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Compartmentalization of a unique ADP/ATP carrier protein SFEC (Sperm Flagellar Energy Carrier, AAC4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece

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Abstract

The longest part of the sperm flagellum, the principal piece, contains the fibrous sheath, a cytoskeletal element unique to spermiogenesis. We performed mass spectrometry proteomics on isolated human fibrous sheaths identifying a unique ADP/ATP carrier protein, SFEC [AAC4], seven glycolytic enzymes previously unreported in the human sperm fibrous sheath, and sorbitol dehydrogenase. SFEC, pyruvate kinase and aldolase were co-localized by immunofluorescence to the principal piece. A homology model constructed for SFEC predicted unique residues at the entrance to the nucleotide binding pocket of SFEC that are absent in other human ADP/ATP carriers, suggesting opportunities for selective drug targeting. This study provides the first evidence of a role for an ADP/ATP carrier family member in glycolysis. The co-localization of SFEC and glycolytic enzymes in the fibrous sheath supports a growing literature that the principal piece of the flagellum is capable of generating and regulating ATP independently from mitochondrial oxidation in the mid-piece. A model is proposed that the fibrous sheath represents a highly ordered complex, analogous to the electron transport chain, in which adjacent enzymes in the glycolytic pathway are assembled to permit efficient flux of energy substrates and products with SFEC serving to mediate energy generating and energy consuming processes in the distal flagellum, possibly as a nucleotide shuttle between flagellar glycolysis, protein phosphorylation and mechanisms of motility.

Keywords: Fibrous sheath; Glycolytic enzymes; Sperm flagellar energy carrier; ADP/ATP carrier; Sperm glycolysis; Principal piece; Energy production; Energy translocation; Sperm motility

Introduction

Mitochondrial ADP/ATP carriers (AACs a.k.a. adenine nucleotide translocases, ANTs) function as antiporters that exchange cytosolic ADP for matrix ATP in mitochondria (Klingenberg, 1981). These proteins, typically contain six membrane spanning domains that span the inner mitochondrial membrane and exchange ADP for ATP in a 1:1 ratio (Duyckaerts et al., 1980). The genomes of most eukaryotes, including yeast, plants and mammals, contain multiple genes encoding ATP/ADP carriers. These proteins have developed several nomenclatures and abbreviations, the most common being AAC (ADP/ATP carrier) or ANT (adenine nucleotide translocase). The AACs are considered the principal link between the energy generating process of oxidative phosphorylation and energy consuming processes of cell metabolism.

In humans, four AAC genes are now known. Patterns of AAC1-3 expression have been noted to vary in different stages of cell division, in cancers, and in cells exposed to various growth conditions and inhibitors. Among normal tissues, AAC1 is thought to be specific to heart and skeletal muscle (Stepien et al., 1992) and AAC1 deficiency has been related to mitochondrial myopathy and cardiomyopathy (Palmieri et al., 2005; Graham et al., 1997). AAC2 is present in proliferating cells, while AAC3 is ubiquitous (Stepien et al., 1992). AAC4 was

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only recently identified through a genome scan and shown to function as an active ADP/ATP carrier in the C^{14}ADP/ATP liposome assay and to catalyze an electrophoretic exchange between ADP^{3-} and ATP-$^{4-}$ (Dolce et al., 2005). GFP-fused AAC4 co-localized to mitochondria in CHO cells leading to the conclusion that AAC4 has properties of a classical mitochondrial adenine nucleotide translocase (Dolce et al., 2005).

The fibrous sheath, a unique cytoskeletal structure specific to the sperm, is located only in the principal piece, a region devoid of mitochondria. The FS has been proposed to function as a protective girdle for the axoneme (Fawcett, 1975; Lindemann et al., 1992) and as a scaffold for enzymes involved in signal transduction, including protein kinase A by anchoring to AKAP3 (Vijayaraghavan et al., 1999; Mandal et al., 1999) or AKAP4 (Fulcher et al., 1995, Turner et al., 1998), the Rho signaling pathway through ropporin (Fujita et al., 2000) and rhophilin (Nakamura et al., 1999), as well as calcium signaling via CABYR (Naaby-Hansen et al., 2002; Kim et al., 2005). Previously, two glycolytic enzymes, glyceraldehyde 3-phosphate dehydrogenase-2 (GAPDH-2, Westhoff and Kamp, 1997; Welch et al., 2000) and hexokinase 1 (HK1, Travis et al., 1998; Mori et al., 1998) have been localized to the human fibrous sheath. Recently, the A isoform of aldolase 1 (ALDOA) and lactate dehydrogenase A (LDHA) have been identified in isolated mouse fibrous sheath (Krisfalusi et al., 2006). Such observations led us posit whether glycolysis and signal transduction indeed occur in the distal flagella of human sperm and if evidence for additional enzymes within these pathways as well as energy intermediates might be found in the human fibrous sheath.

The present study provides biochemical and morphological evidence that AAC4 (SFEC) is present in ejaculated human sperm where it associates with the principal piece of the flagellar cytoskeleton and with glycolytic enzymes. The study has been particularly aided by the well-recognized ultrastructural compartmentalization in the sperm flagellum which consists of mid, principal and end pieces. Each of these regions contains specific organelles and cytoskeletal elements. Mitochondria are restricted to the mid-piece. The principal piece contains the unique circumferential ribs and longitudinal columns of the fibrous sheath. These ribs and columns surround the 9+2 array of axonemal microtubules. The end piece contains microtubules, although they no longer form paired "doublets" in this region. At the core of the axoneme, where ATP is utilized to generate flagellar motion, dynein ATPases associate with outer doublet microtubules and span both mid- and principal pieces. We purified the human fibrous sheath by mechanical and chemical dissection, and utilized mass spectrometric analysis to identify a novel member of the adenine nucleotide translocase family, SFEC, in association with several glycolytic enzymes, previously unreported in the human sperm principal piece. Localization of SFEC (AAC4) with pyruvate kinase and aldolase in the principle piece of the flagellum confirms a growing literature that glycolysis is compartmentalized within the fibrous sheath, the unique cytoskeletal element of the principal piece, and suggests a role for SFEC as an intermediate between flagellar glycolysis and energy consuming processes such as phosphorylation and motility.

Materials and methods

Isolation of human FS

The fibrous sheath was isolated by a multi-step mechanical and chemical sperm dissection procedure (modified from the previous report by Kim et al., 1997). Isolated human sperm tails were extracted for 30 min in 2% (v/v) Triton X-100 and 5 mM DTT with gentle shaking at 4°C. After washing with 50 mM Tris–HCl (pH 9.0) containing 0.2 mM PMSF, the sperm tails were suspended in 25 mM DTT and 4.5 M urea and shaken for 2 h at 4°C. Each step of the procedure was monitored by light and electron microscopy. The purity of the isolated fibrous sheath was confirmed by transmission electron microscopy.

Tandem mass spectroscopic analysis of fibrous sheath

The Coomassie-stained protein bands of the fibrous sheath were cored from 1D SDS-PAGE gels, fragmented into smaller pieces, destained in methanol, reduced in 10 mM dithiothreitol, and alkylated in 50 mM iodoacetamide in 0.1 M ammonium bicarbonate. The gel pieces were then incubated with 12.5 ng/ml trypsin in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were extracted from the gel pieces in 50% acetonitrile and 5% formic acid and microsequenced by tandem mass spectrometry and by Edman degradation at the Biomolecular Research Facility of the University of Virginia.

Northern and dot blot analyses

A human multiple tissue Northern blot containing 2 μg of poly $(A)^+$ RNA from eight selected tissues and a normalized RNA dot blot containing 76 tissues (Clonetech) were probed with a α-^{32}P-labeled 963-bp cDNA containing the entire open reading frame of SFEC. Probes were prepared by random priming with a DNA labeling kit (Roche, Penzberg, Germany). For the Northern and dot blot analysis, hybridization was performed as previously described (Naaby-Hansen et al., 2002). The blot was exposed to X-ray film for 72 h at −70°C.

Expression, purification of SFEC recombinant protein

A truncated construct of human SFEC (aa 4–120) was expressed in bacteria in order to raise a polyclonal antibody. Previous efforts to express the entire SFEC open reading frame were not successful in bacteria presumably because of the existence of putative transmembrane domains in the C-terminus. Gene-specific primers were designed to create an *Nco*1 site at the 5′ end and a *Not*1 site at the 3′ end of the polymerase chain reaction (PCR) product according to the human SFEC cDNA sequences. Primers (Forward primer: 5′-CATGCCATGGAGCCTGCGAAAAA-GAAGGCAGAAAAG-3′: Reverse primer: 5′-ATAGTTTAGCGGCCGCCTGTT-TTTCTTTATTAACTCCAGA-3′) were obtained from GIBCO BRL (Life Technologies, CA). PCR was performed with 10 ng of human SFEC cDNAs as a template to obtain the truncated SFEC cDNA using a program of one 2-min cycle at 94°C followed by 35 cycles of denaturation, annealing and elongation at 94°C for 30 s, 50°C for 1 min and 68°C for 2 min. A product of 351 bp, which begins at bp 129 and ends at bp 479 of the human SFEC nucleotide sequence, were separated on a 1% NuSieve (FMC BioProducts, Rockland, ME) agarose gel and sequenced in both direction using vector-derived and insert-specific primers to confirm the sequences. The cDNA corresponding to the N-terminal 117 amino acids was cloned into the bacterial expression vector pET28b and transformed into *Escherichia coli* strain BLR (DE3) (Novagen, Madison, WI). A single colony was picked from a transformation plate to inoculate 2 l of LB medium containing 50 μg/ml of kanamycin and grown at 37°C until the A_{600} reached 0.5. Recombinant protein expression was induced at 37°C for 3 h with 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). The cells were centrifuged at 5000×*g* for 15 min and suspended in BugBuster Protein Extraction reagent (Novagen, Madison, WI) containing rLysozyme (1KU/ml) and Benzonase (25 U/ml) for the gentle disruption of the cell wall and degradation of DNA and RNA of the *E. coli*. Recombinant SFEC