1. Introduction

“How often have I said to you that when you have eliminated the impossible, whatever remains, however improbable, must be the truth?”

Sherlock Holmes to Dr. Watson in “The Sign of the Four”

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a protein well known to biochemists, to molecular biologists as well as to other scientists of all disciplines and of all ages. It's been studied as a classical protein not only for its role in glycolysis but also as a model protein for enzyme kinetic analyses, crystallographic modeling as well as for gene isolation and characterization. As such, GAPDH was thought of as a “cellular heirloom”; a protein, enzyme and gene from another age and from another time.

However, as with the conventional wisdom from so many areas of life, recent studies demonstrate that mammalian GAPDH is not merely a classical relic, useful only for mundane laboratory exercises or as the obligatory loading control in gene or protein analyses. Instead, as indicated in Fig. 1, it is a multifunctional protein with diverse activities and a distinct subcellular distribution [rev. in 1,2]. New and novel studies indicate that it is directly involved in transcriptional [3,4] and posttranscriptional gene regulation [5–8] and vesicular transport [9–15], receptor mediated cell signaling [16,17], chromatin structure [18,19], and the maintenance of DNA integrity [20]. Further, such investigations have yield significant insight into the cellular response to oxidative stress [21–29] and to apoptosis [30–38]. Surprisingly, other studies also indicate a role for GAPDH in autophagic gene regulation [39,40] as well as its fundamental role in a variety of pathologies including diabetes [41–48], age-related neurodegenerative disorders [rev. in 49–55], malaria [56] and even brucellosis in cattle [57]. Each report indicating the role of GAPDH in human disease is in accord with now classical studies interrelating GAPDH expression and tumorigenesis [rev. in 58–67].

Such diverse GAPDH functions (and the implicit changes in subcellular localization required for those activities) provide us with the proverbial enigma wrapped within a conundrum. GAPDH is a
highly conserved protein with little variance from species to species. It lacks a nuclear localization signal but does contain a nuclear export motif [68]. Genetic analysis indicates that mammalian GAPDH is transcribed from a single structural gene on human chromosome 12 locus or the mouse chromosome 6 [69,70] while a unique species is present in spermatozoa [71]. Alternative transcripts are not produced in somatic cells [72]. Accordingly, as a single mRNA is transcribed and translated, alternative mechanisms need to be utilized for GAPDH regulation.

Therefore, the goal of this review is not only to consider new, timely studies demonstrating the functional diversity of GAPDH but also to examine potential regulatory mechanisms through which its multiple activities may be controlled. Several mechanisms will be considered which may provide the basis through which GAPDH is recruited for different cellular processes. As this review will focus on recent studies with respect to GAPDH, the reader is referred to the author’s prior reviews for a discussion of those previous investigations which first indicated the functional diversity of mammalian GAPDH [1,2].

2. GAPDH and the regulation of intracellular membrane trafficking

Intracellular membrane trafficking requires the formation of multiprotein structures capable of movement from one subcellular localization to another [rev. in 73,74]. Such protein complexes may be formed in a defined temporal sequence based on individual amino acid sequences, protein-protein interactions and subsequent changes in spatial orientation. Once a nascent structure is formed, it may serve as a means to recruit yet other proteins, resulting in the formation of a mature complex. The formation of such complexes may require a unique, initiator protein which “drives” complex formation and may require a defined singling cascade for complex function.

Recent evidence indicates a central role for GAPDH in the formation, regulation and function of Rab2 mediated ER to Golgi cargo shuttling [9–15]. This involves its physical association with the membrane bound Rab2 protein complex [9], the observation that GAPDH glycolytic activity is not required for this new and novel function [12], the central role of GAPDH in altering cytoskeletal structure to facilitate intracellular membrane trafficking [10,15] and, lastly, the crucial role of GAPDH posttranslational modification as the basis for the signaling cascade which underlies Rab2 initiation of ER to Golgi cargo shuttling [10,11,13–15].

2.1. GAPDH and the initiation of ER to Golgi transport

Rab2 recruits a series of cytosolic proteins in a defined temporal sequence to form vesicular tubular complexes (VTC) which are involved in ER to Golgi trafficking [9]. The role of GAPDH in VTC formation and intracellular membrane trafficking was defined by immunoreactive GAPDH localized in vesicular tubular complexes. Kinetic analysis indicated that cytosolic GAPDH was recruited by Rab2 in a dose dependent manner which was blocked by anti-GAPDH antibody neutralization of its membrane association. The physiological relevance of GAPDH in membrane trafficking was indicated by the ability of the anti-GAPDH antibody to prevent ER to Golgi transport.

2.2. GAPDH catalysis and membrane trafficking

As indicated, GAPDH is a well studied protein and enzyme [rev. in 1]. As such, its structure is well known, its glyceraldehyde-3-phosphate (G-3-P) and NAD⁺ binding sites well characterized as well as its active site amino acids long identified. The latter includes cys149 which is an absolute requirement for its glycolytic activity. Accordingly, in studies defining the functional diversity of GAPDH, a critical question relates to the role of its active site in those new functions.

To assess the role of the later in membrane trafficking, a mutant GAPDH construct (C149G) was constructed in which the active site cys149 was replaced by a neutral substitution [12]. The mutant GAPDH protein failed to restore glycolytic activity to GAPDH depleted cytosol indicating that it lacked catalytic function. However, it was recruited by Rab2 to form VTCs at a rate equal to that observed for the wt enzyme. Further, it was efficiently phosphorylated by PKCδ (see below) and facilitated ER to Golgi shuttling in an in vitro trafficking assay equivalent to that observed for the wt protein. These results indicate that the integrity of the GAPDH active site is not required for this new cellular function.

2.3. GAPDH alters cytoskeletal structure to facilitate intracellular membrane trafficking

GAPDH: tubulin interactions provided one of the first bases to indicate the functional diversity of the former [rev. in 1]. However, the physiological significance of these findings remained unclear. As with many aspects of biomedical research, the latter required further investigation and analysis to determine the importance of these protein:protein interactions for normal cell function. In particular, recent evidence demonstrates that tubulin, in the form of microtubular filaments, provides the requisite cytoskeletal structure needed for membrane transport [10]. Further analysis revealed the presence of the motor protein dynein which facilitated intracellular trafficking [15].

These studies also revealed the central role of GAPDH in tubulin regulation and its recruitment of dynein for membrane trafficking. GAPDH facilitated cytosolic to membrane tubulin association and bundled microtubules changing tubulin structure from a perinuclear localization into a cross-linked subcellular network. Again, immunological analysis demonstrated that an anti-GAPDH antibody prevented cytosolic tubulin translocation; microtubule bundling and abrogated intracellular changes in tubulin structure. Preincubation with the anti-GAPDH antibody precluded dynein membrane association and subsequent function, demonstrating that GAPDH was required for the specific recruitment and function of this motor protein in membrane trafficking.

2.4. Sequential GAPDH phosphorylation provides the signaling cascade necessary for Rab2 mediated intracellular membrane trafficking

As illustrated, intracellular membrane trafficking is a complex process. It requires not only the participation of numerous proteins as well as the rearrangement of intracellular cytoskeletal structure followed by the transport of cargo from one subcellular localization to another.

It is axiomatic that such a complex biochemical pathway would require some type of regulatory mechanism to ensure not only proper
complex formation but also the temporal sequence with which each protein is recruited to become part of this subcellular structure. Recent evidence suggests that GAPDH is not only a key component of the complex but also is a focal point for those regulatory mechanisms which exist to control this complex biochemical pathway (Fig. 2).

This involves two separate and sequential post translational phosphorylation steps each catalyzed by a separate and distinct protein kinase: the first at GAPDH tyrosine 41 by Src [13,14], the second at a yet to be defined serine residue by PKC [10,11,15] It is reasonable to suggest that each phosphorylation sequentially changes GAPDH conformation resulting in defined changes in the structure of the membrane bound protein complex. The latter would facilitate the series of reactions necessary for intracellular membrane trafficking.

3. GAPDH and receptor mediated cell signaling

3.1. GAPDH and androgen receptor transactivation

The androgen receptor (AR) is a transcriptional factor which regulates prostate cell genes involved in both proliferation and in differentiation [rev. in 75,76]. When not bound to a ligand such as DHT, it is inactive, localized in the cytosol and associated with the chaperone proteins HSP-90 and HSP-70 [77,78]. Upon ligand binding, it undergoes a conformational change, dissociates from HSP-90 and HSP-70, and binds to a co-activator. The resultant protein complex translocates to the nucleus facilitating AR-induced gene activation. Accordingly, the identification of such co-activators and elucidation of their mechanisms of action are, by definition, functionally significant.

Recent evidence indicates that GAPDH specifically interacts with the androgen receptor as a co-activator and that the resultant cytosolic GAPDH-AR complex is translocated to the nucleus followed by AR transactivation [16]. Transfectional analysis indicated that GAPDH increased the transcriptional activity of the androgen receptor in a dose dependent manner. The specificity of this effect was noted by co-transfection analysis in which GAPDH did not affect the activity of the glucocorticoid receptor nor did it increase estrogen receptor-catalyzed activity. The former was determined by co-immunoprecipitation studies which demonstrated that GAPDH and the androgen receptor formed an intracellular protein complex. The latter was defined by the intracellular localization of the GAPDH:AR complex in the cytoplasm and in the nucleus.

3.2. GAPDH as a macrophtose transferrin receptor

Receptor-dependent uptake of iron is mediated by transferrin, a major plasma iron binding protein [rev. in 79–81]. The latter interacts with cell surface receptors, undergoes endocytosis then is internalized to early endosomes. Previous studies demonstrated that GAPDH is a cell surface protein specifically involved not only in endocytosis [82] but also in the intracellular transport of glutathione conjugates [83]. Recent evidence indicates that GAPDH, as a cell surface protein, specifically interacts with transferrin forming a GAPDH:transferrin protein complex, that this protein structure is internalized then translocated to early endosomes, and that GAPDH is specifically recruited to the cell surface as a function of extracellular iron concentration [17]. Further studies indicate a similar function for the S. aureus membrane GAPDH [84–86].

The functional role of GAPDH as a cell surface transferrin receptor is based on the localization of GAPDH on the surface of the macrophtose membrane. Of note, it was reported that this surface localization is dependent on iron media concentration, i.e., during iron depletion, levels of surface GAPDH increase, implying its active recruitment to the membrane cell surface. In addition, not only did GAPDH and transferrin co-localize but also ELISA demonstrated that GAPDH specifically interacted with transferrin. Co-immunoprecipitation using either anti-GAPDH or anti-transferrin antibodies demonstrated the formation of a GAPDH:transferrin protein complex. Confocal microscopy using surface biotinylated GAPDH revealed that this protein complex was internalized by endocytosis resulting in its movement to early endosomes.

As these findings are similar to those observed with GAPDH:AR binding, as indicated in Fig. 3, this suggests a common mechanism of GAPDH mediated receptor signaling. In both examples, this new and novel GAPDH function is clearly distinct from its classical role in glycolysis. Perhaps the most salient findings may be that GAPDH: receptor complexes are formed in one specific subcellular localization; that these complexes move from that region of the cell to a second, separate and distinct intracellular localization and that there needs to be a high affinity of GAPDH for the respective protein (or vice-versa) which ensures that the complex remains intact as subcellular movement occurs.

4. GAPDH and the maintenance of DNA integrity

The role of GAPDH in DNA structure and function was recognized early as part of the identification of its functional diversity [rev. in 1]. However, as with many of those studies, further investigation was required to define the physiological significance of GAPDH:nucleic acid interactions. In particular, two recent findings provide insight into this significant role of GAPDH in mammalian cells, i.e., its roles in the stabilization of telomere structure [18,19] and in the maintenance of DNA integrity through its modulation of DNA repair capacity [20]. These studies also highlight the continuing question of GAPDH nuclear
localization and the mechanisms through which this protein, which does not contain a nuclear localization sequence, is transported through the nuclear membrane in the absence of oxidative stress [87–91].

4.1. GAPDH and the prevention of telomere shortening

Telomeres fulfill a critical function maintaining not only the integrity of DNA but also that of chromosomal structure. Recent evidence indicates not only the functional role of GAPDH as a determinant of telomere structure but also the mechanisms involved in GAPDH:telomeric DNA binding [18,19]. GAPDH specifically binds telomeric DNA, the resultant GAPDH:DNA complex protects telomeres from shortening during replication; and, GAPDH protects telomeres from ceramide or cancer drug induced shortening.

GAPDH was identified as the major protein bound to single strand telomeric DNA. Ceramide, which induces telomere shortening, inhibited the formation of the GAPDH:telomeric complex. Cell cycle analysis revealed the dynamics of GAPDH:telomeric DNA binding. GAPDH and telomeric DNA co-localize in S phase nuclei. Nuclear GAPDH diminished during cell cycle progression which demonstrated the proliferative-dependent active movement of GAPDH from one subcellular localization to another. As defined by siRNA knockdown, reduction in endogenous GAPDH expression increased telomeric DNA shortening while overexpression of GAPDH abrogated telomeric DNA shortening induced by the cancer chemotherapeutic agents gemicitabine and doxorubicin. NAD⁺ was a competitive inhibitor of complex formation indicating the role of that binding site in this new GAPDH function.

Analyses of the mechanisms through which GAPDH and telomeric DNA interact revealed that GAPDH specifically bound single stranded telomeric DNA in vitro in the absence of other factors. Single base substitution studies indicated that binding of GAPDH required T1, G5 and G6 of the TTAGGG telomeric DNA repeat. Stoichiometric analysis indicated a molar ratio of 2:1 (DNA:GAPDH tetramer). GAPDH formed a nuclear telomeric DNA complex in vivo as defined by co-localization and by co-immunoprecipitation analyses. Mutational analysis revealed several salient aspects of GAPDH:telomeric DNA binding. Substitution of asp³² by alanine in the NAD⁺ binding site and replacement of cys¹⁴⁹ by alanine in the catalytic site abolished DNA binding, GAPDHala32 and GAPDHala149 localized to the nucleus but did not bind to telomeric DNA. Further, whereas wild type GAPDH prevented telomere shortening, each mutant GAPDH failed to preserve telomere structure. Accordingly, not only does this mutational study define this new, nuclear role of GAPDH, but also it demonstrates two salient characteristics of GAPDH function, i.e., changes in its subcellular location as a prerequisite for this new role and its disconnection from its classical glycolytic activity.

4.2. GAPDH and maintenance of DNA integrity by APE-1

Apurinic/Apyrimidinic Endonuclease (APE-1) is a required enzyme not only for the repair of DNA damage [rev. in 92] but also as a transcriptional regulator [93]. APE-1 can exist in two forms, APE-1α, the reduced form which is catalytically active, and APE-1β, the oxidized form, which is catalytically inactive. Formation of APE-1α occurs in vivo at a physiologically significant rate, endangering both the integrity of DNA and the regulation of gene transcription.

Recent evidence indicates that nuclear GAPDH is a major mechanism through which cells ensure that APE-1 remains in its catalytically active form [20]. As with other studies identifying the functional diversity of GAPDH, these investigations were initiated for another purpose, i.e., an analysis of APE-1 structure and function. In the course of these studies, one major protein was detected binding to APE-1. That protein was identified as GAPDH. Further analysis demonstrated that GAPDH was capable of catalyzing the conversion of APE-1α to APE-1β. The functional significance of this GAPDH activity was indicated by the restoration of APE-1 endonuclease activity subsequent to its inactivation by hydrogen peroxide chemical oxidation. The physiological relevance of this activity was demonstrated by the sensitivity of GAPDH knock down cells to methylmethylene sulfonate and bleomycin. Each chemical produces DNA lesions which result in the formation of AP sites. The latter are repaired by APE-1. Further, in those cells treated with GAPDH siRNA to reduce endogenous GAPDH levels, not only was the rate of spontaneous AP sites increased but also there was a simultaneous decrease in APE-1 activity. These results demonstrate that altering the endogenous level of GAPDH interferes with the ability of APE-1 to maintain DNA integrity and may be the first to demonstrate the relevance of GAPDH function to rates of spontaneous mutation in vivo. As such, GAPDH may function as a “guardian protein” protecting APE-1 activity through its ability to reactivate APEIα to APEIβ.

5. GAPDH and the regulation of gene expression

As previously reviewed [2], recent studies indicated the role of GAPDH as an integral part of the Oct-1 OCA-S coactivator protein complex which controls histone gene expression [3,4]. Its functional diversity was dependent on GAPDH-Oct-1 protein interactions, the potentially predominant role of GAPDH in OCA-S complex transcriptional activity, its S phase recruitment, structural changes in nuclear GAPDH required for its role in the OCA-S complex, and, lastly, the stimulatory effect of NAD⁺. The latter may be particularly intriguing as other new functions of GAPDH usually require its NAD⁺ binding site. As such, NAD⁺ is normally a competitive inhibitor of GAPDH functional...
diversity. Since that report, other studies indicate additional roles through which GAPDH may affect gene expression.

5.1. GAPDH and the posttranscriptional regulation of gene expression

Posttranscriptional control of mRNA function may depend on proteins which bind to its 5′ and/or 3′ untranslated regions (UTR). Previous analyses defined GAPDH as a 3′-UTR binding protein involving the interaction of AU rich elements (ARE) in the respective mRNA with the GAPDH NAD⁺ binding site as well as its role as a 5′ UTR binding protein [rev. in 1].

These studies established not only GAPDH:mRNA binding in vitro but also their co-localization in vivo [94]. However, as with many early studies on GAPDH, their potential physiological significance was uncertain. Now, recent investigations suggest that GAPDH may function as an mRNA destabilizing protein, accelerating mRNA decay, thereby diminishing the biosynthesis of the respective protein [7]. Paradoxically, other new reports indicate that GAPDH may function as an mRNA stabilizing protein preventing mRNA decay thereby ensuring the continued biosynthesis of the respective protein [5,6]. With respect to the former, those investigations indicate the utility of GAPDH in maintaining homeostasis while the latter may not be an advantageous new function.

5.2. GAPDH and the posttranscriptional regulation of ET-1 mRNA stability

Vascular homeostasis depends on a number of critical parameters, messenger compounds such as nitric oxide as well as a number of protein; whose expression affects vascular structure and function. The latter includes ET-1, an endothelial vasoconstrictor which is also required for normal embryonic development. It may also be involved in the pathogenesis of heart, renal and pulmonary disease [rev. in 95].

Transcriptional and post transcriptional mechanisms of gene regulation may control ET-1 expression [96]. With respect to the latter, recent studies indicate that AU rich regions in its 3′-UTR may regulate ET-1 synthesis, i.e. decay constants of ARE-containing elements are double those with mRNAs not containing those sequences [7]. Further, binding of cytosolic proteins destabilized ET-1 mRNA leading to its decay. Reduced expression of ET-1 correlated with increased binding of such proteins.

As with many such studies which resulted in the identification of new GAPDH functions, subsequent inquiry focused not on GAPDH but sought to isolate and characterize the cytosolic binding ET-1 binding proteins [7]. A major protein of 40 kDa was detected. Peptide mass fingerprinting identified the protein as GAPDH. Substitution of commercial rabbit muscle GAPDH diminishes binding. Mutant construct analysis identified ARE regions as the GAPDH binding site.

Down regulation by siRNA established the role of endogenous GAPDH in ET-1 expression. Intriguingly, GAPDH depletion reduced its protein levels by 50% while ET-1 expression levels were increased by 50%, suggesting their stoichiometric, albeit inverse, relationship. Diminution of GAPDH was accompanied by a significant reduction in the ability of cytosolic extracts to bind ET-1 mRNA as well as a lower rate of RNA degradation. Analysis of ET-1 mRNA structure suggested that GAPDH binding resulted in the unwinding of ET-1 within the 3′ UTR making it more susceptible to attack by cytosolic ribonucleases. This hypothesis would be in accord with previous studies using circular dichroism analysis which demonstrated that GAPDH binding to the 5′-UTR sequences of hepatitis A virus destabilized its helical structure [97].

The role in ET-1 mRNA binding was determined using amino acid substitution or deletion. The latter analysis demonstrated that only full length constructs bound to the RNA probe demonstrating the requirement for both the catalytic and the NAD⁺ binding sites. In accord with that observation, competition experiments showed that both G-3-P and NAD⁺ inhibited binding. Concentration curves suggested inhibition at Kₘ values. With respect to the former, substitution of serine for cys¹⁵² did not affect RNA binding.

As discussed in Sections 6 and 7, below, GAPDH is a unique cellular target for nitric oxide resulting in its posttranslational modification. For that reason, in these studies the effect of the latter on ET-1 mRNA expression was determined. These studies revealed an intriguing twist, interrelating GAPDH-nitric oxide interactions with its role in the regulation of ET-1 mRNA expression.

Studies with 5-nitrosoglutathione (GSSG) indicated that pretreatment with GSSG inhibited the degradation of ET-1 mRNA in extracts of wt cells but not in the GAPDH⁻/⁻ mutant. These results in accord not only with previous findings that glutathione bound to the Rossman fold in GAPDH [83] but are consistent with the regulatory role of protein S-glutathionylation [s-glu, rev. in 98]. Further, using the nitrothiol GSNO or GSSG, oxidative stress-induced modification of GAPDH⁻⁻/⁻ reduced binding to ET-1 3′-UTR ARE sequences. This reduction was also observed using commercial GAPDH as well as H₂O₂ exposure in vitro which can induce S-glutathionylation. H₂O₂ exposure in vivo resulted in a time and concentration dependent increase in ET-1 mRNA expression. The former was prevented by the antioxidant N-acetylcysteine.

A model illustrating the consequences of GAPDH:ET-1 mRNA binding and the effects of oxidative stress on this interaction is illustrated in Fig. 4. As indicated, GAPDH binding to ET-1 (left) results in the diminution of ET-1 protein levels due to the postulated increase in its mRNA degradation facilitated by GAPDH unwinding of its secondary structure, thereby preventing its vasoconstrictive activity. In contrast (right), oxidative stress induces S-glutathionylation of GAPDH at cys¹⁵², thereby ablatting GAPDH binding to ET-1 mRNA. The resulting increase in ET-1 protein level would facilitate its vasoconstrictive function identifying a new potential mechanism underlying the pathology of nitric oxide.

5.3. GAPDH and colony stimulating factor-1 (CSF-1) gene expression: protein–nucleic interactions as a regulatory mechanism increasing CSF-1 mRNA stability

Colony-stimulating factor (CSF-1) may be involved as a hematopoietic cytokine regulating macrophage cell proliferation and invasive differentiation. Its expression may be clinically significant, associated with a poor prognosis as a marker of tumor cell growth [rev. in 99]. New studies indicate that the CSF-1 gene contains a specific genomic element which may define its mRNA stability and thus its protein expression [5]. In particular, genetic analysis identified exon 10 as a specific region which appears to be a decay element, inducing a pronounced down regulation in protein expression. Further, it contained a 144 nt ARE sequence which formed a ribonucleoprotein complex with a 37 kDa protein. The latter was identified as GAPDH whose deletion by preincubation with an anti-GAPDH antibody prevented complex formation.

Although the above studies identified the GAPDH:CSF-1 nucleic acid interaction, its physiological significance was uncertain. Analyses of the mechanisms through which GAPDH and CSF-1 interacted in ovarian cancer cells revealed that GAPDH was specifically required for the maintenance of CSF-1 mRNA stability thereby maintaining not only its expression but also CSF-1 protein levels [6]. In particular, GAPDH depletion by RNA interference reduced steady state CSF-1 mRNA and protein levels. GAPDH bound to a defined AU-rich region within the 3′UTR of CSF-1 mRNA, in accord with previous findings with respect to GAPDH:RNA binding [rev. in 1].

Of note, this role is different from those previously described for ET-1, i.e., instead of participating in a normal cell function, this GAPDH activity may provide a foundation for the stimulation of tumor cell growth, contributing to the role of GAPDH in human pathology and
disease. This would be in accord with a series of studies indicating the physical interaction of GAPDH with viral mRNA [rev. in 1].

5.4. GAPDH and the posttranscriptional regulation of AT1R mRNA stability

The angiotensin II type 1 receptor (AT1R) binds to, and regulates the function of, angiotensin II, an important cardiovascular protein. As with ET-1, AT1R expression is regulated at many levels including posttranscriptional control. Accordingly, it may not be surprising that not only does the AT1R mRNA contain a 3′ UTR AU rich destabilization element but also that cellular protein binding to that element regulates AT1R translation.

As with other studies, isolation and characterization of the latter revealed a major binding protein of 36 kDa which was identified as GAPDH by peptide mapping [8]. AT1R translation was increased by GAPDH silencing. This is a particularly important study in as much as GAPDH may comprise approximately 10–15% of total cell protein. Given that predominance, it’s binding to cellular macromolecules could be viewed as non-specific in nature, given the laws of mass action. Accordingly, at the present time, knock down studies may be considered as obligatory requirements for the identification of new GAPDH functions.

Subsequent analysis revealed an intriguing twist with respect to the role of GAPDH in AT1R posttranscriptional regulation. In particular, although structural and sequence binding studies indicated a potentially similar GAPDH binding motif to that observed for ET-1 mRNA, there was no similar decrease in AT1R mRNA levels as a consequence of GAPDH silencing. This was the exact opposite finding as that observed for ET-1 mRNA. Accordingly, it was suggested that, in this instance, GAPDH binding inhibited directly AT1R mRNA translation instead of unwinding its structure to facilitate its degradation.
5.5. GAPDH and the posttranscriptional regulation of gene expression

If the above hypothesis is correct, as indicated in Fig. 5, it would indicate that there are three different mechanisms through which GAPDH may regulate posttranscriptional gene regulation, i.e., its binding may increase mRNA stability (CSF-1), relax mRNA structure facilitating the latter’s degradation (ET-1), or, lastly, competitively inhibit its translation (AT1R).

These observations may pose an intriguing intracellular dilemma. In each instance, GAPDH binds to the respective 3-UTR decay elements. Structural analysis would predict that the binding site is presumably identical in each mRNA. Yet, the functional result arising from that binding is uniquely distinct. This would suggest that perhaps the binding sites are actually different, that there needs to be a control mechanism such that GAPDH binding to that single site results in three such disparate results, or that other sequences in the respective mRNA may be downstream regulatory determinants.

6. GAPDH and oxidative stress

Past studies indicated the susceptibility of GAPDH to oxidative stress based on S-nitrosylation of its active site cysteine [rev. in 1,21,24]. However, GAPDH is not merely a passive target whose inactivation results in cell toxicity. Instead, it is a critical focus of the cell’s response to reactive oxygen species. Surprisingly, this may involve the formation of two competing GAPDH protein complexes.

6.1. GAPDH and Siah1: oxidative stress-induced protein interactions as a mechanism to induce cell toxicity

Siah1 is an E3 ubiquitin ligase catalyzing protein degradation [100–102], a major mechanism underlying apoptotic cell death. However, Siah1 is inherently unstable. Accordingly, under normal physiological conditions, its constitutive levels are minimal. New studies indicate a unique relationship between GAPDH and Siah1 [22,23]. GAPDH was identified as an Siah1 binding partner in vivo as defined by a yeast two-hybrid screen using C-terminal GAPDH sequences as bait. Further studies demonstrated the formation of a GAPDH:Siah1 protein:protein cytoplasmic complex as determined by co-immunoprecipitation and immunoblot analysis. Sequence comparison indicated that the former contains a Siah1 binding motif surrounding GAPDH425. Mutation of that amino acid abolished binding.

Formation of the GAPDH:Siah1 complex increased Siah1 stability as determined by its decreased turnover in vivo. Mutation of GAPDH425 in the Siah1 binding site reduced Siah1 half-life. Significantly, oxidative stress-induced S-nitrosylation of GAPDH increased Siah1 binding which was both time-dependent and a function of SNO-GAPDH formation. Mutation of its active site cysteine, which is required for SNO modification, abolished binding. S-nitrosylation of GAPDH increased nuclear translocation of the SNO-GAPDH:Siah1 protein complex. Introduction of GAPDH siRNA abrogated nuclear translocation. The physiological significance of the nuclear SNO-GAPDH:Siah1 protein complex is discussed in the next section on apoptosis.

6.2. GOSPEL, GAPDH and oxidative stress: protein interactions as a regulatory mechanism mitigating GAPDH induced cell toxicity

Recent evidence suggests that GAPDH is not only a key target for oxidative stress but, also paradoxically, that it also may be a focal point for protective mechanisms which seek to mitigate the toxic effects of reactive oxygen species. These studies indicate that cells contain a unique protein, termed GOSPEL (GAPDH’s competitor of Siah Protein Enhances Life). GOSPEL was isolated from a yeast two-hybrid screen using N-terminal GAPDH sequences as bait. GOSPEL is widely expressed and appears to be solely a cytosolic protein [28, commented on in 29].

GOSPEL and GAPDH are both S-nitrosylated in a time-dependent manner. The former, once it is SNO-modified, binds to similarly SNO-modified GAPDH. Kinetic studies indicate that GOSPEL modification occurs earlier than that observed for GAPDH. Mutational analysis suggests that GOSPELcyt47 and GAPDHcyt150 are required as defined by lack of SNO modification of GOSPEL (C47S) and GAPDH (C150S), respectively.

The significance of GOSPEL:GAPDH binding is that it prevents the formation of the SNO-GAPDH:Siah1 complex. GOSPEL competes with Siah1 for GAPDH binding as defined by in vitro competitive binding analyses. Of note, SNO-GOSPEL appears to be a more effective inhibitor of SNO-GAPDH:Siah binding than the latter is of SNO-GOSPEL:SNO-GAPDH binding. Further, reduction of endogenous GOSPEL by siRNA increased cell toxicity upon oxidative challenge.

As indicated in Fig. 6, these studies suggest a complex biochemical pathway through which cells regulate the structure and function of GAPDH subsequent to oxidative challenge. Of particular interest may be the observation that mapping studies indicate that SNO-GOSPEL binds to the N-terminal portion of GAPDH while Siah1 binds to its C-terminal end. This suggests the possibility that a transitory ternary protein complex may be formed as a reactive intermediate. The latter would have a defined half life and specific conformational changes would need to occur for the dissociation of SNO-GOSPEL.

7. GAPDH and apoptosis

Nitric oxide-induced cell injury triggers apoptosis, the defined program of gene expression which results in ordered cell death. Although extensive (and elegant) studies indicated both the nuclear localization and the contribution of GAPDH to apoptosis, its role in programmed cell death was uncertain [rev. in 1]. Now, recent investigations have not only defined that contribution but also have indicated it may comprise two separate and independent functions, each of which may be essential in apoptosis.

7.1. GAPDH-Siah1 in apoptosis-I: protein ubiquitination and degradation in programmed cell death

As indicated in the previous section, oxidative stress induces not only the formation of an SNO-GAPDH:Siah1 complex but also its subcellular nuclear translocation. The physiological significance of complex formation and translocation was defined by its induction of apoptosis. This was demonstrated through its initiation of programmed cell death in cerebellar granule neurons as a function of NMDA-induced oxidative stress as well as the induction of apoptosis in macrophages as a function of lipopolysaccharide (LPS) exposure. The former, a glutamate derivative, activates nNOS while the latter increases inducible NOS (iNOS). Apoptotic induction is eliminated both in cerebellar granule neurons in nNOS knockout mice as well by GAPDH or Siah1 siRNA in wt animals. Similarly, LPS-induced apoptosis is eliminated in macrophages isolated from iNOS knockout mice and reduced in wt macrophages by an iNOS inhibitor, by GAPDH siRNA or by antisense GAPDH [22].

Mechanisms which underlie the role of GAPDH:Siah1 involve ubiquitination of, and degradation of, nuclear proteins. Mutational deletions which eliminate Siah1 activity, which delete its nuclear
localization signal or which preclude GAPDH-Siah1 complex formation abrogate apoptosis. However, of further interest, are studies which indicated that GAPDH-Siah1 induced apoptosis may be independent of GAPDH glycolytic activity. This was defined by in vitro biochemical assay, i.e., catalysis in LPS macrophage exposed cells was equivalent to that of control cells during programmed cell death.

7.2. GAPDH-Siah1 in apoptosis-II: regulation of protein function in programmed cell death

Recent evidence suggests that GAPDH is not only a key target for oxidative stress-induced apoptosis through stimulation of the latter’s E3 ubiquitin ligase activity but also because it initiates an apoptotic program of gene expression [26]. New studies indicate that the latter is based on the binding of S-nitrosylated GAPDH:Siah1 to P300/CBP forming a second nuclear protein complex which is abrogated in iNOS knockout mice. The significance of this second protein:protein interaction is that, once the complex is formed, it initiates a series of reactions leading to the acetylation of SNO-GAPDH at lys160. Mutation of GAPDHlys160 (K160R) or depletion of P300/CBP by RNAi abolished GAPDH acetylation. The latter induces P300 autoacetylation in LPS treated macrophages. Inhibition of iNOS activity, depletion of GAPDH by RNAi or expression of GAPDH K160R (a dominant negative mutant) abolished P300 modification. GAPDH/P300 complex formation and modification initiates an apoptotic pleiotropic cascade which involves downstream regulation of p53, PUMA, Bax and p21. Increases in the expression of gene may be intrinsic to the initiation of programmed cell death. With respect to the latter, ChIP (chromatin immunoprecipitation) identified a p53-p300-sulfonated GAPDH complex at the PUMA promoter region. GAPDH/P300 downstream regulation of p53 and PUMA is required for apoptosis in LPS treated macrophages or GSNO treated neuroblastoma as determined by RNAi depletion of p53 or by overexpression of GAPDH K160R, respectively.

As indicated in Fig. 7, these studies suggest also a complex biochemical pathway through which cells regulate oxidative stress-induced apoptosis through changes in the structure and function of GAPDH. This new and novel effect of GAPDH requires its dual post-translational modification, reminiscent of the twin phosphorylations essential for its role in the regulation of intracellular membrane trafficking. The presumption is that each modification sequentially changes GAPDH conformation as well as the structure of the GAPDH protein complex thereby facilitating the series of reactions necessary for downstream regulation of apoptotic genes. Of further interest are recent intriguing studies indicating that GAPDH may function as a nitrosylase, transferring its NO group to an acceptor protein [103, commented on in [104] as well as being a major protein involved in H2S signaling mechanisms [105]. Each of these new and significant GAPDH functions has the potential to initiate a pleiotropic cascade by selectively affecting gene expression. As such, in toto, these findings suggest that GAPDH is a key focal point for the regulatory mechanisms which control apoptotic induction subsequent to oxidative challenge.

8. GAPDH and autophagy

Apoptosis provides a mechanism through which damaged cells undergo a program of cell death thereby ensuring overall survival. Apoptosis may involve mitochondrial outer-membrane permeabilization (MOMP) and caspase activation. Alternatively, in caspase-independent cell death (CICD), damaged cells are also destroyed from within, again providing a means for the removal of non-viable cells [40]. CICD may also result from mitochondrial dysfunction or as a consequence of substances freed from this organelle by MOMP. In contrast, autophagy represents a restorative pathway through which...
damaged cells may undergo intracellular repair resulting in the recovery of viability and normal cell function [rev. in 106]. As with apoptosis and CICD, this requires a defined program of selective gene expression. Recent evidence suggests that GAPDH may be intimately involved in the autophagic process [39].

As with many GAPDH studies, the goal of these investigations was not to define new functions for GAPDH. Instead, the basic question asked was “What proteins may be involved in cell recovery from CICD when apoptosis is prevented?”. To answer this question, now classical molecular technology protocols were used, screening retroviral cDNA library transfected Jurkat cells for such proteins based on clonal survival after apoptotic stimuli in the presence of apoptotic inhibitors. In this manner, GAPDH was identified as the protein in clones which exhibited the ability to prevent CICD but not apoptosis.

These studies identified two mechanisms through which GAPDH functioned to increase cell survival: the cytoplasmic production of ATP through glycolysis to provide an energy source in the absence of mitochondrial function and the nuclear regulation of autophagic gene expression. With respect to the former, GAPDH increased ATP levels 2-fold above that seen in controls. With respect to the latter, GAPDH induced the transcription of act12, a gene required for autophagy. Act12 induction substitutes for GAPDH. Noteworthy, the use of GAPDH mutant constructs indicates that both functions are required for protection against CICD, i.e., by themselves GAPDH mutants which reduce glycolysis or which prevent autophagic gene expression failed to protect against CICD whereas co-transfection of both mutants provided protection similar to that seen for wt GAPDH.

As overexpression of GAPDH was required for protection against CICD, it may be argued that this represents merely a laboratory finding which may not relate to the functional diversity of GAPDH in vivo. Although in normal cells this may be true, the role of GAPDH in cell protection may provide a cell survival mechanism related to the pathology of human disease. GAPDH expression is elevated in most, if not all, cancers [rev. in 58–60] and is also aberrantly regulated in age-related neurodegenerative disorders [49–55]. As such, this new GAPDH function may be of importance with respect to the etiology of each disease. This may be experimentally verified.

9. Conclusions

“Moonlighting” proteins display activities unrelated to those classical functions through which they were identified and characterized; participate in cell pathways which are separate and distinct from those which they are long associated and accepted; and, perhaps most surprisingly, exhibit distinct changes in their subcellular localization which are a priori requirements for these alternative functions [107].

GAPDH may be considered as the prototype for this new class of proteins. Its cellular functions are varied, its interactions are complex and its dysfunction may contribute to significant human pathologies. These studies indicate the dynamic nature of GAPDH in vivo as contrasted with previous concepts of it as a glycolytic protein of little interest.

However, the mechanism through which this functional diversity is controlled has remained elusive. In spite of past studies which indicated the presence of numerous GAPDH pseudogenes [rev. in 1,108] and isozymes [rev. in 1], its transcription in somatic cells from a single gene [69,70] and the absence of alternate transcripts [72] indicated that such regulation occurred downstream of any transcriptional control process. Alternatively, as with spermatozoid GAPDH [71], unique GAPDH species may exist which fulfill not only a significant function in normal cells but may also be involved in the pathology of human disease [109].

Recent findings indicate that the driving forces which modulate the functional diversity of GAPDH are those which change its three-dimensional conformation thereby modulating its diverse activities. These include specific post-translational modifications, protein:protein interactions and subsequent alteration in its intracellular distribution. Of note, each is highly regulated, occurring within distinct and complex biochemical pathways, providing the rate limiting step through which the latter is controlled.

This is illustrated in Figs. 2 and 7, respectively, in which GAPDH phosphorylation during membrane trafficking and its S-nitrosylation as an apoptotic-inducing signal represent the rate-limiting step in each pathway without which function is limited and subcellular translocation is forestalled. As with Shakespearean drama, events preceding modification are the antecedent Acts I and II, while those events which follow modification (Act III) are the sequelae (Acts IV and V), i.e., the inevitable consequences arising from the latter seminal event. Thus, with respect to the former, binding of the requisite proteins are the antecedent events while changes in tubulin structure are the consequence of GAPDH phosphorylation (and provide the explanation for earlier studies indicating the ability of GAPDH to bundle microtubules, rev. in [1]). With respect to the latter, a more complex pattern emerges with a single antecedent post-translational modification followed by two separate, and significant consequences.

In contrast, GAPDH protein:protein interactions do not appear to involve a post-translational modification. However, binding either to transferrin, the androgen receptor, APE-1 or telomeric DNA would, nevertheless, initiate changes in GAPDH three-dimensional structure thereby affecting function. Of note, the sequences within the GAPDH molecule critical for these interactions are not well characterized and the molecular modeling to understand such changes may be lacking. Other unresolved questions remain. These include the requirement, or the lack thereof, of the GAPDH active-site cysteine, the mechanisms through which GAPDH is distributed normally, those which control its dynamic intracellular movement including its nuclear translocation in the absence of oxidative stress. With respect to the latter, GAPDH is present in the nucleus functioning in the maintenance of chromatin structure, in DNA replication or its repair as well as in transcriptional expression. Those functions require its nuclear translocation which presumably does not involve its S-nitrosylation or, perhaps, its acetylation [110]. Therefore a reactive oxygen species-independent mechanism may exist through which this protein is transported into the nucleus. The latter is particularly evident during cell proliferation in which there appears to be an active cytoplasmic to nuclear to cytoplasmic change in its intracellular distribution. Each of these may bear examination.

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References
