The Human Testis Determining Factor SRY Binds a Nuclear Factor Containing PDZ Protein Interaction Domains*

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The human Y-linked testis determining gene SRY encodes a protein with a DNA binding domain from the high mobility group box family. To date, no function has been assigned to amino acid sequences located outside this DNA binding motif. Here, we identify in a yeast two-hybrid screen a PDZ protein termed SIP-1, as an interacting protein with human SRY. In vitro, biochemical analysis, immunoprecipitation experiments, as well as expression of SIP-1 in human embryonic testis confirm that the two proteins can interact together. Interacting domains were mapped to the C-terminal seven amino acids of SRY and to the PDZ domains of SIP-1, respectively. We hypothesize that SIP-1 could connect SRY to other transcription factors providing SRY for its missing trans-regulation domain.

In mammals, male sex determination is controlled by genetic information encoded on the Y chromosome and leads to the differentiation of embryonic gonads into testes. SRY, a Y-specific gene cloned in 1990 (1), was shown to meet all of the criteria of the testis determining factor (2, 3). SRY encodes a small nuclear protein of 204 residues comprising three distinct domains, with a central domain of about 78 amino acids called the high mobility group (HMG)3 box. This central domain includes a nuclear localization signal (4), and in vitro studies of the human SRY protein have demonstrated its sequence-specific DNA binding through this HMG box (5). To date, no function has been ascribed to the regions of the human SRY protein outside the HMG box, whereas in mouse Sry, the C-terminal part of the protein can function as a transcriptional activator (6). This apparent lack of human SRY transactivation domain and the background-dependent sex determination capabilities of certain mouse Y chromosomes (7) along with the growing list of HMG box containing proteins involved in the assembly of multiprotein complexes (8, 9) suggest that SRY might interact with other proteins. Indeed regulation of target genes by SRY may require collaboration with other factors interacting directly with the SRY protein. Here, using the two-hybrid system we show that a PDZ domain containing nuclear protein interacts with the C-terminal portion of non-rodent SRY. Our two-hybrid results were confirmed by in vitro experiments and by immunoprecipitation that demonstrate that SRY and SIP-1 are associated also in vivo. Finally, immunofluorescence experiments reveal a nuclear expression pattern for SIP-1 in a cell line as well as in human embryo cut at the level of the genital ridges. These results suggest a model in which SIP-1 could provide a mechanism for SRY protein harboring a PDZ binding motif. The nature of this other protein partner and the functional implication of such a complex in human sex determination are now under investigation.

MATERIALS AND METHODS

Library Screenings—Yeast two-hybrid screening was carried out using the HF7c yeast strain harboring His3 and β-gal reporter genes under the control of upstream GAL4-binding sites as originally described (10, 11). The bait consisted of the human SRY open reading frame fused to GAL4. About 1,600,000 clones were screened using a HeLa cell cDNA library (Clontech) constructed in the GAL4 activation domain vector pGADGH. Three identical positive clones interacting specifically with SRY and containing the same 1-kilobase insert were obtained. This insert was next used to screen a human fetal cDNA library and revealed a 3’-overlapping clone with a stop codon, and its sequence to synthesize antisense oligonucleotides for RACE experiment (12). RACE was performed using the 5’-RACE kit (Clontech) according to the protocol provided by the supplier on human testis mRNA. PCR fragments were then subcloned. DNA sequence was obtained using the double-stranded bidirectional sequencing strategy with the Taq DyeDeoxyTerminator kit and an Applied Biosystems 373A automated DNA sequencer (Foster City, CA).

Western Blotting Experiment—Subcellular fractionation of cells was performed by differential centrifugation as described previously (13). For each fraction, 20 μg of protein were separated on 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose for Western blotting. SIP-1 polyclonal antibody was raised against the recombinant SIP-1 protein released from GST after thrombin digestion and next injected into rats. Detection was performed with the enhanced chemiluminescence system (Amersham Corp.).

Co-precipitation Assay—[35S]Methionine-labeled SRY protein was synthesized in vitro transcription-translation (Promega). GST-SIP-1 fusion protein was generated by subcloning the cDNA into pGEX4T-2 (Pharmacia Biotech Inc.) and isolated from Escherichia coli by adsorption to glutathione-Sepharose. GST-SIP-1 protein was mixed with labeled SRY in buffer A (50 mM Hepes (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol) for 1 h at 4 °C, precipitated with glutathione-Sepharose beads which were then washed extensively with buffer A.

Overlay Assays—Filter overlay assays were performed essentially as described (14). SRY protein was used as a probe after in vitro translation using rabbit reticulocytes (Promega) and [35S]methionine according instructions of the supplier. After electroblotting to nitrocellulose filters, proteins were denatured by incubating the filters for 30 min at 4 °C in buffer A (see above) containing 6 M guanidinium hydrochloride. Renaturation was achieved by stepwise dilution of guanidinium hydrochloride to 0.1875 M with buffer A, followed by two washes in buffer A. Filters were blocked for 2 h with 5% (w/v) non-fat dry milk in buffer A and probed for 4 h with buffer A plus 10% glycerol, 1% dry milk, and labeled probe at 4 °C. After probing, filters were washed in phosphate-buffered saline with 0.2% Triton X-100, 1 mM dithiothreitol, and 5 mM phenylmethylsulfonyl fluoride and subsequently analyzed by autoradiography.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U82108.

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1 The abbreviations used are: HMG, high mobility group; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase.
FIG. 1. A, a schematic of the composite SIP-1 cDNA structure is presented. Below the overall structure are bold lines representing each of the three cDNA clones. Clone marked A represents one of the three identical clones that interacted specifically with SRY in the two-hybrid screening. Clone B was obtained by screening of a human fetal brain library with the A-derived insert, and clone C is the result of a 5' rapid amplification of cDNA ends (RACE) on human testis mRNA. The SIP-1 sequence is a composite of the translation of the cDNA clones scoring positive during the two-hybrid assay and of the cDNA from a human fetal brain cDNA library. The length of the reconstructed SIP-1 cDNA (1578 base pairs) is in good agreement with the 1.6-kilobase size detected by Northern blotting (data not shown).

The two PDZ motifs are boxed. The sequence reported in this paper has been deposited in the GenBank database.

C, comparative alignment of the PDZ domains from SIP-1 (PDZ1 and PDZ2) with four other PDZ sequences. The alignment was generated using the PILEUP and PRETTY programs of the GCG sequence analysis software package (GGC, University of Wisconsin, Madison, WI). A consensus is given at the bottom of the alignment.
Human SRY Interacts with a PDZ Domain Containing Protein

RESULTS AND DISCUSSION
The yeast two-hybrid system was employed in order to identify general factors that interact with the human SRY protein. A HeLa cell two-hybrid library was screened using the full-length SRY open reading frame as bait (11). Screening of estimated 1.6 x 10^5 transformants and subsequent complementatory clonings (Fig. 1A) resulted in the cloning of a cDNA encoding a 326-amino acid protein with a predicted molecular mass of 34 kDa (Fig. 1B) and was termed SIP-1 (SRY interacting protein-1). Homology searches (15) revealed two highly conserved (63% identity) PDZ domains (for PSD-95, Disc-large and ZO-1) (16–18), PDZ1 and PDZ2. These domains which are conserved (63% identity) PDZ domains (for PSD-95, Disc-large and ZO-1) (16–18), PDZ1 and PDZ2. These domains which are

Immunoprecipitations—For immunoprecipitation experiments, cell nuclei were prepared as already described (13), sonicated, and incubated in buffer A containing 0.5 mM Pefabloc-SC-AEBSF (Interchim), leupeptin (50 μg/ml), pepstatin (50 μg/ml), and aprotinin (50 μg/ml) for 4 h at 4 °C with the indicated antibody. The immunocomplexes were adsorbed to protein A-Sepharose 4B, washed extensively with buffer A, and resolved by SDS-polyacrylamide gel electrophoresis before immunoblotting.

Immunofluorescence Studies—For cells asynchronously growing NT2-D1 cells were fixed with methanol at –20 °C and rehydrated with phosphate-buffered saline. Immunohistochemical methods were as described using a polyclonal antiserum against SIP-1 protein and subsequent purification (4). SIP-1 immunostaining patterns were visualized with Cy2-conjugated anti-rat labeled goat antibodies (Amersham Corp.). For embryonic tissues a specific SIP-1 antibody was raised after injection of two New Zealand White rabbits with SIP-1 protein mixed with complete Freund's adjuvant (Sigma) in this case. Polyclonal antisera was affinity purified over a CNBr-Sepharose column coupled to SRY protein (Pharmacia, France). 8-Week-old male fetuses were obtained from therapeutic termination (weeks of gestation). Sections of unfixed frozen tissues (7 μm thick) were cut on a cryostat at –25 °C and fixed by immersion in 4% paraformaldehyde. Sections were probed by a 4-h incubation at 37 °C with the SIP-1 antibody (diluted 1/10). Primary antibodies were visualized with biotinylated anti-rabbit (diluted 1/100) and fluorescein isothiocyanate-conjugated streptavidin (diluted 1/200) (both from Amersham Corp.). Nucleus visualization was obtained using Hoechst 33258 DNA staining. Tissues were mounted in FluorSave reagent (Calbiochem).

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To evaluate the direct association between SRY and SIP-1 biochemically, we first expressed SIP-1 as a glutathione S-transferase (GST) fusion protein against which we raised an antibody. The specificity of the resulting antibody was determined by Western blotting analysis. As shown in Fig. 2A, this antibody detects the expected 34-kDa protein in nuclear extracts and insoluble nuclear fractions (i.e. chromatin and nucleoskeleton) derived from a human NT2D1 cell line, a pluripotent embryonic cell line positive for SRY expression (4, 20). In coprecipitation assays, in vitro translated SRY protein (expected size of 27 kDa) associated specifically with SIP-1 fused to GST but not GST alone or with glutathione-Sepharose beads (Fig. 2B). If the reverse experiment using GST-SRY to pull down in vitro translated SIP-1 gave the same association, GST-SRY with a C-terminal truncation including the last 41 amino acids did not (data not shown). To obtain independent confirmation of the direct interaction between the two proteins, in vitro binding assays were also performed (Fig. 2C). Not only does the overlay correlate with bacterially expressed SIP-1 fusion protein, but a 34-kDa protein was also bound when NT2D1 nuclear extracts and insoluble nuclear fractions were used (Fig. 2D). In each case the interaction of SRY with the 34-kDa protein was abolished when the nitrocellulose filter was preincubated with the SIP-1 antibody prior to probe with labeled SRY protein (not shown). Finally, the same protein was recognized with the SIP-1 antibody (Fig. 2D, lanes 8 and 9).

We next examined whether SRY binds to SIP-1 in vivo. We subjected nuclear extracts of either an NT2D1 or an SRY expressing HeLa cell line (4) to immunoprecipitation with previously characterized antibodies directed against SRY (4). Coprecipitation was observed with both cell lines, whereas no SIP-1 protein was precipitated when using wild-type HeLa cells (Fig. 2E). Next, immunostaining pattern of SIP-1 was analyzed with the SIP-1 antibody in NT2D1 cells. These data confirmed a nuclear, punctated localization for the SIP-1 protein (Fig. 3A). This pattern is very similar to the one observed with SRY (4). To involve further SIP-1 as a part of the sex-determining cascade, the question of SIP-1 expression in human embryo testis was addressed. Immunofluorescence experiments with SIP-1 antibody confirmed the nuclear expression of SIP-1 protein in undifferentiated cells from gonadal tissue from a 7-week-old male fetus coincident with the onset of sex determination process (Fig. 3B).

To investigate the role of the different domains of the SIP-1 protein in this protein-protein interaction, we made constructs of various SIP-1 fragments and assayed their interaction with SRY in the yeast two-hybrid assay (Fig. 4A). Both PDZ domains bound efficiently; however, their combination did not increase the strength of the interaction with SRY. On the other hand, the critical region of SRY necessary for its association with SIP-1 was delineated. The two-hybrid assay, in conjunction with the generation of progressive deletions in SRY, revealed that C-terminal portion of SRY is involved in SIP-1 association and is restricted to the last seven amino acids (Fig. 4B). This location provides an explanation at the molecular level for a recently described sex-reversed XY female associated with a mutation in the 3' non-HMG box region of the SRY gene and resulted in a loss of 41 amino acids from the C-terminal region of the human SRY protein (21). Indeed SIP-1 was unable to interact with this truncated product (Fig. 4B). It is worth noting that channel proteins capable of direct interaction with a PDZ motif share a similar sequence of seven amino acids (19). In order to understand the possible conservation of such an interaction mechanism in mammals, a semi-quantitative two-hybrid assay measuring the interaction of SIP-1 with related C-terminal sequences present in diverse mammals was developed (Fig. 4C). Except for the case of the rabbit, non-rodent mammalian SRY protein-derived sequences were tested, and all interacted with SIP-1. The strength and the specificity of these interactions were consistent with their respective evolutionary distance. No clear SIP-1 binding site was identified in rodent Sry. This discrepancy could contribute to the explanation of the inability of the human SRY gene to induce sex reversion in transgenic mice (3) and could result from differences in the genetic control of male sex determination between the two.

In conclusion, a C-terminal sequence located in the human SRY protein has been shown to bind a nuclear protein containing two PDZ domains. This result is in good agreement with the recent demonstration that PDZ domains recognize the C terminus of their target proteins (22, 23). Furthermore, the molecular basis described so far, for peptide recognition by a PDZ motif, is fulfilled by the human SRY C-terminal sequence (22). The presence of two such domains in SIP-1 could contribute to the bridging together of either two SRY molecules or one SRY and a transcription factor which could then provide SRY with its missing trans-regulating domain. SRY, like other HMG box containing proteins, could belong to a higher order nucleoprotein complex, and this may explain the apparent inactivity of SRY in transient transfection assays outside a convenient presertoli cell environment (6). Although the physiological role of
the resulting complex still needs to be established, our results emphasized the fact that the regions of SRY lying outside the HMG box indeed have a functional role. This suggests that the high evolutionary speed noticed by several authors (24–26) is not due to a lack of function of the C-terminal part of SRY but, oppositely, to a co-evolution mechanism between SRY and SIP-1. Thus, the interaction between SRY and SIP-1 may be instrumental in the reproductive isolation.
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