EXPERIMENTAL STUDY

Differential expression of p160 steroid receptor coactivators in the rat testis and epididymis

Junko Igarashi-Migitaka, Akira Takeshita1, Noriyuki Koibuchi2, Shozo Yamada1, Ritsuko Ohtani-Kaneko and Kazuaki Hirata

Department of Anatomy and Cell Biology, St Marianna University School of Medicine, Kawasaki, Kanagawa 216-8511, Japan, 1 Endocrine Center, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, Tokyo 105-8470, Japan and 2 Department of Integrative Physiology, Gunma University School of Medicine, Maebashi, Gunma 371-8511, Japan

(Correspondence should be addressed to A Takeshita, Toranomon Hospital and Okinaka Memorial Institute for Medical Research, 2-2-2 Toranomon, Minato, Tokyo 105-8470, Japan; Email: coactivator@mac.com)

Abstract

Objective: Androgens are critical for the development and maintenance of male sexual characteristics. Their action is mediated through the androgen receptor (AR). Ligand-bound AR interacts with coactivator proteins that mediate transcriptional activation. Such coactivators include three members of the 160 kDa proteins (p160s): SRC-1, TIF2/GRIP1, and p/CIP/RAC3/ACTR/AIB1/TRAM-1. The aim of this study was to investigate the expression of the three p160 coactivators and their association with AR in testis and epididymis.

Methods: We determined the localization of these three p160 coactivators in immature and mature rat testis, and epididymis by immunohistochemistry using the specific monoclonal antibodies. We also performed double immunofluorescence staining to examine whether p160s are colocalized with AR in these tissues.

Results: In seminiferous tubules of mature rat testis, SRC-1 and TRAM-1 immunoreactivity was found predominantly in spermatogonia and spermatocytes. In contrast, TIF2 was expressed predominantly in Sertoli cells. AR was coexpressed with TIF2 in this cell type. In immature rat testis, however, all three coactivators were expressed in both germ cells and Sertoli cells. In the epididymis, SRC-1 and TIF2 immunoreactivities were localized in nuclei of epithelial cells. However, TRAM-1 immunostaining was observed in the luminal portion of the cytoplasm with greater intensity than in the nucleus, especially in the caput epididymidis.

Conclusions: The cell-type-specific expression of p160 coactivators suggests specific roles in male reproductive organs. Further, the strong cytoplasmic localization of TRAM-1 protein in epithelial cells of epididymis suggests that TRAM-1 may have additional role(s) in transcriptional regulation.

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Introduction

Androgens are critical for the development and maintenance of male sexual characteristics. Their action is mediated through the androgen receptor (AR), which belongs to the steroid receptor subfamily of nuclear receptors (NRs) (1, 2). On ligand binding, AR forms a homodimer that binds to specific DNA sequences, named androgen-response elements, located in the promoter regions of target genes. Ligand induces conformational changes in the ligand-binding domain of ARs that enable ARs to interact with coactivator proteins to activate transcription (3). Such coactivators include three members of the 160 kDa proteins (p160s): SRC-1, TIF2/GRIP1 (referred to as TIF2 hereafter), and p/CIP/RAC3/ACTR/AIB1/TRAM-1 (referred to as TRAM-1 hereafter) (4–13). These p160 coactivators then recruit protein complexes containing histone acetyltransferases, such as cAMPresponseelementbinding protein (CREB)-binding protein (CBP), p300, and p300/CREB-binding protein (p/CAF) (9, 11–13), and histone methyltransferases such as coactivator-associated arginine methyltransferase-1 and protein arginine N-methyltransferase-1 (14).

The three p160 coactivators share approximately 30–40% amino acid sequence homology (13), and in vitro transfection assays have shown that they can similarly enhance the transcription of various NRs in a ligand-dependent manner, suggesting a potential functional redundancy among these proteins. However, genetic studies revealed that they have different physiological properties on NR signaling in vivo. Although
both male and female SRC-1-knockout (KO) mice are viable and fertile, they exhibit a partial resistance to steroid and thyroid hormones, reduction in growth in response to hormonal stimulation (15), and delayed development of cerebellar Purkinje cells (16). TIF2-KO mice are hypotentry because of restricted growth of the placenta in females and partial impairment of spermatogenesis in males (17). TRAM-1 (p/CIP)-KO mice show growth retardation, lower levels of insulin-like growth factor 1, reduced female reproductive functions, and impaired mammary gland development (18, 19). The vasoprotective functions of estrogen after vascular injury are also partially impaired (20). In addition, TRAM-1 was amplified and/or overexpressed in breast cancers, ovarian cancers (6), endometrial cancers (21), pancreatic cancers (22, 23), gastric cancers (24), and prostate cancers (25), indicating that TRAM-1 is required for normal somatic growth and may play a role in oncogenesis.

These differential functions of p160 coactivators are thought to be, in part, due to their specific expression in tissues. We screened rat various tissues by immunohistochemistry using monoclonal antibodies against the three p160 coactivators, and observed differential expression, particularly in testis and epididymis (J Igarashi-Migitaka and A Takeshita, unpublished observations). Therefore, to investigate further the specific functions of the three p160 coactivators, we studied the expression of all three p160 coactivators in these tissues. We also performed double immunofluorescence staining to examine whether any of the p160 coactivators are colocalized with AR in these tissues.

Materials and methods

Antibodies

The monoclonal mouse anti-SRC-1 antibody (clone SRC01) raised against a recombinant human SRC-1 protein was purchased from Neo Markers (Fremont, CA, USA). Clone 29 of a TIF2 monoclonal antibody and clone 34 of an TRAM-1/AIB1 monoclonal antibody were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). In both Western blotting and immunocytochemical analysis, the SRC-1 antibody was used at 1:200, and the TIF2 and TRAM-1 antibodies were used at 1:150. These antibodies have previously been used successfully for immunohistochemistry and/or Western-blot analysis (23, 26, 27).

The anti-AR antibody PG-21, an affinity-purified rabbit polyclonal antibody raised to a synthetic peptide corresponding to the first 21 amino acids of rat AR, was purchased from Upstate Biotechnology (Lake Placid, NY, USA). The AR antibody was used at 1:100. Its use as a valid immunological probe for AR from a variety of species, including rat and human, has been established previously (28–30).

Cell culture

The Flp-In™T-Rex™-293 cell line was obtained from Invitrogen (Carlsbad, CA, USA) and was cultured in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum. The Flp-In™T-Rex™-293 cell line is derived from HEK-293 cells. The cell line expresses the Tet repressor and contains a single, integrated Flp-recombination target (FRT) site for generating stable cell lines exhibiting tetracycline-inducible expression of a gene of interest from a specific genomic location.

Specificity of antibodies

The specificity of monoclonal antibodies against the three p160 coactivators was confirmed by Western-blot analysis and immunohistochemistry using stable cell lines that express the p160 coactivators. To generate the three tetracycline-inducible Flp-In™T-Rex™ coactivator-expressing cell lines, the Flp-In™T-Rex™-293 cell line was transfected with the pcDNA5/FRT/TO expression vector containing human SRC-1 (8), mouse GRIP1/TIF2 (31), or human TRAM-1 (13) cDNA and the Flp recombinase expression plasmid, pOG44 (Invitrogen).

Stable transfectants were selected using 100 μg/ml hygromycin B. To induce the expression of the coactivators, tetracycline was added to a final concentration of 1 μg/ml. The cells were incubated for 24 h at 37°C. For Western-blot analysis, cells were rinsed in PBS and harvested. Whole-cell extracts were prepared using T-PER tissue protein-extraction reagent (Pierce, Rockford, IL, USA) containing a Complete Protease Inhibitor Cocktail (Roche Applied Science, Mannheim, Germany). Clarified extracts were obtained by centrifugation at 10,000 × g. 20 μg of extracts were mixed in 20 μl 1 × SDS sample buffer. Then the proteins were separated by SDS/PAGE on a 10% minigel and electrophoretically transferred to nitrocellulose. The membranes were blocked with 5% nonfat dry milk in TBS buffer (50 mM Tris/HCl/150 mM NaCl, pH 7.4) with 0.2% Tween 20 for 1 h. Immunoreactive bands were detected with SuperSignal Substrate System (Pierce) according to the manufacturer’s instructions.

Using the stable cell lines, immunocytochemistry was also performed. The cells were plated onto Biocoat collagen I culture slides (BD Biosciences, Bedford, MA, USA) at a cellular density of 5 × 10⁴ cells/well. Cells were grown to approximately 70% confluence. 1 μg/ml tetracycline was added to the medium, and the cells were cultured for 48 h. After washing with PBS, cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) for 15 min at room temperature. Following washing with PBS, endogenous peroxidase activity was blocked by placing the slides in 0.3% hydrogen peroxide in methanol for 10 min, and immunocytochemistry for the three coactivators was performed using the same methods as for paraflin-embedding of tissues (see below).

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**Experimental animals**

The immature (5 and 15 days old) and mature (60 days old) male Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan). Aged (50 days old) Sprague–Dawley rats were bred and maintained at Gunma University. The rats were housed under controlled temperature and illumination. Food and water were available ad libitum. The animal procedure was approved by the Institutional Animal Care and Use Committee of St Marianna University, Kawasaki, Japan.

**Protein extracts and Western-blot analysis for p160 coactivators in rat testis**

Cellular extract of testis from 60-day-old male rat was used for Western blotting. The testis was excised under sodium pentobarbital anesthesia, and immediately homogenized by ultrasonic disruptor (Tomy, Tokyo, Japan) in cold T-PER tissue protein-extraction reagent containing a Complete Protease Inhibitor Cocktail. Samples were then centrifuged at 9800 g for 5 min, and the supernatant was snap-frozen and stored at −80°C until the experiments. 20 μg of extracts were mixed in 20 μl 1× SDS sample buffer. Then the proteins were separated by SDS/PAGE on a 10% minigel and electrophoretically transferred to nitrocellulose. The membranes were blocked with 5% nonfat dry milk in TBS buffer with 0.2% Tween 20 for 1 h. Immunoreactive bands were detected with SuperSignal Substrate System according to the manufacturer’s instructions.

**Immunohistochemistry**

For immunohistochemistry, 5-, 15-, 60-, and 550-day-old male rats were perfused transcardially under pentobarbital sodium anesthesia, first with physiological saline and then with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Subsequently, the testis and epididymis were excised and post-fixed in the same fixative for 24 h at 4°C. The samples were embedded in Paraplast Plus tissue-embedding medium (Oxford Labware, St Louis, MO, USA) and sectioned at 3 μm.

The sections were mounted on silane-coated slides, deparaffinized in xylene, rehydrated in graded ethanol, and rinsed in water. Then the sections were subjected to microwave antigen retrieval by five 3-min incubations on full power (500 W) in 10 mM citrate buffer (pH 6.0). The sections were allowed to come to room temperature for 20 min before continuing with the immunostaining procedure. After washing in PBS, endogenous peroxidase activity was blocked by placing the slides in 0.3% hydrogen peroxide in methanol for 15 min. The sections were washed with PBS and incubated in 10% nonimmune horse serum in PBS for 30 min to block any nonspecific antibody binding. All incubations were performed at room temperature in a humidified chamber. The sections were incubated for 2 h at room temperature with SRC-1, TIF2, or TRAM-1 antibody. After two 5-min rinses in PBS, the sections were incubated with an affinity-purified biotinylated anti-mouse IgG (H+L), rat adsorbed (Vector Laboratories, Burlingame, CA, USA) at 1:50 dilution in PBS for 60 min. After washing in PBS, sections were incubated for 60 min with Elite avidin–biotin peroxidase complex (Vector Laboratories). After rinsing in PBS, the reaction product was visualized with 0.004% diaminobenzidine (Dojindo, Kumamoto, Japan) in 50 mM Tris/HCl, pH 7.6, and 0.003% hydrogen peroxide. Sections were then rinsed in water and counterstained with Carazi’s hematoxylin.

**Antibody competition**

The antigenic peptide for TRAM-1/AIB1 (BD Biosciences Pharmingen) was used to perform antibody competition. Briefly, preadsorbed AIB1 antibody was prepared by incubating 1 μg TRAM-1 antibody with 20 μg peptide for 24 h at 4°C, then was used as primary antibody as described in immunohistochemistry.

**Double immunofluorescence staining**

For double immunofluorescence staining with AR and the coactivator antibodies, the sections were incubated overnight with rat polyclonal antibody for AR and mouse monoclonal antibody for SRC-1, TIF2, or TRAM-1 in a humidified chamber at room temperature after the microwave antigen retrieval and treating with blocking reagent (Roche Applied Science). The sections were rinsed in PBS. AR antibody binding was visualized using Alexa Fluor 594-labeled anti-rabbit IgG (1:800; Molecular Probes, Eugene, OR, USA). The antibodies for coactivators were detected with an affinity-purified biotinylated anti-mouse IgG (H+L), rat adsorbed (Vector Laboratories). The slides were washed in PBS and incubated with 10 μg/ml Alexa Fluor 488 conjugate of streptavidin (Molecular Probes) in blocking solution for 60 min. After washing by PBS, the sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Roche Applied Science), according to the manufacturer’s instructions. The slides were mounted using mounting medium (PermaFluor, Shandon, PA, USA).

All the immunostained slides were investigated using a Carl Zeiss Axioskop 2 plus microscope, and images were recorded using AxioCam (Carl Zeiss, Tokyo, Japan).

**Results**

**Specificity of antibodies**

The monoclonal antibodies for three coactivators used in the present study were previously characterized to serve as valid probes (23, 26, 27, 32). In fact, the Western blotting
of cellular extracts from rat testis using each antibody detected a major 160 kDa band (Fig. 1A). However, their specificities have not been ascertained fully. Using tetracycline-inducible coactivator-expressing cell lines, the specificities of the antibodies were studied by Western-blot analysis (Fig. 1B). In the absence of tetracycline, each SRC-1, TIF2, and TRAM-1 antibody detected a faint band at approximately 160 kDa. This band presumably represents the endogenous coactivator expression in the 293 cells. In the presence of tetracycline, each antibody detected a strong major band at 160 kDa when the corresponding cDNA expression vector was transfected (Fig. 1B, lanes 2, 10, and 18). Of note, several minor truncated bands, which may be derived from internal ATG codons in the expression plasmids and/or partial degradation of the coactivators during protein extraction, were also observed (Fig. 1B, lanes 2 and 10). The tetracycline-induced expression was also analyzed by immunocytochemistry.

Tetracycline increased the intensity of immunoreactivity of each p160 protein in the nucleus when the corresponding cDNA expression vector was transfected (Fig. 1C and data not shown). From the results of Western blotting and immunocytochemical analysis, the specific immunorecognition by the coactivator antibodies was confirmed. We tested several other commercially p160 antibodies by Western blotting and immunocytochemical analysis as well. However, the antibodies used in the present study only showed one major 160 kDa band (data not shown).

p160 immunohistochemistry in mature rat testis and epididymis

Using immunohistochemistry, SRC-1, TIF2, and TRAM-1 expression were examined within paraffin-embedded 60-day-old male rat testis and epididymis (Fig. 2).
In the seminiferous tubules of the testis, SRC-1 immunoreactivity was found in the nuclei of spermatogonia and spermatocytes (Fig. 2a). TRAM-1 immunoreactivity was also observed in the nuclei of spermatogonia and spermatocytes. Interestingly, TRAM-1 expression was accompanied by some cytoplasmic dot-like staining (Fig. 2c). In Sertoli cells, very faint SRC-1 and TRAM-1 immunoreactivities were observed. In contrast, TIF2 was located specifically in nuclei of Sertoli cells (Fig. 2b).

In the interstitium, SRC-1 was expressed in Leydig cells (Fig. 2a). On the other hand, staining intensity of TRAM-1 immunoreactivity was weaker in the nucleus, with significantly greater cytoplasmic dot-like staining in this cell type (Fig. 2c). TIF2 expression was faint in the interstitium (Fig. 2b).

In the epididymis, SRC-1 and TIF2 immunoreactivities were localized in nuclei of epithelial cells (Fig. 2d, e, g, h, j, and k). In contrast, TRAM-1 immunostaining was observed in both the nucleus and luminal portion of the cytoplasm of epithelial cells (Fig. 2f, i, and l). An especially strong cytoplasmic labeling of TRAM-1 was observed in the caput epididymidis (Fig. 2f).

Immunoadsorption of the TRAM-1 antibody with the excess immune peptide completely abolished the nuclear and cytoplasmic staining of the serial section (Fig. 2f, inset).

**Coexpression of p160 and androgen receptor in testis and epididymis**

We next evaluated the subcellular localization of AR in comparison with p160 coactivators using immunofluorescence staining (Fig. 3). Consistent with previous studies (33–36), AR immunoreactivity in the rat testis was observed in most peritubular myoid cells and Leydig cells, regardless of different stages of spermatogenesis. In contrast, Sertoli cells showed different expression patterns of AR depending on seminiferous tubules, since the intensity of nuclear staining of AR varies as a function of the cycle of seminiferous tubules (35, 36). Similar to the different staining pattern of AR in Sertoli cells, SRC-1 and TRAM-1 in spermatogonia and spermatocytes also showed variable staining intensity, suggesting the stage-specific expression of SRC-1.
and TRAM-1. In contrast, TIF2 in Sertoli cells showed constant staining intensity regardless of different stages of spermatogenesis.

To determine whether AR coexpressed with specific p160 coactivators, double immunofluorescence staining was performed in testis (Fig. 4). The colocalization of AR with TIF2 in Sertoli cell nuclei was confirmed by merging these two images, as shown by the yellow staining pattern. In the both Leydig cells and peritubular myoid cells, AR showed significant colocalization with SRC-1, but not with TIF2 and TRAM-1. Colocalization of AR with p160 coactivators was detected in epididymis as well (Fig. 5). In epithelium of epididymis, AR immnoreactivity was found in nuclei in all regions of the epididymis (Fig. 5, red staining). Colocalization of AR with both SRC-1 and TIF2 was observed in nuclei of the epididymis. In contrast, colocalization of AR with TRAM-1 was weak, as TRAM-1 was located mainly in the cytoplasm. Immunoabsorption of the TRAM-1

Figure 3 The cellular distribution of the three p160 coactivators and AR in mature rat testis. Immunolocalization of the three p160 coactivators was performed using each specific monoclonal anti-p160 antibody, whereas AR was detected by a rabbit anti-AR antibody. The p160 coactivators (green) are shown in the left-hand panel, AR (red) counterstained with DAPI (blue) is shown in the right-hand panel. In seminiferous tubules, SRC-1 and TRAM-1 were expressed in germ cells. Depending on the seminiferous tubules, staining intensities of SRC-1 and TRAM-1 were different, suggesting spermatogenesis stage-dependent expression of these two coactivators. In contrast, TIF2 was specifically and constantly expressed in Sertoli cells although the intensity of nuclear staining of AR differed depending on the spermatogenesis of seminiferous tubules. Scale bars, 100 μm.

Figure 4 Double immunofluorescence staining of the three p160 coactivators with AR in mature rat testis. Immunolocalization of the three p160 coactivators was performed using each specific monoclonal anti-p160 antibody, whereas AR was detected by a rabbit anti-AR antibody. The p160s are shown by green, AR by red, and nuclear DAPI staining by blue. The images of p160 and AR staining were overlaid and orange or yellow colors show where the two molecules were colocalized (Merge; lower right-hand panels). Inset micrographs are high magnifications of merged images indicated by the white squares. Scale bars, 50 μm.
antibody with the excess immune peptide abolished the cytoplasmic staining of the serial section (Fig. 5, inset).

**Developmental expression of p160s as determined by immunohistochemistry**

Finally, to study ontogenic expression of p160 coactivators in rat testis, immunohistochemistry was performed using rat on postnatal days (P) 5, P15, P60, and P550 (Fig. 6). In Sertoli cells, a strong TIF2 immunoreactivity was observed from P5 to P550. Although SRC-1 and TRAM-1 showed minimal expression in Sertoli cells of adult rat testis (P60 and P550), weak but significant levels of SRC-1 and TRAM-1 immunoreactivity were observed in Sertoli cells on P5 and P15.

In germ cells, TIF2 was positive in gonocytes on P5 and in spermatogonia on P15. Then it became faint on P60 in germ cells such as spermatogonia and spermatocytes, whereas SRC-1 and TRAM-1 were positive in germ cells such as gonocytes, spermatogonia, and spermatocytes at all ages.

**Discussion**

To investigate the specific functions of the three p160 coactivators, we determined the tissue distribution of all these coactivators in rat testis and epididymis in the present study. We also examined colocalization of the three coactivators with AR.

TIF2 was specifically expressed in Sertoli cells in adult rat seminiferous tubules. Our observation is consistent with the study of TIF2-KO mice (17). TIF2 transcripts and proteins are expressed in Sertoli cells, but not in germ cells in wild-type mice (17). Male TIF2\(^{-/-}\) mice are hypofertile, have defects in spermiogenesis, and show age-dependent testicular degeneration (17). Such serious fertility impairment has not been reported in SRC-1\(^{-/-}\) or p/CIP\(^{-/-}\) (TRAM-1\(^{-/-}\)) mutants (37), indicating the specific function of TIF2. In agreement with the role of TIF2 in mouse Sertoli cells, some patients with oligospermic infertility possess AR mutations that disrupt the interaction between AR and TIF2 without altered ligand binding (38, 39). TIF2 may be a key regulator of Sertoli cell function.

It is generally considered that stage-specific expression of AR in Sertoli cells is important for spermatogenesis (35, 36). Using double immunofluorescence staining, we showed considerable colocalization of AR and TIF2 proteins in Sertoli cells. It should be emphasized that the level of TIF2 expression in Sertoli cells was stable whereas that of AR expression was variable among cells. Limited expression of AR in Sertoli cells may deter-
mine the ligand-induced interaction with TIF2 to mediate transcriptional regulation.

Whereas TIF2 was colocalized with AR in Sertoli cells, a specific colocalization of SRC-1 with AR in Leydig cells and peritubular myoid cells was observed. This finding suggests that TIF2 may play as a dominant coactivator of AR in Sertoli cells, whereas SRC-1 may be dominant in Leydig and peritubular myoid cells. As such, differential expression of the p160 coactivators in testis may regulate androgen-dependent control of spermatogenesis.

In contrast to our finding of predominant SRC-1 expression in germ cells rather than in Sertoli cells, Mark et al. (40) reported a study of SRC-1/TIF2 compound mutant mice. Their immunohistochemical analysis using polyclonal antibody against SRC-1 showed that SRC-1 is localized in Sertoli cells but not in germ cells, similar to TIF2 expression pattern. The etiology for the difference is not clear, although the difference of species (rat and mouse) and/or development stage (discussed below) may influence the results. Further, differential specificity and sensitivity of antibodies may have caused such differences.

Unlike mature rat testis, all the three coactivators were expressed in both germ cells and Sertoli cells in immature rat testis. Using mRNAs of rat cerebellum, we previously reported that the p160 coactivators as well as corepressors exhibit differential expression during postnatal cerebellar development (41). We also reported hormonal regulation of the expression of SRC-1 mRNA in the anterior pituitary in addition to a gender-related difference (42). Hormones including androgen may regulate the specific p160 expression during development. Further study will be necessary to understand the mechanisms of this issue.

In the epididymis, SRC-1-and TIF2-immunoreactivities were localized in nuclei of epithelial cells. However, TRAM-1 immunostaining was mainly observed...
in the luminal portion of the cytoplasm rather than in the nucleus, especially in the caput epididymis. Recently, nucleocytoplasmic shuttling of p160 coactivators has been reported by several groups (43–45). Qutob et al. (44) showed that p/CIP (mouse TRAM-1) is localized in either the nucleus or cytoplasm, depending on the presence of growth factors in cell culture medium and on the effect of leptomycin B. They demonstrated that cytoplasmic p/CIP associates with tubulin and that an intact microtubule network is required for intracellular shuttling of p/CIP. In addition, they observed that only nuclear p/CIP complexes possess histone acetyltransferase activity (44). Although a common nuclear import signal in the N-terminal region of p160 family members exists, SRC-1 and p/CIP contain different domains for nuclear export (45). Our observations of the specific nuclear (SRC-1 and TIF2) or cytoplasmic (TRAM-1) expression of p160 coactivators in epididymis suggest that specific stimuli may differentially regulate nucleocytoplasmic trafficking among p160 family members. Further, the strong cytoplasmic expression of TRAM-1 protein in epithelial cells of epididymis suggests that TRAM-1 may have additional role(s) to transcriptional regulation, in contrast to SRC-1 and TIF2. Further studies are required to elucidate the role of cytoplasmic TRAM-1 in epididymis.

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