REVIEW

Sperm associated antigen 9 (SPAG9): a new member of c-Jun NH_2-terminal kinase (JNK) interacting protein exclusively expressed in testis

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Abstract. Previously, we cloned and sequenced a novel human sperm associated antigen 9 (SPAG9). Northern blot analysis and RNA *in situ* hybridization experiments revealed testis- and stage-specific expression of SPAG9 mRNA, mainly confined to round spermatid suggesting haploid germ cell expression. Studies on the human and non-human primates (macaque and baboon) have shown a homology of 84.9% and 90.6% at amino acid level and 94% and 96.8% at DNA level, respectively. The presence of high level of homology at amino acid and DNA level indicates that SPAG9 is conserved in human, baboon and macaque sharing common function and common origin in the biological past. In addition, SPAG9 protein revealed structural homology with c-Jun NH_2-terminal kinase (JNK) interacting protein (JIP). The amino acid sequence analysis of SPAG9 predicted coiled coil, leucine zipper and transmembrane domain, speculating the involvement of SPAG9 mediated signal transduction pathways in reproductive processes. (Keio J Med 54 (2): 66–71, June 2005)

Key words: Sperm associated antigen 9 (SPAG9), leucine zipper, JIP, acrosome

Introduction

Mammalian fertilization requires a series of specialized cell-cell interactions that include gamete recognition, adhesion, signaling and fusion. In mammals, the process of sperm-egg fusion is characterized by an increase in intracellular Ca^{2+} and pH[1] and the tyrosine phosphorylation of several proteins.[2,3] While some of these phosphoproteins have been identified on sperm, their role is poorly defined. In somatic cells, induction of tyrosine phosphorylation is associated with activation of down-stream targets including kinases such as MAPK. The MAPK family of serine/threonine kinases occupies a focal point in signal transduction and is involved in the regulation of growth, adhesion, secretion and other physiological events. MAPK signaling pathways are known to relay, amplify and integrate signals from a diverse range of extracellular stimuli. In yeast *Saccharomyces cervisiae*, five MAPK pathways have been described, that regulate mating, sporulation, filamentation, osmoregulation and cell wall biosynthesis.[4–8] Recently, selective activation of physiological processes involving JNK pathways was described in *Xenopus laevis* during oocyte maturation.[9,10]

In mammals, MAPKs can be subdivided into three groups: extracellular signal regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAPK.[11–13] The structural organization of MAPK kinases into specific signaling modules appears to be facilitated by scaffolding proteins such as the JNK interacting protein JIP1, JIP2 and JIP3 in mammalian cells.[14–20] The scaffolding proteins tether various MAPK kinase kinases (MEKKs), MAPK kinases (MKKs) and MAPKs in close proximity so that successive phosphorylation events occur efficiently, thus conferring specificity to a particular combination of kinases. Recently in human sperm, ERK, a member of the MAPK family was shown to be associated with human spermatozoa with direct or indirect function in sperm capacitation.[21]

The mammalian spermatozoon is a terminally differ-

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entiated cell. Its surface is covered by a continuous plasma membrane that is divided into distinct domains in which functional molecules are distributed. The acrosome is an internal organelle located at the anterior head and contains hydrolytic enzymes. The anterior acrosome participates in the acrosome reaction, which is an indispensable event during fertilization. These surface domains and the acrosome are formed during spermiogenesis, during which associated molecules are transported and organized. Many of the molecules thus arranged are functionally immature but gradually become mature during epididymal maturation. Some of them are further altered and redistributed during the fertilization process and play various roles.[22]

Our previous findings have demonstrated an exclusive expression of SPAG9 in haploid round spermatid cells during spermatogenesis in macaque,[23] baboon[24] and human.[25] By homology search in the genetic data base, SPAG9 cDNA was found to be a member of Unigene cluster Hs. 129872 encoded by chromosome 17. Based on structural homology with JIP3, SPAG9 was earlier defined as JIP3γ scaffolding protein[16] and has been recently classified as JIP4 protein.[26] It was found that SPAG9 is structurally distinct from the previously described JIP1[14] and JIP2[20] proteins. Presently we are trying to speculate the role of SPAG9 as a JIP protein and its significance in human sperm-egg interaction.

Domain Structure of SPAG9 Protein

The human *SPAG9* open reading frame (ORF) encodes 766 amino acid residues with a calculated molecular mass of 84 kDa and an isoelectric point (pI) of 4.9. Running the algorithms provided in Simple Modular Architecture Tool on the ExPASy server revealed several features: 1) a characteristic leucine zipper motif (LZ); 2) two coiled-coil domains (coil) and 3) a transmembrane domain (T)[25] (Fig. 1A). There are six putative N-linked glycosylation sites, six putative cAMP-/cGMP-dependent protein kinase phosphorylation sites, six putative protein kinase C phosphorylation sites, eleven putative casein kinase II phosphorylation sites. Deduced amino acid sequence also harboured ten putative myristoylation sites and a characteristic leucine zipper (LZ) motif with six leucine repeats from amino acid 234 to 269. Studies on non-human primates revealed that the macaque *SPAG9* ORF encodes 712 amino acid residues with a calculated molecular mass of 78.19 kDa and an isoelectric point (pI) of 5.44. Further

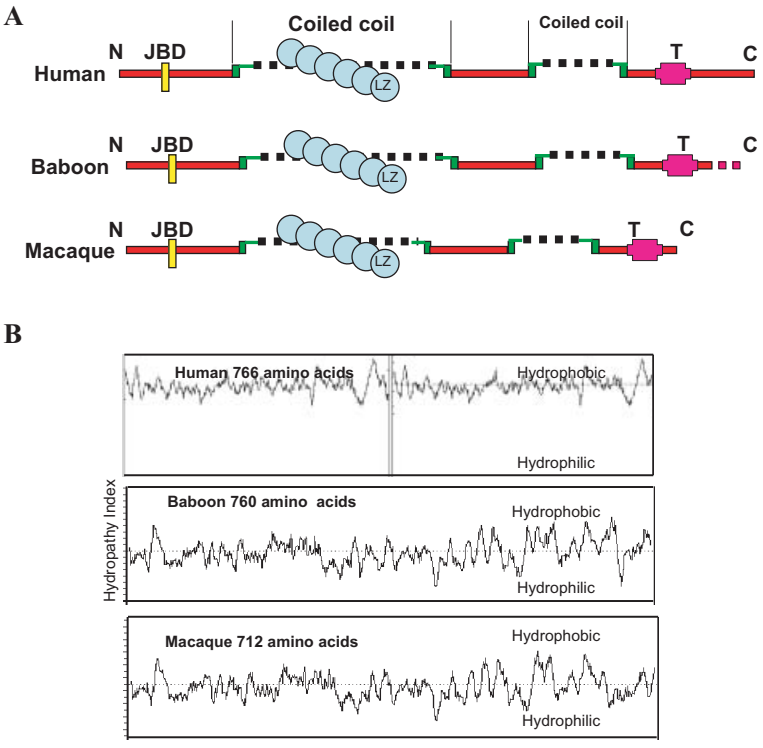

Fig. 1 **(A)** Schematic illustration of the domain structure of the SPAG9. JBD, JNK binding domain; Coil, predicted coiled coil; LZ, leucine zipper; T, predicted transmembrane domain. **(B)** Hydropathy plot of the deduced amino acid sequence of human, baboon, macaque SPAG9 protein.

analysis of macaque SPAG9 protein revealed JNK binding domain, leucine zipper motif, two coiled coil domain and a transmembrane domain (Fig. 1A). In addition, six potential N-linked glycosylation sites, five putative cAMP- and cGMP-dependent protein kinase phosphorylation sites, eleven putative protein kinase C phosphorylation, nine putative casein kinase II phosphorylation sites, one tyrosine sulfation site and ten putative N-myristoylation sites were found at different regions.[23] Similarly, amino acid analysis of baboon SPAG9 also revealed JNK binding domain, leucine zipper motif, two coiled coil domains and a transmembrane domain (Fig. 1A). In addition six potential N-linked glycosylation sites, five putative cAMP- and cGMP-dependent protein kinase phosphorylation sites, seven putative protein kinase C phosphorylation site, eight putative casein kinase II phosphorylation site, one tyrosine kinase phosphorylation and nine putative N-myristoylation sites were found at different regions.[24]

Structural homology analysis with JIP proteins showed presence of signature domains of LZ motif, identified as a structure common to a separate class of DNA binding proteins including nuclear transcription factors.[27] The classical LZ motif of the DNA binding proteins includes leucine repeats with an upstream basic domain called the cluster-spacer-cluster. The LZ motif of the SPAG9 protein does not have any upstream basic domain and thus differs from the classical DNA binding proteins. Literature survey shows membrane associated proteins such as the voltage gated K^+-channels,[28] glucose transporters of vertebrate cells[29] and the fusion (F) glycoprotins of several paramyxoviruses[30] with LZ motifs that are not DNA binding proteins. The function of LZ motifs in SPAG9 may be to aid in dimerization of individual monomers to form a functional dimers for transportation to the cell surface. As SPAG9 is a membrane-associated protein, it is quite likely that LZ motif in SPAG9 is involved in its dimerization and transportation to the sperm surface.

Hydrophobicity

The hydrophobicity plot generated from deduced human SPAG9 protein contained a 658 amino acids extracellular domain, a 20 amino acid transmembrane helical domain and 88 amino acids cytoplasmic domain at the COOH terminus.[25] Three putative antigenic determinant sites were identified based on the three highest points of average hydrophilicity (A_h). As these putative antigenic sites are present within the extracellular domain, it supports the surface localization of SPAG9, which might play a significant role in either sperm egg interaction or could act as a signal transducer from the melieu of the female reproductive tract to the sperm during fertilization. The hydrophobicity plot generated from deduced macaque and baboon SPAG9 revealed a similar pattern to the predicted human SPAG9 protein[23–25] (Fig. 1B). Both the proteins have extracelluar domain, a transmembrane helix and a cytoplasmic domain at the COOH terminus. This analysis suggests that human SPAG9, macaque SPAG9 and baboon SPAG9 proteins have similar over all structures and thus potentially conserved function.

SPAG9 is Evolutionarily Conserved in Human and Non-human Primates

A new era in the elucidation of genome evolution has been heralded with the availability of numerous genome sequences. As more complete genomes are sequenced, conservation of gene order between different organisms is emerging as an informative property of the genomes. Conservation of gene order has been used for predicting function and functional interactions of proteins, as well as for studying the evolutionary relationships between genomes. Conservation of regions also shows which regions are functionally important. The higher the degree of conservation the more likely that a region is involved in the function that is heavily intertwined with many aspects of cellular function or development.

Studies have shown that SPAG9 protein from human and non-human primates (baboon and macaque) shares common N-terminal amino acid sequences which further infer their shared ancestry. Remarkably, our data from macaque, baboon and human showed that SPAG9 protein exhibits conserved amino acid sequences and are highly homologous to each other in the N-terminal region.[23–25] However, C-terminal amino acids sequences of macaque SPAG9 (641–712 aa) is considerably diverged and has only 18.5% homology with hSPAG9 at the (689–766 aa). Similarly, baboon SPAG9 is 90.6% identical to human SPAG9, however, reveals 16.7% homology at the C-terminus of baboon SPAG9 (689–760 aa) when compared to hSPAG9 (689–766 aa) (Fig. 2). C-terminus amino acid difference has also been reported in non-human primate homologue of human SP17.[31] This degree of evolutionary change in SPAG9 is similar to some other proteins for which human and non-human primates sequence have been determined. Of the few sperm proteins that have been sequenced from human and non-human primates, most are identical at the amino acid levels; SP-17 (97%),[31] LDH-C_4 (99.3%),[32] SP-10 (85%).[33] Homology sequence search using SPAG9 also showed homology with PHET (100%),[34] JSAP 1 (JIP 3 – JNK interacting protein 3) (50%),[14] *D. melanogaster* Sunday driver (36%),[35] *C. elegans* Coiled coil protein (30%).[36]

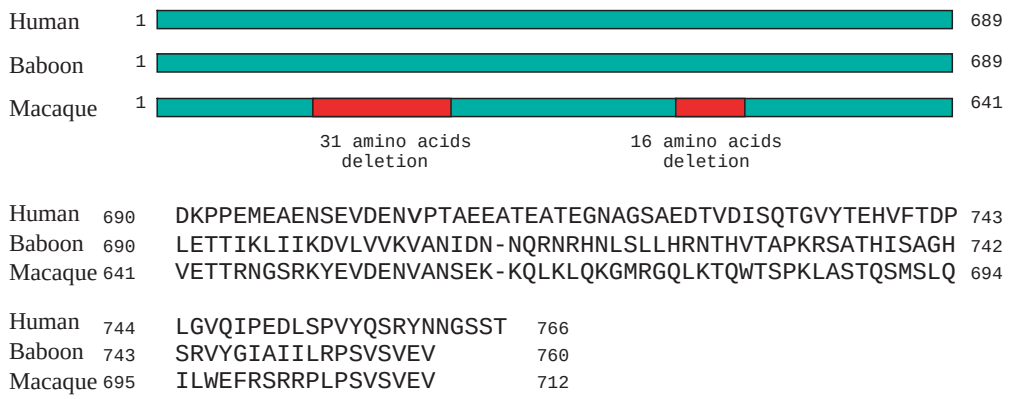

Fig. 2 Amino acids alignment of the human, baboon and macaque SPAG9 protein is shown schematically. The human SPAG9 is 766 amino acids in length and is shown on the top followed by baboon (760 aa) and macaque (712 aa). A comparison of the deduced SPAG9 amino acid sequences of human, baboon and macaque revealed that the protein exhibits a high degree of homology at N-terminal region. Comparing C-terminus amino acids of human SPAG9 (690–766) with baboon (690–760) reveals divergence as shown. Comparing C-terminus amino acids of human SPAG9 (690–766) with macaque (641–712) reveals divergence as shown. Macaque amino acid sequence also revealed inframe deletions of 31 and 16 amino acids.

Structural Homology with c-Jun NH_2-terminal Kinase Interacting Protein

The amino acid sequence analysis of SPAG9 revealed the primary sequence identity to JNK binding domain of JNK interacting protein (JIP). Though many JIP proteins are related in structure and/or function, the genes that encode these proteins are not closely linked in the genome. The human JIP1, and JIP2, which are related in function, are located on human chromosome 11 (11p 11.2-p12, 12 exons) and human chromosome 22 (22q13, 12 exons) respectively,[37,38] whereas human JIP3 gene is located on human chromosome 16 (16p13.3, 30 exons). Sperm specific SPAG9 protein is classified as JIP4,[26] which is structurally related to JIP3. The members of JIP3 group include the JIP3a and JIP3b proteins, which appear to represent the protein products of alternatively spliced transcripts derived from the JIP3 gene. The other JIP3 group of proteins includes JIP3β (accession no. AB11088), which is also structurally related to JIP3.[16,39] Both JIP3 and JIP3β[14] are expressed in the brain, while SPAG9 (JIP4) was shown to be expressed in testis.[23–25,34] Further, it was demonstrated that UNC-16, the *C. elegans* having 30% similarity with SPAG9, interacts with JNK1 and JNKK-1 kinases that have the highest sequence similarity to mammalian JNK3 and MKK7, respectively.[36] Recently, protein highly expressed in testis (PHET), a novel testicular antigen, has been identified and reported to be ectopically over expressed in systemic sclerosis (SSc) dermal fibroblasts. By homology search in the genetic database, PHET cDNA was found to be a member of the UniGene cluster Hs.129872 encoded by chromosome 17q21, but had a unique exon composition and a characteristic mRNA expression profile restricted to the testis. PHET was shown to have 100% homology with SPAG9 and was speculated to be involved in JNK signal pathway.[34]

Localization of SPAG9

Haploid germ cell expression has been a unique feature of the testis specific genes. RNA *in situ* hybridization studies in human, macaque and baboon testis section using antisense SPAG9 riboprobe identified the *SPAG9* transcript on round spermatids of the seminiferous cycle and not on any other cell type.[23–25] The absence of the *SPAG9* transcript in spermatogonia and spermatocytes suggests that the *SPAG9* is transcribed post meiotically in human and non-human primates. In indirect immunofluorescence studies, anti-SPAG9 antibodies revealed a selective acrosomal localization of SPAG9 in macaque[23] and baboon[24] sperm.

Conclusion

In conclusion, testis specific SPAG9 is classified as a new member of JNK interacting protein based on its structural homology with JIP proteins. The amino acid sequence analysis revealed conservation of SPAG9 protein in human and non-human primates predicting functional interactions of proteins, as well as the evolutionary relationships between genomes. Primary sequence analysis of SPAG9 revealed identity to JNK binding domain and predicted coiled coil, leucine zipper and transmembrane domain. Therefore, it is suggested that SPAG9 may function as scaffold protein in JNK cascade. As a JIP family member SPAG9 merits further evaluation as JNK scaffolding protein in upstream of MAPK module, speculating the involvement of SPAG9

mediated signal transduction pathways in reproductive processes.

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