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NHERF2/SIP-1 Interacts with Mouse SRY via a Different Mechanism than Human SRY*

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In mammals, male sex determination is controlled by the SRY protein, which drives differentiation of the bipotential embryonic gonads into testes by activating the Sertoli cell differentiation program. The morphological effects of SRY are well documented; however, its molecular mechanism of action remains unknown. Moreover, SRY proteins display high sequence variability among mammalian species, which makes protein motifs difficult to delineate. We previously isolated SIP-1/NHERF2 as a human SRY-interacting protein. SIP-1/NHERF2, a PDZ protein, interacts with the C-terminal extremity of the human SRY protein. Here we showed that the interaction of SIP-1/NHERF2 and SRY via the SIP-1/NHERF2 PDZ1 domain is conserved in mice. However, the interaction occurs via a domain that is internal to the mouse SRY protein and involves a different recognition mechanism than human SRY. Furthermore, we show that mouse and human SRY induce nuclear accumulation of the SIP-1/NHERF2 protein in cultured cells. Finally, a transgenic mouse line expressing green fluorescent protein under the control of the mouse Sry promoter allowed us to show that SRY and SIP-1/NHERF2 are co-expressed in the nucleus of pre-Sertoli cells during testis determination. Taken together, our results suggested that the function of SIP-1/NHERF2 as an SRY cofactor during testis determination is conserved between human and mouse.

In mammals, testicular differentiation is under the control of the Y chromosome-encoded master switch gene SRY (1–3), which instructs the supporting cell precursors to become Sertoli cells rather than granulosa cells (4). The differentiation of Sertoli cells is thought to then drive the differentiation of the remaining cell lineages down the testis developmental pathway giving rise to an embryonic testis able to produce anti-Mullerian hormone and male steroid hormones (5).

The SRY gene is the founding member of the SOX gene family, all of which encode a 79-amino acid DNA binding domain called the HMG domain. The HMG domain shares homology with the high mobility group proteins, which include the T cell-specific transcription factor (6). The SRY DNA binding capacity and nuclear localization are crucial for sex determination. Mutations within the HMG box of the SRY gene from XY sex-reversed patients affect either its DNA binding (7), DNA bending (8), or nuclear localization (9). Most of the sex-reversing mutations described in the human SRY open reading frame are located in the HMG domain. However, several mutations have been described that affect either the N-terminal domain (10, 11) or the C-terminal domain, where the two mutations described affect the last 50 amino acids either by a stop codon (12) or by a frameshift mutation (13). These mutations lead to the deletion of the protein region that interacts with SIP-1 (also called NHERF2 or E3KARP), a PDZ domain containing protein that we previously isolated and characterized for its interaction with the human SRY protein (14).

An intriguing property of the SRY protein is its rapid evolution across mammalian species. When comparing SRY proteins from non-rodent species, the regions outside the HMG box are evolving dramatically fast, and nonequivalent mutations are accumulating with a very high rate (15). The mammalian SRY proteins can be subdivided into two groups, one including the non-rodent mammals where the HMG domain is central, and the second including the rodent species, where the N-terminal domain is restricted to two amino acids directly followed by the HMG domain and a long C-terminal domain made of at least two different regions. The first domain (residues 82–144, the “bridge domain”) is the most conserved between mouse species (16), and the second (the “glutamine-rich domain”) contains a number of glutamine-rich clusters, which display length variation among mouse species (16, 17).

Recent transgenic experiments, where expression of the human SRY protein was controlled by mouse Sry regulatory regions, show the capacity of the human protein to induce testis determination in a mouse context (18). These results suggest two possible hypotheses in regard to the functionality of the different SRY protein domains.

1) Only the HMG box is important for SRY function. The regions outside the HMG domain would be involved only in the conformation and the stability of the HMG domain. Rapid evolution of SRY genes across mammalian species argues in favor of this hypothesis, but sex-reversing mutations outside the HMG box in humans argue against it.

2) Cryptic conserved protein domains are present outside the HMG domain, which interact with others factors involved in SRY function.

Starting from this last hypothesis, we show in this study that the interaction between SRY and SIP-1 proteins is conserved in both humans and mice. In mice, this interaction involves the bridge domain located between the HMG domain and the glutamine-rich domain. By using transfection of expression vectors in cultured cells, we show that mSRY and SIP-1 interact in vitro. Moreover, by using a transgenic mouse line expressing GFP under the control of the mouse Sry gene

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The abbreviations used are: HMG, high mobility group; GST, glutathione S-transferase; dpc, days post-coitum; CT, C-terminal; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HA, hemagglutinin; FCS, fetal calf serum; GFP, green fluorescent protein; m, mouse; h, human.
promoter, we show that SIP-1 is co-expressed in pre-Sertoli cells where Sry is acting.

MATERIALS AND METHODS

Cell Culture and Transfection—HEK293T and NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 2 mM glutamine, penicillin (100 units/ml)/streptomycin (100 µg/ml) at 37 °C in a 5% CO2 atmosphere. Cells were transfected with various expression vectors using JetPEI reagent (Qbiogen) following the manufacturer’s protocol.

Plasmid Constructs—The mouse SRY (mSRY) open reading frame (19) was PCR-amplified from a cosmid containing the mSry gene from the Mus musculus 129 strain (RZPD) and was inserted into the pcRII TOPO vector (Invitrogen) and sequenced by using an ABI 377 sequencer. The mSRY ORF was then subcloned into the pcDNA3 (Invitrogen) HA tag-containing vector for cell transfection experiments. mSRY from Mus musculus was PCR-amplified from a NotI genomic subclone of a 13-kb genomic clone L961 already described (20). Human SRY (hSRY) open reading frame was PCR-amplified from H2.1 genomic subclone (1) and inserted into the pcDNA3 (Invitrogen) HA tag-containing vector. For in vitro translation, the mSRY ORF and all other mSRY-derived constructs were PCR-amplified, inserted in pcRII TOPO, sequenced, and subcloned into the pGloMy vector (a gift from M. De Pamphilis, Dr. M. Martin, and Dr. R. Beauchamp, respectively).

SIP-1 open reading frame was cloned in pEGFP-C3 (Clontech). Vectors pCRII TOPO, sequenced, and subcloned into the pcDNA3. For the GFP-SIP-1 expression vector, the 94–100 residues. For SIP-1, the open reading frame of the human cDNA was cloned in pcDNA3. For the GFP-SIP-1 expression vector, the SIP-1 open reading frame was cloned in pEGFP-C3 (Clontech). Vectors pSIS-YAP65, pRN3-EZRN-GFPi, and pGEX-EBP50 were gifts from Dr. M. De Pamphilis, Dr. M. Martin, and Dr. R. Beauchamp, respectively. For the GST-SIP-1 bacterial expression vector, the open reading frame was cloned into the pGEX4T1 plasmid (Amersham Biosciences). The four domains of SIP-1 (PDZ1, Hinge, PDZ2, C-terminal) were PCR-amplified, inserted in pcRII TOPO, sequenced, and then subcloned into the pGEX4T1 vector.

Recombinant Proteins Production—Bacteria carrying GST expression vectors were grown at 37 °C in LB broth until an A600 0.6 and then induced with isopropyl-1-thio-β-D-galactopyranoside (0.1 mM) for 4 h at 20 °C. Bacteria (1 liter) were harvested, washed with cold phosphate-buffered saline (PBS), and resuspended in 40 ml of lysis buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2% N-lauroylsarcosine. Bacterial suspensions were then sonicated for 1 min. Triton X-100 was added up to 4%. Lysates were centrifuged for 30 min at 10,000 × g (4 °C). Supernatants were collected and incubated overnight (4 °C) in the presence of the immunoprecipitating antibody: 1 µg/ml mouse anti-HA (12CA5; Roche Applied Science), 1 µg/ml rabbit anti-SIP-1 (14), or 1 µg/ml unrelated antibody. Extracts were then incubated for 30 min (4 °C) with 5 µl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) saturated with 1 mg/ml BSA. Agarose beads with immunocomplexes were washed three times with 1 ml of extraction buffer and resuspended in 20 µl of 1× Laemmli buffer lacking DTT to prevent immunoglobulin chains dissociation. Immunoprecipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose with a Trans-Blot apparatus (Bio-Rad). The nitrocellulose sheet was cut coverslips in 24-well plates and co-transfected the day after using JetPEI (Qbiogen) in 100-mm plates with 8 µg of expression vector, harvested after 48 h, and washed in cold PBS. Cells were then sonicated (2 s) in 1 ml of extraction buffer: 50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM DTT, 1 µM distamycin A (Sigma), 25 mM β-glycerolphosphate, 2 mM NaPPi, 0.5 mM sodium vanadate, and complete protease inhibitor mixture (Roche Applied Science). Total extracts was then centrifuged at 10,000 × g for 10 min. Supernatants were collected and incubated overnight (4 °C) in the presence of the immunoprecipitating antibody: 1 µg/ml mouse anti-HA (12CA5; Roche Applied Science), 1 µg/ml rabbit anti-SIP-1 (14), or 1 µg/ml unrelated antibody. Extracts were then incubated for 30 min (4 °C) with 5 µl of protein A/G PLUS-agarose (Santa Cruz Biotechnology) saturated with 1 mg/ml BSA. Agarose beads with immunocomplexes were washed three times with 1 ml of extraction buffer and resuspended in 20 µl of 1× Laemmli buffer lacking DTT to prevent immunoglobulin chains dissociation. Immunoprecipitated proteins were subjected to SDS-PAGE and transferred to nitrocellulose with a Trans-Blot apparatus (Bio-Rad). The nitrocellulose sheet was cut to remove the nonreduced immunoglobulins migrating at a molecular mass of ~150 kilodaltons. Western blot analysis was performed using the anti-HA antibody (1:500) (previously mentioned), after SIP-1 immunoprecipitation, and anti-SIP-1 antibody (1:100), after HA immunoprecipitation, and then detected using the ECL Plus kit (Amersham Biosciences).

Immunofluorescence Analysis of Cultured Cells and Embryonic Gonads—5 × 106 (NIH3T3) or 106 (HEK293T) cells were plated on coverslips in 24-well plates and co-transfected the day after using JetPEI (Qbiogen) with the following amounts of expression vectors: 1 µg for mouse and human SRY expression vectors and 10 ng for GFP-SIP-1 expression vector. Cells were fixed 12 h later with 4% paraformaldehyde in PBS at room temperature for 10 min and permeabilized with 0.5% Triton X-100, 5% FCS in PBS. The cells were incubated in wash buffer (0.05% Triton X-100, 5% FCS in PBS) with anti-HA antibody (1 µg/ml) for 1 h at room temperature and washed three times with wash buffer. The preparation was then incubated with secondary antibody for 30 min at room temperature (goat anti-mouse Alexa 568, Molecular Probes, dilution 1:1000) and washed three times. Nuclear DNA was stained with Hoechst 33286.

Fixation and processing of the embryonic gonads of transgenic mice (21) for immunofluorescence was done as described previously (22). Purified SIP-1 antibody (14) was used at a 1:100 dilution, and mouse anti-GFP (Molecular Probes) was used at a 1:1000 dilution.

Subcellular Fractionation of Mouse Genital Ridges—Urogenital ridges were dissected from Swiss Webster 11.5 dpc mouse embryos, and the genital ridge and adjacent mesonephros were roughly separated.

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Embryos were sexed as described previously (22). Nuclear and cytoplasmic extracts were prepared using a nuclear extract kit from Active Motif. Protein extracts (20 μg per lane) were probed, after SDS-PAGE and transfer on nitrocellulose, with rabbit anti-SIP-1, mouse anti-tubulin (Sigma) (dilution 1:1000) and mouse anti-p300 (Santa Cruz Biotechnology) (dilution 1:200) and detected using ECL Plus (Amersham Biosciences).

RESULTS

mSRY Protein Interacts in Vitro with SIP-1 via a Molecular Mechanism Different from hSRY—To evaluate a potential interaction between mSRY and SIP-1, GST-pull-down experiments were performed. After cloning the open reading frame in an in vitro translation vector, 35S-labeled mSRY protein was produced and used in an interaction assay together with bacterially produced human GST-SIP-1 protein. As a positive control, in vitro-translated 35S-hSRY protein was used in the same assay. As show in Fig. 1A, mSRY (lane 3) was retained on the GST-SIP-agarose beads similar to hSRY (lane 1) and in contrast to the negative control GST-agarose beads that displayed no detectable interaction (lanes 2 and 4). These results were confirmed for mSRY using a yeast two-hybrid system as described previously for hSRY (data not shown).

In order to identify the interaction domain of both proteins, subclones of GST-SIP-1 were produced and used in the interaction assay. An interaction between mSRY and the PDZ1 domain of SIP-1 (GST-PDZ1) was detected (Fig. 1B, lane 1), whereas no signal was obtained with the other domains of SIP-1. Despite the strong homology shared by the SIP-1 PDZ1 and PDZ2 domains, an interaction was not detected between mSRY and GST-PDZ2 (Fig. 1B, lane 3), suggesting an important selectivity of interaction. The same results were obtained with hSRY (lanes 5–8), showing that the target domain for both SRY proteins is the PDZ1 domain of SIP-1. The negative control using GST alone showed no interaction (data not shown). In our original study using the yeast two-hybrid interaction test, hSRY was also interacting with PDZ2, in contrast with our results presented here. To verify that GST-PDZ2 was properly folded and functional, we have tested its interaction with YAP65, a protein described to interact with the PDZ2 domain of the NHERF family (24). As shown in Fig. 1B (lane 9), YAP65 is interacting with GST-PDZ2. The same control test for GST-CT was also obtained in a GST-pull-down experiment with the EZRIN protein that interacts with the C-terminal domain of the NHERF proteins family (25, 26) (data not shown).

We next defined the interaction domain of mSRY with SIP-1. Serial deletions starting from the mSRY C terminus were constructed and used to produce in vitro-translated radiolabeled mSRY proteins. Fig. 1C showed that in contrast to hSRY, mSRY did not interact with SIP-1 via its C-terminal residues; mSRY lacking the last 29 residues still interacted with SIP-1 (lane 2, mSRY, 1–367). Fig. 1C (lane 3, mSRY, 1–230) also shows that mSRY from the M. domesticus strain, which contains only eight glutamine-rich cluster motifs, interacts with SIP-1. Moreover, mSRY protein that completely lacks the glutamine-rich domain, mSRY-1–144, also interacts with GST-SIP-1 (Fig. 1C, lane 4) showing that the glutamine-rich domain is not required for the interaction. We then localized the SIP-1 interaction motif to the bridge domain located between the HMG box and the glutamine-rich domain. By using further serial deletions of mSRY, we have observed a loss of interaction with SIP-1 using mSRY-(1–93) (Fig. 1C, lane 6), whereas mSRY-(1–103) does interact (Fig. 1C, lane 5). These results suggest that the interaction of mSRY with the PDZ1 domain of SIP-1 is dependent on a motif located between amino acids 93 and 103 in mSRY.

Human and mouse SRY interact with the PDZ1 domain of SIP-1, which is a “class one PDZ motif” having (S/T)X(Y/V/L) as a C-terminal consensus recognition sequence. Moreover, PDZ domains can interact with an internal PDZ-binding motif followed by a potential β-sheet structure that could mimic the C-terminal extremity of a protein (27). Analysis of mSRY sequence between amino acids 93 and 103 revealed a TKL96–98 motif, which is also found in the SIP-1 interaction motif of hSRY. To determine the importance of the TKL motif, a Q96–98 dele-
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mSRY and SIP-1 Co-immunoprecipitate from Cultured Cells.—Next, we examined whether mSRY and SIP-1 interacted in cultured cells. We transfected HEK293T cells with HA-tagged mSRY and SIP-1 expression vectors, and cellular extracts were subjected to immunoprecipitation. Precipitated complexes where then separated by SDS-PAGE and detected by Western blot. When anti-HA antibody was used for immunoprecipitation (mSRY precipitation), the Western blot revealed the presence of SIP-1 in the precipitated complexes (Fig. 3A). Similarly, when anti-SIP-1 antibody was used for immunoprecipitation, mSRY was also detected (Fig. 1B). These results suggest that mSRY and SIP-1 interact when both are expressed in the same cell.

mSRY, like hSRY, Induces Nuclear Accumulation of SIP-1.—To study the potential impact of mSRY on the subcellular distribution of SIP-1, the HA-mSRY expression vector was co-transfected in HEK293T or NIH3T3 cells with a GFP-SIP-1 expression vector. In the absence of mSRY (Fig. 4g), GFP-SIP-1 was distributed in both the nuclear and cytoplasmic compartments (Fig. 4h), and also beneath the plasma membrane in spotted foci. However, in presence of either HA-mSRY (Fig. 4e) or HA-hSRY (Fig. 4d), GFP-SIP-1 protein was concentrated in the nucleus (Fig. 4, b and e, respectively).

We then assessed if interaction between the mSRY and the PDZ1 domain of SIP-1 was necessary for nuclear localization. A construct, which lacks the SIP-1 interaction motif (HA-mSRY-(Δ94–138)) was co-transfected with GFP-SIP-1. Although the HA-mSRY-(Δ94–138) protein was clearly localized in the nucleus (Fig. 4j), GFP-SIP-1 displayed the same cellular localization that occurs in the absence of SRY: a distribution in both the cytoplasm and nucleus (Fig. 4k) instead of the nuclear accumulation observed with wild type mSRY and hSRY.

Taken together, our results show that mSRY and SIP-1 interact via an intramolecular process when co-expressed. The results further show that via this interaction, mouse or human SRY proteins are sufficient to induce a change in the distribution of SIP-1 so that it is concentrated in the nuclear compartment.

mSRY and SIP-1 Are Co-expressed in Pre-Sertoli Cells during Male Sex Determination.—Our next step was to determine whether mSRY and SIP-1 were expressed in the same cells and in the same cellular compartment during mouse sex determination. Because an antibody against mSRY was not available, we used a mouse transgenic line (21) where a GFP was expressed under the control of the mouse Sry promoter (prom Sry-GFP). In this mouse line, GFP is specifically expressed in the undifferentiated gerinal ridges within the gonadal supporting cell lineage precursors, which will differentiate into Sertoli cells in the testis and granulosa cells in the ovary. Frozen sections from 11.5 dpc (the time of the peak of mSRY expression (19)) male and female genital ridges were processed for co-immunofluorescent staining using anti-GFP (to enhance the GFP signal) and anti-SIP-1 antibodies. Fig. 5A shows that SIP-1 protein is expressed in all somatic cells in male (b) and female (c) genital ridges. Furthermore, SIP-1 protein is expressed in GFP-positive cells in the male (white arrows), which are the mSRY expressing pre-Sertoli cells. Moreover, faint nuclear staining for SIP-1 was discernible in these cells, and also in non-Sry expressing cells. To verify the observed nuclear staining, subcellular fractionation of genital ridges from male and female 11.5 dpc embryos was performed. Cytoplasmic and nuclear fractions were then analyzed for the presence of SIP-1 using Western blot detection. As shown in Fig. 5B, SIP-1 protein was present in both the nuclear and cytoplasmic fractions in male and female gonads, confirming the SIP-1 nuclear staining observed by immunofluorescence. SIP-1 is thus co-expressed with mSRY in the nucleus of pre-Sertoli cells within the male genital ridge.
Interaction of SIP-1/NHERF2 with SRY Is Conserved in Mouse

Transgenic experiments in mice have shown that human and mouse SRY were interchangeable (18), suggesting that both proteins act through a common molecular process, despite the lack of homology outside the HMG domain. This finding predicts that physiologically significant proteins that interact with SRY in humans should also interact with SRY in mice. We previously identified SIP-1, an SRY-interacting protein in human, and we show here that SIP-1 also interacts with mouse SRY.

Our results using GST-pull-down experiments suggest that the SIP-1/SRY interaction in both humans and mice occurs via the PDZ1 domain of SIP-1. We did not detect any interaction between the SIP-1 PDZ2 domain and human or mouse SRY. This result is in contrast with our previous study showing interaction between hSRY and both the PDZ1 and PDZ2 domains of SIP-1 (14). This result is not because of a mis-folding of the bacterially produced GST-PDZ2 because it interacts with YAP65 as already described (24). This discrepancy can be rather explained by the fact that our original studies were performed using a yeast two-hybrid assay; this test is qualitative and poorly quantitative because of the utilization of multicopy expression vectors in yeast, which thus are able to detect faint interactions between two proteins. In this study, the interaction assay is more stringent, thus detecting only strong interaction between proteins.

In these assays, we used the human SIP-1 protein. However, the mouse SIP-1 PDZ1 domain shares 99% identity (across 79 amino acids) with the human SIP-1 PDZ1 domain, strongly suggesting that interaction between mSRY and mSIP-1 is similar to mSRY and hSIP-1. Moreover, the interaction between mSRY and SIP-1 involves a different molecular process than hSRY and SIP-1. In this study, we demonstrate that SIP-1 interacts with mSRY via a motif that is internal to the mSRY protein (amino acids 93–103) rather than via a C-terminal motif as for hSRY. This internal motif is within the bridge domain located between the HMG box and the glutamine-rich domain and contains a TKL motif (amino acids 96–98), which is characteristic of PDZ binding domains. Most interestingly, the last three amino acids TKL of hSRY are crucial for the interaction with SIP-1. Nevertheless, deletion of the TKL motif from mSRY did not eliminate the interaction with SIP-1, suggesting a more complex mechanism of interaction than for hSRY. Deletion of the amino acids adjacent to the TKL motif (amino acids 100–138) also is not enough to abrogate interaction with SIP-1, whereas deletion of the TKL motif plus adjacent 94–138 amino acids was sufficient to abrogate interaction with SIP-1. These results suggest that the interaction between mSRY and SIP-1 involves protein-protein contacts within the bridge domain of mSRY. Future experiments involving systematic mutagenesis of the mSRY bridge domain should reveal which amino acids are involved.

The internal motif recognition mechanism for PDZ domains has been described previously for the interaction of neuronal nitric-oxide synthase with the PDZ domain of syntrophin (27). The neuronal nitric-oxide synthase protein sequence forms a β-finger structure mimicking a C terminus, which docks at the syntrophin PDZ motif domain. Further studies are necessary to determine whether the bridge domain of mSRY could have such a structure.

A recent study has shown that mouse and human SRY proteins interact with a KRAB domain protein, KRAB-0 (28). Interestingly, mSRY interacts with KRAB-0 via amino acids 92–124, which is the same region that interacts with SIP-1. It would be interesting to see whether SIP-1 and KRAB-0 are mutually exclusive for their interaction with SRY protein or whether they can participate with the same protein complexes. Because evolution maintained the interaction of SIP-1 with SRY as for KRAB-0, it suggests a conserved function regarding SRY action even though different mechanisms of contact for the human and mouse protein are involved.

By using immunostaining experiments, we showed that mSRY like hSRY induced a nuclear translocation of SIP-1. In cell lines, in the
absence of SRY, SIP-1 is distributed in the different cell compartments and can be found in the membrane and cytosolic and nuclear fractions (data not shown). In contrast, SIP-1 can be detected only in the nuclear fraction in a cell line that expresses endogenous hSRY (NT2/D1) (14). Here we show that GFP-SIP-1 concentrates in the nucleus of HEK293T and NIH3T3 cells co-transfected with SRY (mouse and human). In these same cells in the absence of SIP-1, SIP-1 is distributed in a spotted pattern in the cytoplasm or under the plasma membrane, in the cytoplasm and in the nucleus. The fact that SRY induced a nuclear concentration of SIP-1 begs the question of the mechanism involved. Does SRY interact directly in the cytoplasm with SIP-1 and then co-transport it to the nucleus, or does SIP-1 continually shuttle between the nucleus and cytoplasm but is retained by interaction with nuclear SRY? Experiments with leptomycin B, an inhibitor of nuclear export, should distinguish between these possibilities. However, from the experiments presented here we can conclude that SRY protein (mouse and human) is sufficient to induce nuclear accumulation of SIP-1.

By using a transgenic mouse line where GFP is expressed only in Sry-expressing cells (GFP expression is under the control of the mSRY promoter) (21), we observed that SIP-1 is present in GFP-expressing pre-Sertoli cells. Most of the protein is localized in the cytoplasm, and strong staining for SIP-1 was detected under the plasma membrane. Nevertheless, nuclear staining was detectable. This nuclear staining was not sex-specific and can be detected in 11.5 dpc XX gonads in GFP-expressing pre-granulosa cells. (The Sry-GFP transgene is also expressed in female gonads.) Moreover, nuclear staining for SIP-1 is also visible in other gonadal cells, suggesting that this protein is involved in other nuclear processes.

To verify the presence of nuclear SIP-1 in genital ridges, we performed subcellular fractionation from the male and female genital ridge at 11.5 dpc. Our results show that SIP-1 protein was present in nuclear extracts from male and female genital ridges, thus confirming the results of the immunofluorescence experiments. Even though these data are not demonstrating in vivo interaction of mSRY and SIP-1, they at least show that SIP-1 is located at the right time and at the right place to possibly interact with mSRY. Further experiments using the Sry transgene unable to interact with SIP-1 should demonstrate the role of the SRY/SIP-1 interaction in the sex-determining pathway.

SIP-1 is the second member of the NHERF family; SIP-1 is also known as NHERF2, and EBP50 is also known as NHERF1 (29). These two proteins display high homology: 49% identity between NHERF2/SIP-1 and NHERF1/EBP50. The homology between the PDZ motifs is even higher; the PDZ1 motifs of SIP-1 and EBP50 show 72% identity. This observation suggests the possibility of an interaction between EBP50 and SRY. By using GST-pull-down assays, we have observed an interaction between GST-EBP50 and mouse or human SRY. However, EBP50 is not expressed in the same cells as mSRY in E11.5 genital ridges (data not shown).

What is the function of the SRY/SIP-1 interaction? The fact that SIP-1 is present in many cellular compartments suggests the possibility of multiple cellular functions. Most intriguingly, NHERF1/EBP50 has been shown to participate in the GSK3/β-catenin transduction pathway (23). Our preliminary data suggests that SIP-1 also interacts with β-catenin5 and thus could have a similar function.

SIP-1, an adapter, may provide a link between SRY, lacking a clear transactivation domain, and co-activator or co-repressor proteins. This mechanism would be similar to that proposed for the SRY/KRAB-0 interaction, which leads to a potential gene silencing mechanism (28). As discussed before, SIP-1 and KRAB-0 could be mutually exclusive in their interaction with SRY leading to different types of chromatin-regulating complexes, depending on the different present adapter proteins. This hypothesis would explain the fact that SRY does not have a clear transcription activating or inhibiting domains; association with different interacting proteins would allow SRY to have either gene activating or silencing capacity depending on the chromatin environment.

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