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Recombinant human sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) is expressed at high yield as an active homotetramer in baculovirus-infected insect cells

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ABSTRACT

The sperm-specific glyceraldehyde-3-phosphate dehydrogenase (GAPDHS) isoform is a promising contraceptive target because it is specific to male germ cells, essential for sperm motility and male fertility, and well suited to pharmacological inhibition. However, GAPDHS is difficult to isolate from native sources and recombinant expression frequently results in high production of insoluble enzyme. We chose to use the Bac-to-Bac baculovirus-insect cell system to express a His-tagged form of human GAPDHS (Hu his-GAPDHS) lacking the proline-rich N-terminal sequence. This recombinant Hu his-GAPDHS was successfully produced in *Spodoptera frugiperda* 9 (Sf9) cells by infection with recombinant virus as a soluble, enzymatically active form in high yield, >35 mg/L culture. Biochemical characterization of the purified enzyme by mass spectrometry and size exclusion chromatography confirmed the presence of the tetrameric form. Further characterization by peptide ion matching mass spectrometry and Edman sequencing showed that unlike the mixed tetramer forms produced in bacterial expression systems, human his-GAPDHS expressed in baculovirus-infected insect cells is homotetrameric. The ability to express and purify active human GAPDHS as homotetramers in high amounts will greatly aid in drug discovery efforts targeting this enzyme for discovery of novel contraceptives and three compounds were identified as inhibitors of Hu his-GAPDHS from a pilot screen of 1120 FDA-approved compounds.

Introduction

Glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (GAPDHS[1]; GenBank Accession ID: NM_014364.4), the sperm-specific isoform of human GAPDH [1–3], is under active study as a male contraceptive target [4–6]. Gene targeting studies in mice provided compelling evidence that GAPDHS is required for sperm function and male fertility in mice [6,7], and that males lacking this isozyme are infertile and produce sperm with very low ATP levels and no progressive motility [6]. In contrast to the somatic form of GAPDH, the sperm-specific GAPDHS possesses a unique proline-rich extension at the amino terminus which varies between species (a 72 amino acid extension in human [1], of which 42 residues comprise the proline-rich region (50% proline) [3]), and may mediate binding to the fibrous sheath in the sperm flagellum [1,3,8].

The ability to identify potential drugs targeting human GAPDHS has been hindered by the difficulties in producing sufficient soluble and active enzyme for study. Isolation of native GAPDHS from sperm has proved challenging due to its tight association with the fibrous sheath. The expression of recombinant GAPDH enzymes in *Escherichia coli* has met with varied success, with high production of insoluble material frequently observed [9]. A recent study found that expression of rat GAPDHS, either as a full-length protein or a truncated protein lacking the proline-rich N-terminus, resulted in protein that was predominantly insoluble [5]. We have had similar difficulties obtaining high yields of soluble protein when expressing recombinant mouse or human GAPDHS in *E. coli*. Although native GAPDH enzymes typically function as homotetramers (molecular mass ~150 kDa), the expression of rat GAPDHS in *E. coli* resulted in mixed tetramers consisting of 1 subunit of rat GAPDHS and 3 subunits of *E. coli* GAPDH [5]. The generation of GAPDH heterotetrameric forms combining purified GAPDH dimers from two different species has been reported [10,11].

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[1] *Abbreviations used:* GAPDHS, glyceraldehyde-3-phosphate dehydrogenase, spermatogenic.

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Recombinant forms of GAPDH enzymes from a number of human parasites have also been expressed in *E. coli* and shown to be tetrameric [12–16]. There are few reports on the expression of recombinant GAPDH enzymes in host systems other than *E. coli*, and expression of a GAPDH enzyme in *Pichia pastoris* yielded inactive enzyme [9]. The eukaryotic baculovirus-insect cell system has been used to produce soluble oligomeric forms of human GAPDH [17] and GAPDH from *Schistosoma mansoni* [9]. The *S. mansoni* GAPDH expressed in Sf9 cells consisted of both monomeric and tetrameric forms, although it was not determined whether the tetrameric form consisted of a single type or mixed subunits.

Herein, we describe the expression and biochemical characterization of the sperm-specific human GAPDHS protein in baculovirus-infected insect cells. In this system, the protein was highly expressed as a biologically active and soluble form. Extensive analysis by mass spectrometry and Edman sequencing demonstrated that the protein was a homotetrameric species. The availability of this form of GAPDHS will greatly facilitate ongoing efforts to identify novel inhibitors that may have potential as male contraceptives.

Materials and methods

Construction of the recombinant human GAPDHS baculovirus

Unless stated, all procedures were performed according to manufacturer's procedures (Bac-toBac™, Invitrogen). The full-length gene for human sperm-specific gyceraldehyde-3-phosphate dehydrogenase (GAPDHS) was synthesized (GeneArt, Inc.). A truncated GAPDHS sequence lacking the proline-rich domain was generated by creating an EcoR1 restriction site upstream of residue 69 of the synthetic gene, and this region comprising residues 69–408 was subcloned in-frame into the EcoRI and XhoI sites of a phosphatase (CIP)-treated pFastBac HT baculovirus shuttle vector (Invitrogen). The resulting recombinant gene encodes 30 amino acids at the N-terminus, comprising a hexahistidine tag, a spacer region and a TEV protease cleavage site plus the truncated GAPDHS gene (Fig. 1). The resulting clonal DNA was sequenced across the two flanking restriction sites to verify correct gene insertion.

The sequence-verified clone was transformed into DH10Bac, an *E. coli* strain carrying both a baculovirus genome-containing plasmid and a plasmid encoding a transposase enzyme. The GAPDHS gene was inserted via Tn7 transposable elements immediately downstream of the polyhedrin promoter in the baculoviral DNA. Positive white colonies from the lacZ DH10Bac strain were restreaked twice to ensure clonal recombinant virus. Mini-prep DNA from the resulting white colony isolates, bacmid DNA, was PCR verified for the presence of the GAPDHS insertion, and then used in the initial *Spodoptera frugiperda* (Sf9) transfection.

Sf9 cell maintenance

Spodoptera frugiperda (Sf9) cells (ATCC) adapted to HyQ SFX-Insect serum free medium (HyClone) supplemented with 2 mM L-glutamine and 1× penicillin/streptomycin were cultured at 27 °C in either monolayer or suspension culture. Sf9 suspension cultures were maintained at a cell density of 0.5×10^6 to 3×10^6 cells/ml as 80 ml cultures in 250 ml shaker flasks shaken at 115 rpm in an ATR Multitron shaking incubator (ATR, Inc.).

Insect cell transfection and recombinant virus isolation

To generate viral particle formation, newly attached Sf9 cells in 6-well plates (9×10^5 cells/well) were transfected with the PCR-verified recombinant GAPDHS bacmid DNA (1–2 μg) in Cellfectin™ (Invitrogen) and incubated in unsupplemented Sf-900 II SF medium (Invitrogen for 5 h at 27 °C). After aspiration of the transfection mixture, TNM-FH medium (HyClone) supplemented with 10% FBS, 2 mM L-glutamine and 1× penicillin/streptomycin was added. Conditioned medium was collected 5 d after transfection and the supernatant was reserved as P1 viral stock. P2 and P3 viral amplification was accomplished in 75 cm^2 T-flasks at a multiplicity of infection (MOI) of 0.1 assuming titers of 1×10^6 and 2×10^7 plaque forming units (pfu)/ml for P1 and P2 viral stocks, respectively. The resulting P3 viral stock was titered via the end-point dilution method [18], and determined to be 2×10^8 pfu/ml.

Expression optimization of recombinant Hu his-GAPDHS in Sf9 cells

Optimization of protein expression was performed in 6-well plates by varying multiplicity of infection (MOI, values of 1, 2, 5, 10 and 20) and time of expression (24, 48, 72 and 96 h). Cells were seeded at 2×10^6 cells/well, and baculovirus stock added in 2.5 ml supplemented (as described above) HyQ SFX-Insect medium. At regular time points, cells were washed with PBS, harvested, and protein expression in the conditioned medium and cell pellet assessed by SDS–PAGE.

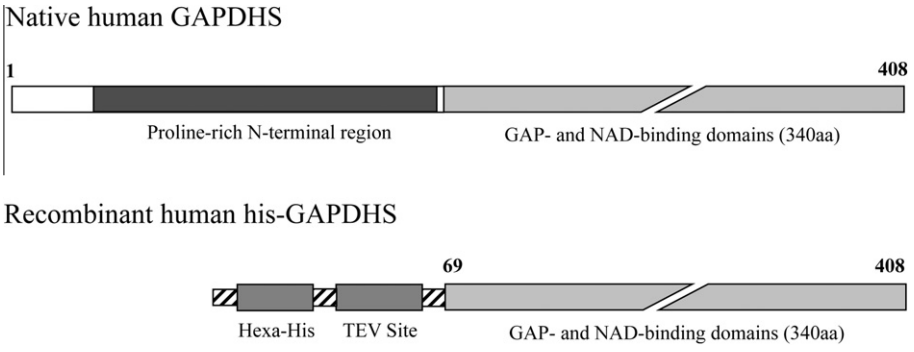

Fig. 1. Schematic diagram of full-length human GAPDHS and the recombinant human his-GAPDHS expressed in insect cells. The sperm isozyme possesses a proline-rich domain at the N-terminus that is not present in the somatic GAPDH isozyme. In human GAPDHS, this extension comprises a 19 amino acid conserved sequence, a 3 amino acid intervening proline cysteine region and a 42 amino acid proline-rich region (50% proline) from residues 27–67 [3]. Numbering in this figure refers to the sequence for human sperm-specific gyceraldehyde-3-phosphate dehydrogenase (GAPDHS) (GenBank Accession ID: NP_055179.1). For the form of GAPDHS expressed in insect cells herein, the N-terminal extension containing the proline-rich region was deleted (residues 1–68) and replaced by the 30 amino acid FastBac vector sequence which included a hexahistidine purification tag and TEV protease cleavage sequence.