Molecular cloning and characterization of a novel splicing variant of PIASx[1]

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KEY WORDS human; testis; spermatogenesis; PIAS-NY; sequence analysis; gene expression

ABSTRACT

AIM: To investigate molecular mechanism of testis development and spermatogenesis. **METHODS:** A human testis cDNA microarray was hybridized with probes from human adult testis, embryo testis and human sperm, and the differential expressed clones were sequenced and analyzed. Expression of PIAS-NY gene was analyzed by RT-PCR. **RESULT:** A new isoform of PIAS family, named PIAS-NY, was isolated from human testis cDNA liabrary. It was strongly expressed in adult testis and weakly expressed in both embryo testis and human sperm. Analysis of the open reading frame of PIAS-NY indicated that PIAS-NY was a polypeptide of 405 amino acid residues, and the sequence from the 15th amino acid to the end of PIAS-NY protein was the same as the N-terminal amino acids of PIASx-α and PIASx-β protein. PIAS-NY protein contained two conserved putative LXXLL signature motifs and a zinc binding motif. Tissue distribution analysis revealed that PIAS-NY was predominantly expressed in testis, weakly in the pancreas, and almost imperceptibly in the other organs. **CONCLUSION:** PIAS-NY may play important role in testis development and/or spermatogenesis.

INTRODUCTION

Signal transducer and activator of transcription (STAT) plays important roles in numerous cellular processes including immune responses, cell growth and differentiation, cell survival and apoptosis, and oncogenesis. STAT does not act alone. A number of proteins have been identified that interact with STAT and modulate the activity of STAT at various steps of the activation-inactivation cycle[1]. Protein inhibitor of activated STAT (PIAS) family of molecules is discovered in yeast two-hybrid screens designed to identify STAT-interacting proteins[2]. There are at least five mammalian genes encoding PIAS protein: PIAS1, PIAS3, PIASxα (also named androgen recepter-interacting protein, ARIP3), PIASxβ, and PIASy[3]. The PIAS family are thought to have at least three functions: (1) Regulate the transcriptional activity of STATs in the nucleus. PIAS1 and PIAS3 inhibit STAT1- and STAT3-mediated transcription by blocking their DNA binding activity[4]. In contrast, The PIASy protein represses STAT1 without affecting DNA binding. Both PIASxα and PIASxβ can inhibit STAT4-mediated gene activation. They are component of a large transcriptional co-repressor complex (the STAT4-DNA binding complex)[5]. (2) Act as an androgen receptor (AR) transcriptional co-regulator. PIASxα modulates the transcriptional activity of AR. Other PIAS proteins, such as PIAS1 and PIASy, have recently been demonstrated to function as co-regula-

[1] Project supported by grants from State Key Basic Research and Development Program (No G1999055901) and National Natural Science Foundation of China (No 30170485).
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Received 2003-10-23 Accepted 2004-04-01

tors for AR[6-11]. (3) Function as small ubiquitin-related modifier (SUMO)-1-tethering proteins and zinc finger-dependent E3 SUMO protein ligases[12-14]. Several reports have shown that PIAS proteins enhance SUMO modification of proteins that includes AR, p53 and glucocorticoid receptor, and they are therefore proposed to function as SUMO E3-like factors[14]. Both PIAS1 and PIASxα act as specific SUMO-E3 ligases for AR in intact cells as well as *in vitro*[12].

Spermatogenesis involves a number of unique processes, including mitotic, meiotic and post-meiotic phases[15]. The highly ordered process requires a precise and well-coordinated program that regulates the constantly changing patterns of gene expression. All of PIAS family members are reported to be expressed in testis[3,10]. It is possible that these PIAS proteins co-regulated the progression of spermatogenesis.

On the basis of the adult testis cDNA microarray prepared by our laboratory, we compared the genes expressed in the embryo and adult testis at a high throughput. Herein we describe the isolation and tissue distribution of a PIASx (include PIASxα and PIASxβ) splice variant named PIAS-NY. To find the possible involvement of PIAS-NY in testis development and/or spermatogenesis, we test its expression in different developmental stages of male testis and discuss its characterisic and tissue distribution.

MATERIALS AND METHODS

Preparation of human testis cDNA microarray The testis cDNA microarray was constructed as described before[16,17]. Briefly, this microarray contained 9216 cDNA clones which were derived from a Human Testis 5'-STRETCH PLUS cDNA library (Clontech, Palo Alto, CA, Source of insert cDNA came from 25 Caucasians, aged from 20 to 65 years). The inserts were amplified by PCR using 5'-CCATTGTGTTGGTACCCGGGAATTCG-3' as a forward primer and 5'-ATAAGCTTGC TCGAGTCTAGAGTCGAC-3' as a reverse primer. PCR products were used to make human testis cDNA microarray.

Clones of interest sequencing and analyzing Clones of interest were selected and extracted by DNA extraction system (Qiagen, Hilden, Germany) and sequenced by ABI 377. All sequences were blasted in GenBank, and nucleic acids and putative proteins were analyzed by the software of gene runner.

Analysis of PIAS-NY gene expression in different tissues by RT-PCR To determine the tissue distribution of PIAS-NY, primers specific to 5′ end of PIAS-NY overlapping an intron were used to amplify cDNAs of 16 human tissues using the Human MTC Panel I and II kit (Clontech, Palo Alto, CA). Primers were synthesized at BioAsia company (Shanghai, China): P1: 5'-GCAGTCAAACCTACCCAAC-3', P2: 5'-AAGCAGCACAGAACCAAC-3', the upstream primer, was located at specific 5'-region, and P2, the downstream primer, was in the common region of PIAS-NY, PIASxα, and PIASxβ. The PCR product size was 436 bp. PCR reaction mixture 20 μL contained: 2 μL cDNA template, 2 μL 10×reaction buffer, 1.5 μL $MgCl_2$ (25 mmol/L), 1.5 μL dNTP (2 mmol/L), 1 μL each primers (5 pmol/μL), 0.1 μL *Taq* polymerase (5 U/μL, promega, Shanghai, China), and 10.9 μL water. PCR was carried out using PE2400 (Perkin-Elmer, USA) and the condition used were 1 cycle at 94 ºC for 5 min, 35 cycles at 94 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 30 s, followed by 1 cycle at 72 ºC for 10 min. G3PDH was used as comparative control. Its upstream primer was 5'-TGAAGGTCGGAGTCAACGGA TTTGGT-3', and downstream primer was 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The desired fragments were 983 bp. PCR reaction mixture was the same as used above except for primers. The PCR condition used were 1 cycle at 95 ºC for 1 min, 36 cycles at 95 ºC for 30 s, 68 ºC for 3 min, followed by 1 cycle at 68 ºC for 3 min. The products of PCR were analyzed by 1.5 % (w/v) agarose gel electrophoresis.

Analysis of PIAS-NY expression in human adult, embryo testis and sperm using RT-PCR Human testis RNA and sperm RNA were isolated using TRIzol Reagent (GIBCO BRL, Grand island, NY). Human embryo testis (aged 6 month), adult testis (aged 43 years) and human sperm from normal fertile man were obtained legally from Peoples' Hospital of Jiangsu Province, and Body Donor Center of Nanjing Medical University. The informed consents were provided by the patients and donors. Reverse transcription was performed in 15 μL of reaction mixture. First 1 μL of total RNA(about 3 μg), 1 μL of random primer (0.2 mg/L Sangon, Shanghai, China) and 7 μL of DEPC water were mixed and incubated at 70 ºC for 5 min; then 3 μL of M-MLV RT 5×buffer, 0.75 μL of dNTP (20 mmol/L), 0.35 μL of Rasin(50 U/μL), 1 μL of M-MLV Reverse Transcriptase (Promega, Shanghai, China), 1 μL of DEPC water were added and incubated at 37 ºC for 1 h, and then 95 ºC for 5 min. PCR reaction mixture, condition, and primers were the same as used above.

The primer sequences for human β-actin cDNA were: P1: 5'- CGGTTGGCCTTGGGGTTCAGGGGG-3'; P2: 5'-ATCGTGGGGGCGCCCCAGGCACCA- 3'. The desired fragment was 247 bp. The products of PCR were detected by staining with ethidium bromide after electrophoresis on a 1.5 % (w/v) agarose gel.

RESULTS

cDNA microarray hybridization The results of hybridization with embryo and adult testis probes revealed that there was a clone named PIAS-NY highly expressed in adult testis. The signal intensities hybridized with adult testis probe, embryo testis probe, and human sperm probe were 72.15, 5.08, and 11.67 respectively. The expression level in adult testis was about 14.2-fold stronger than that of embryo (Fig 1).

Sequence identification and analysis of PIAS-NY gene The full nucleotide and amino acid sequences of PIAS-NY were displayed in Fig 2. The 1421 bp PIAS-NY cDNA contained a complete ORF of 1218 bp with a methionine start codon at position 67 and a TGA stop codon at position 1284. The first start codon was preceded by an in-frame stop codon TAG at position 52, suggesting that ATG at position 67 was the start codon for PIAS-NY protein. PIAS-NY cDNA encoded a 405-amino acids protein with predicted molecular weight 45 kDa, and isoelectric point, 9.45. Subcellular localization analysis of PIAS-NY protein (PSORT version II, French and PLOC, Japan) revealed that PIAS-NY was localized in nucleus. Blast search in the human genome database localized PIAS-NY gene to human chromosome 18 (NT_010966.13|Hs18_11123). PIAS-NY had the GenBank accession number AF361054.

Homologous analysis between PIAS-NY and other PIAS family members BLASTN 2.2.6 (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) search revealed that PIAS-NY was highly homologous to three other genes, all of which were identified in humans: BC015190, AF077953, and AF077954 (Fig 3). Just like PIAS-NY, the three genes also localized in human chromosome 18 (NT-010966.13|Hs18-11123), one clone in chromosome 18 contains the genomic sequences of the three proteins. Compared with other moleculars of PIAS family, PIAS-NY lacked exon 11,12,13,14,15 and 16 at 3′ end, while a unique exon existed at the 5′ end. PIAS-NY had a shorter ORF and coded a small protein (405 amino acid), which had a similar NH_2 terminus to the above three genes. The deduced amino acid of PIASX (BC015190) was the same as PIASxα. Blast search in the human genome database localized PIAS1 and PIASy to human chromosome 19. PIAS3 was located in chromosome 1.

BLASTP 2.2.6 search showed that PIAS-NY had high homology with PIAS family members. PIAS-NY also showed extensive sequence identity with Rattus norvegicus MIZ/ARIP protein and identity rate was 93 %. Clustal w1.81 (http://www.ebi.ac.uk/Clustalw/) was used to compare amino acid sequences of all six PIAS family members. The result revealed that the sequence from the 15th amino acid to the end of PIAS-NY protein was the same as the N-terminal amino acids of PIASxα and PIASxβ protein. PIAS-NY protein showed 75 %, 75 %, and 64 % sequence identity to PIAS1, PIAS3, and PIASy protein respectively.

Sequence analysis revealed several interesting structural features of the PIAS family of proteins. A conserved putative LXXLL signature motif and zinc

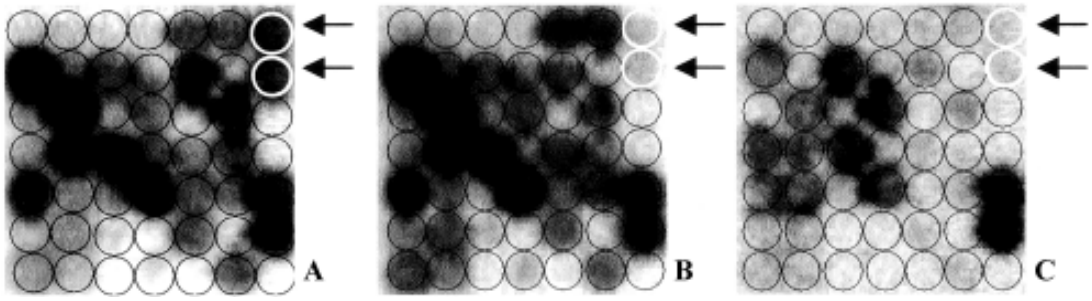

Fig 1. cDNA hybridization images showing differential expression of PIAS-NY in (A) adult human testis, (B) 6-month-old fetal testis and (C) human sperm(red circle). The hybridization intensity in adult testis, fetal testis and human sperm was 72.15, 5.08, and 11.67, respectively.

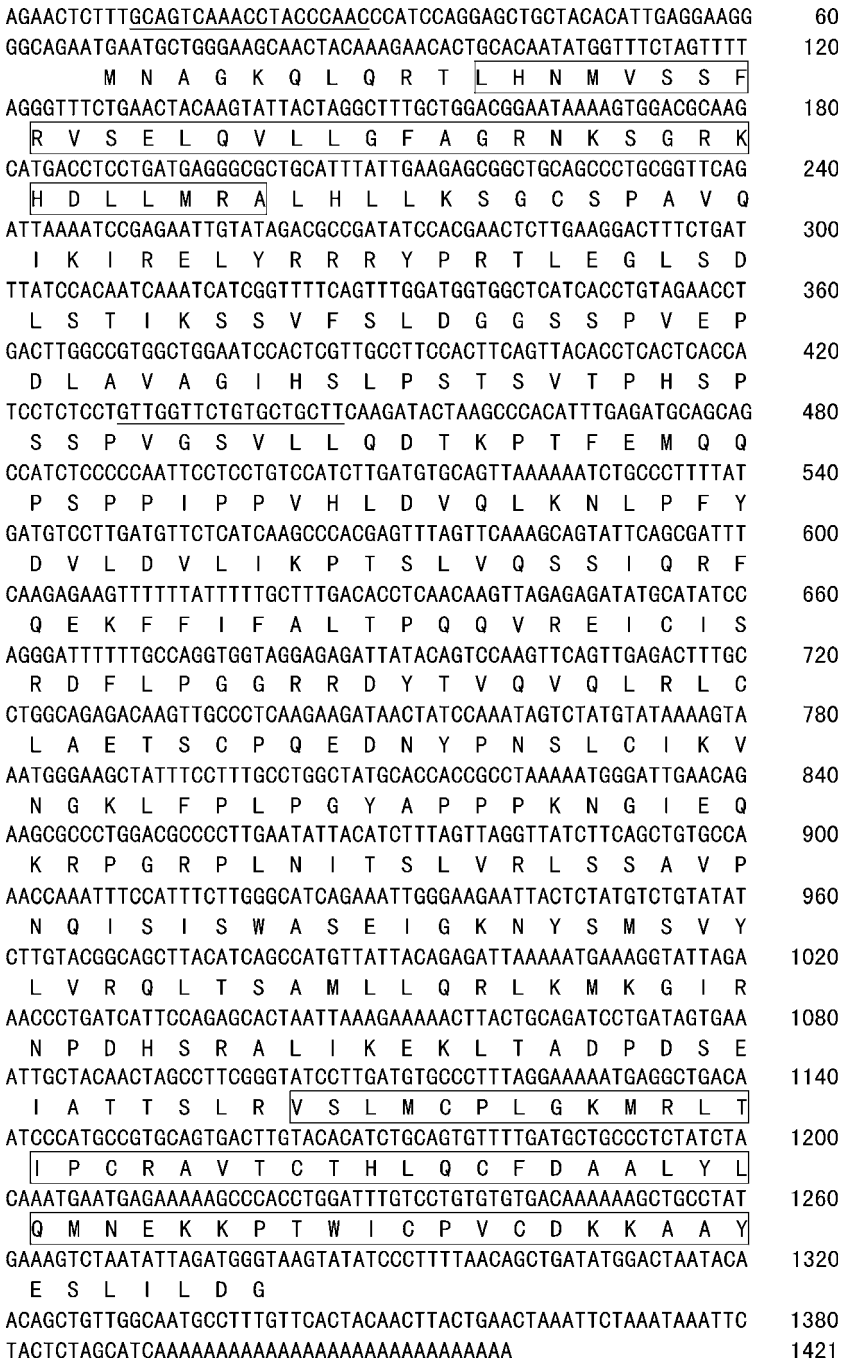

Fig 2. Nucleic acid and deduced amino acid sequences of the cDNA for PIAS-NY. Underlining shows PCR primers for the determination of expression profile. The upstream primer is located in the specific region of PIAS-NY. The downstream primer is homologous with that of PIASxα and PIASxβ. Boxes show a signature motif in transcriptional co-activators mediates (upper) and zinc binding domain (under).

binding motif were present in the NH_2-terminal region of all PIAS proteins. The COOH-terminal regions of PIAS proteins were the least conserved and consisted of a highly acidic region and a serine/threonine rich region. Unlike other PIAS proteins, PIASy lacked the COOH-terminal serine/threonine rich domain and PIAS-NY only contained LXXLL and ZBD motif. Program Smart analysis predicted that PIAS-NY contained SAF box and zinc finger motif (Fig 4).

mRNA expression of PIAS-NY Expression pattern of PIAS-NY was examined by RT-PCR in 16 human tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and

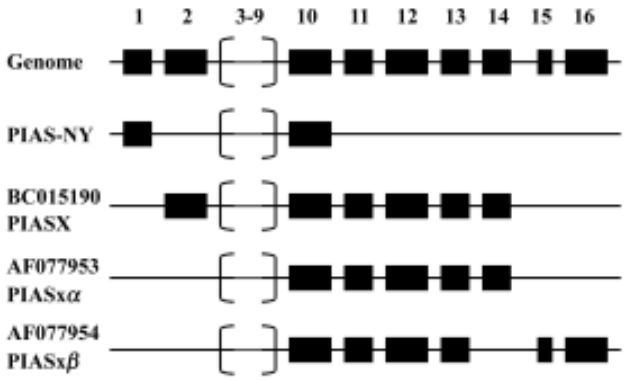

Fig 3. Transcript and splicing comparison of PIAS-NY with homologous genes. Seven identical exons (exons 3-9) in four sequences are boxed and omitted. Exon 1 only existed in PIAS-NY, Exon 2 only existed in PIASX and exons 15, 16 only existed in PIASxβ. Exon 10 in PIAS-NY is longer than the others and PIAS-NY lacks exons 11-16. PIASx-β lacks exon 14 and PIASx-α has a longer exon 14 than PIASX.

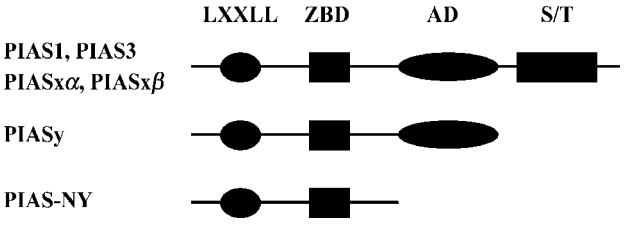

Fig 4. The domain structure of PIAS proteins. LXXLL: a signature motif in transcriptional co-activators mediates, ZBD: zinc binding domain, AD: acidic domain, S/T: Serine-threonine rich region.

leukocyte. The gene was predominantly expressed in testis, weakly in the pancreas, almost imperceptibly in the other organs (Fig 5). Besides the specific PCR product of 436-bp, there were two other DNA fragments about 600 bp and 700 bp respectively. RT-PCR showed PIAS-NY was differentially expressed in human embryo and adult testes, which confirmed the hybridization result of cDNA microarray with stronger signal in adult than in embryo. The product detected in human sperm was very weak (Fig 6).

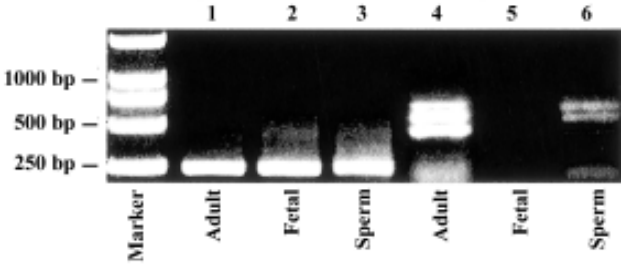

Fig 6. RT-PCR analysis of PIAS-NY expression in human adult, embryo testis, and human sperm. β-Actin was used as an internal control. Line 1-3: β-actin, Line 4-6: PIAS-NY.

DISCUSSION

The present study reports the identification of a new isoform of PIAS family designated PIAS-NY by using cDNA microarray constructed from human testicular cDNA library. PIAS-NY, PIASxα, and PIASxβ are differential splicing products of the identical PIASx gene. The relation among PIAS-NY, PIASxα, and PIASxβ is indicated by following lines of evidence: 1) The nucleic acid sequence of PIAS-NY (1179 bp) presents a highly homologous to that of PIASxα, and PIASxβ. Furthermore, The sequence from the 15th amino acid to the end of PIAS-NY protein is the same as the N-terminal amino acids of PIASx-α and PIASx-β protein. 2) Blast conserved domain shows that , within

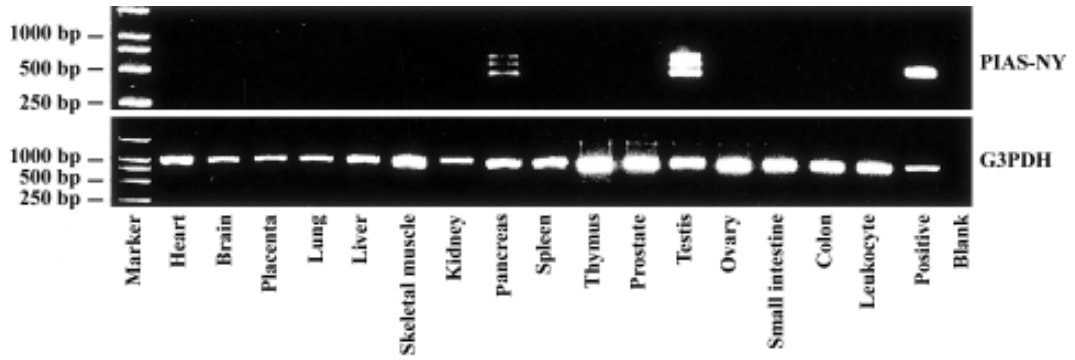

Fig 5. RT-PCR analysis of mRNA expression. PIAS-NY mRNA expression (upper panel) and G3PDH mRNA expression in various human tissues were accessed using a multiple-tissue cDNA. PIAS-NY Positive: PIAS-NY plasmid, G3PDH positive: control cDNA. PIAS-NY is predominantly expressed in testis and weakly expressed in pancreas.

the coding region, PIAS-NY, PIASxα, and PIASxβ shared LXXLL and zinc finger domains but differ in the C-terminal amino acids. 3) Unigene library search demonstrats that PIAS-NY could be within the same Unigene claster as PIASxα and PIASxβ (Unigene number: Hs. 441069) which is located in chromosome 18 as determined by mapping analysis. Moreover, 600 bp and 700 bp bands beside PIAS-NY anticipative band implied there were two other unidentified splicing variants of PIASx in testis.

The expression pattern of PIAS-NY is similar to that of the PIASxα and PIASxβ. PIASxα expresses solely in human testis[3]. There is no report about the tissue distribution of PIASxβ. Our results of tissue distribution show that PIAS-NY was also predominantly expressed in human testis though there was weak expression in pancreas. In adult rat testis, PIASxα and PIASxβ mRNAs are localized to the germ cells and Sertoli cells in the seminiferous epithelium. And the expression of PIASxα and PIASxβ is higher in spermatocytes than in other germ cells. Moreover, the expression level of these genes increases significantly in adult mouse testes compared with infant mouse[3]. Given the expression of PIAS-NY in human sperm, we hypothesize that PIAS-NY is at least expressed in germ cell. Hybridization of cDNA microarray with probes from human adult testes and embryo testes shows a 14.2-fold higher expression of PIAS-NY in adult than in embryo testis, indicating its development-dependent expression. PIASxα and PIASxβ proteins are detected in the nuclei of Sertoli cells, in spermatogonia, and in primary spermatocytes up to late pachytene stage of development[10]. Subcellular localization analysis and characterization of PIAS-NY protein (such as LXXLL and zinc finger domain) reveal that it is also located in nucleus. Similar tissue and cell distribution, stage–specific expression and subcellular localization suggest that PIAS-NY may play a crucial role in testicular development and/or spermatogenesis just like its splicing variants.

Androgen is essential for male sexual development and the initiation and maintenance of spermatogenesis. Most of the signaling effects of androgens are mediated through the androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors[18]. It has become clear that the transcriptional activity of AR is affected by co-regulators that influence a number of functional properties of AR, including ligand selectivity and DNA binding capacity. Recently, many of such co-factor proteins have been identified. These proteins include CBP/p300, ARA54, ANPK, FHL2, hsp40, Calreticulin, Cyclin D1, HBO1, and so on[19-26]. PIAS family members such as PIAS1, PIASy, and PIASxα have also been reported to be co-regulator of AR by multiple mechanisms[8-11].

PIAS1 is isolated in yeast two-hybrid screen as a factor capable of interacting with AR in an androgen-dependent manner. The interaction with AR occurs through the PIAS1 NH2 terminus, which contains three LXXLL motifs. PIAS1 is predominantly expressed in the testis with expression observed in the Sertoli and Leydig cells as well as in spermatogenic cells[6]. In addition, when co-expressed with PIAS1, other PIAS family members including PIASxα, PIASxβ, and PIASy counteract PIAS1 co-regulation of AR transactivation[7]. Gross *et al* reported that LXXLL motif in PIASy was required for suppression of AR transactivation but not for PIASy binding to the AR DNA bingding domain[8]. PIASxα is also found to be an AR co-regulator and, like PIAS1, is primarily expressed in the testis. PIASxα interacts with AR both in intact mammalian cells and *in vitro*, and it is capable of modulating AR-dependent transcriptional activity through facilitating AR NH2/COOH-terminal interaction rather than DNA binding affinity[9,11].

On the other hand, PIAS1 and PIASxα can also function as E3 ligase for small ubiquitin-related modifier (SUMO) conjugation[12,13]. The AR has been shown to be modified by small ubiquitin-like modifier 1 (SUMO1) *in vivo*. Blocking sumoylation can increase the transactivation ability of AR, suggesting that SUMO modification negatively regulates AR activity. PIAS1 and PIASxα repress the AR-dependent transactivation in a manner dependent on their zinc finger domains, which are required for their SUMO-E3 ligase activity[12].

Similar to PIAS1 and PIASxα, PIAS-NY predominantly expresses in testis. These results suggest that PIAS-NY mainly play its roles in testis. Sequence of PIAS-NY is highly homologous to PIAS1, PIASy, and PIASxα gene. Moreover, PIAS-NY also has LXXLL and zinc finger domains which were considered to be useful in interaction with AR. So we speculate that PIAS-NY may be a new co-regulator protein of AR and may play important role in testis development and maintenance of spermatogenesis. The function and molecular basis of PIAS-NY in AR transcriptional regulation need further elucidation.

In conclusion, we cloned PIAS-NY for PIAS isoform with LXXLL and zinc finger motif from hu-

man cDNA library. Characterization analysis of PIAS-NY sequence, tissue distribution, and stage–specific expression suggest that PIAS-NY may have important role in testis development and/or spermatogenesis.

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