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The HMG box of SRY is a calmodulin binding domain

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Abstract The HMG box domain of the testis determining factor, SRY, includes a basic amphiphilic sequence common to calmodulin (CaM) binding proteins. By affinity chromatography, native gel electrophoresis and fluorescence spectroscopy, we show the calcium-dependent binding of SRY to CaM. Binding occurs via the HMG box and an SRY peptide of residues 57–80 binds CaM like the intact domain. SRY/CaM complex formation is specifically inhibited by the SRY DNA binding site sequence, AACAAT, but not a mutated sequence. Fluorescence spectra of the SRY/CaM complex indicate 1:1 stoichiometry and that binding is accompanied by a conformational change in SRY. The A domain of HMG1 also binds CaM and we propose that CaM binding is a property of the wider HMG box family, including SOX and TCF/LEF proteins. These results suggest that CaM may regulate the DNA binding activity of HMG box transcription factors.

Key words: HMG box; SRY; Calmodulin; DNA binding

1. Introduction

During mammalian development, expression of the testis determining factor, SRY, is necessary and sufficient for the development of the indifferent gonad into testes [1]. The DNA binding domain of SRY shares homology with a number of known or suspected transcription factors of the HMG box class [2]. Experiments in vitro suggest that SRY binds linear DNA in a sequence-specific manner [3,4] and causes a bend [5,6]. Mutations in the HMG domain of SRY encoded by XY gonadal dysgenesis patients affect the DNA binding or DNA bending activity of SRY in vitro which is consistent with these activities being necessary for testis development [7]. SRY is expressed in pre-Sertoli cells of the developing genital ridge, but to date, the downstream target genes of SRY or the cellular factors that mediate its action are not known.

Calmodulin (CaM) is a major intracellular calcium receptor responsible for the inhibition or activation of numerous enzymes. Many nuclear functions are under CaM control and some transcriptional activators are known to be modulated indirectly through CaM-dependent kinases [8,9]. CaM has more recently been shown to influence transcription by interacting directly with basic helix-loop-helix (bHLH) DNA binding proteins through differential inhibition of the DNA binding activity of these domains [10]. Given the ubiquitous expression of CaM and its binding partner promiscuity, the possibility exists that CaM interacts with other transcription factors to modulate gene expression.

A basic amphiphilic α-helix is a structural feature common to many calmodulin-binding peptides and proteins [11]. The 3-D structure of the HMG box of SRY and HMG1 consists of three α-helices stabilised by a hydrophobic core of aromatic amino acids [12–14]. In the HMG box of SRY, we observe strongly basic sequences in helix 1 and 3 whose core resembles the CaM binding region of CaM-dependent protein kinase II, RRKLK [11]. On the basis of this similarity, we tested whether SRY could interact directly with CaM.

2. Materials and methods

2.1. Materials

The following were obtained from commercial sources: calmodulin agarose and protein kinase II peptide 290–309 (LKKFNARRKLKGAILTTMLA) were obtained from Sigma, St. Louis, MO; hog brain calmodulin from Boehringer Mannheim. SRY peptides spanning the HMG domain were synthesised by Nichola O'Reilly, ICRF laboratories, London.

2.2. Recombinant protein production

SRY was synthesised in *E. coli* BL21 (DE3) cells harbouring plasmid pT7-FHK-SRY, which contains the tag sequence MDYKDDDDKHHHHHHRRASV at the N terminus of human SRY. This tag specifies a phosphorylation site for heart muscle kinase (underlined). Cells were grown, induced, harvested, sonicated and nucleic acids removed as previously described in the Qiagen manual. 1 μg SRY (about 80% pure) was phosphorylated by heart muscle kinase and [γ-^{32}P]ATP as described previously [15].

The HMG box of SRY (residues 55–143) was synthesised in *E. coli* BL21 (DE3) cells harbouring plasmid pT7-hSRYbox88 and purified to homogeneity by FPLC as described elsewhere (Borden et al., in preparation). The A domain of rat HMG1 was synthesised in *E. coli* BL21 (DE3) cells harbouring plasmid pT7-7 HMG1a (a gift from M.E. Bianchi) and purified as described [6]. Protein concentrations were measured by UV absorption spectroscopy at 280 nm assuming absorption coefficients of 21.2 and 8.9 mM^{-1} cm^{-1} for SRY and HMG1 respectively.

2.3. Calmodulin affinity chromatography

^{32}P-labelled SRY (5×10^6 cpm, 1 μg) in 1 ml 50 mM HEPES pH 7.9, 100 mM NaCl, 1% bovine serum albumin (buffer A) containing 1 mM $CaCl_2$ was applied to a 0.3 ml calmodulin agarose column equilibrated in buffer A/1 mM $CaCl_2$ and 0.5 ml fractions were collected. The column was first washed with 5 ml buffer A/1 mM $CaCl_2$ then with 5 ml buffer A/5 mM EDTA. The column was additionally washed with 1.5 ml buffer A/0.1% NP40 and 1.5 ml buffer A/1% SDS to elute residual protein. Radioactivity in fractions was determined by Cerenkov counting and fractions with high counts were analysed by SDS-PAGE. 200 μl was removed and precipitated with an equal volume ice-cold 20% TCA and the precipitate collected by centrifugation and resuspended in SDS-PAGE sample buffer. Proteins were separated on a 12.5% Phast SDS-PAGE gel (Pharmacia) and the air-dried gel was autoradiographed.

2.4. Native polyacrylamide gel electrophoresis (PAGE)

Gel and buffer composition conditions were essentially as for SDS-PAGE in standard Tris-glycine buffers [16] but lacking SDS, and consisted of a 12.5% separating gel and a 5% stacking gel. Components were added together (10 or 16 μl final volume of 20 mM Tris pH 6.8, 10% glycerol, 1 mM DTT, 0.1% bromophenol blue) and incubated at 4°C for 1 h prior to electrophoresis at 4°C on a BioRad

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minigel system at 20 mA constant current. Typically 2–6 μg CaM, 1–4 μg HMG box or 5 μg peptide were used. In competition experiments, various amount of DNA (0.25–1 μM) were used. DNA oligonucleotide encoding the consensus SRY binding site, 5′-GTTAACGTAACAATGAATCTGGTAGA [17], was annealed to its complement. As a negative control, the oligonucleotide 5′-GTTAACGTCCGCGGTAATCTGGTAGA, which SRY does not bind, was used.

2.5. Measurement of fluorescence spectra

Fluorescence spectra were recorded at 20°C on a Spex Fluoromax Fluorimeter using 1 ml cells. The buffer was 25 mM Tris pH 7.0, 0.1 M KCl, with added 1 mM $CaCl_2$. For titrations with CaM, an excitation wavelength of 295 nm was used. At this wavelength, the emission maximum of CaM, which contains no tryptophan residues, is about 308 nm, whereas that of SRY HMG domain (3 Trp residues) is at 347 nm and HMG1 A domain (1 Trp residue) is at 337 nm. Hence, adding CaM to SRY under these conditions adds negligible fluorescence at 350 nm. DNA used was 12mer oligonucleotide with the sequence GATAACAATGGC, bearing the high affinity SRY DNA binding site, TAACAAT [17].

3. Results

^{32}P-labelled SRY was loaded onto a CaM-agarose column in the presence of $CaCl_2$ and the column was washed first with $CaCl_2$ buffer, then with EDTA and collected fractions were analysed by SDS-PAGE/autoradiography. SRY migrates at 27 kDa by SDS-PAGE and Fig. 1 shows that SRY bound to the CaM column in the presence of Ca^{2+} (fractions 1–5) and was eluted by EDTA (fractions 5–8). This suggests that SRY interacts with CaM-agarose and that binding is calcium dependent.

To determine if CaM binding occurs in free solution and via the HMG box of SRY, purified SRY HMG box was electrophoresed in the presence or absence of CaM, and the Ca^{2+} dependence of this interaction was assessed (Fig. 2a). Stained protein bands were not observed in lanes containing SRY alone (lanes 1, 4), because SRY migrated toward the cathode. In the presence of $CaCl_2$, CaM migration is retarded (lane 5) relative to that in the presence of EGTA (lane 2). For mixtures of SRY and CaM in the presence of EGTA, only CaM was observed (lane 3), whereas in the presence of $CaCl_2$, the migration of CaM was further retarded due to the formation of a Ca^{2+}-CaM-SRY complex (lane 6). The specificity of interaction between CaM and SRY was analysed using a CaM binding peptide of CaM protein kinase II in a competition assay. At equimolar concentrations of PKII peptide and SRY, the SRY-CaM complex was dramatically reduced (lane 7). Given the K_d of PKII peptide-CaM is reportedly 1 nM [18] we estimate the relative K_d of SRY-CaM interaction is greater than 10 nM.

To further localise the CaM binding region of SRY, eight peptides spanning the HMG domain were investigated (Fig. 2b). In the absence of CaM, peptides were not detected in the gel (result not shown). In the presence of CaM, peptides from two regions of the SRY HMG domain showed an interaction: residues 57–80 (lane 1) and a second, weakly binding region, 131–144, which gave multiple bands (lane 9). No CaM binding was detectable with the other six SRY peptides (lanes 3–8). Binding affinity of SRY peptides 57–80 (QDRVKRPMNAFIVWSRDQRRKMAL) to CaM was similar to that for the whole HMG box; binding of SRY peptides 131–144 (PRRKAKMLPKNCSL) was about five-fold weaker. Two SRY mutations, R62G and R133W, encoded by XY gonadal dysgenesis patients, fall in the conserved RRK regions of the

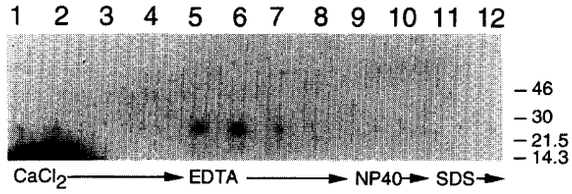

Fig. 1. Ca^{2+}-dependent affinity chromatography of SRY on calmodulin agarose column. SDS-PAGE/autoradiography of eluted column fractions. Lanes 1–4, SRY eluted with buffer A/$CaCl_2$; lanes 5–8, SRY eluted with buffer A/EDTA; lanes 9 and 10, SRY eluted with buffer A/0.1% NP40; lanes 11 and 12, SRY eluted with buffer A/1% SDS. On the right side of the gel are the molecular mass standards in kDa. FHK-SRY migrates at its predicted molecular weight of 27 kDa.

CaM binding SRY peptides, 57–80 and 131–144 respectively [19]. Fig. 2b, lanes 2 and 10, shows that in each case the mutant peptide bound CaM. Mobility shift differences can be explained by the effect of the mutation upon the charge of the peptide. CaM binding is therefore unlikely to be the basis for defective SRY function in these patients.

The effect of DNA upon the CaM binding activity of SRY was tested. Mixtures of SRY and 1 μM high affinity SRY DNA binding site (probe B), whose core is TAACAAT, formed DNA/SRY complexes (Fig. 2c, lane 1) whereas non-binding site DNA (probe N), with the core GGCGCCT, failed to (lane 2). Mixtures of SRY and CaM in the presence of $CaCl_2$ produced CaM/SRY complex (lane 3) but co-incubation with increasing probe B DNA (0.25, 0.5, 1.0 μM) results in reduced CaM/SRY complex formation (lanes 4, 5, 6). This loss of CaM/SRY complex is associated with the concomitant appearance of free CaM and of DNA/SRY complex. At 1 μM, probe B completely abolishes CaM/SRY complex formation (lane 6). This abolition is sequence-dependent as 1 μM probe N has no effect on CaM/SRY complex formation (lane 7); inhibition is detected at 10 μM (not shown). These results demonstrate that the affinity of SRY for its DNA binding site is significantly larger than for CaM. We conclude that specific DNA binding by SRY inhibits its CaM binding activity.

A second HMG box, the A domain of HMG1, was purified and tested for CaM binding. Binding affinity was comparable with SRY (Fig. 2d, lane 1). The HMG1 mutant, W49R, has much of its protein structure destroyed, with $< 10\%$ residual α [20]. This protein was stably expressed and bound CaM with activity comparable to wild type HMG1 A domain (Fig. 2d, lane 2).

We used the fluorescence properties of SRY and HMG1 to further investigate the CaM-HMG box interaction. Addition of CaM to 2 μM HMG1 increased the fluorescence of the HMG1a domain four-fold and saturation was reached at equimolar CaM indicating 1:1 stoichiometry. Emission maximum was shifted from 337 nm in free HMG1 to 332 nm in the complex (result not shown). We also observed 1:1 binding stoichiometry for the SRY/CaM complex at 1 μM and 0.2 μM, where signal strength becomes weak (result not shown).

Addition of CaM greatly increases the fluorescence of SRY HMG box, and causes the emission maximum to shift from 347 nm in the free protein (Fig. 3b, curve 1) to 338 nm in the complex (Fig. 3b, curve 2). This shift was calcium-dependent

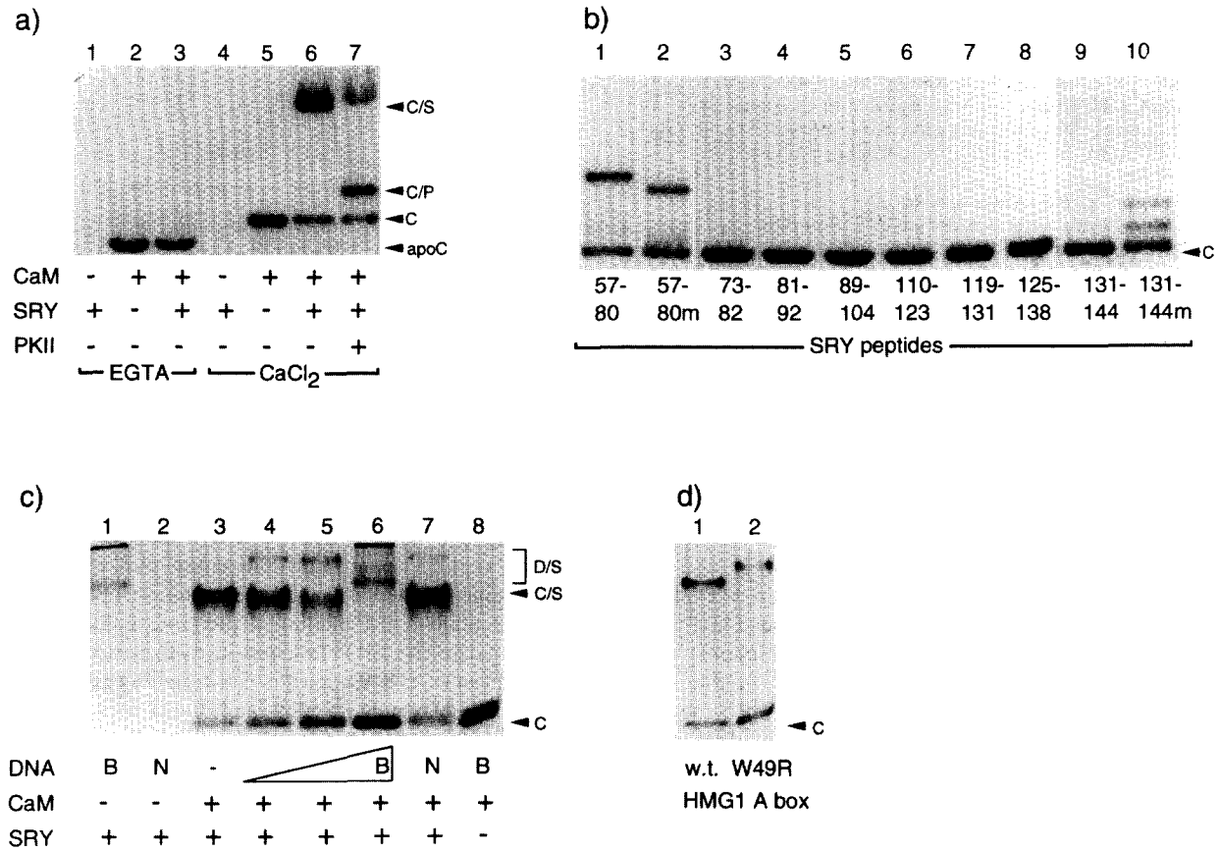

Fig. 2. Non-denaturing polyacrylamide gel electrophoresis of the HMG box of SRY and HMG1 with CaM. The anode is at the bottom of the gel. Proteins were visualised by staining with Coomassie brilliant blue. (a) Ca^{2+}-dependent complex formation of SRY HMG box with calmodulin. Where indicated, 35 μM CaM, 40 μM SRY HMG box and 40 μM PKII peptide were used in the presence of 5 mM EGTA or 5 mM $CaCl_2$. Arrows (bottom to top) indicate the position of apoCaM, Ca^{2+}-CaM, Ca^{2+}-CaM/protein kinase II peptide complex and CaM/SRY HMG box complex. (b) Peptides from two regions of SRY HMG box bind CaM. 37 μM CaM and 100 μM SRY HMG box peptides were mixed in the presence of $CaCl_2$. Peptides span SRY amino acid residues indicated. 57–80m refers to a mutated form of peptide 57–80 with the R62G substitution while 131–144m refers to the peptide 131–144 with the R133W substitution. (c) Effect of DNA upon CaM/SRY complex formation. DNA was mixed with 17 μM CaM and 20 μM SRY HMG box in the presence of $CaCl_2$. DNA specifying the SRY binding site 'B' or a non-binding site sequence 'N' was present at 1 μM in lanes 1, 2, 6, 7 and 8 and 'B' is present at 0.25 μM in lane 4 and 0.5 μM in lane 5. Arrows (bottom to top) indicate the position of CaM, CaM/SRY and DNA/SRY. (d) Interaction of 10 μM wild type HMG1 A domain or W49R mutant HMG1 A domain with 17 μM CaM in the presence of 5 mM $CaCl_2$.

because (i) addition of excess EGTA reversed the effect and (ii) adding apoCaM to either SRY or HMG1 HMG box caused a negligible increase in fluorescence intensity whereupon addition of Ca^{2+} caused an increase in fluorescence (result not shown). Upon adding DNA bearing the SRY binding site, AACAAT, the fluorescence of the preformed SRY/CaM complex is quenched (Fig. 3b, curve 3). This decrease to less than that for free SRY, with a slight blue shift, indicates the preferential formation of an SRY/DNA complex. The SRY/DNA complex is shown to have an emission maximum only slightly blue-shifted compared with free SRY, but is quenched about 50% at saturation (Borden et al., in preparation). Taken together with the electrophoretic data, we conclude that DNA and CaM compete for SRY such that only binary complexes are formed.

These results allow us to place some limits on the affinity of SRY for CaM. Stoichiometric binding to CaM was obtained at 200 nM SRY and given that DNA containing AACAAT ($K_d = 15$ nM) competes out equimolar CaM, the K_d for CaM must be in the 15–200 nM range. This is consistent with the electrophoretic data, where SRY binding site DNA competes out CaM but non-binding site DNA (K_d of 300 nM [6]) fails to compete.

4. Discussion

These findings illustrate that the HMG domain of SRY can bind CaM with 1:1 stoichiometry and in a calcium-dependent manner. Binding is competed by PKII peptide which suggests that SRY, like PKII peptide, binds to CaM in a conventional way, with the two globular domains of CaM engulfing the α-helix of the target [21]. The moderate affinity we observe ($K_d < 200$ nM) is significantly weaker than the highest affinity CaM-dependent enzymes ($K_d = 1$ nM) but closer to MyoD ($K_d = 20$ nM) and remains potentially relevant at the physiological level given that CaM concentrations in mammalian tissues vary between 2 μM and 30 μM [22]. The HMG1 A domain, which bears only 25% sequence identity to SRY [23], also binds CaM suggesting that CaM binding is likely to be a feature of the wider HMG1 box superfamily, including SOX

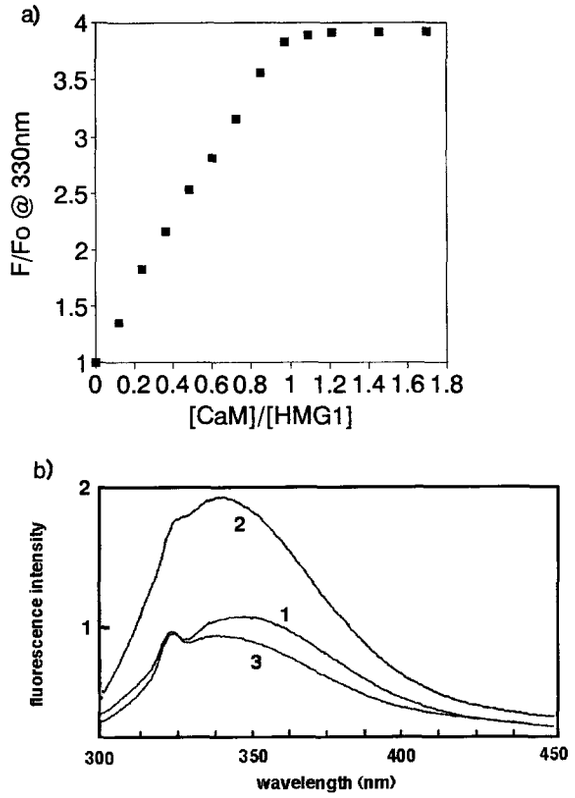

Fig. 3. Fluorescence measurements of the interaction of HMG box of HMG1 or SRY with CaM. (a) Titration of 2 μM HMG1 with increasing CaM concentrations Fo is the fluorescence of HMG1 alone at 330 nm and F is the observed fluorescence upon CaM addition. (b) Fluorescence of 1 μM SRY (curve 1) or combined with 1.25 μM CaM (curve 2) or SRY-CaM complex as in curve 2 following addition of 0.8 μM DNA (lower spectrum). Asterisk denotes Raman peak from water.

[19] and TCF/LEF1 [24] proteins. Although primary sequence may not be strongly conserved amongst HMG domain proteins, basic amphiphilic helix is retained.

CaM binding is likely to involve SRY amino acids 57–80, comprising N-terminal extended chain (residues 57–64) and helical region (helix 1 is residues 65–81). These regions make extensive contacts with DNA, namely residues R59, R62, M64, N65, F67 and I68, R72 and R75 [12]. Presumably CaM shares at least part of its SRY binding site with DNA, given the competition we observe between these molecules.

The effect of CaM and DNA on the fluorescence of HMG proteins is dramatically different, with DNA quenching the fluorescence, and CaM greatly enhancing it. The possibility of Förster energy transfer from the SRY fluorescence to DNA is negligible, indicating that there is some change in the environment around the tryptophan residue(s) on forming the specific DNA complex. The only tryptophan present in HMG1 A domain is W49 (W97 in SRY), which lies near the end of the second helix and is completely conserved among HMG box proteins. This residue stacks on another conserved aromatic residue (F23 in HMG1 and W70 in SRY) and forms part of a hydrophobic cluster in the 'hinge' region of the molecule. Changes in the stacking of this tryptophan with its neighbour due to changes in the hinge angle upon complex formation would readily account for changes in the fluorescence emission of this residue, with no requirement for a direct contact with either DNA or CaM. The simplest explanation for our results is a conformational change in the HMG protein upon forming these complexes. The HMG1 A domain mutant, W49R, is largely unfolded yet it retains its CaM binding activity suggesting that CaM binding is a property of primary and possibly secondary, but not tertiary structure elements of the HMG box. So while CaM affects the tertiary structure of the HMG box, it is not dependent upon it for binding.

CaM transcription factor interactions involving bHLH proteins have been demonstrated in vivo, and shown to inhibit DNA binding [10,25]. The results described in this paper demonstrate similar properties for a different class of nuclear proteins, albeit with lower affinities. The propensity for CaM to bind helical basic peptides is well-known, and affinity for HMG proteins remains to be demonstrated in vivo. HMG1, which is abundant in the nucleus, could translocate CaM to other CaM binding transcription factors, e.g. SOX or bHLH proteins, in order to regulate their function in concert with calcium or cell cycle.

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