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Somatic and Sperm-Specific Isoenzymes of Glyceraldehyde-3-phosphate Dehydrogenase: Comparative Analysis of Primary Structures and Functional Features

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Abstract—The elucidation of the interdependence between structural features and functions of somatic and sperm-specific isoenzymes of glyceraldehyde-3-phosphate dehydrogenase (GAPD and GAPDS, respectively) was the goal of comparative analysis of their primary structures. GAPDS was shown to lack the sequence similar to the atypical nuclear export signal motif (NES) of the somatic isoenzyme GAPD. This finding is confirmed by experimental data on the absence of interaction between GAPDS and antibodies 6C5 recognizing the NES motif in the sequence of GAPD. The lack of NES correlates with functional peculiarities of the sperm-specific enzyme that is tightly bound to the fibrous sheath of the sperm flagellum. The sequences of the two isoenzymes were examined for the short motifs that might participate in apoptosis, endocytosis, and DNA repair. Sites of phosphorylation by different protein kinases have been revealed in both isoenzymes, and their characteristic features are discussed. These observations can serve as the basis for subsequent search for new ways of regulating the two isoenzymes.

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D-Glyceraldehyde-3-phosphate dehydrogenase (GAPD, EC 1.2.1.12) is a glycolytic enzyme catalyzing oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate coupled with the reduction of NAD to NADH. Mammals possess two isoforms of the enzyme: somatic (GAPD) and sperm-specific enzyme (GAPDS). The polypeptide chain of GAPD consists of 334 amino acid residues and includes NAD-binding and C-terminal domains. GAPDS is expressed in spermatids and bound tightly to the fibrous sheath of the flagellum of the spermatozoid. GAPDS contains an additional N-terminal domain of 74 amino acid residues. In mammals, GAPD and GAPDS are encoded by genes located in chromosomes XII and XIX, respectively. There is no information concerning the existence of alternative splicing or alternative transcription initiation sites for these genes.

A very high concentration of GAPD in the cell (5-15% of the total concentration of cytoplasmic proteins) gave a rise to the suggestion that this protein can function not only as the glycolytic enzyme. Actually, it was shown that apart from glycolysis, GAPD is involved in many other processes, including endocytosis [1], membrane fusion [2], replication and reparation of DNA [3], as well as in the induction of apoptosis [4, 5]. The latter process is connected with the translocation of GAPD through the nuclear membrane, which is probably accompanied by the dissociation of the cytoplasmic tetrameric GAPD yielding dimers and monomers [5]. Accumulation of GAPD in the cell nucleus can be caused by numerous factors (action of oxidants (peroxides and NO) [5], K^+-depolarization [6], aging of the cell culture [7], treatment by some anticancer medication (mercaptopurine or cytosine arabinoside) [7])

Abbreviations: AP2) adaptor protein 2; APC) anaphase-promoting complex; CRM1) exportin-1; FS) frequency score; GAPD and GAPDS) somatic and sperm-specific glyceraldehydes-3-phosphate dehydrogenases, respectively; HMM) Hidden Markov Models; NES) nuclear export signal; SUMO-1) small ubiquitin-related modifier 1.

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and is followed by degradation of the cell. It was demonstrated that GAPD is a constituent of the nuclear protein complex that is capable of binding to double-strand DNA containing modified nitrogen bases (for example, thioguanine) [8]. The site that is responsible for the interaction of GAPD with the proteins of the nuclear export system and determining the intracellular localization of GAPD (nuclear export signal, NES) was identified in the C-terminal domain of the protein [9]. GAPD is exported from the nucleus by the protein CRM1 (exportin-1), requiring NES for the binding GAPD [10]. The nuclear export signal of human GAPD is a sequence of 13 amino acid residues (^{259}KKVVKQASEGPLK^{271}). The replacement of K259 with asparagine results in the accumulation of GAPD in the nucleus. Thus, residue K259 is necessary for the functioning of the NES.

The monoclonal antibodies 6C5 against denatured and monomeric forms of GAPD that were obtained and investigated in our laboratory [11] were shown to interact with the NES sequence [12], which allows its identification in all proteins.

The presence in the same organism of the enzyme form capable of moving inside the cell and the form fixed tightly on the cell structure gives a unique possibility for the comparative analysis of the significance of separate motifs of the GAPD sequence for specific functions of the enzyme. Comparison of the motifs in the sequence of GAPD and GAPDS allows elucidation of the functions that are characteristic for the enzyme that is capable of moving between different cell compartments, as well as specific functions of GAPDS.

Thus, the main goal of this work was the elucidation of the motifs responsible for certain functions in the amino acid sequence of GAPD and GAPDS, comparative analysis of the motifs of these two proteins, and selection of the most interesting findings for subsequent experimental verification. The second goal was to search for proteins containing motifs similar to NES of GAPD. This analysis was important for adequate use of the monoclonal antibodies 6C5 against GAPD while investigating the intracellular localization of the enzyme with the use of immunofluorescence staining, since the presence of identical epitopes could result in an incorrect interpretation of the results. At the same time, the search for NES motifs could reveal new proteins moving between the nucleus and cytoplasm and elucidate the role of GAPD in the regulation of cell functions. To solve this problem, we used Hidden Markov Models profiles (HMM), one of the most sensitive methods for searching for similar sites.

MATERIALS AND METHODS

HMM profiles (Hidden Markov Models). Analogs of the NES motif of GAPD were sought using the HMM profiles. The main advantage of this method over other search methods (particularly over the BLAST method [13]) consists in the fact that HMM profiles allow minimizing the influence of differences in non-conservative sequence positions (insignificant for protein functioning) on the results of the search [14]. HMM profiles training for detection of fragments similar to GAPD nuclear export signal and revealing of proteins containing such fragments was executed using EMBOSS package programs [15]. Multiple alignment of sequences of human somatic GAPD orthologs used for HMM profile training was built with using the ClustalW program [16].

Minimotif Miner program. Short motifs within the sequences of GAPD and GAPDS were detected by the Minimotif Miner program [17]. This program provides for analysis of protein sequences for short functional fragments that are necessary for posttranslational modifications, interaction with other proteins, transport to different cell compartments, etc. (motif search is carried out by patterns). The advantage of the Minimotif Miner program over similar methods (for instance, PROSITE [18]) is the possibility of searching for a large number of motifs important in protein–protein interactions. Since the detected motifs are rather short, false-positive findings may occur. The program estimates the reliability of the findings by calculating FS (frequency score) value for each of them. FS shows how the frequency of motif occurrence in the protein query relates to the frequency of motif occurrence in the entire proteome. FS values exceeding unity indicate that the detected motif occurs more frequently in the protein query than in random sequence of the same length. The Minimotif Miner program also filters detected motifs using the data on the cell localization of a protein and the information concerning the species taxonomy.

RESULTS AND DISCUSSION

Comparative analysis of amino acid sequences of GAPD and GAPDS. GAPD and GAPDS exhibit 68% identity in amino acid sequences. The most similarity is observed in the sequences involved in the catalysis of the glycolytic reaction: for example, the cysteine residues in the active site of the enzyme (Cys151 and Cys155 in human muscles GAPD, which correspond to Cys149 and Cys153 in GAPD from most other sources) and the residues in their proximity. Besides, the sequences involved in the NADH binding are also highly conservative.

GAPDS contains the additional N-terminal domain consisting of 74 amino acid residues that is absent in the somatic enzyme. This domain contains approximately 31% proline residues (average proline content in human proteins is less than 5%). Besides, this domain is non-conservative (significant differences are observed in mouse and human sequences). However, a high content of proline residues is characteristic for the N-terminal domains of

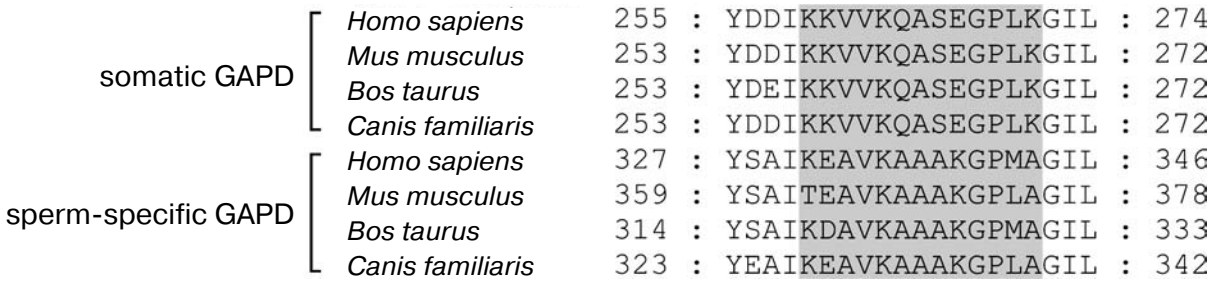

Fig. 1. Alignment of the NES motifs of mammalian GAPD with the corresponding motifs of mammalian GAPDS (NES motifs of GAPD and the corresponding motifs of GAPDS are highlighted).

mammalian GAPDS. It was demonstrated that the main function of this domain is the binding of the enzyme to the fibrous sheath of the flagellum of the spermatozoid [19].

One of the most conservative motifs in the sequence of GAPD is the NES motif. Similar motifs are present in GAPD of all multicellular organisms, and residues K259, A265, and G268 are the most conservative in these motifs. A high conservatism of this motif supports the idea that it is a signal sequence.

It should be noted that NES motif of GAPD significantly differs from the corresponding sequence of GAPDS. Besides, in the case of GAPDS, this motif is not conservative (Fig. 1). Considering that the amino acid sequence of GAPDS has no motifs similar to NES of somatic GAPD, it can be concluded that GAPDS lacks nuclear export signal. This can be due to the fact that the enzyme attached to structural proteins has no possibility to move through the nuclear membrane.

Thus, the monoclonal antibodies 6C5, whose antigen determinant is the NES motif of GAPD, will not react with GAPDS. The experimental data obtained in our laboratory support this assumption [20].

Detection of motifs within the GAPD sequence. To detect the presence of short motifs specific for cytoplasmic and nuclear proteins, the sequences of two isoenzymes were analyzed using the Minimotif Miner program. Among all detected motifs, we selected those that were conservative within mammalian GAPD and contained amino acid residues on the surface of protein globules (monomers). The selection was made using the results of the X-ray analysis of tetrameric GAPD from human liver and the model of tertiary structure of GAPDS (without N-terminal domain) presented in MODBASE [21]. Since there are no data concerning the structure of the N-terminal domain, its motifs were not considered apart from those that are specific for GAPDS.

Table 1. Motifs revealed in the sequences of somatic and sperm-specific GAPD

Motif	FS	Sequence	Isoenzyme
AP2-binding motif	27.814 for GAPD; 22.770 for GAPDS	DPF	somatic and sperm-specific
Phosphate-, FAD-, NADH-binding motif	9.666 for GAPD; 7.913 for GAPDS	G?G??G	somatic and sperm-specific
Motif responsible for protein degradation in the S-phase of the cell cycle	0.463 for GAPD; 0.759 for GAPDS	P?L	somatic and sperm-specific
Motif of the modification by SUMO-1 protein	2.038	V/I/L/A/F/P-K?E	somatic
Motif recognizing the unpaired thymidine in DNA	1.225	F?E	somatic
MAD2-binding motif	20.5	K/R-I/V-L/V-?????P	sperm-specific
Clathrin-box motif	3.315	L-I/V/L/M/F-I/V/L/M/F-D/E	sperm-specific
Motif interacting with WW-domains	12304.697	PPPPP-L/R	sperm-specific (N-terminal domain)

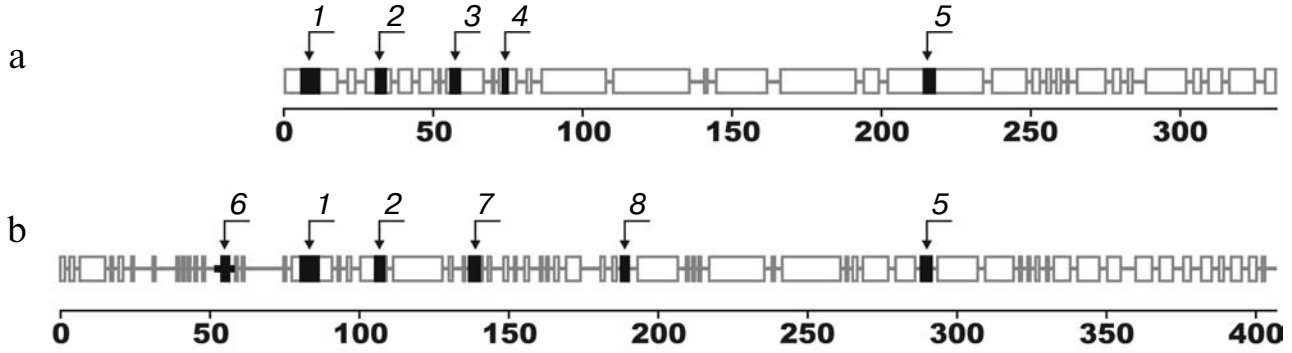

Fig. 2. Localization of the motifs in the sequences of human GAPD (a) and GAPDS (b). Absolutely conservative sites of mammalian enzymes are shown by rectangles: *1*) AP2-binding motif; *2*) phosphate-, NAD-, and FAD-binding motif; *3*) motif of the modification by SUMO-1; *4*) motif recognizing the unpaired thymidine; *5*) motif that is responsible for protein degradation in the S-phase of the cell cycle; *6*) WW-domain interacting motif; *7*) clathrin-box motif; *8*) MAD2-binding motif.

The detected motifs are given in Table 1, and the maps of the sequences of GAPD and GAPDS displaying the motifs and conservative fragments are shown in Fig. 2.

Three conservative motifs that are common for GAPD and GAPDS and located within homologous fragments of the sequences have been revealed: phosphate-, FAD-, NADH-binding motif, the AP2-binding motif, and the motif responsible for protein degradation in the S-phase of the cell cycle.

AP2-binding motif (FS 27.814) is a part of many auxiliary proteins involved in the formation of clathrin-coated transport vesicles (including endocytosis process) and serves for the interaction with AP2 (Adaptor Protein 2). The function of this motif is recruiting proteins to this process [22]. It is known that GAPD participates in endocytosis. Obviously, the AP2-binding motif of GAPD provides its interaction with AP2, thus fulfilling a regulatory function. Presumably, GAPDS also participates in the formation of transport vesicles.

Phosphate-, FAD-, and NADH-binding motif (FS 9.666) as a part of both GAPD and GAPDS interacts with NADH according to the structures of GAPD and GAPDS. Since this motif is involved in glycolysis, it is present in both isoenzymes.

The motif responsible for protein degradation in S-phase of the cell cycle (FS 0.463) is specific for nuclear proteins. Its presence in the sequence of GAPDS (which is not a nuclear protein [20]) and FS value close to 1 suggest that this protein fragment and the motif sequence are very likely to coincide through accidental reasons.

Within the sequence of GAPD, two conservative specific motifs have been revealed: a motif for modification by the protein SUMO-1 and a motif recognizing unpaired thymidine within DNA. The motif for modification by SUMO-1 (FS 2.038) (Small Ubiquitin-related Modifier), as well as ubiquitin, is a small protein that is capable of covalent binding to other proteins. Ligation of a protein to SUMO-1 affects the intracellular localization and protein–protein interactions of the modified protein. For instance, the modification of one of the adenoviral proteins by SUMO-1 results in its accumulation in the nucleus. In unmodified state this protein is translocated from the nucleus to the cytoplasm via the NES [23]. Ligation of SUMO-1 to GAPD also may activate its translocation into the nucleus, thus regulating such processes as DNA repair and induction of apoptosis.

Motif recognizing unpaired thymidine within DNA (FS 1.225) is revealed within the prokaryotic protein MutS, which participates in the repair of unpaired thymidine and G–T pairs. MutS recognizes mismatches anticipating their repair [24]. It was shown that GAPD also participates in DNA repair. This motif is likely to provide mismatch recognition. However, FS value close to 1 suggests that this GAPD fragment and the motif sequence are very likely to coincide through accidental reasons.

Within GAPDS, three conservative specific motifs have been revealed (the MAD2-binding motif, the motif interacting with WW-domains, and the clathrin-box motif). One of them (the motif interacting with WW-domains) is a part of the N-terminal domain.

The motif interacting with class II WW-domains (FS 12304.697) is supposed to be located in the N-terminal domain of GAPDS. WW-domains are constituents of many non-homologous proteins and interact with proline-rich ligands. As a rule, WW-domains are parts of cytoskeletal proteins or proteins participating in transmitting signals to the nucleus. Due to the fact that GAPDS is associated with the cytoskeleton (with fibrous sheath of the sperm flagellum), the detected motif may provide interaction with a cytoskeleton protein containing WW-domain, and thus affect intracellular GAPDS localization.

A fragment similar to MAD2-binding motif (FS 20.5) was found in CDC20 protein; it promotes anaphase in the complex with APC (anaphase-promoting complex). MAD2 controls mitotic spindle assembly, inhibiting the CDC20–APC complex (thus preventing

anaphase) in the case the kinetochores are not attached to the spindle. Previously, it was demonstrated that one of the dehydrogenases (GAPD from *Caenorhabditis elegans*) is able to interact with APC [25]. The presence of MAD2-binding motif in the sequence of GAPDS suggests that it participates in the control of spermatid spindle assembly.

Therefore, motifs participating in glycolysis and formation of clathrin-coated transport vesicles are common for both GAPD and GAPDS. Besides, an additional motif that is capable of interacting with clathrin was detected within the GAPDS sequence. Presumably, the formation of transport vesicles in spermatids differs from that in somatic cells. Therefore, GAPDS has the additional motif. Participation of GAPDS in the process of vesicle formation should be examined experimentally.

In GAPD, two specific motifs related to its nuclear function (the motif for DNA repair and replication, and the motif determining the intracellular localization of the protein) have been revealed. The absence of these motifs in GAPDS suggests that the nuclear functions are not characteristic for GAPDS. Presumably, GAPDS participates in the assembly of spermatid spindle. It appears that somatic GAPD does not participate in this process (in other cell types). The presence of all specific motifs within the sequences of both isoenzymes should be proved experimentally.

Motif interacting with WW-domains detected within the N-terminal domain of GAPDS may provide binding of this protein to the fibrous sheath of sperm flagellum. It was shown earlier that recombinant GAPDS lacking the N-terminal domain was not bound to the fibrous sheath [19]. Thus, the presence of this motif within the sequence of GAPDS may contribute to identification of the fibrous sheath protein to which GAPDS is attached (only spermatozoid structural proteins containing WW-domains should be examined).

Detection of phosphorylation sites within sequences of GAPD and GAPDS. The phosphorylation sites, as well as short motifs, were sought using the Minimotif Miner application. Among all findings, those located on the surface of GAPD globules were selected (see above). The detected phosphorylation sites are given in Table 2.

It was previously shown that GAPD from rabbit muscle contains phosphotyrosine [26]. This may be a result of phosphorylation by EGFR or ABL protein kinases (other protein kinases phosphorylate serine or threonine residues). Phosphorylation of GAPD by these protein kinases possibly regulates its glycolytic activity according to requirements of the organism (for example, under the action of the epithelial growth factor) or serves for regulation of its non-glycolytic functions.

Phosphorylation by CamKII protein kinase may have similar aims. It has been already proved experimentally in our laboratory that CamKII is able to phosphorylate GAPD [27, 28], which confirms the correctness of the motif detection.

Within the GAPD sequence, the following phosphorylation sites were found that are absent in GAPDS: phosphorylation site by EGFR (epidermal growth factor receptor) protein kinase, protein kinase C, and CamKII protein kinase (calmodulin-dependent kinase II). GAPDS is probably regulated by mechanisms different from those involved in the regulation of GAPD, which follows from the fact that there are no sites of phosphory-

Table 2. Possible phosphorylation sites of somatic and sperm-specific GAPD

Protein kinase	Sequence	Coordinates	Isoenzyme
ABL	I/V/L-Y?-P/F	43	somatic and sperm-specific
RSK	K/R-??-S/T	233, 308	somatic and sperm-specific
JAK-2	Y??-L/I/V	254	somatic and sperm-specific
EFGR	E/D-Y-I/L/V	92	somatic
Protein kinase C	S/T-?-K/R	24, 58, 142	somatic
RSK	K/R-??-S/T	79, 144	somatic
CamKII	R??-S/T	79	somatic
Casein kinase II	S/T-??-D/E	102	somatic
PLK	D/E-?-S/T-I/L/F/W/M/V/A	140	somatic
SRC	Y?E?E	348	sperm-specific
Tyrosine kinase	R/K-??-D/E-???-Y	395	sperm-specific
ABL	I/V/L-Y????-P/F	147	sperm-specific
JAK-2	Y??-L/I/V	173	sperm-specific
Casein kinase II	S/T-??-D/E	349	sperm-specific
PLK	D/E-?-S/T-I/L/F/W/M/V/A	144	sperm-specific

lation by EGFR, C, and CamKII protein kinases in the GAPDS sequence.

Thus, the presence of some detected phosphorylation sites within the GAPD sequence has been proved experimentally. Experimental checking of other suggested sites would clarify the role of previously unknown phosphorylation sites in the regulation of glycolytic and non-glycolytic functions of the enzyme.

Search for proteins containing NES-like motifs. Two human proteins containing conservative motifs similar to NES of GAPD have been revealed: the protein ECM29 (204.3 kD) and flotillin-2 (41.7 kD). It was demonstrated that 38% of ECM29 is localized in the cell nucleus [29]. Presumably, the NES-like fragment of the ECM29 sequence also serves as the nuclear export signal and thus affects translocation of the protein between the nucleus and the cytoplasm.

Finding of the NES motifs in other proteins, such as ECM29 and flotillin-2, is of importance while investigating the intracellular localization of GAPD with the use of immunofluorescence microscopy. Presumably, these proteins can interact with the monoclonal antibodies 6C5 against NES motifs of GAPD. Consequently, the use of the 6C5 antibodies can lead to incorrect results while immunofluorescence staining of the cells. Since the affinity of the 6C5 antibodies to GAPD can be much higher than to other mentioned proteins, this effect can be observed only under high concentration of the antibodies. This assumption was confirmed by the preliminary data on the immunofluorescence staining of spermatozoa with the 6C5 antibodies that do not interact with GAPDS. Spermatozoa are not stained with low concentrations of the 6C5 antibodies used for immunofluorescence staining of somatic cells. However, the addition of the same antibodies in high concentrations results in staining of cytoplasmic membrane, acrosome membrane, and the neck of the spermatozoa [20]. Presumably, these parts of spermatozoa contain proteins bearing the NES motifs interacting with the 6C5 antibodies. This assumption will be tested experimentally to reveal the proteins possessing NES-like motifs.

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