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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.

Introduction

Mitochondrial ADP/ATP carriers (AAC's 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

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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 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 catalyse an electrophoretic exchange between ADP^{3-} and $ATP\text{-}^{4-}$ (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 5mM DTT with gentle shaking at 4° C. After washing with 50mM Tris-HCl (pH 9.0) containing 0.2mM PMSF, the sperm tails were suspended in 25mM DTT and 4.5M urea and shaken for 2 hours 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 hours 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′-CATGCCATGGAGCCTGCGAAAAAGAAGGCAGAAAAG-3′: Reverse primer: 5′-ATAGTTTAGCGGCCGCCTGTTTTTCTTTATTAACTCCAGA-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 second, 50 °C for 1 min and 68 °C for 2 min. A product of 351 bp, which begins at bp129 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 liters of LB medium containing 50μg/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 hour with 1mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). The cells were centrifuged at 5,000 g for 15 min and suspended in BugBuster Protein Extraction reagent (Novagen, Madison, WI) containing rLysozyme (1KU/

ml) and Benzonase (25 units/ml) for the gentle disruption of the cell wall and degradation of DNA and RNA of the E.coli. Recombinant SFEC containing six residues of histidine on the C-terminus of the protein was confirmed using anti-histidine antibody. The recombinant SFEC protein was purified as previously described (Kim et al., 2005). The purity of the isolated recombinant protein was confirmed by Coomassie and SYPRO Ruby stain (Bio-Rad).

Generation of anti-SFEC antibody

Approximately 100 μg of purified recSFEC protein in PBS emulsified with an equal volume of Freund's complete adjuvant was subcutaneously and intramuscularly injected into each female Sprague Dawley rat. Animals were boosted two times at intervals of 21 days with 50 μg of recombinant protein in incomplete Freund's adjuvant and serum was collected 7 days after the second boost. Rats were sacrificed after confirmation of antibody production by Western blot analysis of the recombinant SFEC, human sperm and isolated fibrous sheath proteins.

Electrophoresis and Western analyses

The reaction of SFEC antibody was tested by Western blots on recombinant SFEC, human sperm, isolated FS proteins and mitochondrial proteins isolated from human heart. After isolation of a motile fraction by the swim up procedure, sperm proteins were extracted at 4°C for 2 hours by 1% Nonidet P40 and 0.5% sodium deoxycholate containing protease inhibitors: 2mM PMSF, 5mM iodoacetamide, 5mM EDTA, 3 mg/ml L-1-chlor-3-(4-tosylamido)-7-amino-2-heptanon-hydrochloride, 1.46 mM pepstatin A, and 2.1 mM leupeptin. Insoluble material was removed by centrifugation at 10,000 x g for 5 min, and the supernatant containing solubilized human sperm protein was applied to one dimensional SDS PAGE. Isolated human fibrous sheath and human mitochondrial protein (obtained from Molecular Probes) were solubilized with sample buffer for one dimensional SDS-PAGE.

The proteins resolved by one dimensional SDS-PAGE (4–20% gradient gel) were transferred onto nitrocellulose membrane and detected by the anti-SFEC antibody. The excess protein-binding sites on the membrane were blocked with PBS containing 5% (w/v) non-fat milk powder and 0.2 % (w/v) Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany) for 1 h. The membrane was probed overnight at 4 °C with a rat polyclonal antiserum raised against SFEC protein. Anti-SFEC antibody was diluted 1:2000 with blocking solution. Preimmue sera were used at an identical dilution for control experiments. The membrane was then incubated for 45 min with an anti rat immunoglobulin IgG-secondary antibodies linked to horseradish peroxidase (Jackson ImmunoResearch lab., West Grove, PA. USA), diluted 1:5000 in blocking solution. The blot was developed with a chemiluminescent substrate (Pierce, Rockford, IL) or 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Kirkegaard and Perry Lab, Gaithersburg, MD. USA).

Indirect immunofluorescence localization of SFEC, pyruvate kinase and aldolase on human sperm

Swim-up human sperm were labeled with 25nM Mito-tracker Red CMX Ros (Molecular Probe, Eugene, OR) for 10 min at room temperature. Sperm were washed with PBS two times, diluted to a concentration of 1 x 10^6 sperm/ml, and then spotted on glass slides. Before air-drying, the sperm were fixed with 4% formaldehyde for 10 min at room temperature. After washing 3 times in PBS, the samples were permeablized with cold methanol for 15 min, then blocked in 10% normal goat serum in PBS overnight at 4°C. The sperm were then incubated with a 1:50 dilution of the rat anti recombinant human SFEC antibody or pre-immune serum in blocking solution for 2h at room temperature. For an additional control, the primary antibody was pre-absorbed with SFEC recombinant proteins. The slides were then washed 3 times x 5 min in PBS, and the secondary antibody, goat anti-rat IgG FITC conjugated (Jackson

ImmunoResearch), was applied at a 1:200 dilution in 10% normal goat serum in PBS for 1 hour at room temperature. The slides were washed 3x5 min in PBS, and a Slow Fade-Light Antifade Kit (Molecular Probes, Inc.) was used to reduce the fading rate of the fluorescein. Indirect immunofluorescence microscopy using goat anti-pyruvate kinase and anti-aldolase antibodies (Novus Biologicals, Inc. Littleton, CO) were performed as described above except using different blocking serum and secondary antibody. Sperm were blocked by 10% normal donkey serum in PBS and donkey anti-goat IgG FITC conjugated secondary antibody (Jackson ImmunoResearch) was used.

Results

Isolation of Human Fibrous Sheath Ribs and Columns

Fibrous sheaths were isolated by mechanical and chemical methods (modified from a previous report by Kim et al., 1997). Each step of the isolation was monitored by light and electron microscopy. The purity of the human fibrous sheath preparation was verified by electron microscopy after studying multiple sections and fields. This final fibrous sheath fraction consisted of longitudinal columns and ribs without other contaminating sperm tail components such as the axonemal complex, outer dense fibers or mitochondrial sheaths (Figure 1A). Although these fine structural observations confirm the structural homogeneity of the preparation with respect to fibrous sheath elements, the possible co-purification of molecules from other cytoplasmic compartments can not be excluded by fine structural assessment alone.

Microsequences of glycolytic enzymes in the isolated fibrous sheath

Protein extracts of the purified fibrous sheath fraction were resolved by one dimensional SDS-PAGE, revealing at least 17 Coomassie protein bands ranging from 15.5 to 140 kDa (Figure 1B). Each protein band was assigned a nomenclature C253–C269, and subsequently cored and microsequenced by tandem mass spectrometry. Analysis of peptide masses derived from isolated human fibrous sheaths confirmed the presence of GAPDH-2, one of two previously reported glycolytic enzymes in the human fibrous sheath (Welch et al., 2000), while another enzyme, hexokinase (HK1, Travis et al. 1988), was not identified in the isolated fibrous sheath preparation perhaps indicating that the HK1 was not bound to the fibrous sheath as tightly as GAPDH2 (Travis et al., 2001). Importantly, seven glycolytic enzymes, previously unreported as components of the human fibrous sheath, were identified in the preparation. These (Table 1) were isoforms of aldolase A, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate kinase known to be present in somatic tissues; the testis specific isoform of lactate dehydrogenase, LDH C, as well as the somatic isoform LDH A; and sorbitol dehydrogenase, from the polyol pathway. Table 2 summarizes each peptide obtained by mass spectrometry microsequencing the human fibrous sheaths and summarizes the percent of the predicted amino acid sequence of each glycolytic enzyme covered by the microsequences.

SFEC (AAC4) was identified in the purified fibrous sheath

In addition to the identification of glycolytic enzymes within the purified fibrous sheaths a hypothetical protein, ***DKFZp434N1235***, was sequenced from band C265 and identified as a member of the ADP/ATP carrier family (AAC). This previously unannotated protein was cloned from human testis cDNA and the amino acid and nucleotide sequences deposited in GenBank under the name SFEC (Genbank accession no, ***AY550240***), sperm flagella energy carrier [Note on nomenclature: the SFEC sequence was deposited by the authors in GenBank on February 17, 2004, whereas the identical protein was deposited as AAC4 on November 15, 2004; By priority date, SFEC terminology takes precedence. However, for purposes of standard nomenclature we suggest the term AAC4 be used for this protein when referring to it in somatic tissues and the term SFEC be employed when referring to sperm].

Human SFEC contained three repeating motifs (Figure 3) as well as the PX(D/E)XX(K/R) sequence, both characteristics of all known mitochondrial carriers (Walker and Runswick, 1993;Nelson et al., 1998) as well as the hexapeptide signature RRRMMM **(**Figure 2) which is present in all ADP/ATP carriers but absent from other mitochondrial carriers (Pebay-Peyroula et al., 2003). The orthologous murine gene (RIKEN cDNA 1700034J06) was also identified (Fig. 4) and deposited in GenBank as mSFEC (GenBank accession no, ***AY550241***). Human and mouse SFEC protein sequences share 83% identity and 89% similarity (Figure 4). Comparison with other human AACs (Figure 4) revealed the highest homology (69% identity and 79% similarity) with ADP/ATP carrier 1, (AAC1, ANT1), from human heart/skeletal muscle (Cozens et al., 1989) and a 67% identity and 80% similarity to AAC3 (ANT3) of human liver (Cozens et al., 1989). It also revealed a 67 % identity and 79 % similarity with a human fibroblast isoform, AAC2 (Ku et al., 1990). The SFEC protein was localized to human chromosome 4q28.1, whereas the other known ADP/ATP carrier proteins localize to chromosome 4q35.1 (AAC1, Haraguchi et al., 1993); chromosome Xp22.32 and Yp (AAC3, Slim et al., 1993;Schiebel et al., 1993), and Xq24 (AAC3, Schiebel et al., 1993), respectively. The mouse SFEC protein localized to chromosome 3B, which is syntenic to locus 4q28.2 in the human (Dehal et al., 2001). The mouse AAC1 and AAC2 were localized to chromosome 8 and chromosome X, respectively (Ellison et al., 1996). However, there is presently no known mouse homologue for the human ANT 3 (Levy et al., 2000).

The characteristic feature of SFEC in both mouse and human is the presence of unique N and C-termini domains (Figure 4). The conservation of these N and C extensions in these two species and their absence in AAC1-3 of both species indicates that SFEC is a novel member of the family of ADP/ATP carrier proteins. The thirteen amino acid N terminus in both mouse and human does not contain any known motifs but may be important in the fibrous sheath localization of this molecule in the sperm flagellum (see below).

SFEC (AAC4) is a testis abundant protein

Gene expression of SFEC on the Northern analysis and dot array using a 32P-labeled cDNA of the full length open reading frame (315 amino acids) demonstrated that the SFEC mRNA transcripts were expressed in the testis but not in spleen, thymus, prostate, small intestine, colon, leukocytes or ovary (Figure 5). Recombinant SFEC was expressed and purified (Figure 6A). Generation of an antibody against truncated SFEC (amino acid 4–120) was performed in rats. Western blot analysis demonstrated that the anti-SFEC antibody recognized the recombinant protein as well as a 32 kDa band in the isolated FS fraction and in human sperm protein extracts. This is the same molecular weight as the FS band originally microsequenced (Figure 6B). However, the anti-SFEC antibody did not recognize human heart mitochondrial proteins (Figure 6B) while anti-ANT1 antibody detected a 32 kDa ANT1 band (Figure 6C). No band was observed in the negative control experiment in which the anti-ANT1 antibody was replaced by normal goat serum (Figure 6C).

AAC4 (SFEC) localizes to the principal piece of the sperm flagellum

Indirect immunofluorescence localized SFEC to the principal piece of the sperm flagellum in 100% of sperm (Figure 7). Interestingly, in addition to the principal piece, the entire (21.5%, 83/386) or part of the mid piece (9.8%, 38/386) were also stained by anti-recombinant SFEC antibody. The restricted principal piece localization of SFEC in 69% of sperm differed from the known mitochondrial association of ANT family members. No staining was observed in the negative control experiment in which the anti-SFEC antibody was replaced by either pre-immune sera or immune sera pre-absorbed with recombinant SFEC protein (Figure 7).

Pyruvate kinase and aldolase localize to the human sperm principal piece

The glycolytic enzymes, aldolase (Figure 8) and pyruvate kinase (Figure 9), localized to the principal piece of the sperm flagella. No staining was found in the negative control experiment in which the anti-aldolase or pyruvate kinase antibodies were replaced by normal goat serum.

Prediction of SFEC structure

Protein Data Base crystal structure entry 1okc (Pebay-Peyroula et al., 2003) is a bovine mitochondrial ADP/ATP carrier in complex with carboxyatractyloside, an ANT inhibitor. The sequence identity of 1okc to SFEC and to other ANTs ranges from 56–90%, making 1okc suitable for homology modeling. The high conservation of sequences in the ATP/ADP binding pocket among SFEC, 1okc and other human ANTs predicts an identical ATP/ADP pocket in SFEC and other human ANTs (Figure 10). A substitution of tryptophane for tyrosine-124 in the entrance or “ladder” to the ATP cleft is a marked difference between human SFEC and the other three human ANTs (Figure 10).

Discussion

SFEC (AAC4) localized to the flagellar principal piece

Indirect immunofluorescence revealed that SFEC, a unique ADP/ATP carrier protein characterized by extensions of N- and C- termini, localized solely to the flagellar principal piece in 69% of human sperm. These immuno-localization studies were performed more than 10 times using Mito-tracker labeling to determine the precise distal boundary of the mid-piece mitochondrial sheath. SFEC’s localization solely to the principal piece in the majority of the sperm flagella was confirmed in all cases. Together with the recovery of SFEC microsequences in the isolated preparation of human fibrous sheath ribs and columns, this evidence establishes that this member of the ADP/ATP carrier protein family, whose other members are found associated with the inner mitochondrial membrane, is located in the non-mitochondrial region of the human sperm flagellum.

This unique localization of SFEC in the human sperm flagellar cytoskeleton was not anticipated in the previous report of Dolce et al., (2005) who showed that ACC4 (SFEC) functions in the C^{14}ADP/ATP liposome assay as an active ADP/ATP carrier to catalyze an electrophoretic exchange between ADP^{3-} and ATP-$^{4-}$. By real time PCR AAC4 was identified only in human liver, brain and testis (Dolce et al., 2005). Dolce and associates also found that 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. It is possible that AAC4 functions in brain and liver as a mitochondrial protein, since it does possess a functionally active ADP/ATP binding domain that is nearly identical to ACC1-3 as well as six transmembrane domains. The non-mitochondrial, cytoskeletal location of SFEC in the sperm fibrous sheath may be due to a specific interaction mediated by the unique N and C terminal regions. The previous non-mitochondrial localization of a yeast AAC protein family member, peroxisomal adenine nucleotide transporter (Ant1p), in the peroxisomal membrane (Palmieri et al., 2001; Lasorsa et al., 2004) suggested a possible role in transporting cytosolic ATP into the peroxisomal lumen in exchange for AMP generated in the oxidation of fatty acids (Palmieri et al 2001).

SFEC Co-localizes with Glycolytic Enzymes

Studies since the 1960s have demonstrated that glycolytic enzymes cofractionate with an insoluble sperm component (Mohri et al, 1965; Storey and Kayne, 1975, 1978; Gillis and Tamblyn, 1984). The identification of glycolytic enzymes, including the A isoform of aldolase 1 (ALDOA) and LDHA, in isolated mouse fibrous sheath (Krisfalusi et al, 2006) coupled with

the identification of glycolytic enzymes not previously reported in purified human fibrous sheaths (Table 1 and 2, this study) support the concept that this unique cytoskeletal element in the principal piece of the mammalian sperm flagellum has evolved as a site for glycolysis. These observations extend the earlier localizations of hexokinase (Travis et al., 1998, Mori et al., 1998; Travis et al., 2001) and GAPHD-2 (Westhoff and Kamp, 1997; Welch et al., 2000) in the fibrous sheath of the sperm principal piece as well as demonstrations that glycolysis occurs in the principal piece compartment, separate from oxidative phosphorylation (Miki et al, 2004; Mukai and Okuno, 2004). Recently, a proteomic analysis of mouse sperm flagella identified glycolytic enzymes including aldolase 1, triosephosphate isomerase, phosphoglycerate kinase 2 and glycerol 3-phosphate dehydrogenase (Cao et al., 2006). However, in that study there was no determination of subcellular localization and the protein fraction examined by mass spectrometry contained all sperm tail components such as mitochondria, outer dense fibers, axoneme and fibrous sheath due to technical difficulties in the isolation of each component from mouse sperm (Cao et al., 2006). The present findings confirm in the human that the fibrous sheath is a compartment for glycolysis perhaps serving as a “scaffold” to which glycolytic enzymes bind and detach or as a stable multi-protein complex. This concept that glycolysis is organized in the mammalian sperm flagellum parallels recent studies that identified enzymatic activities for phosphoglycerate mutase (PGM), enolase and pyruvate kinase (PK) in isolated *Chlamydomonas* flagellum (Mitchell et al., 2005), while another proteomic study of *Chlamydomona* flagella identified these glycolytic enzymes as well as aldolase, GAPDH, and phosphoglycerate kinase (Pazour et al., 2005). These findings have led to the conclusion that ATP synthesis throughout the *Chlamydomonas* flagellar compartment is essential for motility.

Functionally related enzymes in the glycolytic pathway may be spatially juxtaposed in the fibrous sheath to increase the efficiency of flux of metabolites through the pathway, a process traditionally referred to a channeling. In *Drosophila* flight muscles glycolytic enzymes that catalyze consecutive reactions along the glycolytic pathway have been co-localized along sarcomeres at M-lines and Z discs and this co-localization is required for normal flight (Wojtas et al., 1997; Sullivan et al., 2003). In flight muscles aldolase, glycerol-3-phosphate dehydrogenase (GPDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase, phosphoglycerate kinase and phosphoglycerol mutase (PGLYM) have an identical pattern of co-localization. In mutants null for GPDH, the other enzymes do not co-localize, indicating interdependency of localization (Sullivan et al., 2003). Glycolytic enzymes identified in the fibrous sheath of the sperm flagellum and their association with the sperm’s cytoskeleton provides a readily accessible model in which to further study enzyme-enzyme interactions in these pathways.

Evidence of Polyol and Lactate pathways in the fibrous sheath suggest several anaerobic pathways converge in the distal flagellum

The present finding of sorbitol dehydrogenase (SDH) in the isolated fibrous sheath suggests that the principal piece of the sperm flagellum may utilize sorbitol originating in the seminal plasma (O’Shea and Wales, 1965; Murdoch and White, 1968; Frenkel et al., 1975) and in female reproductive tract secretions (Casslen and Nilsson, 1984) as an energy source for sperm motility. SDH lies within the polyol metabolic pathway in which glucose is reduced first to sorbitol by aldose reductase, and the resulting sorbitol is subsequently oxidixed by SDH to fructose (Hers, 1956; King and Mann, 1958). The polyol pathway has been reported in the testis (Kobayashi *et al*., 2002) and glucose, fructose and sorbitol are known energy sources for sperm metabolism (Frenkel et al., 1975; Leese et al., 1981). The glucose transporter isoform GLUT5 has been shown to function also as a fructose transporter and to be highly expressed in human testis and localized to the plasma membrane of mature spermatozoa including the entire sperm flagellum (Burant et al., 1992).

Under aerobic conditions, glycolysis is a prelude to the citric acid cycle of mitochondrial respiration. However, if the oxygen concentration is not sufficient, as in rapidly contracting muscle, pyruvate is converted into lactate, or ethanol, as in yeast under anaerobic conditions (alcoholic fermentation), with only a small net energy yield from glucose as compared with the yield from oxidative phosphorylation. The finding of both LDHA and LDHC in the isolated fibrous sheath indicates that pyruvate is further catabolized by LDHA or/and LDHC to form lactate generating nicotinamide adenine dinuleotide (NAD^+) when the amount of oxygen is limited in the distal sperm flagellum. Identification of both LDHA (somatic form) and LDHC (testis specific form) as well as GAPDH-2 (testis specific form) and GAPDH (somatic form) in the fibrous sheath support the concept that both somatic and testis specific glycolytic isoforms are compartmentalized in the principal piece of the flagellum.

The mitochondria, where ATP generation occurs from oxidative phosphorylation, are localized solely in the sperm mid-piece. Yet the flagellum extends another 45 μm or so beyond the annulus at the distal end of the mid piece in human sperm. The localization within the fibrous sheath of the enzymes of glycolysis, the anaerobic LDH pathway wherein pyruvate is converted to lactate and the polyol pathway leads to the conclusion that the principal piece provides for convergent pathways of anaerobic ATP production along the flagellum beyond the mid-piece.

SFEC's role in the principal piece of the sperm flagellum

SFEC (ACC4) has been shown to be an active adenine nucleotide carrier (Dolce et al., 2005). This is in accord with the computer model for SFEC (Fig. 10) that predicts identical conformations of the ATP/ADP binding pocket among SFEC and the other three human AACs. It is not yet apparent if SFEC functions in the distal flagellum as an ATP reservoir, an ATP carrier, and/or contains sites of protein-protein interaction with specific enzymes in the glycolytic pathway. One prediction from our model that enzymes in the fibrous sheath glycolytic pathway will be spatially linked posits that SFEC may directly interact with those specific enzymes, eg. phosphoglycerate kinase and pyruvate kinase, that catalyse ATP yielding reactions. The homology model for SFEC (Fig 10) predicts unique residues at the entrance to the nucleotide binding pocket of SFEC that are absent in other human ADP/ATP carriers, suggesting contraceptive opportunities for selective drug targeting.

The presence of glycolytic pathway enzymes together with a unique adenine nucleotide translocase SFEC in the fibrous sheath suggest new possibilities for energy production and translocation mechanisms in the principal piece of the sperm flagellum related to motility and capacitation. Protein phosphorylation is essential for capacitation (Visconti and Kopf, 1998) and the presence of local ATP generating mechanisms in proximity to ATP utilization in the capacitation dependent phosphorylation pathways suggest possible pathways and protein-protein interactions for SFEC. The reliance of the sperm on glycolysis as the principal source of ATP for motility (Miki et al, 2004; Mukai and Okuno, 2004) suggests that the sperm has evolved mechanisms for adapting to low oxygen tensions, a feature likely to occur during transport through varying regions of the tortuous female reproductive tract. This thought is in concert with the presence of a unique soluble adenylate cyclase in the sperm, sAC, which is bicarbonate sensitive (Chen et al., 2000). sAC shows higher homology to cyclases of cyanobacteria than to somatic cyclases raising the possibility that it represents an "ancestral cyclase" retained in the sperm. By analogy, SFEC, with its unique N and C terminus extensions and its expression in the sperm, is hypothesized to be an ancestral isoform of the ACC family leading to the prediction that ACCDs which associate with the cytoskeleton and with glycolytic enzyme complexes remain to be found in other organisms and model systems.

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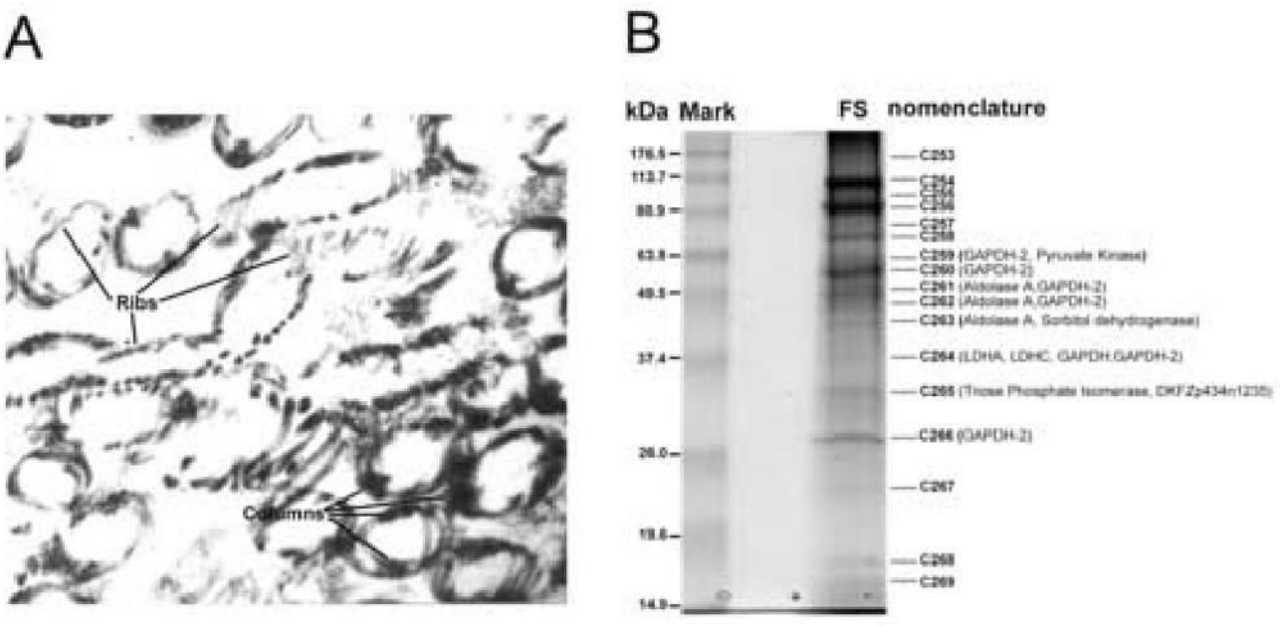

Figure 1.

A, Transmission electron micrograph of the longitudinal columns (C) and transverse ribs (R) of isolated human sperm fibrous sheath confirming the purity of the isolated preparation. No other sperm tail components including the outer dense fibers, axoneme and mitochondrial sheaths were observed. Original magnification, x 30,000 **B**, SDS-PAGE(12.5%) of the human fibrous sheath proteins revealing at least 17 Coomassie blue stain bands. A nomenclature of C253 to C269 was established for each band before coring. Peptides for seven glycolytic enzymes, including somatic and testis specific forms, were recovered including: GAPDH-2, GAPDH, LDHA, LDHC, aldolase A, triosephosphate isomerase, pyruvate kinase as well as sorbitol dehydrogenase. C265 contained peptides for ***DKFZp434N1235***, identified as a unique member of the ADP/ATP carrier protein family (AAC), also known as adenine nucleotide translocases (ANT).

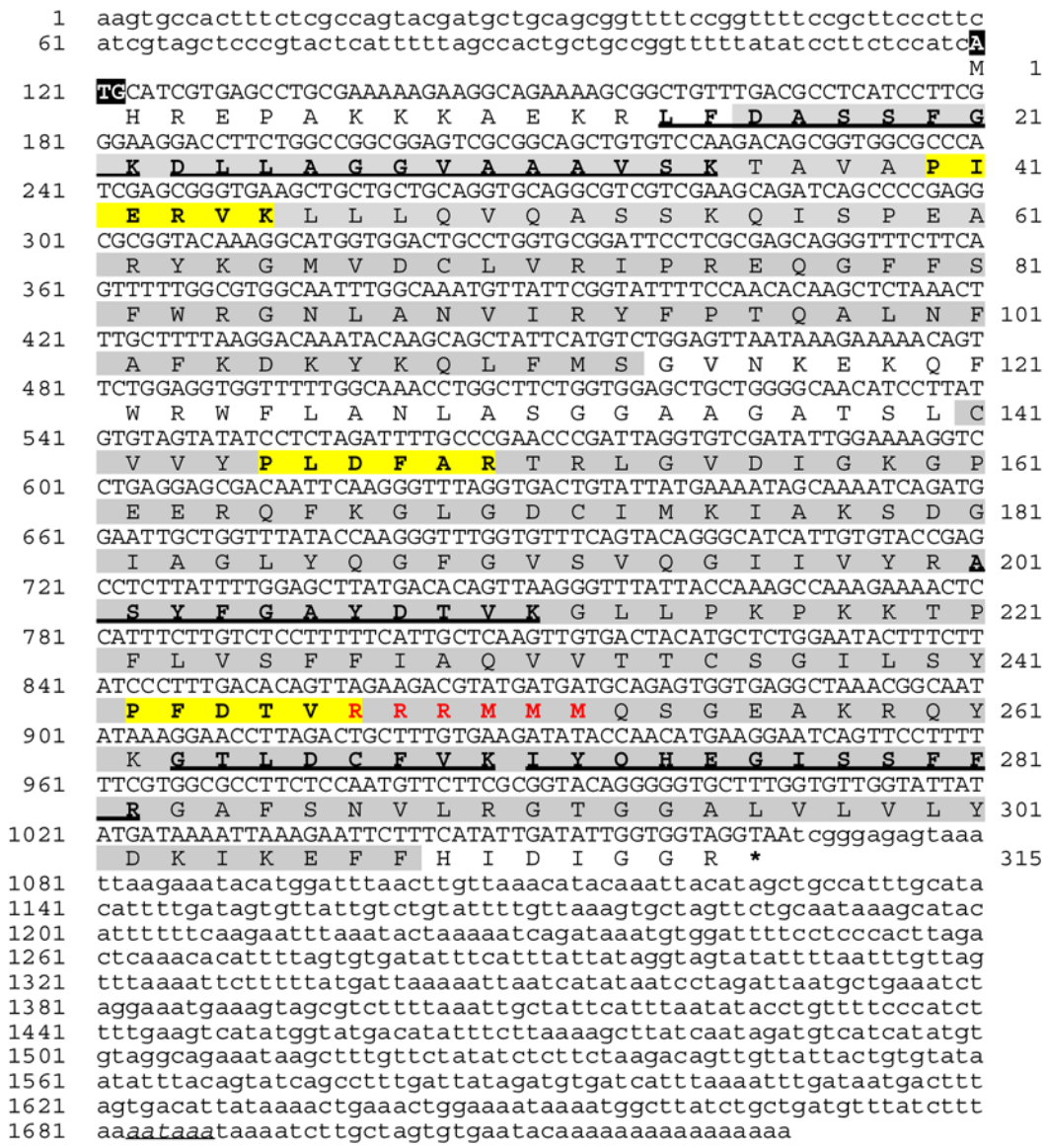

Figure 2.

A. Complementary DNA and deduced amino acid sequences of human SFEC protein (GenBank™. accession number ***BC022032***). Numbering of nucleotide base pairs is on left and numbering of amino acids is on right. The 1727-bp cDNA sequence is shown with the 945-bp ORF in uppercase letters and the 119-bp 5′ UTR and 660-bp 3′ UTR in lowercase letters. Peptide sequences originally obtained by mass spectrometry are bold underlined. Homology domains placing SFEC in the mitochondrial carrier protein family are shaded. These consist of three similar motifs (unit1: aa 16–118; unit2: aa141–217; unit3: aa218–308). The consensus motif of PX(D/E)XX(K/R) (bold in yellow block) lies in each repetitive motif at amino acids 40–45 (PIERVK), 145–150 (PLDFAR) and 242–247 (PFDTVR). This motif is characteristic for all mitochondrial carriers (Walker and Runswick, 1993; Nelson *et al*., 1998). SFEC contains a hexapeptide carrying the signature of RRRMMM at amino acid 247–252 (bold in red), a feature of all ADP/ATP carriers but absent from other mitochondrial carriers (Pebay-Peyroula *et al*., 2003).

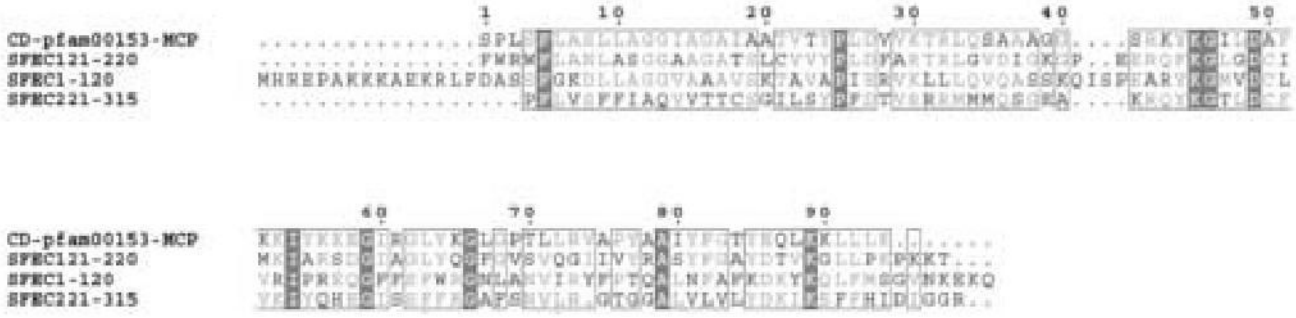

Figure 3.

Alignment of SFEC with mitochondrial carrier protein (pfam153) using the conserved domain architecture retrieval tool (CDART, Geer et al., 2002). SFEC contains three repeat motifs characteristic of mitochondrial carrier proteins. In addition PX(D/E)XX(K/R) consensus sequences are located at amino acids 40–45 (PIERVK), 145–150 (PLDFAR) and 242–247 (PFDTVR), another characteristic of all mitochondrial carriers (Walker and Runswick, 1993; Nelson *et al*., 1998).

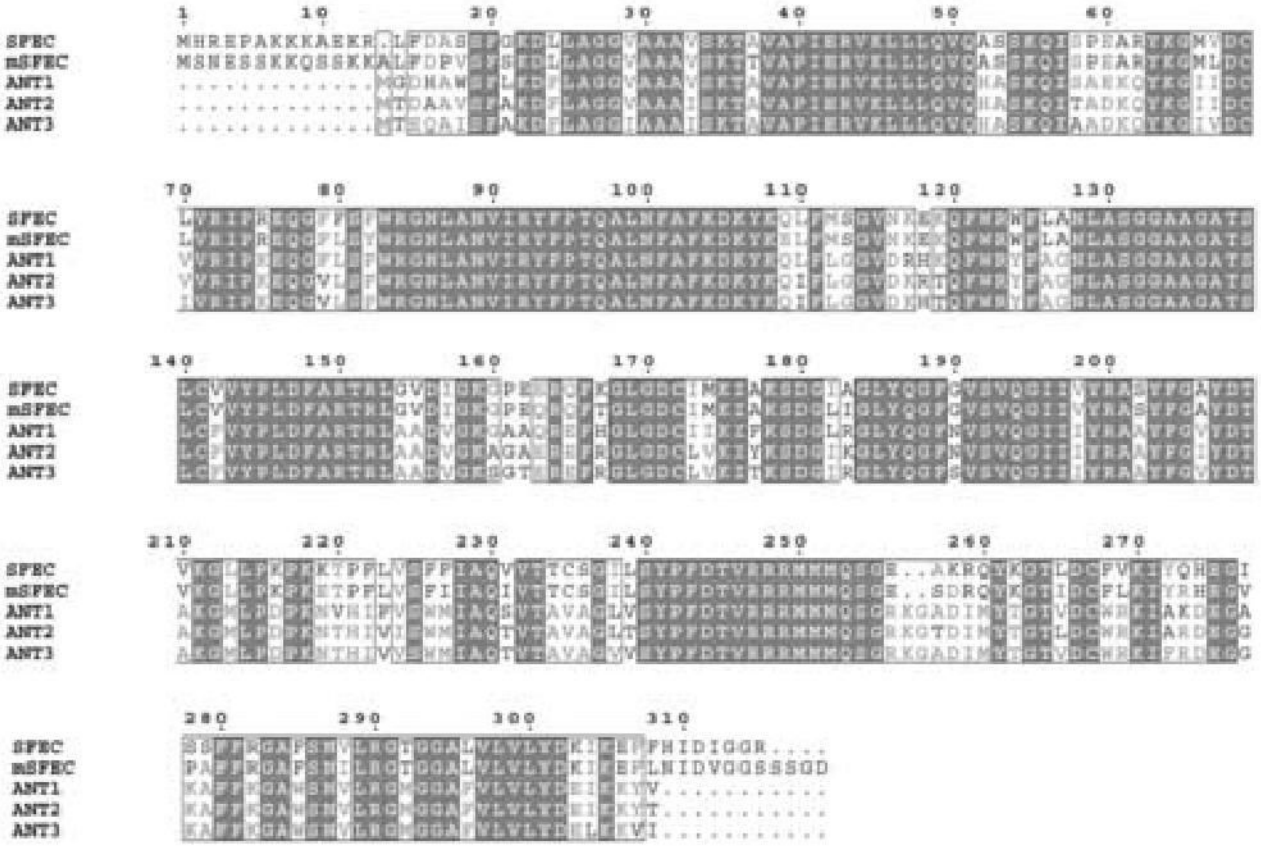

Figure 4.
Alignment of human and mouse SFEC with other human adenine nucleotide carrier proteins (adenine nucleotide translocases, ANTs) using the conserved domain architecture retrieval tool (CDART, Geer et al., 2002). Both human and mouse SFEC differ from the human (as well as mouse) ANTs by their characteristic N and C terminal extensions.

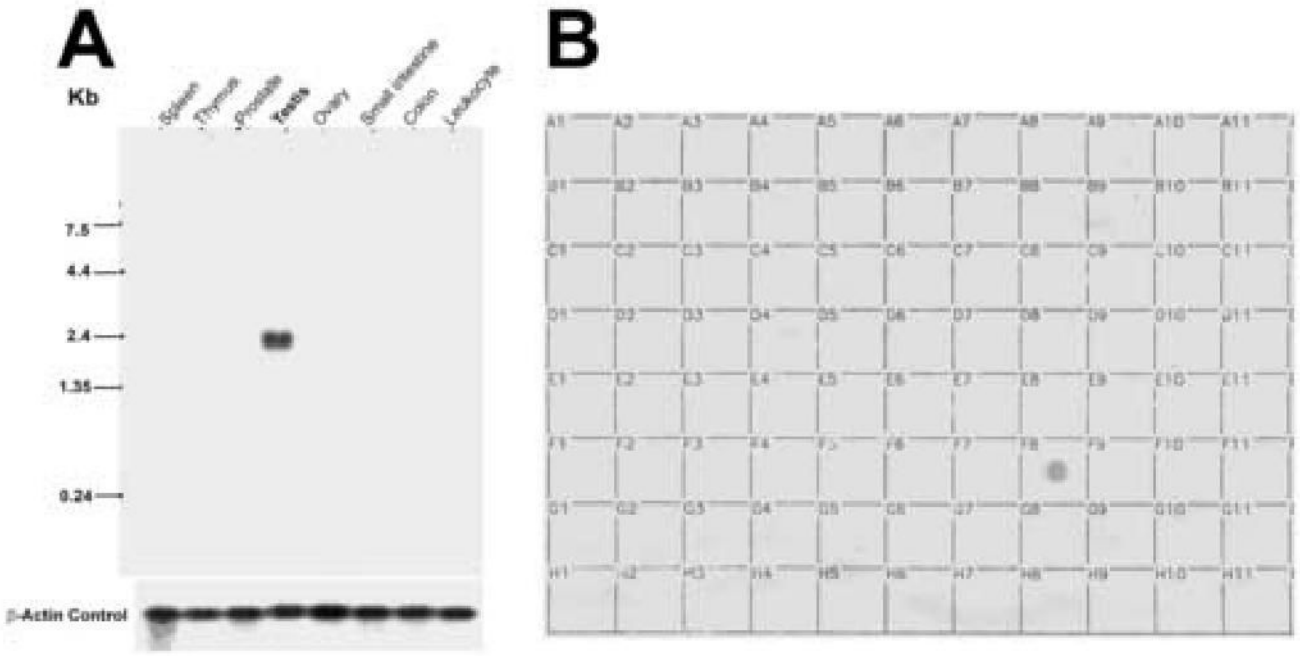

Figure 5.

A, Northern blot (Clontech) containing poly(A)$^+$ mRNA from human tissues (2 μg per lane) was hybridized with radiolabeled SFEC cDNA and exposed for 72 h. RNA markers are indicated on the *left*. A single SFEC transcript of 2.4 kb was apparent only in testis. The blot was subsequently stripped and rehybridized with human β-actin as a probe to assess the levels of RNA in each lane (*lower panel*). **B**, Dot blot containing poly(A)$^+$ RNA from 76 human tissues (obtained from Clontech) was hybridized with a radiolabeled full length of SFEC cDNA, and the signal was visualized by autoradiography. A hybridization signal was found only in testis (*box F8*) after 72-h exposure. The distribution of mRNAs from 76 human tissues in the dot blot was as follows. *A1*, whole brain; *B1*, cerebral cortex; *C1*, frontal lobe; *D1*, parietal lobe; *E1*, occipital lobe; *F1*, temporal lobe; *G1*, paracentral gyrus of cerebral cortex; *H1*, pons; *A2*, left cerebellum; *B2*, right cerebellum; *C2*, corpus callosum; *D2*, amygdalla; *E2*, caudate nucleus; *F2*, hippocampus; *G2*, medulla oblongata; *H2*, putamen; *A3*, substantia nigra; *B3*, accumbens nucleus; *C3*, thalamus; *D3*, pituitary gland; *E3*, spinal cord; *A4*, heart; *B4*, aorta; *C4*, left atrium; *D4*, right atrium; *E4*, left ventricle; *F4*, right ventricle; *G4*, interventricular septum; *H4*, apex of the heart; *A5*, esophagus; *B5*, stomach; *C5*, duodenum; *D5*, jejunum; *E5*, ileum; *F5*, ilocecum; *G5*, appendix; *H5*, ascending colon; *A6*, transverse colon; *B6*, descending colon; *C6*, rectum; *A7*, kidney; *B7*, skeletal muscle; *C7*, spleen; *D7*, thymus; *E7*, peripheral blood lymphocytes; *F7*, lymph node; *G7*, bone marrow; *H7*, trachea; *A8*, lung; *B8*, placenta; *C8*, bladder; *D8*, uterus; *E8*, prostate; *F8*, testis; *G8*, ovary; *A9*, liver; *B9*, pancreas; *C9*, adrenal gland; *D9*, thyroid gland; *E9*, salivary gland; *F9*, mammary gland; *A10*, leukemia HL-60; *B10*, HeLa S3; *C10*, leukemia K-562; *D10*, leukemia MOLT-4; *E10*, Burkitt's lymphoma Raji; *F10*, Burkitt's lymphoma Daudi; *G10*, colorectal adenocarcinoma SW480; *H10*, lung carcinoma A549; *A11*, fetal brain; *B11*, fetal heart; *C11*, fetal kidney; *D11*, fetal liver; *E11*, fetal spleen; *F11*, fetal thymus; *G11*, fetal lung.

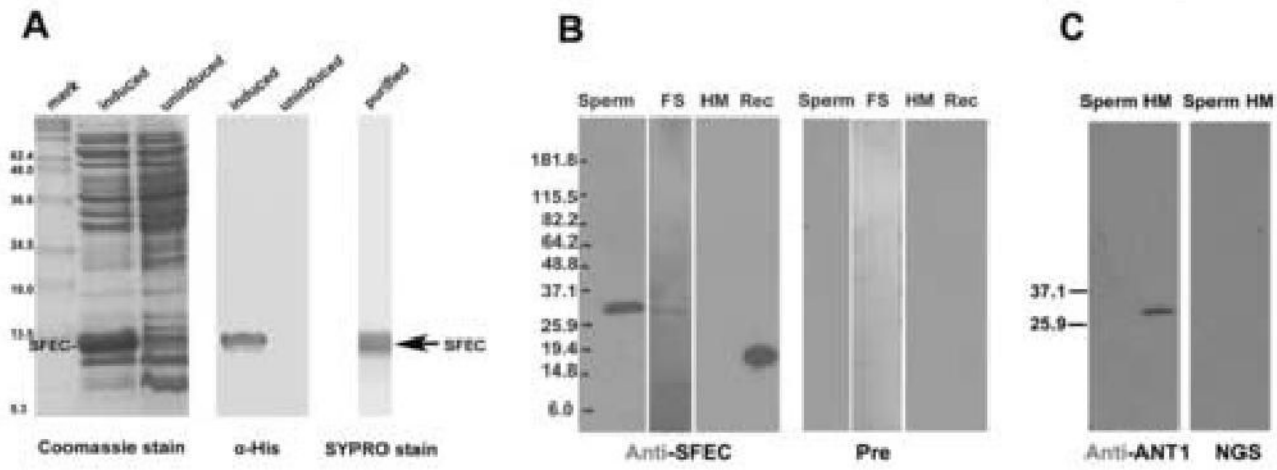

Figure 6.

Expression, purification of SFEC and Western analysis with rat anti-recSFEC antibody demonstrating that SFEC is a component of FS in sperm protein extracts , undetected in heart mitochondrial.. **A** Coomassie blue stain of induced and uninduced truncated recombinant SFEC (117 amino acids; aa 4–120) expressed in BLR (DE3) host cell after cloning into the pET28b vector. Western analysis of recombinant proteins showing that anti-histidine antibody detects the expected molecular weight of ~13 kDa on the induced truncated SFEC protein. Affinity purified recombinant SFEC protein stained by SYPRO Ruby stain (Bio-Rad) confirming the purity of immunogen before injection into rats. **B.** Western blots of anti-recSFEC antibody on the human sperm (A), isolated FS (B), mitochondria from human heart (C) and recombinant SFEC (D) proteins. Immue serum recognized the recombinant SFEC and a band at 32kDa in human sperm proteins extracted by 1% Nonidet P40 and 0.5% sodium deoxycholate (A). Proteins from isolated FS contained the 32 kDa band (C) identical to that initially identified as SFEC by mass spectrometry (B). Human heart mitochondrial proteins, although having the highest amino acid sequence identity with SFEC (67%), were not recognized by rat anti-recSFEC antibody. Pre immune serum did not recognized any protein bands**. C.** Western blots of anti-human ANT1 antibody on human sperm and human heart mitochondrial proteins (HM). The human heart mitochondrial protein was recognized at 32kDa by anti-ANT1 antibody, serving as a positive control for the mitochondrial protein extract while sperm proteins were not recognized by goat anti-human ANT1 antibody. Normal goat serum controls did not recognize any proteins.

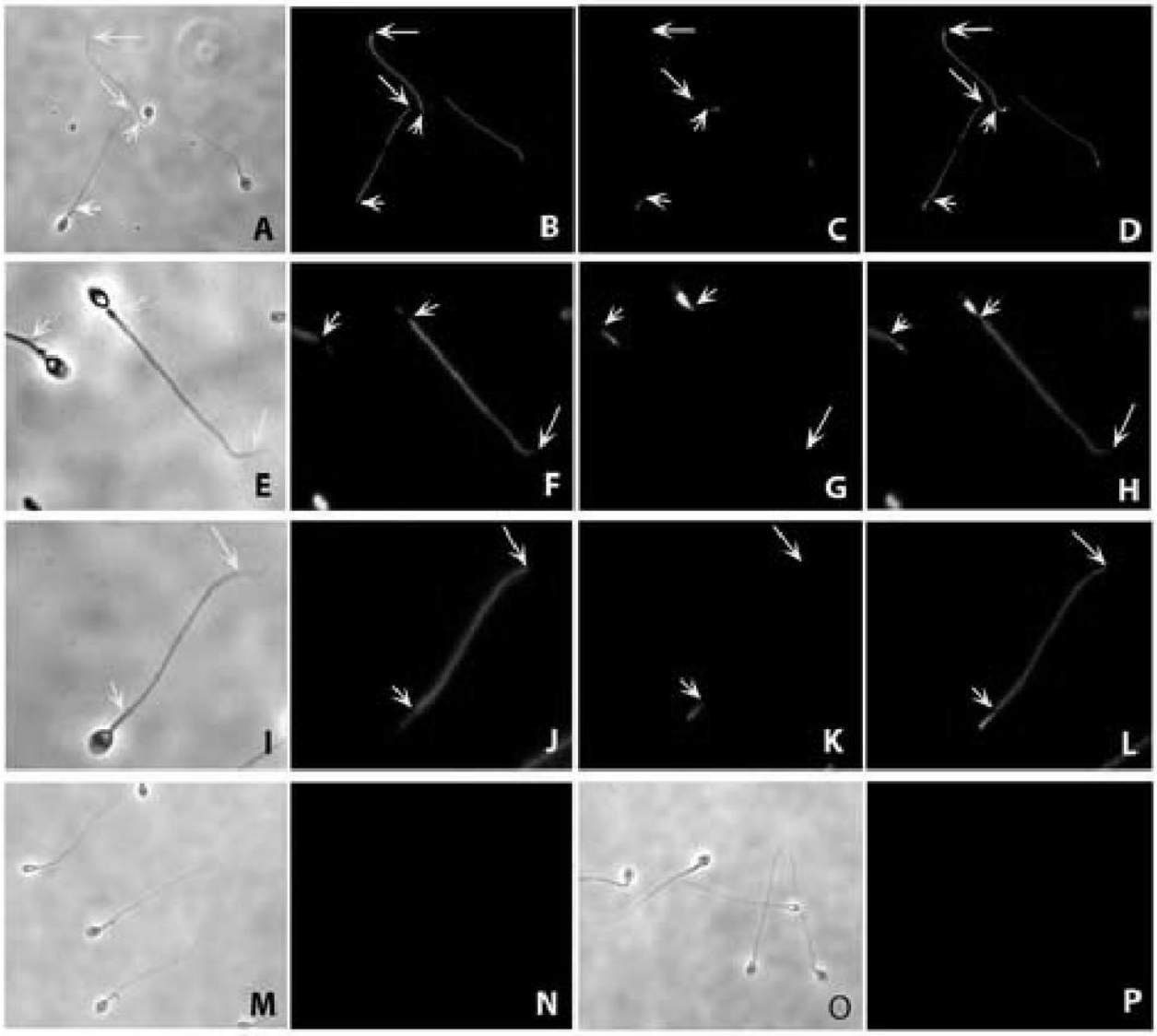

Figure 7.

Indirect immunofluorescent localization of SFEC to the entire principal pieces of the human sperm flagellum using rat antiserum against recombinant human SFEC. **A,E,I,M** and **O** are phase contrast images of the corresponding FITC images **B,F,J,N** and **P,** respectively. The mid piece of the sperm flagellum was identified by labeling with Mito Tracker Red CMX Ros (**C.G,K**). **D,H,L:** Merged images of FITC with Mitotracker. The entire mid piece (**J**) or part of the middle piece (**F**) as well as the principal piece was recognized by anti-rat SFEC antibody. Pre-immune serum showed no immunofluorescence in human sperm (**N**). Post immune sera pre-absorbed with recombinant SFEC protein revealed no immunostaining (**P).** Short arrows indicate the posterior end of the mid piece whereas the long arrows indicate the posterior end of the principal piece. The principal piece is the region between long and short arrows where major immunostaining is seen (**B, D, F, H, J** and **L**).

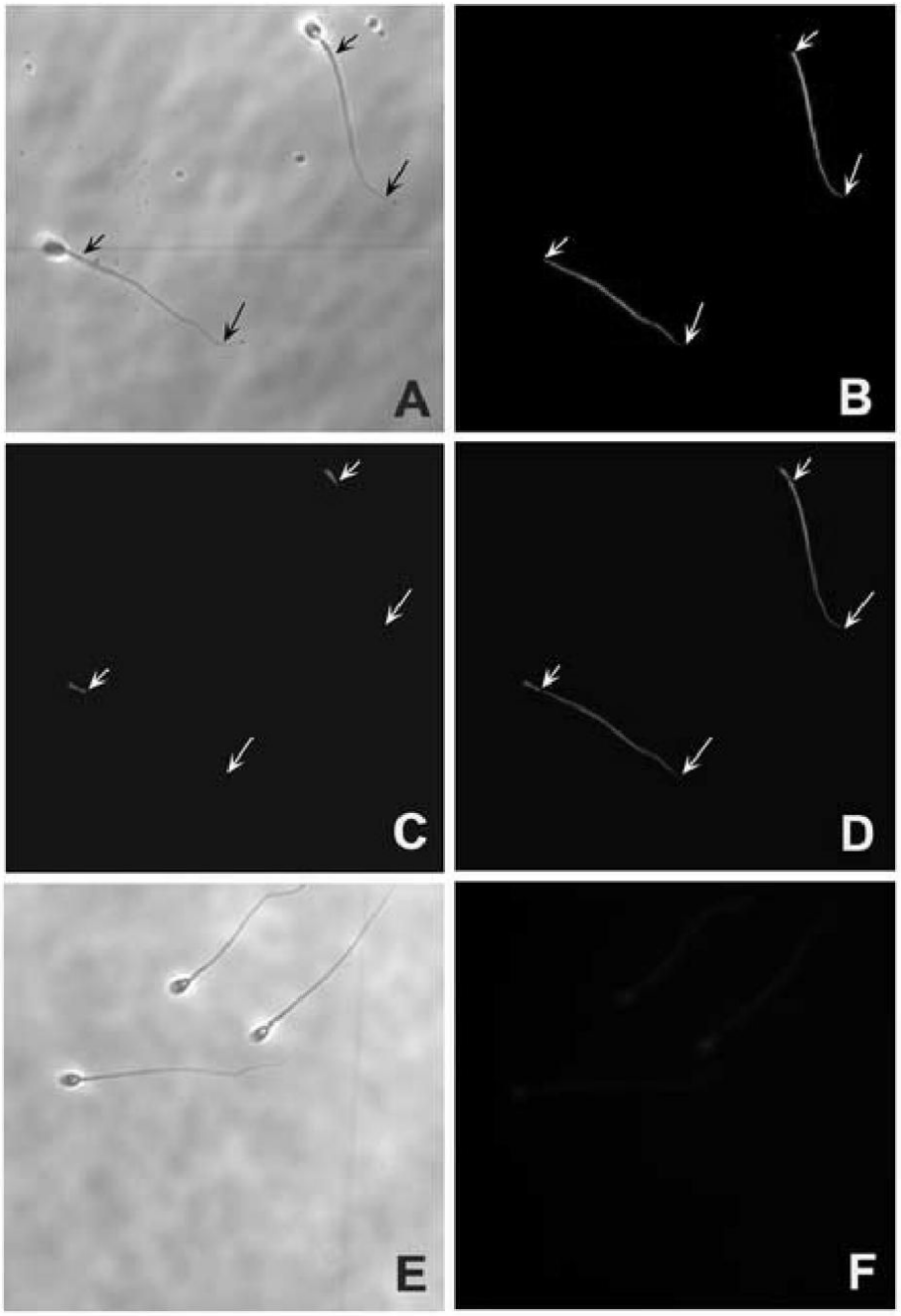

Figure 8.

Indirect immunofluorescent localization of aldolase (rabbit muscle) to the entire principal pieces of the human sperm flagellum using goat anti-aldolase antibody (Novus Biologicals, Inc. Littleton, CO). **A** and **E** are phase contrast images of the corresponding FITC images **B** and **F** respectively. The mid piece of the sperm flagellum was identified by labeling with Mito Tracker Red CMX Ros (**C**). **D:** Merged images of FITC with Mitotracker. The entire mid piece principal piece (**B** and **D**) was recognized by anti-goat aldolase antibody. Normal goat serum showed no immunofluorescence in human sperm (**F**). Short arrows indicate the posterior end of the middle piece whereas the long arrows indicate the posterior end of the principal piece.

The principal piece is the region between long and short arrows where major immunostaining is seen (**B** and **D**).

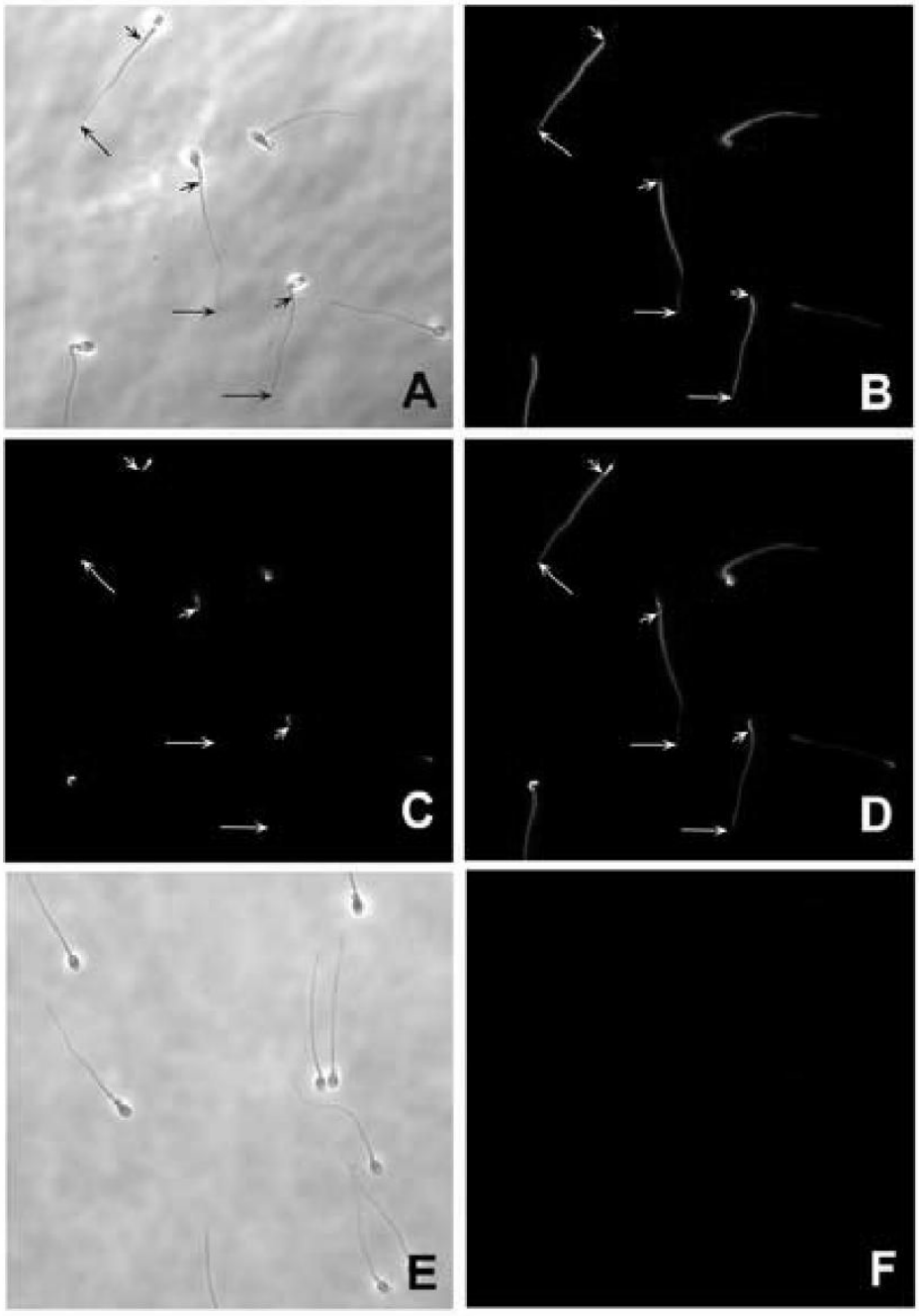

Figure 9.

Indirect immunofluorescent localization of pyruvate kinase (rabbit muscle) to the entire principal pieces of the human sperm flagellum using goat anti-pyruvate kinase antibody (Novus Biologicals, Inc. Littleton, CO). **A** and **E** are phase contrast images of the corresponding FITC images **B** and **F** respectively. The mid piece of the sperm flagellum was identified by labeling with Mito Tracker Red CMX Ros (**C**). **D:** Merged images of FITC with Mitotracker. The entire middle piece principal piece (**B** and **D**) was recognized by anti-goat pyruvate kinase antibody. Normal goat serum showed no immunofluorescence in human sperm (**F**). Short arrows indicate the posterior end of the mid piece whereas the long arrows indicate the posterior end of the

principal piece. The principal piece is the region between long and short arrows where major immunostaining is seen (**B** and **D**).

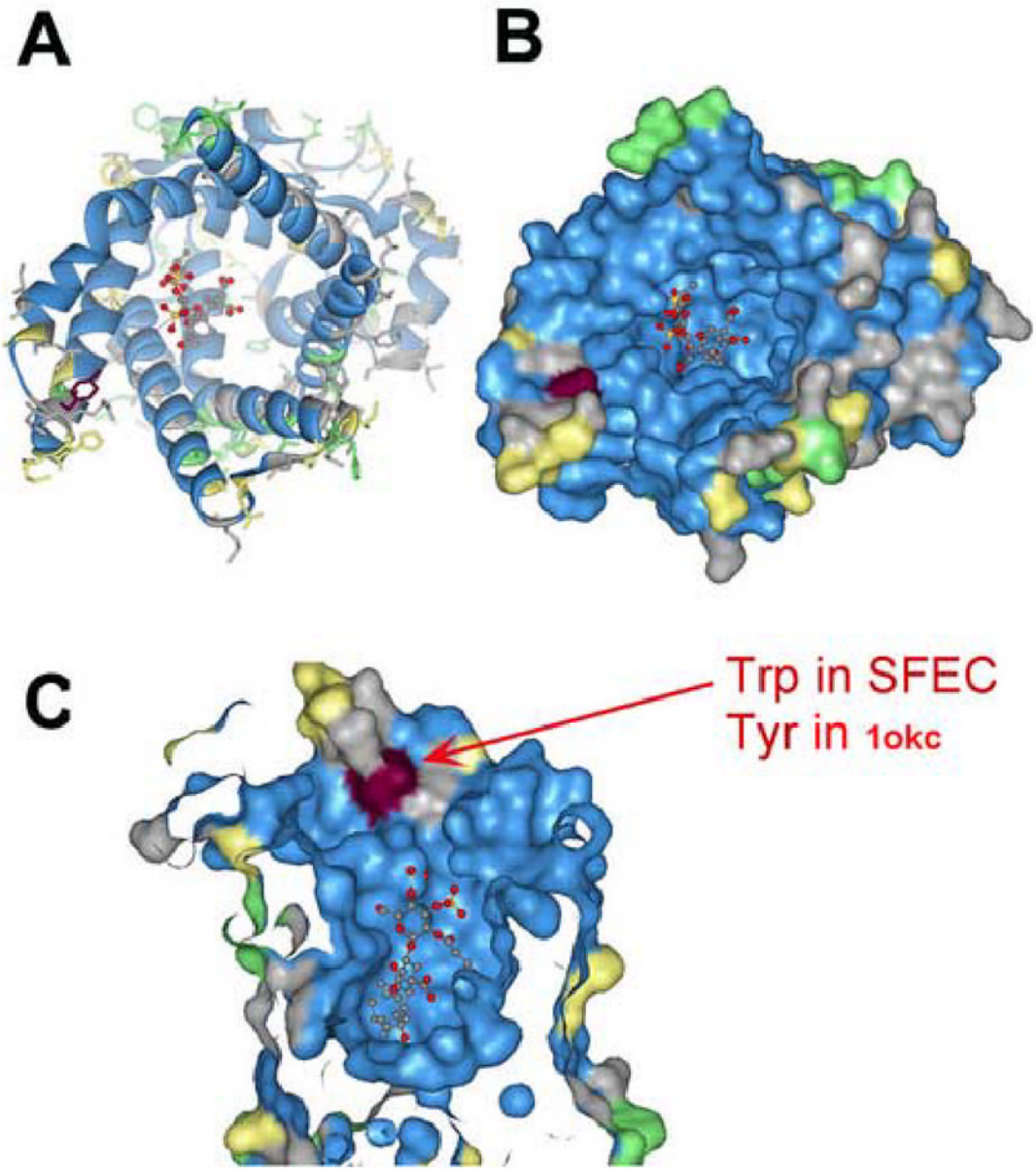

Figure 10.

Modeling of the structure of SFEC by comparison with a known ANT1 (1okc) demonstrated that one amino acid, tryptophan in SFEC replaces tyrosine in ANT1 in the entrance to the binding niche. This architecture may afford an opportunity for selective inhibition. **A**. Ribbon presentation, **B.** Surface presentation, **C.** Surface presentation zoomed into binding niche. Blue: identical amino acid; Grey-yellow: Strong or weak homologous amino acid; Green: no homology.

Table 1

Eight glycolytic enzymes identified in the present FS proteomics including GAPDH -2 which is previously reported in the FS.

Name	Nomenclature (Identified Band)	Respective NCBI# and Molecular Weight (kDa)	Gene locus	Expression	Testis EST#	References
Aldolase A	C261, C262, C263	4557305, 39.4	16q22-q24	Multiple tissues	***BQ433120.1***	Izzo et al., (1988)
Triose Phosphate Isomerase (TPI)	C265	4507645, 26.7	12p13	Multiple tissues	***BQ223970.1***	Maquat et al., (1985)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	C264	7669492, 36.4	12p13	Multiple tissues	***BG393651.1***	Ercolani et al., (1988)
Glyceraldehyde 3-phosphate dehydrogenase2 (GAPDH-2)	C259, C260, C262, C264, C266	7657116, 44.5	19q13.1	Testis specific	***BG718767.1***	Welch et al., (2000)
Pyruvate Kinase	C259	4505839, 57.9	15q22	Multiple tissues	***BM478612.1***	Tsutsumi et al., (1988)
Lactate dehydrogenase-A (LDH-A)	C264	5031857, 36.7	11p15.4	Multiple tissues	***BG506200.1***	Chung et al., (1985)
Lactate dehydrogenase-C (LDH-C)	C264	4504973, 36.2	11p15.5-p15.3	Testis specific	***BQ435561.1***	Edwards et al., (1989)
Sorbitol Dehydrogenase (SDH)	C263	1583520, 38.3	15q15.3	Multiple tissues	***BG393670.1***	Iwata and Carper (1995)

Table 2

Peptides identified by tandem mass spectrometric analysis of isolated human fibrous sheaths. Except sorbitol dehydrogenase, multiple peptides were obtained for each glycolytic enzyme covering 8.3 to 72.3% of the entire protein. The Sequest algorithm was used to interpolate the spectra.

Enzymes	MS Peptides	Positions	Protein coverage by residue count
Aldolase A	GILAADESTGSIAK	29–42	31.0%
	ADDGRPFPQVIK	88–99	
	GVVPLAGTNGETTTQGLDGLSER	112–134	
	IGEHTPSALAIMENANVLAR	154–173	
	FSHEEIAMATVTALR	244–258	
	ALQASALK	305–312	
	YTPSGQAGAASESLFVSNHAY	343–364	
Triose phosphate isomerase (TPI)	KFFVGGNWK	6–14	72.3%
	KQSLGELIGTLNAAK	19–33	
	VPADTEVVCAPPTAYIDFAR	34–53	
	IAVAAQNCYK	60–69	
	VTNGAFTGEISPGMIK	70–85	
	DCGATWVVLGHSER	86–99	
	RHVFGESDELIGQK	100–113	
	VAHALAEGLGVIACIGEK	114–131	
	LDEREAGITEK	132–142	
	VVLAYEPVWAIGTGK	161–175	
	TATPQQAQEVHEK	176–188	
	SNVSDAVAQSTR	195–206	
	IIYGGSVTGATCK	207–219	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GALQNIIPASTGAAK	214–228	8.3%
	LISWYDNEFGYSNR	323–336	
Glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH-2)	AEVEPQPQPEPTPVR	34–48	41.2%
	EEIKPPPPPLPPHPATPPPK	49–68	
	VVAVNDPFIDPEYMVYMFK	101–119	
	NGQLVVDNHEISVYQCK	135–151	
	FGIVEGLMTTVHSYTATQK	240–258	
	GAHQNIIPASTGAAK	273–287	
	LTGMAFRVPTPDVSVVDLTCR	300–320	
	LAQPAPYSAIK	321–331	
	AGIALNDNFVK	371–381	
	LISWYDNEYGYSHR	382–395	
	VVDLLR	396–401	
Pyruvate kinase	NTGIICTIGPASR	44–56	13.4%
	TATESFASDPILYRPVAVALDTK	93–115	
	GADFLVTEVENGGSLGSK	189–206	
	GVNLPGAAVDLPAVSEK	208–224	
Lactate dehydrogenase-A (LDH-A)	DQLIYNLLKEEQTPQNK	6–22	29.5%
	DLADELALVDVIEDK	43–57	
	LKGEMMDLQHGSLFLR	58–73	
	DYNVTANSK	82–90	
	LVIITAGAR	91–99	
	VIGSGCNLDSAR	158–169	
	VTLTSEEEAR	306–315	
	SADTLWGIQK	319–328	
Lactate dehydrogenase-C (LDH-C)	LIEDDENSQCK	12–22	19.3%
	ITSGKDYSVSANSR	77–90	
	YLIGEK	172–177	
	QVIQSAYEIIK	233–243	
	INLNSEEEALFK	306–317	
	SAETLWNIQK	319–328	
Sorbitol dehydrogenase	LENYPIPEPGPNEVLLR	22–38	4.8%