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New Nuclear Functions of the Glycolytic Protein, Glyceraldehyde-3-Phosphate Dehydrogenase, in Mammalian Cells

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Abstract Recent studies establish that the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is not simply a classical metabolic protein involved in energy production. Instead, it is a multifunctional protein with defined functions in numerous subcellular processes. New investigations establish a primary role for GAPDH in a variety of critical nuclear pathways apart from its already recognized role in apoptosis. These new roles include its requirement for transcriptional control of histone gene expression, its essential function in nuclear membrane fusion, its necessity for the recognition of fraudulently incorporated nucleotides in DNA, and its mandatory participation in the maintenance of telomere structure. Each of these new functions requires GAPDH association into a series of multienzyme complexes. Although other proteins in those complexes are variable, GAPDH remains the single constant protein in each structure. To undertake these new functions, GAPDH is recruited to the nucleus in S phase or its intracellular distribution is regulated as a function of drug exposure. Other investigations relate a substantial role for nuclear GAPDH in hyperglycemic stress and the development of metabolic syndrome. Considerations of future directions as well as the role of GAPDH post-translational modification as a basis for its multifunctional activities is suggested. J. Cell. Biochem. 95: 45–52, 2005.

Key words: glyceraldehyde-3-phosphate dehydrogenase; GAPDH; transcriptional regulation; membrane fusion; cancer chemotherapy; telomere structure; metabolic syndrome; post-translational modification

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) was intensively investigated as a classical metabolic enzyme involved in energy production. However, numerous studies established that it is, in reality, a multifunctional protein in mammalian cells [rev. in Sirover, 1999]. These investigations revealed that GAPDH displays a distinct membrane, cytosolic and nuclear localization. In those subcellular locales it functions in endocytosis and membrane fusion, vesicular secretory transport and translational control; nuclear tRNA transport, DNA replication, and DNA repair. GAPDH is implicated in apoptosis which may be predicated on its translocation into the nucleus. The key role of GAPDH in many of these processes was established by mutational studies, the use of antisense GAPDH constructs and transfection analysis utilizing the *GAPDH* gene. Alteration of *GAPDH* gene structure resulted in reduced endocytosis [Robbins et al., 1995]; depletion of GAPDH mRNA inhibited apoptosis while overexpression of the *GAPDH* gene induced programmed cell death [Ishitani and Chuang, 1996; Tajima et al., 1999, respectively].

The discovery of new GAPDH functions is usually established through the unexpected identification of its participation in a seemingly unrelated cell pathway. This is followed by its rigorous characterization as part of that cell process. It is also accompanied by the investigators initial puzzlement of how this energy related protein could be involved in the particular pathway [McKnight, 2003; Reusch, 2003]. This pattern of amazement is reflected in new studies that focus on the role of GAPDH in several important nuclear functions that are the subject of this review. In particular, the last

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few years have seen a series of intensive investigations characterizing fundamental nuclear processes all of which resulted in the identification of GAPDH as a critical protein required for that specific function. These include the transcriptional regulation of histone gene expression, the role of GAPDH in nuclear membrane fusion, its binding as a modulator of telomere structure and in the recognition of fraudulent nucleotides in DNA arising from cancer chemotherapy. Further, mechanisms of GAPDH function in vivo may have significant relevance to a new emerging health question that has become the focus of intense concern and study. This relates to the current alarm with respect to obesity in relation to the development of diabetes and coronary heart disease [Abraham, 2004; Scaglione et al., 2004]. In this regard, new studies demonstrate that hyperglycemic stress activates a complex signaling pathway resulting in the post-translational modification of nuclear GAPDH and the subsequent inhibition of cytosolic GAPDH [Du et al., 2000, 2003; Nishikawa et al., 2000]. It was postulated that this may provide a molecular foundation for metabolic syndrome, a precursor for diabetes and cardiovascular disease [Nathan, 1994; Reaven, 1995; Hanson et al., 2002]. Lastly, as stated, the focus of this review is on nuclear GAPDH structure and function. Accordingly, intriguing studies on the role of GAPDH in vesicular secretory transport are regrettably outside the scope of this review [Tisdale, 2001, 2002, 2003].

New Nuclear Functions of Mammalian GAPDH

As discussed, the traditional view of GAPDH focused on its cytosolic role in energy production. During that time, the likelihood of its nuclear localization was not a major focus of examination. However, as its multidimensional nature became appreciated, the importance of its nuclear localization and activities has grown in significance. This is reflected in its new nuclear roles, the control of its subcellular localization in relation to these new functions, as well as the potential mechanisms through which mammalian cells regulate GAPDH structure to fulfill these new responsibilities.

Regulation of histone gene expression by mammalian GAPDH. The importance of histones in chromatin structure is well established as is the strict control of their expression [rev. in Davie and Spencer, 1999; Khorasanizadeh, 2004]. Examination of histone gene regulation identified a protein, Octomer binding protein-1 (Oct-1), that was required for transcription of the *H2B* gene in S phase [Fletcher et al., 1987]. Paradoxically, it was determined that not only was the level of Oct-1 expression in the cell cycle invariant but also that Oct-1 was unable by itself to stimulate transcription using the H2B promoter. For that reason, it was postulated that an accessory protein, the Oct-1 CoActivator in S phase (OCA-S), was a necessary requirement for Oct-1 regulation of the *H2B* gene [Luo and Roeder, 1995].

Using stimulation of Oct-1 transcription in an in vitro assay, OCA-S was chromatographically purified from a HeLa cell nuclear extract [Zheng et al., 2003]. Seven proteins comprised the OCA-S complex including a protein with an $M_r = 38$ kDa. In what has become a common occurrence, the p38 protein was identified as GAPDH. Subsequent analysis demonstrated that anti-GAPDH antibodies co-immunoprecipitated Oct-1 as well as the other OCA-S proteins. Depletion of GAPDH by immunoprecipitation diminished transcription in vitro. Reduction of GAPDH expression by RNA interference reduced H2B transcription in vivo. Addition of NAD^+ stimulated transcription while NADH addition blocked transcription. These results are intriguing in that previous studies demonstrate, for the most part, that the multifunctional activities of GAPDH involve the NAD^+ binding site as demonstrated by competition studies [rev. in Sirover, 1999]. In this instance, NAD^+ is a stimulatory factor. This may indicate that the redox status of the GAPDH protein may be important. Flow cytometry and co-immunoprecipitation studies revealed the cell cycle recruitment of GAPDH to the H2B promoter. En toto, these findings demonstrate not only that GAPDH is part of the OCA-S complex but also that its presence is a physiological necessity.

Another indication of the role of GAPDH in transcription was revealed in studies of the promyelocytic leukemia protein (PML). Initial studies demonstrated an association between PML and GAPDH [Carlile et al., 1998]. Double labeling immunocytochemical analysis using confocal microscopy and co-immunoprecipitation was used to define interaction of PML and GAPDH. In the latter, immunoprecipitation was preformed with anti-GAPDH antibodies. Subsequent immunoblot analysis successfully

detected PML. In the reverse experiment, immunoprecipitation was performed with an anti-PML antibody then GAPDH was successfully identified by the immunoblot protocol. Intriguingly, RNase treatment demonstrated that the PML and GAPDH interaction was dependent on the presence of RNA.

The potential physiological significance of PML/GAPDH interactions was indicated by a recent study that revealed the association of PML nuclear bodies with transcriptionally active genomic regions [Wang et al., 2004]. These investigations reported that a histone encoding gene cluster associated with PML bodies in S phase. However, it was concluded that PML bodies were not necessary for transcription but were present at those sites. These findings present the initial studies indicating a significant role for GAPDH in, at a minimum, the control of histone gene expression especially during the cell cycle.

Regulation of telomere structure by mammalian GAPDH. As chromosomal termini, telomers represent an important loci responsible for the maintenance of cellular genetic information [rev. in Chakhparonian and Wellinger, 2003; Neidle and Parkinson, 2003]. Shortening of telomeric structure may provide a mechanism for aging while maintenance of telomere length may be involved in tumorigenesis. A major focus of investigation is the identification of specific proteins involved in these nucleoprotein complexes. A number of such proteins have already been identified, including telomere repeat binding factor (TRF)-1 and -2, Pot1, and hRap1 [Broccoli et al., 1997; Li et al., 2000; Baumann and Cech, 2001]. Of particular interest are proteins that may regulate telomere length and those that may be targets of compounds whose mechanism of action involves the regulation of telomerase activity or telomeric structure.

Recent evidence suggests that the sphingolipid ceramide regulates the former [Ogretmen et al., 2001]. Analysis of the latter indicated that ceramide treatment inhibited the telomeric binding of a protein with an $M_r = 36–40$ kDa [Sundararaj et al., 2004]. Protein analysis identified the protein as GAPDH. Once it was identified as the unknown protein, a series of strict experimental protocols were used to rigorously define its identity as well as its requirement for telomere integrity. These studies included the use of commercially available GAPDH which complexed to a telomere probe; competition analysis with NAD^+ which inhibited telomere binding, and the overexpression of GAPDH which abrogated telomere shortening after drug exposure.

Immunolocalization using confocal microscopy and immunoblot analysis revealed the cell cycle dependent nuclear distribution of GAPDH. Ceramide treatment prevented GAPDH nuclear localization. Lastly, and intriguingly, two-dimensional gel analysis revealed differences in intracellular GAPDH structure. Cytoplasmic GAPDH was characterized by a pI of 7.0–7.5 while its nuclear counterpart displayed a pI of 8.7. These findings are the initial demonstration of a new role for nuclear GAPDH involving its NAD^+ site, its proliferative dependent nuclear regulation, and specific changes in its structure that may be involved with this new function.

Role of GAPDH in nuclear membrane fusion. Previous studies detailed the significant role of GAPDH in membrane cell fusion. Mutational analysis revealed defects in endocytosis in vivo based on a $C \rightarrow T$ change resulting in a $Pro_{234} \rightarrow Ser$ substitution [Robbins et al., 1995]. Analysis in vitro identified membrane fusion catalysis by a non-catalytic isoform of GAPDH [Glaser and Gross, 1995]. New studies indicated that this capability was also a characteristic of the nuclear species [Nakagawa et al., 2003]. In these studies, anti-immune patient serum known to react with nuclear envelope (NE) proteins was tested for its ability to suppress NE assembly using an in vitro *Xenopus* egg extract nuclear assembly system. One serum, K199, strongly suppressed nuclear assembly and contained antibodies that reacted with proteins in *Xenopus* egg extract. Five antibodies were purified that reacted with proteins of $M_r = 35–54$ kDa. Protein analysis identified these proteins as GAPDH, fructose-1,6-bisphosphate aldolase and the regulation of chromosome condensation-1 (RCC1) protein. The role of GAPDH in nuclear membrane fusion was verified by the use of commercially available anti-GAPDH antibodies whose addition suppressed nuclear assembly activity. Addition of purified GAPDH resulted in its recovery.

Previous studies identified that a phosphatidylserine binding site of GAPDH was required for its participation in membrane fusion [Kaneda et al., 1997]. This site corresponded to amino acid residues 70–94 of GAPDH which was

termed GAPDH70. Addition of anti-GAPDH70 antibody reduced nuclear assembly activity. The observed inhibition was not detected when GAPDH70 was premixed with the antibody. Intriguingly, studies using an antibody against amino acid residues 390–403 of phosphatidylserine decarboxylase not only reacted with GAPDH in *Xenopus* egg cytosol but also diminished nuclear assembly. Confocal microscopy using the anti-GAPDH70 antibodies was utilized to identify the specific step in which GAPDH participates in nuclear assembly. Analysis of chromatin surface revealed that the nuclear envelope was not enclosed. Accordingly it was suggested that GAPDH participated in the nuclear membrane fusion step.

These results suggest a new nuclear function for GAPDH. It would be of interest to determine whether the non-catalytic isoform of GAPDH is the species which participates in nuclear membrane fusion. Further, as recent studies suggest that tubulin binds to and inhibits the membrane fusion activity of this isoform [Glaser et al., 2002], the effect of this protein interaction on nuclear assembly may be of interest. Lastly, with respect to cell pathology, mutations in lamin A, an important nuclear envelope protein, were identified as the molecular defect underlying Hutchinson-Gilford progeria syndrome [De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003; Mounkes et al., 2003]. Considering the role of GAPDH in membrane fusion, it may be reasonable to suggest that GAPDH:lamin A interactions may play a role in the cellular phenotype of that human genetic disorder.

Role of GAPDH in the recognition of fraudulent DNA nucleotides. Cancer chemotherapy relies in part on the use of structural analogues which are substrates for cell anabolic pathways. Included among these antimetabolites are purine agonists, cytosine arabinoside and 5-fluorouracil. As nucleotide precursors, these compounds will perturb nucleic acid structure and function when incorporated into DNA or RNA, thereby achieving tumor cell kill.

As such perturbations in DNA structure may be recognized by cellular DNA repair pathways, thiopurine cytoxicity was initially examined in six mismatch repair (MMR) proficient and deficient acute lymphoblastic leukemia cell lines [Krynetski et al., 2001]. Each was sensitive to thiopurine cytotoxicity but was independent of MMR (MutSα). Using synthetic duplexes containing a thioguanine residue (G^s) or DNA mismatches, a protein complex was identified by gel shift assay. GAPDH was identified as 1 of the 4–5 major proteins in that complex. Further analysis revealed the nuclear translocation of GAPDH subsequent to mercaptopurine exposure.

Protein mass spectroscopy was used to identify the 5 major proteins comprising this complex [Krynetski et al., 2003]. These included high mobility group (HMG) proteins B1 and B2, heat shock cognate (HSC) protein 70, protein disulfide isomerase (ERp60) and GAPDH. Immunoprecipitation analysis with anti-HMGB1 antibody resulted in a supershift of G^s duplexes. In contrast, complex formation was abrogated using an anti-GAPDH antibody. The general specificity of this complex with respect to the recognition of therapeutically incorporated fraudulent nucleotides was examined using cytosine arabinoside and 5-fluorouracil containing oligomers. The identical complex was observed in the gel shift assays.

As indicated, the formation of the fraudulent DNA recognition protein complex is accompanied by GAPDH nuclear translocation. This presumes that the GAPDH protein contains both nuclear localization and nuclear export sequences. Recently, a novel exportin1 or chromosome region maintenance (CRM)1-dependent nuclear export signal (NES) comprising 13 amino acids (KKVVKQASEGPLK) was identified in the C-terminal GAPDH domain [Brown et al., 2004]. Truncation or mutation of this sequence abrogated CRM1 binding and caused nuclear accumulation of GAPDH. Lys^{259} was identified as an important residue necessary for proper functioning of the NES. As such, these studies present the first identification and initial characterization of this new DNA recognition complex. The role of this complex in the response to the incorporation of structural analogues into DNA has not been determined. However, it was noted that apoptotic changes were detected 24 hr. after mercaptopurine exposure. Accordingly, this complex may participate in an initial cytotoxicity response as a first step towards programmed cell death.

GAPDH and Metabolic Syndrome

Metabolic syndrome is considered as a precursor for type 2 diabetes and cardiovascular disease. Also termed the insulin resistance syndrome, it is characterized by perturbations in glucose metabolism and insulin utilization producing hyperglycemic stress [Reaven, 1995;

Hanson et al., 2002]. Vascular damage results from the exquisite sensitivity of endothelial cells to hyperglycemic stress [Caballero et al., 1999]. These investigations revealed the seminal role of mitochondrial reactive oxygen species (ROS) as the molecular basis for vascular damage induced by metabolic syndrome [Uemura et al., 2001; Gross et al., 2003]. This correlated with previous studies that demonstrated that ROS formation diminished GAPDH glycolytic activity through a transitory reversible interaction [Knight et al., 1996].

Recent studies identified a unique twist interrelating metabolic syndrome with both the classical role of GAPDH in energy production as well as its new nuclear functions [Du et al., 2000, 2003; Nishikawa et al., 2000]. This new signaling pathway starts with the formation of mitochondrial ROS during hyperglycemic stress in endothelial cells. This is followed by their nuclear translocation inducing DNA damage including single strand breaks activating poly (ADP-ribose) polymerase. The significance of the latter is that this enzyme ADP-ribosylates nuclear GAPDH. Subcellular localization studies demonstrated the modification of cytosolic GAPDH indicating the movement of modified GAPDH within the cell.

These findings identified a new signaling pathway that is initiated by hyperglycemic stress and finishes with the unique diminution of intracellular GAPDH function. Its significance may relate to the pleiotropic effect of ADP-ribosylation on GAPDHs multiple nuclear, cytosolic and membrane functions. It is known that such alteration of GAPDH severely diminishes glycolytic catalysis causing an increase in the formation of advance glycation end (AGE) products and accumulation of glucosamine [Giardino et al., 1996; Shinohara et al., 1998]. In contrast, the effect of this post-translational modification on other GAPDH functions is unknown at the present time. Thus, further studies examining these activities as a function of hyperglycemic-induced oxidative stress may prove intriguing.

DISCUSSION

The new findings chronicled in this review identify four unanticipated roles for nuclear GAPDH (Table I). Taken individually or together, these novel activities may signify a quantum leap in the importance of GAPDH in mammalian cells. Combined with our previous knowledge of nuclear GAPDH function in tRNA transport, DNA replication, DNA repair and apoptosis, these cumulative findings may indicate that GAPDH is one of the most indispensable proteins in mammalian cells. This assertion does not even take into consideration its cytosolic and membrane functions.

The question then arises how a cell regulates not only the subcellular localization but also the intranuclear distribution of GAPDH so that it may fulfill its diverse functions. GAPDH is encoded by a single gene without alternate splicing. The latter would seem to obviate control mechanisms based on mRNA synthesis. However, Tsuchiya et al. [2004] identified a novel proapoptotic GAPDH protein resulting from a unique promoter upstream from the coding region (−154 to −84). A similar transcriptional control mechanism may underlie the role of GAPDH in the response to hypoxia [Graven et al., 1999]. In those studies, a hypoxia inducible factor-1 (HIF-1) binding site was identified (−130 to −112). A further study identified a second hypoxia response element in the *GAPDH* gene at position −217 to −203 [Lu et al., 2002]. Other control regions identified previously include a phorbol response element (gTRE), a c-fos/c-jun binding site as well as insulin response elements [Alexander-Bridges et al., 1992].

TABLE I. Nuclear Protein Complexes

Activity	Proteins	Nuclear GAPDH Translocation	Effect of NAD^+/NADH	GAPDH Isozyme
Telomer binding	TRF-1, TRF-2, Pot1, Hrap1, GAPDH	Cell cycle dependent	NAD^+-inhibition of GAPDH binding	Nuclear-pI 8.3–8.7, Cytoplasmic-pI 7.0–7.5
Transcriptional control	Oct-1, UNG, Hsp70, Stl1, LDH, GAPDH	Cell cycle dependent	NAD^+-stimulation of transcription NADH-blocked transcription	N.D.
Recognition of fraudulent DNA	HMGB1, HMGB2, HSC70, Erp60, GAPDH	Mercaptopurine dependent	N.D.	N.D.
Membrane fusion	Fructose-1,6-bisphosphate aldolase, RCC1, GAPDH	N.D.	N.D.	N.D.

These results demonstrate a complex mechanism of transcriptional control of GAPDH expression. However, irrespective of the stimulus, it would appear that each would regulate the synthesis of the identical transcript. Thus, it would be reasonable to suggest that other mechanisms may be responsible for the regulation of subcellular GAPDH isoforms.

Accordingly, an alternate approach is to consider post-translational control mechanisms in the regulation of new and previously described nuclear GAPDH functions (Fig. 1). This hypothesis postulates the presence of two separate sets of GAPDH isoforms. Termed $GAPDH_C$ and $GAPDH_N$, respectively, the former is characterized by a cytosolic localization with a pI of 7.0–7.5 while the latter is present in the nucleus and displays a pI of 8.6–8.7. The bases of this hypothesis are two reports identifying unique cytoplasmic and nuclear GAPDH isoforms [Saunders et al., 1999; Sundararaj et al., 2004]. The former identified for the first time increases in basic nuclear GAPDH species during apoptosis. The latter identified nuclear GAPDH isoforms with a pI of 8.6–8.7. These appear to be distinct from those species detected in the cytosol.

As indicated in Figure 1, this model predicts the transformation of $GAPDH_C$ into $GAPDH_N$ as a consequence of nuclear translocation. Subsequently, $GAPDH_N$ functions in each of its four indicated new nuclear functions as well as its previously identified nuclear activities [rev. in Sirover, 1999]. This model predicts also the existence of an enzymatic mechanism through which $GAPDH_C$ is modified to $GAPDH_N$ at some specific point during its translocation to the nucleus. Considering the basic change in pI, it may be reasonable to suggest phosphorylation as this post-translational modification. This

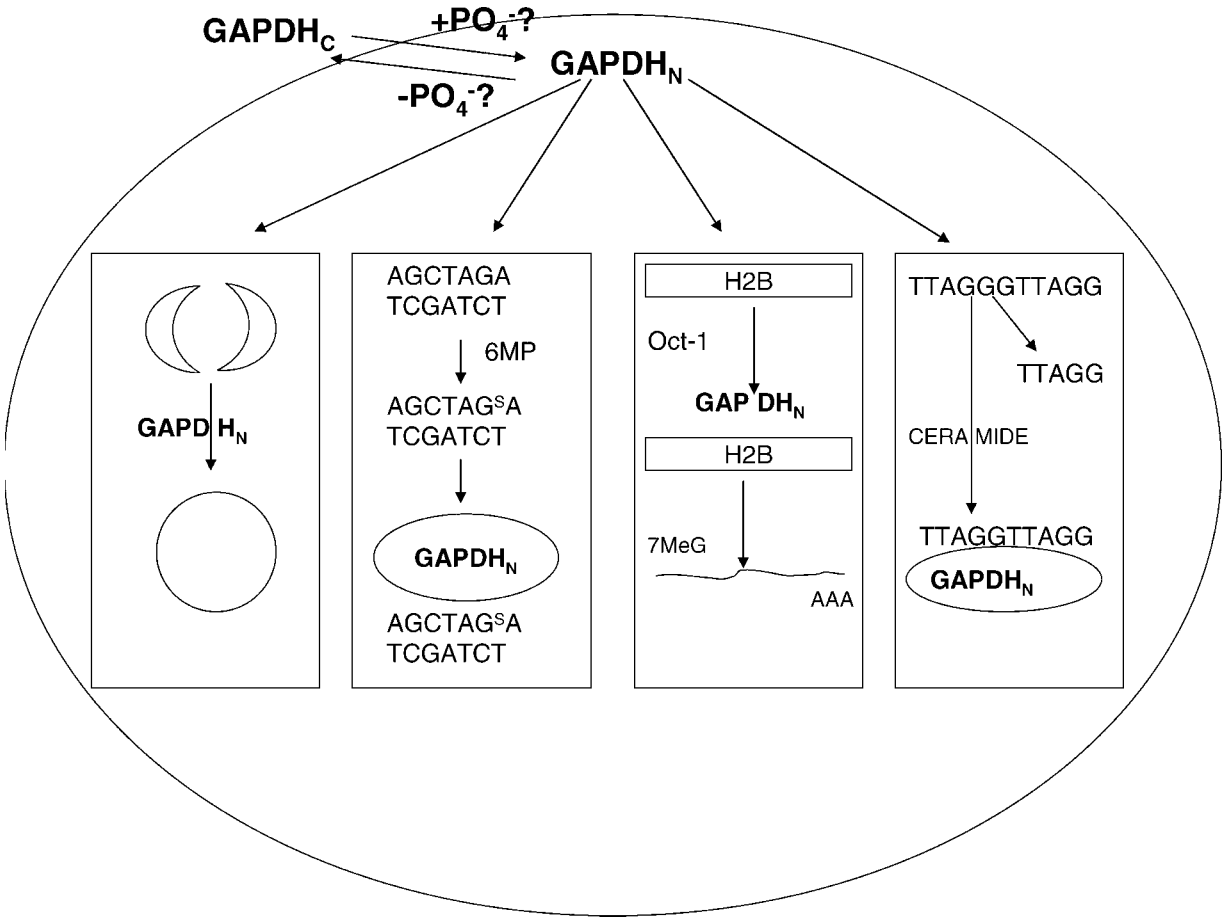

Fig. 1. Post-translational modification of GAPDH. Nuclear translocation of $GAPDH_C$ and itsconversion to $GAPDH_N$ is indicated as is the new functions of $GAPDH_N$ (Left to right: Membranefusion, binding to fraudulent DNA, regulation of histone 2B gene expression, maintenance oftelomere structure). Previously reported functions of $GAPDH_N$ are not illustrated [rev. in Sirover, 1999].

would be in accord with previously described GAPDH phosphorylation by protein kinases [Reiss et al., 1986; Ashmarina et al., 1988; Tisdale, 2002] or by its autophosphorylation [Kawamoto and Caswell, 1986]. Irrespective of the specific post-translational modification, a further support for this control mechanism is the defined cell cycle regulation of GAPDH subcellular localization defined in these new studies (Table I) and in previous investigations [Schmitz, 2001; Corbin Gong et al., 2002]. Such changes in its intracellular distribution may be more easily facilitated by changes in protein structure rather than by increased gene transcription. This may be tested experimentally.

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