The human SRY protein is present in fetal and adult Sertoli cells and germ cells

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ABSTRACT  Sex determination in mammals is controlled by the Y chromosome located SRY gene. Despite recent advances towards understanding the mechanisms that regulate sex determination in mammals, the expression profile of the SRY protein in human tissues is unknown. To localize the SRY protein and determine its cellular distribution, we prepared monoclonal antibodies (mAb) against the recombinant SRY protein. One antibody, LSRY1.1, recognizes a SRY-specific epitope and was used to localize the protein in different cells and tissues. The mAb recognizes a protein of 27 kDa in total lysates of HeLa SRYB3 cells. Immunocytochemical staining showed a nuclear localization of the protein. Immunohistochemical studies performed on gonadal tissue of a fetus, a one month-old boy and an adult man, demonstrated the presence of SRY protein in the nucleus of Sertoli and germ cells. In addition two 46,XX SRY(+) males had the SRY protein in their gonadal tissues. All other samples were negative, including all female tissue studied and the testis of a 46,XX SRY(-) male. The presence of SRY protein in fetal and adult gonadal tissues including germ cells suggests that SRY may have other male-specific functions in addition to sex determinism.

KEY WORDS: SRY protein, monoclonal antibody, testis, germ cells, Sertoli cells

Introduction

The mechanism regulating mammalian sex determination depends on a molecular switch that is genetically controlled by a gene on the Y chromosome. The factor controlling this molecular switch has been termed Testis Determining Factor (TDF) in humans and Tdy in mice. Using 46,XX males that carried a portion of the Y chromosome translocated to an X chromosome, the SRY (Sex determining region Y chromosome) gene was cloned (Gubbay et al., 1990; Sinclair et al., 1990). Several lines of evidence indicate that Sry corresponds to TDF: 1) individuals with a 46,XY karyotype but presenting a female phenotype with complete gonadal dysgenesis, carry mutations in the SRY open reading frame (Berta et al., 1990; Jäger et al., 1990). Several lines of evidence indicate that Sry corresponds to TDF: 1) individuals with a 46,XY karyotype but presenting a female phenotype with complete gonadal dysgenesis, carry mutations in the SRY open reading frame (Berta et al., 1990; Jäger et al., 1990). 2) In normal developing mouse embryos, expression of Sry occurs in the genital ridge around 10.5-12.5 days post coitum (dpc), just before the differentiation of the genital ridge into testis (Koopman et al., 1990). 3) Mice with a female karyotype developed testis when a 14 kb fragment of DNA containing the Sry gene was introduced as a transgene, demonstrating that Sry is both necessary and sufficient to determine testis formation (Koopman et al., 1991).

The SRY protein has a 79-amino acid HMG (High Mobility Group) domain, a conserved motif present in many DNA-binding proteins including several transcription factors (Ner, 1992). SRY binds to DNA and induces substantial bending in target DNA sequences, suggesting that SRY may act as a transcriptional regulator of other downstream sex-determining genes (Ferrari et al., 1992; Giese et al., 1992; Van der Wetering and Clevers, 1992).

In mice, fetal expression of Sry is limited to male gonadal somatic tissues and does not require the presence of germ cells (Koopman et al., 1990). High levels of Sry mRNA are also found in adult mouse testis, but this transcript has a circular structure, is not
associated with polysomes and is therefore probably not translated (Sinclair et al., 1990; Rossi et al., 1993; Cao et al., 1995). A high level of expression of mRNA has been observed in murine meiotic and post-meiotic germ cells (Rossi et al., 1993). A similar pattern of mRNA expression has been described for the human SRY gene, and the protein has been demonstrated to be present in the male genital ridge before testis formation (Clépet et al., 1993; Poulat et al., 1995). In the present work, we describe the production of a monoclonal antibody directed against SRY and demonstrate the localization of this protein in cell lines and in human gonadal tissue. Our results show that SRY expression is restricted to the nucleus of gonadal tissue. Furthermore, the data demonstrate that SRY is expressed not only during fetal development, a time when it is postulated it exerts its regulatory action, but it is also present in adult gonadal tissue, suggesting that SRY may have additional functions.

Results

Production of monoclonal antibodies against the recombinant SRY protein

In order to produce monoclonal antibodies, female Balb/c mice were immunized with 50 µg of recombinant SRY protein, purified from bacterial extracts (Smith and Johnson, 1988). Fusion of spleen cells from the SRY immunized mouse with NSO/2 murine myeloma cells lines gave 12 SRY-positive hybridomas screened by ELISA using recombinant SRY protein as antigen. After two cycles of subcloning, a hybridoma which produced a monoclonal antibody specific for the SRY protein was selected (mAb LSRY 1.1). The mouse isotype immunoglobulin of LSRY 1.1 was IgG-2a.

According to the published sequence of the SRY gene, the deduced molecular weight of the SRY protein corresponds to 25 kDa (Sinclair et al., 1990). This protein contains an HMG domain which is present in all the proteins of the Sox gene family. Since we used the complete sequence of the SRY protein as an immunogen, constructs containing GST-SRY, GST-HMG or the HMG domain alone were used to determine the specificity of the LSRY 1.1 mAb. The LSRY 1.1 monoclonal reacted with the complete GST-SRY protein only (data not shown) establishing that this antibody does not recognize the HMG domain.

Cellular localization of the SRY protein

To confirm the specificity of the monoclonal antibody, we performed western blots on total lysates of HeLa SRYB3 cells, a stable cell line transfected with the human SRY gene under the control of the SV-40 promoter (Poulat et al., 1995). As control, we used total extracts of normal HeLa 7A, a cell line derived from a female human cervix carcinoma, which does not carry the Y chromosome and thus it does not express the SRY protein. Figure 1 shows that the LSRY 1.1 monoclonal antibody detects a protein of approximately 27 kDa from lysates prepared from HeLa SRYB3, (Fig. 1, lane 2), a size corresponding to the expected molecular weight for SRY (Desclozeaux et al., 1998). No reaction was observed with total extracts from the HeLa 7A cell line (Fig. 1, lane 1). LSRY 1.1 monoclonal antibody detects a 25 kDa protein in Western blots using the recombinant SRY protein as a target (data not shown). The difference in size from 25 kDa and 27 kDa between the recombinant SRY protein and the protein obtained from the cell line, corresponds to unphosphorylated and phosphorylated SRY proteins respectively (Desclozeaux et al., 1998).

Flow cytometry analysis of permeabilized and non-permeabilized HeLa B3SRY cells showed that LSRY 1.1 recognizes an intracellular protein (data not shown). To determine the intracellular localization of the SRY protein, fixed and permeabilized HeLa SRYB3 cells and control HeLa 7A cells were labeled by indirect immunofluorescence with the LSRY 1.1 mAb and observed by confocal fluorescent microscopy. Figure 2 shows that the LSRY 1.1 antibody reacts with an antigen located in the nucleus of the HeLa B3SRY cells (Fig. 2d). No labeling can be observed in the control HeLa 7A cells (Fig. 2b) or in control experiments done with the second antibody (Fig. 2a and c). The heterogeneous level of staining observed in the HeLa SRYB3 cells could reflect cells at different stages of the cell cycle.

Expression of SRY in human tissues

The presence of SRY mRNA transcripts has been demonstrated in the human cell lines, DU145 and HepG2 as well as in different human tissues (Clepet et al., 1993; Tricoli et al., 1993). However, the presence of the SRY protein has not been clearly demonstrated. We used the LSRY 1.1 mAb to screen for the presence of SRY protein in the DU 145 and Hep G2 cell lines. In spite of the fact that the mRNA was clearly demonstrated by RT-PCR (data not shown), no protein could be detected with our antibody by immunoblot, confocal microscopy or flow cytometry (data not shown).

In order to investigate the presence of SRY protein in human tissues, immunohistochemical analysis was performed on different tissues with the LSRY 1.1 mAb. First we investigated the presence of SRY protein in testis tissue from a 26 week old fetus. LSRY 1.1 mAb presented a positive reaction (brown spot) in the nucleus of Sertoli cells and germ cells (Fig. 3b). No staining was observed in ovarian tissue (Fig. 3a) of the same age. A similar pattern of SRY expression was observed in the Sertoli and germ cells from a 1 month old boy (Fig. 3c). Furthermore, testis tissue from a normal adult male (32 years old), showed a positive labeling with the LSRY 1.1 mAb in the nuclei of Sertoli cells as well as in round spermatids (Fig. 3d). No staining was observed in liver, kidney or lung (data not shown).

Immunohistochemical studies were performed also in gonadal tissues of three sex reversal patients with a 46,XX chromosome
complement who had been previously investigated for the presence or absence of the SRY gene (Abbas et al., 1993; Vilain et al., 1994).

Patient 1 was a 46,XX SRY-negative male as demonstrated after PCR analysis for the presence of the SRY transcript in different tissues, including the gonads (Vilain et al., 1994). Figure 3e shows that in this case there was no reaction with the LSRY 1.1 monoclonal antibody.

Figure 3f shows the reaction of mAb LSRY1.1 with gonadal tissue of a second 46,XX true hermaphrodite that contains a 35 Kb Y-specific sequence including the SRY gene on a X chromosome. DNA from this individual was used to isolate the SRY gene (Sinclair et al., 1990). This patient presented both an ovotestis and a testis. In the ovotestis, we observed that the nuclei of granulosa-like cells and of the ovogonia were positive with this antibody.

Finally, the third patient was a 46,XX SRY(+) male. This patient had a portion of Y chromosome on the X chromosome and gonadal tissue that was identified as testis. The LSRY1.1 antibody recognized an antigen in the nuclei of Sertoli and germ cells as demonstrated for the normal male, (Fig. 3g).

Discussion

The master gene in the mammalian sex determination pathway is SRY. There are a relatively large number of studies describing expression of the SRY transcript in a number of species, however the presence of the SRY protein has not been well-defined. In the present study, the localization of the SRY protein was demonstrated, in both foetal and adult tissues, using a mAb prepared against SRY. Since monoclonal antibodies were prepared using the complete recombinant SRY protein as immunogen, it was possible that some of the selected hybridomas could produce antibodies directed against the HMG box, a domain that SRY shares with other proteins of the SOX family. One of the mAb obtained here (LSRY 1.1), showed no reaction when tested against several proteins of this family or against an isolated HMG domain. Western blot analysis performed with this antibody against the isolated recombinant protein as well as lysates of HeLa SRYB3 cells (a cell line of female origin transfected with the SRY gene), showed that the antibody recognizes a 25 kDa and a 27 kDa protein, the expected sizes for the unphosphorylated and phosphorylated proteins respectively (Poulat et al., 1995; Desclozeaux et al., 1998).

The monoclonal antibody was used to analyze endogenous SRY expression in several human cell lines. In spite of the presence of SRY transcripts, no protein was detected in the DU145 cell line, a cerebral metastasis of a human prostate carcinoma, and in the HepG2 cell line, a human hepatocarcinoma (Clepet et al., 1993; Tricoli et al., 1993). The SRY protein was present in HeLa SRYB3 cells as demonstrated by both Western blotting and immunofluorescence. Consistent with the hypothesis that SRY may function as a transcriptional regulator, staining was observed in cell nuclei.

In normal human testis, the SRY protein was observed in gonads from a 26 week old fetus, testis from a 1 month old boy and in adult testis. This suggests that SRY protein is present from the period of testicular formation early in fetal life until adult testis. This contrasts with the profile of expression of murine Sry where transcripts are present from 10.5 dpc until 12 dpc. The expression profile in man is similar to SRY expression in sheep gonads, were expression of SRY mRNA is maintained from the moment of testis determination until adult life (Payen et al., 1996).

At all ages, the human SRY protein was present in the nuclei of both Sertoli and germ cells. In adult tissues, the protein was present in round spermatids. These observations are similar to the findings of Rossi et al. (1993), who described the expression of murine Sry in meiotic and post-meiotic germ cells. Since, 46,XY individuals who carry mutations in the SRY gene are sex-reversed females, with gonads that consist of a streak of fibrous tissue lacking germ cells, one cannot exclude the possibility that SRY may have an other male-specific functions in addition to sex determination.

The presence of SRY protein was investigated in gonads obtained from three sex-reversed individuals presenting a 46,XX chromosomal complement and a male phenotype or female with genital ambiguities (true hermaphrodite). Two individuals carried a
though the SRY protein is present. The true hermaphrodite ana-
tory, however, there are a number of hypotheses that may explain
progenitor cells differentiate to become Sertoli cells. The presence
portion of the Y chromosome including the SRY gene on an X
chromosome. One is a XX male and the other a XX true herma-
phrodite. The third male was a 46,XX individual who did not carry
the SRY gene (Vilain et al., 1994). The immunochemical results
clearly demonstrated the presence of the antigen in the gonad of
the two SRY(+) individuals and the absence of SRY protein in the
SRY(-) male. In the 46,XX SRY(+) true hermaphrodite with an
ovotestis, positive staining was observed both in the testicular
portion of the gonad and also in some regions of the ovarian tissue.
Brown spots were observed in both the ovocyte and also in
granulosa-like cells surrounding the ovocyte. This result is consis-
tent with the hypothesis that Sertoli and granulosa cells share a
common cell progenitor, the colleomic epithelium cells (Barber,
1988). In the presence of the testis determining gene, SRY, these
progenitor cells differentiate to become Sertoli cells. The presence
of SRY protein in granulosa-like cells may at first seem contradic-
tory, however, there are a number of hypotheses that may explain
why these cells have not differentiated into Sertoli cells even
though the SRY protein is present. The true hermaphrodite ana-
lyzed in this study, carries the SRY gene on the short arm of her
paternal X chromosome (Abbas et al., 1993). This case and her
46,XX male brother carry only 35 kilobases of the Y chromosome
including the SRY gene, translocated into the pseudoautosomal
region (Sinclair et al., 1990; Abbas et al., 1993). Incomplete testis
determination of these individuals is considered to be the result of
inactivation of the translocated X chromosome. In a previous study,
cells from a 46,XX SRY(+) male were analyzed, and the translo-
cated X chromosome was observed to be late replicating in
approximately 50% of cells (Fechner et al., 1994). As the late-
replicating chromosome is presumed to be the inactive X chromo-
some, selection of cells in which the Y-bearing X chromosome has
been inactivated may play a role in the incomplete testis determina-
tion in 46,XX true hermaphroditism. This mechanism could
result in insufficient SRY protein, necessary to induce completely
Sertoli cell formation. Dosage effects appear to be an important
cause of sex reversal (Bardoni et al., 1994). X-inactivation may
also result in the disruption of the timing of SRY expression. The
timing of expression of the murine Sry gene appears to be critical
for normal testis determination (Eicher et al., 1995).

Materials and Methods

Reagents
Dulbecco’s Modified Eagle Medium (DMEM), high glucose DMEM,
trypsin, penicillin, streptomycin and glutamine were purchased from Gibco-
BRL (Life Technologies, France). Foetal Calf Serum (FCS), Glutathione
agarose, reduced glutathione, IPTG, thrombin, Triton X-100, Tween-20
and complete Freund’s adjuvant were purchased from Sigma (France).
Goat anti mouse polyclonal antibody conjugated to FITC was obtained from
DAKO (France). Goat anti mouse polyclonal antibody conjugated to horse-
radish peroxidase was obtained from BIOSYS (Compiegne-France). ECL
detection system was obtained from Amersham (Buckinghamshire, En-
gland).

Cell lines
The human cervix carcinoma, HeLa 7A, was obtained from the Ameri-
can Type Culture Collection. These cells were cultured in DMEM containing
10% FCS, 100 I.U. penicillin, 100 mg/ml of streptomycin, and 2 mM
glutamine. HeLa SRYB3, a cell line that expresses SRY under the control
of the SV-40 promoter was a kind gift from Dr. Philippe Berta (Centre de
Recherche de Biochimie Macromoléculaire, Montpellier, France); (Poulat
et al., 1995). HeLa SRYB3 cells were cultured in high glucose DMEM,
supplemented with 10% FCS, 100 I.U. penicillin, 100 mg/ml of streptomycin,
and 2 mM glutamine.

Production of the recombinant SRY
The SRY open reading frame was cloned into the pGEX-2T expression
vector (Smith and Johnson, 1988; Sinclair et al., 1990). The protein was
produced as a fusion protein with Glutathione-S-transferase (GST), that
contains a site of hydrolysis for thrombin.

E. coli bacterial strain HP101 was transformed with the vector contain-
ing the fusion protein as previously described by Smith and Johnson,
(1988). Cells were grown at 30°C and induced by IPTG (0.1 mM) for 2 h.
The cells were pelleted at 2,500g and then lysed by sonication in PBS 1%
Triton X-100 (10 mM phosphate, 150 mM NaCl, pH 7.2).

Protein purification
SRY recombinant fusion protein was purified by affinity chromatography
on a column of Glutathione-agarose beads. The lysates containing recom-
binant protein were run through the column and then the column was
washed with PBS 1% Triton X-100. The fusion protein was eluted by
competition with 50 mM TRIS containing 5 mM reduced glutathione pH 8.0.
Recombinant SRY protein was hydrolyzed from GST by treatment with thrombin at room temperature in 50 mM TRIS pH 8.0, 150 mM NaCl and 2.5 mM CaCl$_2$. The solution containing both proteins was passed again through the Glutathione-agarose column and the recombinant SRY was obtained as the eluate. Protein purity was assessed by 10% SDS-PAGE after staining with Coomassie Brilliant blue.

**Antibody production**

Balb/c female mice were immunized with three weekly injections of 50 µg each of recombinant SRY in complete Freund’s adjuvant. Antibodies titers were checked four weeks after the first injection. The mouse presenting the highest antibody titer was sacrificed and spleen cells were fused with NSO/2 murine myeloma cells using standard procedures (Köhler and Milstein, 1976). SRY reacting hybridomas were screened by an ELISA using the recombinant SRY as antigen.

**Immunofluorescence assay**

Immunofluorescence was performed on adherent HeLa 7A and HeLa SRYB3 cells grown on coverslips. Cells were fixed with 3.2% paraformaldehyde in PBS and permeabilized in PBS 0.05% Triton X-100, and washed with PBS. Immunofluorescence was done by incubating the cells for 30 min at room temperature with an appropriate dilution of the antibody, then washed in PBS 2% FCS, and incubated for 30 additional min with a FITC-labeled goat anti mouse IgG antibody. The coverslips were mounted and observed on Carl Zeiss confocal microscope.

**Immunohistochemistry**

Gonadal tissues were obtained by extraction (in accordance with the Comité Consultatif National d’Ethique Pour les Sciences de la Vie et de la Santé, Ref AB/hd N° 90-293, July 1990). Formalin fixed tissues were embedded in paraffin and sections of 4 mm were cut on a microtome. After deparaffinization and rehydration, the selected sections were submitted to microwave oven retrieval (immersed in 0.1 mM citrate buffer pH 6.0), 2x5 min at 95°C.

Immunostaining was done in a DAKO hem MATE TM system using the anti-SRY mAb as a first antibody and a secondary antibody (mixture of anti-mouse and anti-rabbit biotinylated immunoglobulins, DAKO), followed by peroxidase conjugated streptavidin. Antigen localization was done after reaction of diaminobenzidine and H$_2$O$_2$. Nuclei were detected after 1 min of green methyl coloration.

**Western blotting**

Cellular lysates were prepared as follows: cells washed with cold PBS were lysed directly in Laemmli sample buffer at 95°C (40 mM TRIS-HCL, pH: 6.8, 1% SDS, 50 mM DTT, 7.5% Glycerol, 0.3% bromophenol blue) and the lysate passed through a tuberculin needle by DNA binding protein extraction. The samples were then heating at 95°C for 5 min and then centrifuged at 12000xg for 5 min at 4°C. Proteins were separated on a 15% SDS-PAGE and transferred to nitrocellulose. Free sites on the nitrocellulose sheets were saturated in PBS-Tween (PBS with 0.1% Tween-20) for 1.5 h, and incubated overnight at 4°C with SRY 1.1 monoclonal antibody in PBS-Tween. After 3 washes in PBS-Tween, the nitrocellulose sheets were incubated for 1 h with goat anti-mouse IgG peroxidase-labeled antibody diluted appropriately in PBS-Tween. Antibody detection was carried out by using the ECL detection system.

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**References**


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