recognition by CaM-binding proteins. The potency of each antagonist upon nuclear accumulation and the IC₅₀ value for the inhibition of CaM-stimulated phosphodiesterase activity have been previously described (23, 38, 39, 40, 42). SOX9 protein is exclusively localised in the nucleus of untreated cells (Fig. 5A, column 1). SOX9 cytoplasmic staining could be seen in 3 µM CDZ and W7 (Fig. 5A, column 2) treated cells. In contrast, cells treated with the low affinity CaM binder W12, showed no SOX9 cytoplasmic staining (Fig. 5A, column 2). To quantify SOX9 nuclear accumulation in COS7 cells from the immunofluorescence data, CLSM image analysis was performed on SOX9-expressing COS7 cells at each concentration of antagonist (Fig. 5B). At 3 µM of CDZ, a two-fold reduction of nuclear accumulation of SOX9 was detected when compared to untreated cells (Fig. 5B). This decrease was significant (p = 0.001). CDZ concentrations of 30 µM and 300 µM did not show any further decreases in nuclear
accumulation of SOX9 when compared to 3 µM of CDZ (data not shown). For W7, a significant reduction in SOX9 nuclear accumulation was observed at 3 µM compared to untreated cells, but the extent of reduction was not as dramatic as that observed for CDZ treatment (p = 0.009) (Fig. 5B). For W12, no significant difference in SOX9 nuclear accumulation was observed compared to untreated cells (Fig. 5B). Thus the known potency of CaM antagonists correlates with their ability to reduce SOX9-CaM interaction and affects SOX9 nuclear import.

Given the observed calcium dependency of SOX9 interaction with CaM in-vitro (refer to Fig. 2), EGTA was used to indirectly determine the effect of calcium concentration upon nuclear accumulation of SOX9. Cells treated with 1 mM or 3 mM EGTA for 20 minutes showed a dose-dependent reduction of SOX9 accumulation in their nuclei to 28% and 18% of that in untreated cells respectively (Figure 5C, D). This demonstrated a direct dependence of SOX9 nuclear import upon a divalent cation. Taken together with the effects of the CaM antagonists, the data suggests that calcium-bound CaM is required for SOX9 nuclear import.

To investigate the effect of CaM antagonists on SOX9 transcriptional activity, CDZ was added to COS7 cells previously transfected with pcDNA3-SOX9 and the SOX reporter construct pS\textsubscript{10}E1bCAT, which is an adenovirus E1b promoter CAT reporter plasmid containing 10 copies of the SOX consensus element (AACAAT) to confer SOX9 responsiveness to the E1b promoter. The transcription control plasmid, pCMV-lac, was also included as an internal control for basal transcription levels. SOX9 transcriptional activity was then assayed for CAT expression. In the untreated cells, we obtained a ~13-fold activation of the (SCE)\textsubscript{10}-E1b-CAT reporter when compared with E1bCAT basal transcription control reporter (Fig. 6, column 1, row 1). The addition of CDZ
resulted in a reduction of transcriptional activity in the transfected cells. Transactivation by SOX9 was reduced by 50% in cells treated with 9 µM CDZ when compared to the untreated cells. Consistent CMV promoter-driven β-galactosidase activity throughout all samples in this assay indicate that the decrease in transcription activity was not due to general changes in polymerase II transcription with increasing concentrations of CDZ used.

In addition to WT SOX9, a previously characterized point mutant, A158T, from a XY female with campomelic dysplasia (CD) was investigated (19). This substitution of a small alanine molecule with a bulky threonine molecule destabilized the hydrophobic core of the HMG domain which is believed to stabilize SOX9 conformation (Fig.1). This mutant displayed normal DNA bending properties although its ability to bind DNA was reduced (19). Immunofluorescence localization of the A158T mutant protein showed a 50% reduction in nuclear accumulation (despite binding to importin-β with the same affinity as that of the WT SOX9 protein), suggesting a defect in an importin-β-independent nuclear import pathway (19).

Consistent with our previous observations (19), we observed that transcriptional activity of the A158T mutant was significantly lower than that of the WT SOX9 protein when the same amount (13ng) of pcDNA3-SOX9–A158T was transfected into COS7 cells (Fig. 6, column 2, row 1). Hence, the decreases in transcriptional activity due to the addition of CDZ were barely observable (Fig. 6, column 2, row 1). In order to compare the sensitivity of the wildtype SOX9 and A158T mutant expressing cells, an increased amount (2-fold) of pcDNA3-SOX9–A158T was used to obtain transcriptional activity at levels comparable with that of the WT protein (Fig. 6, column 3, row 1). The A158T mutant showed a steeper drop in transcriptional activity in response to CDZ treatment
compared to WT SOX9 (Fig. 6, column 3, row 1). Linear regression analysis indicated that the transcriptional activity of WT SOX9 was inhibited by 50% at 9.75 µM CDZ. In contrast, only 5.5 µM of CDZ was calculated to give a 50% decrease in the A158T mutant-expressing cells. Hence, transcriptional activation by nuclear import-defective SOX9 mutant A158T was apparently more sensitive to CDZ when compared to WT SOX9, suggesting a CaM defect in this CD/SRA patient.

In order to assess directly CaM binding to the A158T mutant, we compared the fluorescence profiles of WT and mutant HMG domains in the presence and absence of bound CaM (Fig. 7A). The intensity of tryptophan fluorescence is sensitive to the environment about the indole chromophore and can provide information concerning changes in protein structure and dynamics. SRY fluorescent emission data was also included to enable comparison (28). Using an excitation wavelength of 295 nm which excites tryptophan residues only, CaM-bound WT SOX9 blue-shifted the emission maximum by 10 nm from the free unbound state to the bound state. A similar value (9 nm) was observed for SRY as previously reported (28). In the case of the mutant A158T, a blue shift was recorded although the change in emission maximum was only 2 nm – 5 fold lower than that of WT SOX9 (Fig. 7A). This small shift observed in the A158T mutant suggests that A158T has a different structure compared to the WT SOX9 protein when bound to CaM.

In order to investigate further the nature of the microenvironments in the WT SOX9 and mutant HMG domains, acrylamide quenching was used to measure the accessibility of tryptophan residues to a quenching agent. In the absence of CaM as shown previously and confirmed here (18), similar fluorescence quenching profiles of both the WT and A158T SOX9 mutant indicate no changes in the accessibility to
acrylamide (Fig. 7B) suggesting that the tryptophan residues in both proteins are equally accessible to solvent. The effect of the addition of CaM on the tryptophan accessibility to acrylamide is shown in Figure 7C. In the CaM-SOX9 wild-type complex, only a minor change in the tryptophan acrylamide quenching profile was observed while the quenching profile of the mutant A158T SOX9-CaM complex changed significantly compared to the uncomplexed situation. An analysis of the quenching profiles in terms of a two-site (one site accessible, one site inaccessible) model (see Materials and Methods) showed that in the WT SOX9 and A158T SOX9 proteins, all of the tryptophan fluorescence was accessible to quenching. In the WT SOX9 protein, the effect of CaM complexation was to slightly increase (by 5%) the average accessibility of the two tryptophan residues to solvent. However in the A158T SOX9-CaM complex, the analysis suggested that complex formation resulted in complete shielding of about 50% of the tryptophan fluorescence from the acrylamide quencher and a large increase (by a factor of 2) in the accessibility of the remaining 50% to solvent. It is clear from this analysis that the conformation of the A158T SOX9-CaM complex is different from that of the WT SOX9-CaM complex.
Discussion

CaM binds to strongly basic sequences within the N-terminus of the HMG box of SRY. This region is characteristic of certain CaM binding domains such as CaM dependent kinase, and is highly conserved amongst SOX proteins (28). The SOX9/CaM interaction identified in this study is likely to be mediated through this highly conserved N-terminal region of the HMG box, which suggests that CaM binding is a property of all SOX proteins. Furthermore, amino acids within this region of SRY and SOX9 also act as functional NLSs when fused to β-galactosidase (29).

The structure of the SRY HMG box suggests that the N- and C-terminal NLS are in close proximity to each other (32, 43). Both importin α and β bind the SRY N-terminal NLS poorly while the C-terminal NLS interacts strongly with importin β (19-21). This suggests that the mechanism by which the N-terminal NLS mediates nuclear import is unlikely to involve the Ran/Importin pathway. The fluorescence properties of SOX9 HMG box suggest that it adopts a similar, but not identical conformation to that of SRY in the free, DNA-bound or CaM-bound form. The SOX9 HMG tryptophan residues are characterised by a relatively non-polar environment (336 nm), which is slightly altered upon CaM or DNA binding as indicated by fluorescence maxima. The addition of CaM to the SOX9 HMG box however, causes an increase in the quantum yield, which is quenched upon addition of DNA bearing the SOX9 high-affinity binding site. The large changes in quantum yield and emission maxima of the SOX9 HMG box upon CaM or DNA binding suggests that the SOX9 HMG box undergoes conformational changes in moving between these states (i.e. CaM binding and DNA binding). Based on our SOX9 HMG model (32), the W143 residue located at the end of helix 2 stacks on W115 residue (Fig. 1). These residues form part of the hydrophobic core, which is predicted to
maintain the angles between helices 1 and 2 and helices 2 and 3. Hence, any changes in the environment between the tryptophan residues are predicted to affect the angles between the 3 helices upon CaM or DNA binding. Fluorescence studies of SOX9 suggested that like SRY, the binding of CaM or DNA to SOX9 is mutually exclusive. The unique conformation adopted by SOX9 HMG upon CaM binding may facilitate interaction with binding partners within the cytoplasm as part of a nuclear import process. Once in the nucleus, partner protein steroidogenic factor 1 (SF1), which binds the N-terminal region of SOX9 HMG box, could together with DNA, facilitate the release of CaM from SOX9. Accompanying conformational changes in SOX9 may facilitate further protein-protein interactions such as those formed between SOX9, SF1, Wilm's tumour protein (WT1) and GATA4 during transcription of the MIS gene (6, 44).

By *in-vitro* binding, nuclear accumulation and transcriptional activation assays, we observed reduced SOX9 activity when treated with CaM antagonists, the more potent antagonists producing a more profound effect. The most potent CaM antagonist, CDZ, reduced SOX9 CaM binding, nuclear accumulation and transcriptional activity at low CDZ concentrations. That SOX9-CaM complex formation was reduced at low CDZ *in-vitro* supports a mechanism for SOX9 nuclear importin *in-vivo*. This is likely to be via direct binding of the SOX9 N-terminal NLS/CaM binding domain to CaM. Like CDZ, W7 also reduced nuclear import although to a lesser extent than CDZ, while W12 showed no effect in the range tested. Treatment of SOX9-expressing COS7 cells with the Ca\(^{2+}\)-ATPase inhibitor, CPA, resulted in a slight inhibition in nuclear accumulation of SOX9. This is in contrast to the observation made by Sweitzer and Hanover (23), where no change in nuclear accumulation of the SV40-like NLS was observed upon CPA treatment in their assay system over the same concentration range (23). CPA inhibits Ca\(^{2+}\)-ATPase and prevents the uptake of calcium into intracellular stores resulting in an
elevation of cytoplasmic calcium. The observed decrease in nuclear accumulation of SOX9 in COS7 cells treated with CPA could be due to the elevated cytoplasmic calcium concentration, which is known to inhibit conventional Importin/Ran-dependent nuclear import (23).

Our studies extend earlier findings of Sweitzer and Hanover who, using a permeabilised cell assay system, showed that synthetic peptides, coupled to fluorescent probes, were able to mimic the effects of bipartite NLS sequences. Treatment with CaM and calcium antagonists, had a profound effect on the nuclear accumulation of the peptides (23), perhaps through CaM binding directly to these peptides, although this was not tested. Our study suggests that CaM plays a direct role in mediating SOX9 nuclear import by binding the N-terminal bipartite NLS of SOX9. The sequence conservation of this NLS among SOX proteins implies that CaM binding could be a general mechanism of nuclear import for all SOX transcription factors. The p21<sub>CIP1</sub> protein directly binds to CaM (26) and, analogous to the inhibition of SOX9 nuclear accumulation in COS7 cells, the nuclear accumulation of the transcription factor Cdk4 that interacts with p21<sub>CIP1</sub> in NRK cells was inhibited upon treatment of cells with CaM antagonists (25, 26). Hence, CaM may play a role in mediating nuclear import of different classes of transcription factors.

CaM binding may be influenced by direct and indirect effects upon the SOX9 HMG domain. CaM can bind the N-terminal NLS of SOX9 and through some unknown process, SOX9 undergoes a conformational change, which facilitates an importin-independent nuclear import process. CaM binding to the N-terminal NLS of SOX9 might also influence (perhaps block) binding of importin β to the C-terminal NLS of SOX9. Such indirect effects have been demonstrated in an XY gonadal dysgenesis patient.
carrying a SRY mutation in the CaM binding region, R62G. This SRY mutant protein showed impaired nuclear import by directly reducing N-terminal NLS function. In addition, there was a reduction in importin β binding to the C-terminal NLS. SRY/SOX mutations outside the CaM binding region could also affect the CaM recognition process leading to nuclear import defects (21). Indeed the SOX9 mutation, A158T, which lies outside the NLS regions, affected nuclear localisation but did not affect importin β binding (19). Using tryptophan fluorescence spectroscopy, we have extended our earlier studies (19) and shown here that direct interaction between CaM and the SOX9-A158T results in a change in conformation of the mutant protein. Taken together with the increased inhibition of A158T transcriptional activity with CaM antagonist drugs, this data suggests that the A158T mutant adopts an unusual conformation in the presence of CaM which could be more sensitive to disruption by CDZ.

Residual SOX9 nuclear import in the presence of 3 μM CaM antagonist treatment might be attributed to the importin-dependent activity of the C-terminal NLS. Hence, both CaM and importin nuclear import pathways are probably required to give maximal SOX9 nuclear accumulation and may in fact contribute equally (Fig. 8). This would explain why CaM antagonists were unable to inhibit nuclear import of SOX9 by much more than 50%. Transcriptional activity of SOX9 was reduced to a lesser extent than was nuclear import (Fig 5, 6). With CDZ, import was reduced to 50% at 3 μM whereas transcription was reduced to 50% at 9.75 μM. This could be due to SOX9 binding co-operatively to multimerised SOX sites in the CAT reporter used here (data not shown), analogous to co-operative binding of SOX9 to targets in-vivo such as Col11a2 (45).

Co-incident with the onset of SRY expression in the developing mouse XY gonad, the subcellular location of SOX9 changes from cytoplasmic to nuclear (11). This
differential onset of expression suggests that a highly specific mechanism acts to regulate nuclear transport. Hence, components of the importin/RanGTP- or CaM-dependent pathways may act to regulate nuclear accumulation of SOX9 into Sertoli cells to allow a sufficient amount of SOX9 protein to activate the anti-Mullerian hormone gene, MIS, SF1 and other genes. The subcellular localisation and expression of CaM varies during testis development with CaM levels increasing just prior to the proliferation of Sertoli cells and spermatagonia (46, 47). The calcium-CaM status of the Sertoli cells could be critical at this time when cells in the XY gonad are being committed as Sertoli cells. A disruption in CaM recognition, as in SOX9-A158T, could ultimately be responsible for the XY gonadal dygenesis in this patient.
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References


**Footnotes**

1 Abbreviations:

$^{35}$S-Met, $^{35}$S-methionine; CaM, calmodulin; CAT, chloramphenicol acetyltransferase; CDZ, calmidazolium chloride; CLSM, confocal laser scanning microscopy; CPA, cyclopiazonic acid; HMG, High mobility group; MIS, mullerian inhibitory substance; NLS(s), nuclear localisation signal(s); NPC, nuclear pore complex; NRK, normal rat kidney; PKII, protein kinase II; Sf1, steroidogenic factor 1; SV, Simian Virus; SOX, Sry related HMG box; WT1, Wilm’s tumour 1.
Figure Legends

**Figure 1. CaM binding domain is highly conserved.** An alignment of the HMG box of SOX proteins (representative from each SOX group), the HMG box of LEF1 and the A box of HMG1. The region in grey represents the CaM-binding domain mapped in SRY. The bipartite N-terminal NLS, and the SV40-like C-terminal NLS are boldly underlined. Tryptophan residues are thinly underlined. Amino acid residues, which form helices 1, 2 and 3 of SRY HMG box, are indicated corresponding to the dark boxes representing helix 1, 2 and 3.

**Figure 2. Interactions between SOX9 and CaM in-vitro. (A)** SDS-PAGE analysis of *E.coli* recombinant SOX9 HMG box protein. *E.coli* cell lysates before and after ion-exchange chromatography were resolved on a 10-20% polyacrylamide gradient gel at 20 V/cm for 3 h and stained with Coomassie Brilliant Blue. 100 µg of *E.coli* soluble extract (Lane 1) and 2-4 µg of purified SOX9 HMG box are shown (Lane 2). The position of the SOX9 HMG box is indicated on the right and molecular weight standards are indicated on the left. (B) Ca^{2+}-dependent complex formation of SOX9 HMG box with CaM. Where indicated, 3 µM CaM, 43 µM SOX9 HMG box and 1 µg PKII peptide were used in the presence of 5 mM EGTA or 5 mM CaCl_{2}. Reactions were incubated for 1 h on ice prior to native PAGE electrophoresis. Proteins were visualised by staining with Coomassie brilliant blue. Arrows indicate the position of CaM, Ca^{2+}-CaM, Ca^{2+}-CaM/PKII complex and Ca^{2+}-CaM/SOX9 HMG box complexes. (C) Glutaraldehyde-crosslinking of CaM and SOX9. Full-length SOX9 was synthesised *in-vitro* and CaM (µM) and/or 0.01% Glutaraldehyde (Glut) was added in the presence of CaCl_{2} (5 mM) or the chelating agent, EGTA (5 mM). The reactions were incubated for 20 min at room
temperature, resolved on a 12.5% SDS-PAGE and analysed following autoradiography. Molecular weight standards are indicated on the left. (D) Glutaraldehyde-cross linking of CaM (5 µM) and SOX9 in the presence of increasing CaCl₂ (µM). Reactions were resolved on 10% SDS-PAGE and analysed following autoradiography. Arrows indicate the migration position of SOX9 alone and Ca²⁺-CaM-SOX9 complex.

**Figure 3. Fluorescence measurements of the interaction of the HMG box of SOX9 with CaM and DNA.** (A) Fluorescence of 0.58 µM SOX9 HMG box (Curve 1) combined with 0.7 µM CaM (Curve 2) or SOX9-CaM complex as in curve 2 following addition of 0.94 µM DNA, either wildtype DNA (Curve 3) or non-specific, mutant DNA (curve 4). Fluorescence spectra were recorded at 20°C on a SPEX Fluorology-2 frequency domain spectrofluorometer using a 0.5 ml cell. Excitation of tyrosine and tryptophan residues was detected using an excitation wavelength of 280 nm. (B) Fluorescence of 0.58 µM SOX9 HMG box (Curve 5) combined with 0.94 µM DNA, either wildtype DNA (Curve 6) or mutant DNA (Curve 7).

**Figure 4. Inhibition of the Ca²⁺-dependent complex formation of SOX9 HMG box with CaM upon treatment with CDZ.** Reaction mixtures were made to a final volume of 20 µl, containing 1 µg (3 µM) CaM, 3 µg (43 µM) SOX9 HMG box and increasing amounts of CDZ (0.3-30 µM) in the presence of 5 mM CaCl₂ (Lanes 1 to 4). Reactions were incubated for 1 h on ice prior to native PAGE electrophoresis. Proteins were visualised by staining with Coomassie brilliant blue. Arrows indicate the position of Ca²⁺-CaM and Ca²⁺-CaM-SOX9 HMG box complex.
Figure 5. Qualitative and quantitative measurements of SOX9 nuclear localisation in COS7 cells treated with various CaM antagonists and EGTA. (A) 500 ng of pcDNA3-SOX9 was transfected into COS7 cells using lipofectamine (see methods), and grown for 24 h. Cells were then treated with varying concentrations of CaM antagonists, CDZ, W7, W12 or the Ca$^{2+}$ antagonist, CPA and grown for a further 24 h. SOX9 protein was detected by indirect immunofluorescence as previously described (20). Nuclear accumulation of SOX9 protein in COS7 cells, is shown after treatment with 0 and 3 µM of CDZ (Panels 1-2), W7 (Panels 3-4), W12 (Panels 5-6) and CPA (Panels 7-8). (B) Confocal laser scanning microscopy (CLSM) analysis of SOX9 protein treated with varying concentrations of antagonists. Measurements represent the average of two separate experiments, where each point represents the average of 8–22 separate measurements for each of nuclear (Fn), and cytoplasmic (Fc) fluorescence with background (auto-fluorescence) subtracted. For each untreated SOX9-tranfected COS7 cell, F(n/c) was given a value of 1. Significant differences compared to untreated cells are indicated (*, p < 0.05, **, p < 0.01, *** p <0.001). (C) Transfection and indirect immunofluorescence analysis of COS7 cells were carried out as in Fig. 5A. 24 h after transfection, the cells were treated with 1 and 3 mM EDTA for 20 min before fixation. (D) CLSM analysis of SOX9 protein treated with 1 and 3 mM EGTA. Measurements were carried out as in Fig. 5B.

Figure 6. The effect of CDZ treatment on SOX9 transcriptional activity. COS7 cells were transiently transfected with pcDNA3-SOX9 or pcDNA3-SOX9-A158T expression plasmid (at varying concentrations as indicated on the top of each column), pS$_{10}$E1bCAT SOX reporter plasmid and pCMV-lac transcription control plasmid. Cells
were grown for 24 h and treated with varying concentrations of CaM antagonist CDZ. Each transfection was done in duplicate and the experiment performed three times. Each graph shows the fold induction of transcription as compared to the E1b CAT reporter plasmid (indicates basal transcription levels), transcriptional activity of SOX9 (Row 2, measured as ng CAT/50 µg protein extract) and the CMV-lac reporter (Row 3, measured as units β-galactosidase activity/µg protein extract), in the presence of varying concentrations of CaM antagonist.

**Figure 7**: The effect of CaM binding on the tertiary structure of the HMG box in SOX and SRY proteins as determined by tryptophan fluorescence spectroscopy and acrylamide quenching. (A) Change in the average tryptophan micropolarity upon complexation with CaM as assessed by the shift in average emission wavelength ($\lambda$). The experiments were carried out twice each on two different preparations of wildtype and mutant SOX9-HMG. (B) Tryptophan accessibility to aqueous phase acrylamide quenching in wild-type and mutant SOX proteins. The ratio of intensities of the tryptophan emission from the HMG box in the absence and presence of acrylamide are plotted as a function of acrylamide concentration (ex 295 nm, em 350nm). The quenching profiles of both proteins in the absence of CaM are essentially identical. (C) Tryptophan accessibility to aqueous phase acrylamide quenching in wild-type and mutant SOX-CaM complexes. Note the large difference between the wild-type SOX9-CaM and mutant A158T SOX9-CaM complexes indicative of an altered conformation in the mutant complex.

**Figure 8**: A model for signal-mediated nuclear import of SOX transcription factors. Nuclear import of SOX9 can occur via two pathways. One involves the
Importin/Ran dependent pathway where Importin β interacts directly with the C-terminal NLS signal in the HMG box of SOX9 (9). Importin β docks the complex to the periphery of the NPC and mediates translocation through the NPC. Upon binding of RanGTP to importin β, the importin β-SOX9 complex dissociates, possibly facilitated by DNA binding. At times of elevated cytoplasmic calcium levels in the range of 1-10 µM, the second pathway could be invoked where importin-mediated nuclear import is inhibited and Ca^{2+}-CaM binds to the CaM binding domain of SOX9, which encompasses the N-terminal NLS. SOX9-CaM is translocated through the NPC by an unknown mechanism as depicted by (?). Following translocation, the SOX9-CaM-Ca^{2+} complex dissociates by an unknown mechanism (as depicted by [?]) possibly facilitated by sequence-specific DNA binding by SOX9. Mutations in SOX9 which reduce CaM binding (such as A158T) or CaM antagonists which bind to CaM inhibiting binding to intracellular calcium result in partial inhibition of nuclear import. However, nuclear import of SOX9 can still occur via the alternate importin/Ran-dependent pathway.
Figure 1
Figure 2

(A) 

(B) 

(C) 

(D) 

Figure 2
Figure 4

1 2 3 4

Ca\textsuperscript{2+}-CaM/
SOX9

Ca\textsuperscript{2+}-CaM

µM CDZ
0 0.3 3 30
Figure 5

(A) Untreated 3 µM, 24 h

(B) Fluorescence (nuclear/cytoplasmic) vs. CaM antagonist (µM)

(C) Untreated 3 mM, 20 min

(D) Fluorescence (nuclear/cytoplasmic) vs. EGTA (mM)

Figure 5
Figure 6
Figure 7
Figure 8
A SOX9 defect of calmodulin-dependent nuclear import in campomelic
dysplasia/autosomal sex reversal
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