Letter to Editor

Identification of testis-expressed cell cycle regulating proteins with special reference to meiosis

Uma Chandran, Malini Laloraya and Pradeep Kumar G

Molecular Endocrinology & Reproduction Division, Rajiv Gandhi Centre for Biotechnology, Thycaud PO, Poojappura, Thiruvananthapuram - 695 014, Kerala, India

Summary

Two percent of human males are infertile because of severe defects in sperm production. In the clinical cases spermatogenic arrest is an interruption of germ cell differentiation that may result in either oligozoospermia or azoospermia and can be diagnosed in testicular biopsy. Although spermatogenesis requires many gene products, mutation or absence of the genes expressed at different levels of spermatogenesis may lead to spermatogenic arrest and infertility. Identification of new genes specifically involved in spermatogenesis and analysis of the phenotypes could provide an insight into this developmental process and a more rational basis for treatment of male infertility. Using differential display proteomics followed by genomic assays and molecular modeling, we have identified a few testis-specific genes that may regulate cell cycle in germ cells. We are currently concentrating on a class of testis-specific proteins named Cyclin-Like Proteins (CLPs), which are classical cyclin box-bearing proteins with typical folds. Using RT-PCR based approach, we have sequenced the full length CDS of mouse testicular CLP-1. With major thrust on this molecule, we are aiming at elucidating the intricate molecular control of meiosis and germ cell differentiation. We will also attempt to examine whether there are defects in the CDS of CLP-1 gene associated with male subfertility.

Key words: male infertility, testis-specific proteins, cyclin-like proteins,

Introduction

Cyclins are proteins that physically interact with cyclin-dependent kinases (cdks) and modulate their kinase activity. Cyclins open up active sites of cdks and start downstream regulation by phosphorylating specific proteins within the cells. Cyclins or cdks cannot act independently. Cyclins got the name because they appear and disappear at defined times during cell cycle.

Most of the cyclins contain a 100 amino acid conserved region at the centre of the linear protein sequence, and it is referred to as cyclin box. Within this conserved domain, approximately 30-35 amino acids in the midregion are highly conserved. Studies indicated that the cyclin box region of cyclins is essential for binding and activation of the catalytic subunit to which they are bound (Lees and Harlow, 1993).

Infertility is defined as lack of conception after at least 12 months of unprotected intercourse (WHO, 1993). It is estimated that 50-80 million people of the world are probably facing one or other type of problems with fertility. There are about 2 million new infertile couples reported each year and the number is increasing. It is established that inadequacies on the part of the male contribute to a significant percentage of the infertility problem, and is estimated to be as high as 40% by some investigators (WHO, 1993). The causes may reside in the genital tract itself, in higher nervous centers, endocrine organs or in the control of testicular functions. Major causes of infertility in men include varicocele, obstruction of the spermatic ducts, agglutination of sperm, high semen viscosity, necrospermia, low volume of ejaculate, ejaculatory dysfunction and high sperm density; when no cause is attributable, the man is said to be having idiopathic infertility (Greenberg et al., 1978).

A novel gene family, named ancient conserved domain proteins(ACDP), which encodes four protein members in humans and mice, was cloned and sequenced (Wang et al., 2003, 2004). This gene family is evolutionarily conserved in diverse species ranging from bacteria, yeast, *Caenorhabditis elegans* and *Drosophila melanogaster* to mammals. All ACDP proteins possess a conserved sequence motif of 31 amino acids that is present in the cyclin box region of all cyclins. Property-based homology modeling of ACDP proteins reveals 20 turns and 21 helices in its architecture. Generally, this pattern is typical of cyclins. These helix-rich domains showed high

Correspondence to be addressed to: Dr. G. Pradeep Kumar Email: pradeg@gmail.com

structural homology with domains 1 and 2 of bovine cyclin A3 (Brown et al., 1995).

ACDP genes are known in different names. They are termed ACDP (Ancient Conserved Domain Protein) because of the presence of highly conserved ACD domain in evolutionarily divergent species from bacteria to human, ACDK (activators of cdk) because they are found to activate cdk-1 and cdk-2 *in vitro* and also Cyclin M because of the presence of conserved cyclin box region. As the function is still unknown, but the presence of cyclin box-like region and the structural similarity to cyclins led us to refer these proteins as Cyclin Like Proteins (CLP). Of the four splice variants identified, CLP-1 expression is restricted to brain and testis where as the other variants are expressed in various other tissues also.

Differential display proteomics and genomics studies of CLP-1 indicated its absence in human male factor infertility (oligozoospermia and aspermia) cases. Even though the exact functions of CLP-1 are not known several lines of evidence suggest that these genes may play an important role in biological processes. The cyclin box-like region, the sequence conservation and the presence of multiple members within a species may imply functional importance associated with this gene. Since CLP-1 is present in testis and its absence is related to infertility, it may be a candidate gene regulating meiotic cell cycle regulation or spermatogenesis. In this study, we attempted to evaluate the expression of CLP-1 and to elucidate the full length sequence of its transcript in mouse testis. Localizion of the CLP-1 protein in spermatozoa collected from testis and different segments of the epidydimis was also performed.

Material and Methods

Animal

Healthy male mice (*Mus musculus,* Swiss strain, 3-4 month old) bred in the institute animal facility, housed in temperature 27±1^{o}C and humidity controlled conditions under 14 hour light:10 hour dark and provided with food and water *ad libitum,* were used for the study.

Total RNA preparation

Total RNA from the mature male mouse testis was prepared using TRI reagent method (Sigma), according to the manufacturer's instruction.

First Strand Synthesis

From the total RNA, first strand cDNA was prepared using the Ready To Go T-primed first strand synthesis Kit which utilizes the Molney Murine Leukemia Virus (M-MuLV) reverse transcriptase and an oligo (dt)18 primer to generate the first strand cDNA. The prepared cDNA were stored at –20^{0}c.

Polymerase Chain Reaction

A set of mouse CLP-1 specific primers were generated from the sequences derived from mouse gene bank submission at NCBI for the CLP-1 message in brain using Primer3 software (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR of the first strand was performed using the CLP-1 gene specific primers. The testicular cDNA was amplified in PE Gene AMP 9600 PCR instrument by using specially designed CLP-1 forward and reverse primers .The samples were subjected to 35 PCR cycles of 30s at 95^{0}C for denaturing, 30s at 65^{0}C annealing temperature for annealing and 60 sec at 72^{0}C for extension.

Agarose gel electrophoresis

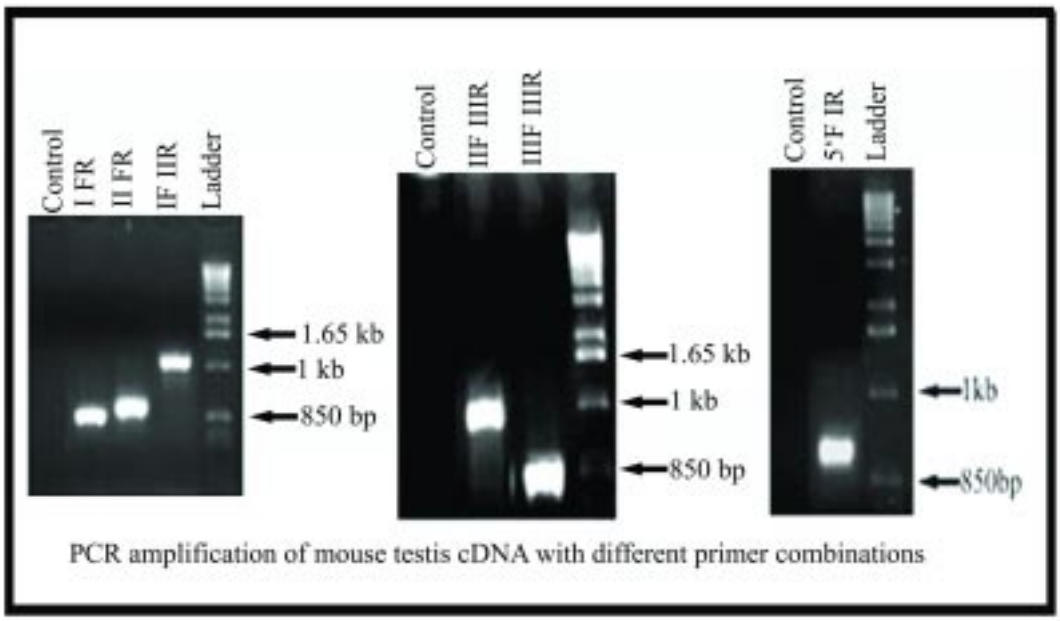
The PCR product was mixed with 2ml of gel loading buffer .The DNA was separated on a 1% agarose gel with 5ml of 10mg /ml of ethidium bromide. Gel was run in 0.5%TBE running gel buffer using the Pharmacia LKB GNA 100 sub marine Gel Electrophoresis at 100V constant voltage .The PCR product was visualized on UV Trans illuminator from UVP and images captured using the chemiImager 4400 from the Alpha Innotech Crop., U.S.A. The illuminated bands were cut and used further for DNA sequencing.

Elution of the PCR product

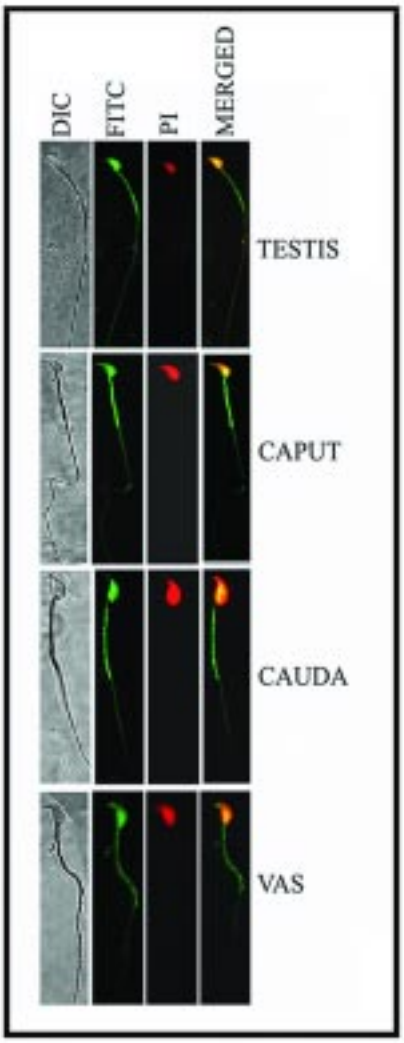
The band of interest was sliced of using sterile blade and eluted using Qia quick gel extraction kit (QIAGEN), according to manufacturer's instruction.

Automated sequencing of DNA

Automated sequencing reaction was performed by using the Big Dye Terminator v3.1 Cycle Sequencing Kit. The cycle sequencing was performed in a PE GeneAMP 9600 PCR instrument by using the conditions in which the initial temperature of 94^{0}C for 2 min., followed by twenty five cycles each of at 94 ^{0}C for 10 sec, annealing at 54 ^{0}C for 10 sec, and final extension at 60 ^{0}C for 4 min. The dye-terminated products were precipitated and were run in an ABI 3700 48 capillary automated DNA sequencer. The sequences were analyzed by using NCBI nucleotide-nucleotide BLAST.



PCR amplification of mouse testis cDNA with different primer combinations



Clustal W alignment

After sequencing, the obtained sequences (both nucleotides and amino acids) were aligned using the bioinformatics software ClustalW (http://www.ebi.ac.uk/clustalw/).

Immunoflourescence staining

Mature mouse spermatozoa were coated on poly L-lysine coated cover slips and processed with cyclin B2 primary antibody (1:100), goat anti-rabbit FITC conjugated secondary antibody (1:200) and finally stained with propidium iodide (1:2000). Processed cover slips were mounted on glass slides, observed and photographed using Leica TCS-SP2 AOBS confocal microscopy system at RGCB, Trivandrum.

Results and Discussion

We have designed primers based on the sequence information of mouse brain cyclin M1, which is available on NCBI gene bank. Primer pairs against the 5'- and 3'-regions of the mouse brain cyclin M1 did not yield any product from mouse testicular cDNA amplification attempts. Then, we attempted to amplify fragments of this gene using four sets of internal primers by dividing cyclin M1 sequence (2818 bp gene, 1761 cds) into 600-700 bp overlapping segments. The four sets of primers were used individually and in combination (Fig. 1).

The PCR products were recovered and were subjected to dye termination chemistry and the products were sequenced on an ABI 3700 automated DNA sequencer. Sequencing results were analyzed using NCBI blast and ClustalW. The analysis aided to generate full length CDS of CLP-1, by manually joining the overlapping sequences. The generated 1835 bp sequence was submitted to NCBI gene bank (Ac. No. DQ885890).

As there was no specific antibody against CLP-1, we used a cross reacting antibody cyclin B2 to localize the expression of this molecule, in spermatozoa of mice collected from testis, different segments of epididymis and vas deferens, adopting immunoflourescence technique (Fig. 2). This study showed the presence of this particular molecule in the head as well as tail regions of spermatozoa.

The full length CDS of CLP-1 of mouse testicular cDNA showed high similarity to mouse brain Cyclin M1. When predicted amino acid sequences of both were analyzed using clustalW, they showed difference by only two amino acids. Further studies will evaluate the role of this molecule in brain and testis.

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