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GAPDH binds Akt to facilitate cargo transport in the early secretory pathway

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) undergoes numerous post-translational modifications, which impart new function and influence intracellular location. For example, atypical PKC α phosphorylates GAPDH that locates to vesicular tubular clusters and is required for retrograde membrane trafficking in the early secretory pathway. GAPDH is also required in the endocytic pathway; substitution of Pro²³⁴ to Ser (Pro²³⁴Ser) rendered CHO cells defective in endocytosis. To determine if GAPDH (Pro²³⁴Ser) could inhibit endoplasmic reticulum to Golgi trafficking, we introduced the recombinant mutant enzyme into several biochemical and morphological transport assays. The mutant protein efficiently blocked vesicular stomatitis virus-G protein transport. Because GAPDH binds to microtubules (MTs), we evaluated MT binding and MT intracellular distribution in the presence of the mutant. Although these properties were not changed relative to wild-type, GAPDH (Pro²³⁴Ser) altered Golgi complex morphology. We determined that the GAPDH point mutation disrupted association between the enzyme and the serine/threonine kinase Akt. Interestingly Rab1, which functions in anterograde-directed trafficking, stimulates GAPDH-Akt association with membranes in a quantitative binding assay. In contrast, Rab2 does not stimulate GAPDH-Akt membrane binding but instead recruits GAPDH-aPKC. We propose a mechanism whereby the association of GAPDH with Akt or with aPKC serves as a switch to discriminate between anterograde directed cargo and recycling cargo retrieved back to the ER, respectively.

Keywords

GAPDH; Intracellular trafficking; Akt; Microtubules; Rab1; Rab2

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Author contribution

E.J. Tisdale designed and performed experiments, analyzed the data, and wrote the paper. N. Talati prepared recombinant proteins and performed experiments. C.R. Artalejo contributed to experimental design and data analysis. A. Shisheva contributed to experimental design and data analysis. All authors reviewed the contents of the manuscript.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme found in all tissues that catalyzes the nicotinamide adenine dinucleotide-mediated oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3 diphosphoglycerate [1]. Although once thought to be a simple housekeeping protein, GAPDH is now recognized as a multi-functional protein: various post-translational modifications of GAPDH contribute to the enzymes diverse activities that also influence GAPDH intracellular location and protein-protein association [2,3]. Indeed, we found that GAPDH is phosphorylated by atypical PKC (α PKC) and that the resulting phosphoGAPDH influences microtubule dynamics in the early secretory pathway [4]. Moreover, vesicular stomatitis virus G-protein (VSV-G) trafficking between the ER (endoplasmic reticulum) and the cis Golgi compartment required Src-dependent tyrosine phosphorylation of residue 41 in GAPDH [5]. In addition to phosphorylation, GAPDH is reversibly S-nitrosylated by nitric oxide at the active site cysteine, which promotes binding with Siah1 and subsequent translocation of the complex into the nucleus [6]. GAPDH has also been found modified by the acetyltransferase p300/CREB binding protein (CBP) at Lys¹⁶⁰ [7], whereas hyperglycemia-induced GAPDH inhibition was the result of poly ADP-ribosylation [8]. In contrast to these protein modifications that induce GAPDH structural/activity changes, Robbins and coworkers found that a single amino acid substitution in GAPDH (Pro²³⁴Ser) generated by random chemical mutagenesis rendered CHO cells defective in endocytosis [9]. However, the mechanism by which the GAPDH mutant altered endocytosis was not clearly defined.

Both the endocytic and the secretory pathways utilize the process of vesicular trafficking to move molecules within the dynamic endomembrane system [10–12]. The ongoing cargo and membrane exchange process involves vesicle fission and fusion, events regulated by the Rab family of small GTPases. To date, more than 70 different Rab proteins have been identified [13]. For example, Rab1 and Rab2 are required for transport in the early secretory pathway. Rab1 regulates ER-Golgi transport and intra-Golgi trafficking, whereas Rab2 facilitates retrograde transport from pre-Golgi intermediates back to the ER [13–16]. Golgi function and organization is controlled by a variety of signaling molecules and signal transduction pathways [17,18]. Protein kinases may also participate in inter-compartment cargo transport. For example, the serine/threonine kinase Akt (protein kinase B) phosphorylates Sec24, a component of COPII, suggesting a potential role in ER to Golgi transport [19]. Additionally, Du et al. reported that an Akt-dependent event was necessary for transport of the sterol regulatory element binding protein-2 in the early secretory pathway [20]. Akt has also been shown to regulate vesicle-mediated transport of ceramide from the ER to the Golgi [21]. Interestingly, several studies have reported that Akt binds to GAPDH [22, 23]. The Akt protein family regulates a wide range of biological processes: cell growth, cell differentiation, survival, and cellular metabolism [24,25]. Akt is activated at the plasma membrane and recruited to multiple intracellular compartments where a variety of substrates are phosphorylated, which in turn either positively or negatively regulates substrate function. In that regard, studies in cardiac muscle showed that Akt-bound to GAPDH increased GAPDH catalytic activity [26]. In ovarian cancer cells, GAPDH-Akt is inhibited from nuclear translocation [22].

Here, we investigated the effect of GAPDH (Pro²³⁴Ser) on VSV-G transport in the early secretory pathway. The mutant enzyme efficiently blocked membrane trafficking in an in vitro biochemical and a morphological transport assay. Therefore, we hypothesized that GAPDH (Pro²³⁴Ser) must be affecting a molecule(s) that functions in both secretion and endocytosis. Because GAPDH binds directly to MTs and MT integrity is critical for efficient membrane trafficking, we evaluated the effect of GAPDH (Pro²³⁴Ser) on the cytoskeleton in tissue culture cells. Although the MT network appeared relatively organized and intact, we observed a dramatic change in Golgi morphology suggesting that the GAPDH mutant influenced the activity of a molecule(s) that regulates membrane transport and/or Golgi structure. Interestingly, unlike wild-type GAPDH, the mutant enzyme does not associate with Akt, a kinase that also plays a role in endocytosis [27,28]. Moreover, an inverse relationship between Akt and aPKC recruitment to membrane was observed in a quantitative membrane binding assay: Rab1 recruited Akt whereas Rab2 recruited aPKC. Our results suggest a mechanism in which GAPDH-Akt and GAPDH-aPKC associations play a distinct role in Golgi homeostasis by regulating anterograde directed cargo versus retrograde-directed cargo transport in the early secretory pathway.

2. Materials and methods

2.1. Construction of GAPDH mutant

The site-directed mutation was made in GAPDH by a two-step PCR procedure involving two complementary mutagenic oligonucleotides in combination with flanking 5' and 3' primers. In the first reaction, overlapping 5' and 3' fragments were produced using pDual GAPDH as the template [29]. The 5' mutagenic oligonucleotide (5'-GGCATGGCCTTCCGTG.

TCTCGACTGCCAACGTG-3') and the 3' wild-type antisense oligo-nucleotide primer containing an EcoRI site (5'-GGCGGGGAAATTCTTACTCCTTGGAGGCCAT-3') was used to produce the 3' portion of the molecule. The 5' portion of the molecular was generated using a 5' oligonucleotide that included a *Bam*HI site or Nde I site (5'-GGCGGATCCATGGGGAAGGTGAA.

GGTCGGAGTCAACGG-3') in combination with the 3'-mutagenic oligonucleotide primer (5'-CACGTTGGCAGTCGAGACACGGAA GGCCATGCC-3'). The two PCR products were combined to generate the full-length mutant in a second reaction using the respective 5' sense and 3' antisense primers. Full length GAPDH (Pro²³⁴Ser) was subcloned into pcDNA4/HisMax-TOPO (Invitrogen, Carlsbad, CA) or into pGEX-2T (GE Healthcare Life Sciences), or into pET100/D-TOPO (Invitrogen) and then the sequence verified by automated DNA sequence analysis.

2.2. Purification of recombinant proteins

BL21 (DE3) pLysS cells (EMD4 Millipore, Billerica, MA) that contained the recombinant plasmid pET100/D-TOPO GAPDH/GAPDH (Pro²³⁴Ser) or pGEX-2T GAPDH/GAPDH (Pro²³⁴Ser) were grown at 37 °C to 0.6_{A600}, and then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 4 h at 32 °C. The liquid culture was centrifuged at 4300g for 30

min and the pellet resuspended in cold PBS, 0.1% Triton X-100, 1 mM PMSF, sonicated, centrifuged at 17,211 *g* for 20 min, and then the supernatant applied to a 1 ml column of Ni²⁺-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) equilibrated in Buffer A (10 mM Hepes (pH 7.9), 5 mM MgCl₂, 0.1 mM EDTA, 50 mM NaCl, and 0.8 mM imidazole). The column was washed with 10 volumes of Buffer A containing 25 mM imidazole. The tagged protein was eluted with Buffer A supplemented with 200 mM imidazole. Alternatively, the lysate was applied to a PBS washed glutathione-Sepharose 4B column and eluted with 5 mM reduced glutathione [29]. The eluted recombinant proteins were dialyzed against the appropriate buffer, concentrated using a Centrprep centrifugal filter device (EMD Millipore), and then the total protein concentration determined by BCA protein assay (Pierce, Rockford, IL).

2.3. Analysis of transport, in vitro

Normal rat kidney (NRK) cells were infected for 4 h with the temperature sensitive VSV strain (ts045), and then biosynthetically radiolabeled with 100 μ Ci Expre^{35S}35S (PerkinElmer Life Sciences, Downers Grove, IL) for 10 min at the restrictive temperature (39.5 °C) to maintain the VSV-G mutant protein in the ER. The cells were perforated by swelling and scraping, and then employed in the ER to cis/medial Golgi transport assay [5,15]. Transport reactions were performed in a final volume of 40 μ l in a buffer which contains 25 mM Hepes-KOH (pH 7.2), 75 mM KOAc, 2.5 mM MgOAc, 5 mM EGTA, 1.8 mM CaCl₂, 1 mM N-acetylglucosamine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate and 0.2 IU rabbit muscle creatine phosphokinase), 5 μ l rat liver cytosol, (~20 μ g of total protein), and 5 μ l of semi-intact cells (~5 $\times$ 10⁷ cells/ml, ~25–30 μ g total protein) resuspended in 50 mM Hepes-KOH, 90 mM KOAc (pH 7.2). The reactions were supplemented with the indicated concentration of purified recombinant GAPDH or GAPDH (Pro²³⁴Ser), incubated at 32 °C for 40 min, and then transferred to ice to terminate transport. The cells were pelleted 30 s in a microfuge, solubilized in buffer and digested with endoglycosidase H (endo H) (New England Biolabs, Ipswich, MA). The samples were analyzed by SDS-PAGE and the fraction of ts045 VSV-G protein processed to the endo H resistant forms (% of total) quantitated by a Storm PhosphorImager (GE Healthcare Life Sciences, Piscataway, NJ).

2.4. Morphological assay/transfection/indirect immunofluorescence

NRK cells plated overnight on coverslips were infected with VSV ts045 as above, and then shifted to ice and permeabilized with digitonin (20 μ g/ml) for 5 min [30]. Coverslips with permeabilized cells were inverted and placed in tissue culture wells that contained the transport cocktail described above, preincubated on ice for 10 min with 100 ng purified recombinant GAPDH or 100 ng GAPDH (Pro²³⁴Ser), or no addition (control) and then incubated for 40 min at 32 °C. To terminate transport, the cells were transferred to ice and fixed in 3% formaldehyde/PBS for 20 min, blocked for 1 h in PBS/5% NGS normal goat serum (NGS). For transfection experiments, NRK cells were plated overnight on coverslips (5 $\times$ 10⁵ cells /75 mm dish), and then transiently transfected with pcDNA4/HisMax-TOPO-GAPDH or pcDNA4/HisMax-TOPO-GAPDH (Pro²³⁴Ser) using Lipofectamine (Life Technologies, Grand Island, NY) for 36 h at 37 °C in a 5% CO₂ incubator. The cells were fixed for 20 min with 3% formaldehyde, washed 3X with PBS, and then blocked for 1 h in

PBS/5% NGS. The fixed cells were then incubated for 30 min at RT with the indicated primary antibody; monoclonal antibody to VSV-G (P5D4) (Sigma-Aldrich, St. Louis, MO), anti-GM130 (BD Biosciences, San Jose, CA), anti- α/β tubulin polyclonal (Cell Signaling, Danvers, MA), or anti-p58 cytoplasmic tail [31], washed with PBS, incubated with Texas-red conjugated anti-mouse or FITC conjugated anti-rabbit (Pierce) for 30 min at RT, washed and mounted in Mowiol (EMB Millipore, Billerica, MA) containing DABCO (Sigma-Aldrich), and then viewed with a Zeiss Axio Imager epifluorescence microscope (Carl Zeiss, Gottingen, Germany) and photographed with an AxioCam MRm camera (Zeiss Microimaging, Thornwood, NY). In some cases, AxioVision Z-stack software was used to capture 15 image of $\sim 3.6 \mu\text{m}$ (Zeiss Microimaging). The image threshold was obtained for both channels and the extent of true co-localization in the boxed area was analyzed using the Manders overlap co-localization coefficient (co-localization module, Zeiss Microimaging). A value of 1 is high co-localization and value of 0 is low co-localization.

2.5. Immunoprecipitation

NRK cells (3×10^6) were transfected with empty vector (control), or pcDNA4/HisMax-TOPO-GAPDH or pcDNA4/HisMax-TOPO-GAPDH (Pro²³⁴Ser), as described above. Forty-eight hours post-transfection, cells were scraped off the dish, lysed in TBS/1% Triton X-100, and equal total protein (5 mg/ml) processed for immunoprecipitation and western blotting, as previously described [29] using anti-Xpress antibody (Invitrogen).

2.6. MT binding assay

MTs were assembled from 1 μg of pure bovine brain tubulin (Cytoskeleton, Inc., Denver, CO) in 20 μl of Buffer B (100 mM Pipes, (pH 6.8), 2 mM MgCl_2 , and 0.5 mM EGTA) containing 20 μM paclitaxel (Sigma-Aldrich) for 20 min at 37 °C [32]. The MTs were then incubated with 100 ng of purified recombinant His6/Xpress-GAPDH or His6/Xpress-GAPDH (Pro²³⁴Ser) in a total volume of 50 μl for 30 min at 37 °C. The reaction mixtures were layered onto 250 μl of a 20% sucrose cushion in Buffer B containing 20 μM paclitaxel, and then centrifuged for 30 min at $100,000 \times g$ in a Sorvall M120 Discovery ultra-microcentrifuge using a S120-AT2 rotor. The resultant supernatant and pellet were separated by SDS-PAGE and transferred to nitrocellulose. The blot was probed with the appropriate antibody as indicated under “Results,” washed, further incubated with HRP-conjugated anti-mouse or anti-rabbit antibody, and then developed with ECL.

2.7. Quantitative membrane binding assay

HeLa membranes ($\sim 30 \mu\text{g}$ of total protein) prepared as previously described [4,5] were added to a reaction mixture that contained 27.5 mM Hepes (pH 7.4), 2.75 mM MgOAc , 65 mM KOAc , 5 mM EGTA, 1.8 mM CaCl_2 , 1 mM ATP, 5 mM creatine phosphate, 0.2 IU rabbit muscle creatine phosphokinase, rat liver cytosol ($\sim 25 \mu\text{g}$ total protein, depleted of GAPDH [29]), 1 μM GST-GDP Dissociation Inhibitor-1 or -2 [33], and 2.0 μM $\text{GTP}\gamma\text{S}$ [32,34]. Purified recombinant GAPDH or GAPDH (Pro²³⁴Ser) prepared as described above, was added at the concentrations indicated under “Results,” and the reaction mix incubated at 32 °C for the specified times in the presence or absence of purified recombinant in vitro isoprenylated Rab1 or Rab2 [16,29]. The binding reaction was layered onto a 20% sucrose cushion and centrifuged at $35,000 \times g$ for 20 min at 25 °C. The proteins in the recovered

pellet were separated by SDS-PAGE and transferred to nitrocellulose in 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol. The membrane was blocked in Tris-buffered saline that contained PhosphoBLOCKER (Cell Biolabs, Inc., San Diego, CA) or 5% nonfat dry milk and 0.5% Tween-20, incubated with the indicated primary antibody, washed, further incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at RT, washed, developed with enhanced chemiluminescence (GE Healthcare), and then quantified by densitometry using the ImageQuant program (GE Healthcare). Several films of different exposure times were analyzed to ensure that the signals were within the linear range of detection sensitivity.

2.8. GST-pull down

Purified recombinant GST-GAPDH or GST-GAPDH (Pro²³⁴Ser) or GST (5 µg) were preabsorbed to 20 µl glutathione Sepharose 4B for 1 h at room temperature by rotation, collected by centrifugation at 1700×g for min, and then washed 3X with TBS and 1% Triton X-100 to remove any unbound protein. The beads were resuspended in 50 mM Tris (pH 7.5), 5 mM MgCl₂, 100 mM NaCl, 10 µM GTPγS (ICN Biochemicals Inc., Irvine, CA), and then 5 µl rat liver cytosol added (~20 µg of total protein and depleted of GAPDH) [29] and incubated for an additional 2 h at RT. The beads were washed 4X with 1 ml of 50 mM Tris (pH 7.5), 10 mM MgCl₂, and 100 mM NaCl, and the beads with bound proteins boiled in sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was blocked as above, and the membrane probed with an anti-GAPDH monoclonal antibody (Chemicon Intl.), anti-GST monoclonal (Novagen, Madison, WI), anti-phosphoAkt (Ser473) polyclonal antibody (Cell Signaling), or with anti-Akt 9 (pan) polyclonal antibody (Cell Signaling), washed, incubated with an HRP-conjugated secondary antibody, and then developed with ECL.

2.9. Assay for GAPDH activity

Purified recombinant GST-GAPDH or GST-GAPDH (Pro²³⁴Ser) (1 µg) were first incubated in 10 mM sodium pyrophosphate (pH 8.5), 20 mM sodium phosphate, 0.25 mM β-NAD (MP Biomedicals, Inc.), and 3 µM DTT at 25 °C for 5 min to establish a baseline. The reaction was initiated by the addition of 0.4 µM glyceraldehyde-3-phosphate (Sigma-Aldrich) and the catalytic reduction of NAD to NADH was monitored at the indicated times described under “Results” by an increase in absorbance at 340 nm. The GAPDH units correspond to the mass (µM) of GAPDH converted/min/mg. The mass of NADH generated was calculated using $\epsilon=0.622$ [29].

2.10. Blue native gel electrophoresis

Purified recombinant GAPDH and GAPDH (Pro²³⁴Ser) (100 ng) was separated on a 4–16% NativePage Bis-Tris gel as outlined by the manufacturer (Novex/Thermo Fisher Scientific) and the relative molecular size assigned based on migration of NativeMark protein standards (Novex/Thermo Fisher Scientific) [35].

3. Results

3.1. GAPDH (Pro²³⁴Ser) inhibits VSV-G transport from the ER to the Golgi complex

To determine if GAPDH (Pro²³⁴Ser) had an effect on the early secretory pathway, we performed an in vitro ER to Golgi complex transport assay [5,36]. For this assay, NRK cells were first infected with ts045 VSV-G, a type I transmembrane protein that is retained in the ER at 39.5 °C and acquires two N-linked oligosaccharides. The plasma membrane of the infected cells is then perforated to release soluble content, but retain functional ER and Golgi compartments. Upon temperature shift to 32 °C and addition of a transport cocktail, ts045 VSV-G exits the ER in a synchronized manner. ts045 VSV-G transport to the Golgi results in processing to endo H-resistant forms (R₂) that are detected by mobility shift after SDS-PAGE and subsequently quantitated by Phosphorimager analysis. As shown in Fig. 1A, VSV-G transport was reduced by ~50% (S) when 100 ng of GAPDH (Pro²³⁴Ser) was added to the assay. In contrast, VSV-G trafficking was not affected when the transport assay was performed in the presence of recombinant wild-type GAPDH. Similarly, pulse chase experiments of GAPDH (Pro²³⁴Ser) transfected/ts045 VSV-G infected intact cells accumulated ~49% endo H sensitive VSV-G forms compared to ~8% in GAPDH-WT transfected cell (data not shown).

Additionally, ts045 VSV-G transport was monitored by indirect immunofluorescence to observe the morphological phenotype of VSV-G accumulation in the presence of the GAPDH mutant [5,30]. As above, NRK cells were infected with virus for 2 h at the restrictive temperature, permeabilized, and incubated with recombinant wild type GAPDH or GAPDH (Phe²³⁴Ser) for 30 min at 32 °C. Cells treated with the GAPDH mutant accumulated ts045 VSV-G in perinuclear and peripheral punctate structures that failed to transport into the centrosomal region (Fig. 1B). These morphological results are consistent with the effects of GAPDH (Pro²³⁴Ser) on the processing of ts045 VSV-G to endo H-resistant forms measured in the biochemical assay and suggest that the mutant GAPDH protein blocks cargo transport before fusion with the cis Golgi compartment.

Incubation of semi-intact cells at 15 °C leads to the accumulation of ts045 VSV-G in vesicular tubular clusters (VTCs) [15,30,37]. VTCs are pleomorphic structures that sort anterograde-directed cargos from recycling proteins and trafficking machinery that is returned to the ER [37,38]. GAPDH (Phe²³⁴Ser) associated with VTCs, assessed by co-distribution with anti-p58, a marker protein of VTCs (boxed area: Manders coefficient of 0.922) (Fig. 2A) [15]. To establish if GAPDH (Pro²³⁴Ser) inhibited transport from these structures, semi-intact cells were preincubated for 60 min at 15 °C, and then shifted to 32 °C in the presence of the purified recombinant GAPDH wild-type or GAPDH mutant. Wild-type GAPDH treated cells processed ts045 VSV-G to endo H-resistant forms with no lag time (Fig. 2B). In contrast, within ~5 min of incubation at 32 °C, transport of ts045 VSV-G was > 80% insensitive to the GAPDH mutant suggesting that GAPDH (Pro²³⁴Ser) functions at an early event in cargo transport from the VTC to the cis Golgi compartment (Fig. 2B).

3.2. GAPDH (Pro²³⁴Ser) causes Golgi fragmentation without altering microtubule organization

The ability of GAPDH (Pro²³⁴Ser) to affect endocytosis and secretion implied that the mutant protein was either dysfunctional or that the mutant protein was interfering with the activity of a molecule(s) that participates in both pathways. To address the possibility that the point mutation rendered GAPDH nonfunctional, we assessed the biochemical properties of the mutant protein. First, the enzymatic activity of purified recombinant GAPDH or GAPDH (Pro²³⁴Ser) was determined by measuring an increase in absorption at 340 nm resulting from NAD reduction. The dehydrogenase activity of the mutant protein was comparable to wild type GAPDH (Fig. 3A). Similar to GAPDH, the mutant GAPDH assembled into a tetrameric form, as determined by subjecting the recombinant proteins to Blue Native gel electrophoresis and mobility analysis relative to known marker proteins (Fig. 3B). These results indicate that the biochemical properties of GAPDH (Pro²³⁴Ser) are comparable to wild type GAPDH, which does not interfere with membrane trafficking.

In our previous studies, we reported that GAPDH influenced MT dynamics in the early secretory pathway [4]. Based on those observations and the fact that MT-based vesicle transport also facilitates endocytic membrane-trafficking events [39–41], we assessed the MT binding ability of the mutant protein. Purified recombinant GAPDH wild type or GAPDH (Pro²³⁴Ser) were incubated with paclitaxel assembled MTs for 20 min at 37 °C. As shown in Fig. 4A, the GAPDH mutant co-sedimented with MTs in the high speed pellet indicating that the point mutation does not affect MT association. We then evaluated the effect of ectopic overexpression of GAPDH (Pro²³⁴Ser) on MT organization. Although the MT distribution appeared normal and intact (Fig. 4B), indirect immunofluorescence staining for the cis Golgi complex marker protein GM130 showed that the organelle was extended, containing tubular projections and fragments (Fig. 4B, a', b'). It appears that GAPDH (Pro²³⁴Ser) does not block ER to Golgi transport by altering MT stability, but by influencing the activity of a molecule(s) that are essential for maintaining the structure of the Golgi complex and/or membrane trafficking.

3.3. Akt does not bind to GAPDH (Pro²³⁴Ser)

Since the substituted proline²³⁴ in GAPDH is adjacent to the threonine that binds Akt [22], we first performed immunoprecipitation experiments of total cell lysates from wild-type and mutant transfected cells to learn if Akt interacts with GAPDH (Pro²³⁴Ser). Although Akt was found in the GAPDH wild type immunoprecipitate, we did not detect Akt associated with GAPDH (Pro²³⁴Ser) in the recovered immune complex (Fig. 5A). Similarly, Akt did not associate with GAPDH (Pro²³⁴Ser) in GST-pull down experiments (Fig. 5B). These combined results are highly suggestive that the proline to serine substitution at position 234 eliminated GAPDH-Akt interaction and may contribute to the dominant-negative effect of the GAPDH mutant on VSV-G transport.

Based on our previous observations that Rab2 stimulates GAPDH binding to membranes, we employed a quantitative membrane binding assay to learn if Akt was co-recruited [29,34]. For this assay, salt-washed HeLa cell membranes were incubated with GAPDH-depleted rat liver cytosol [29] and GTP γ S without or with purified recombinant GAPDH (Fig. 6A) or

GAPDH (Pro²³⁴Ser) (Fig. 6B). The reaction was then centrifuged through a 20% sucrose cushion and the pellet containing membranes and associated proteins were subjected to SDS-PAGE and Western blot analysis. Although Rab2 dependent membrane-associated GAPDH was observed, the amount of Akt bound to membrane was trivial and comparable to control membranes (Fig. 6A). However, when purified recombinant Rab1 was added to the binding assay, the level of membrane-bound Akt increased considerably (Fig. 6A). In contrast, a negligible amount of Akt was present on membranes treated with the GAPDH mutant and Rab1 (Fig. 6A). Importantly, the absence of Akt recruitment was not due to the inability of GAPDH (Pro²³⁴Ser) to associate with membranes, as the mutant protein was detected in both Rab1 and Rab2 treated membranes (Fig. 6B). Moreover, the membrane-associated Akt was activated, as assessed by anti-phospho-Akt (Ser⁴⁷³) (Fig. 6A). As we previously reported, Rab2 stimulated aPKC membrane binding whereas Rab1 incubated membranes failed to recruit aPKC above the level detected in control (Fig. 6A, B). These collective results suggest that either aPKC or Akt binds to membrane associated GAPDH, which is determined by the respective Rab protein.

4. Discussion

GAPDH has multiple intracellular activities in addition to the well-defined enzymatic role in glycolysis. The various post-translational modifications that have been identified on GAPDH influence the activity and location of the enzyme [2,3]. Consequently, the modifications and alterations in GAPDH subcellular location contribute to various pathological conditions including neurodegeneration and cancer [42–44]. Therefore, it is important to understand the mechanism by which GAPDH functions in normal cellular activities including intracellular membrane transport, a process where dysfunction contributes to both diseases. The first indication that GAPDH played a role in membrane trafficking came from the studies of Robbins et al. [9]. CHO cells mutagenized with ethyl methanesulfonate and selected for accumulation of a fluid phase marker were found to express a point mutation (C²³⁴→T) in GAPDH that caused the phenotypic change. The authors proposed three different models to account for their results, though the precise mechanism how the GAPDH mutant altered endocytosis was not elucidated.

Because GAPDH is required for transport in the early secretory pathway, we wanted to learn if the GAPDH (Pro²³⁴Ser) could interfere with secretion. To that end, the purified recombinant mutant protein was introduced into an in vitro biochemical and a morphological assay that measures membrane trafficking. In both assays, we found that transport of the reporter molecule was blocked before entry into the cis Golgi compartment. Based on those combined results, we focused our efforts on molecules known to have activity in the early secretory pathway and endocytosis that may become dysfunctional in the presence of the GAPDH mutant to help us elucidate the mechanism. MTs were a reasonable candidate since GAPDH binds at the carboxyl-terminus of the α -tubulin subunit [45]. Moreover, the integrity of the Golgi complex correlates with the organization of the MT network and associated motors [46,47]: Golgi fragmentation observed in GAPDH (Pro²³⁴Ser) treated cells could be the result of MT disassembly. However, GAPDH (Pro²³⁴Ser) MT binding properties were similar to wild-type GAPDH and the MT network assessed by indirect immunofluorescence appeared normal.

Several studies had reported that Akt associates with GAPDH. Baba et al. found that insulin stimulation of cardiac muscle resulted in Akt activation and binding to GAPDH [26]. In those studies, Akt phosphorylation of GAPDH was found to increase GAPDH catalytic activity. Interestingly, Huang and coworkers also reported that Akt2 binds to GAPDH in ovarian cancer cells and that Thr²³⁷ in GAPDH was critical for interaction and phosphorylation [22]. GAPDH does not contain the typical recognition motif for Akt (RXRXX(S/T) [48]. However, several laboratories have reported Akt phosphorylation of proteins that like GAPDH lack arginine at position 5 in the motif [49–51]. Our results indicate that substitution of the proline adjacent to the Thr²³⁷ eliminates GAPDH-Akt interaction. Proline has been found to be a critical residue in several ligand binding domains including the Src Homology 3 domain (SH3) and the EPS 15 Homology domain (EH domain) [52,53]. The side chain of proline has a cyclic structure, which imparts conformational rigidity that influences the secondary structure of the protein. Because of these properties, ligand binding to proline rich regions is weak, but results in more rapid ligand binding/release rates [54]. For that reason, signaling molecules often associate with proline containing domains.

We reported previously that Rab2 binds directly to GAPDH and requires GAPDH for transport [29,55]. Conversely, we have not been able to detect a direct interaction between Rab1 and GAPDH (data not shown). Rab2 like Rab1 is required for membrane trafficking in the early secretory pathway [14]. However, Rab2 mediates retrograde vesicle formation from VTCs that sort anterograde directed cargo from recycling proteins [15,16]. In contrast, Rab1 plays a role in anterograde trafficking in the early secretory pathway. Although Rab1 and Rab2 are 40% identical, the proteins have partial overlapping but predominantly distinct and highly regulated downstream effectors and functions. For example, Rab2 requires aPKC for vesicle formation and MT and motor recruitment [16,32]. Because Rab1 plays a role in anterograde trafficking in the early secretory pathway, Akt activity may be required for forward transport. It is notable that Rab1 also participates in early endosome sorting, an event that utilizes Akt [56]. Our results indicate that Rab2 does not stimulate GAPDH-Akt membrane binding. Interestingly, it has been shown that aPKC interacts with Akt and the interaction inhibits Akt activation [49,55]. However, Akt does not affect aPKC activity [57]. Based on those observations, it is possible that GAPDH-aPKC does not co-bind Akt or aPKC-Akt interaction is nonactivating and therefore Akt dissociates. This could explain why Akt is not found on membranes with Rab2-GAPDH-aPKC.

Why would Rab1 need GAPDH-Akt? Similar to aPKC and Src phosphorylation of GAPDH that promotes complex formation with Rab2 [32,58]. Akt phosphorylation of GAPDH could promote temporal and spatial interaction of downstream effectors required to discriminate between Rab1-mediated anterograde directed traffic and Rab2-mediated retrograde trafficking (mechanistic model shown in Fig. 7). For example, potential downstream effectors include the 14-3-3 proteins that bind to Akt substrates [59]. It is conceivable that 14-3-3 protein interactions with membranes/vesicles containing Rab1 and GAPDH-Akt, which subsequently blocks COPI binding, allows anterograde-directed vesicle transport to and/or through the Golgi complex [60]. Indeed, recent proteomic studies indicate that a distinct form of GAPDH binds specifically to 14-3-3 proteins [61]. Studies are in progress to

address our hypothesis and to determine the specific role of GAPDH-Akt in early secretory events.

5. Conclusions

Golgi function and organization is controlled by a variety of signaling molecules. Our studies suggest that two serine/threonine kinases, which associate with GAPDH on secretory organelles act like a switch that determines the directionality of membrane transport. GAPDH-Akt contributes to Golgi integrity by influencing anterograde transport in the early secretory pathway, whereas Rab2 utilizes aPKC kinases to recycle itinerant cargo to the ER. Therefore, the GAPDH (Pro²³⁴Ser) mutant has served as a valuable tool to recognize the potential role of Akt in secretion and to further clarify the function of GAPDH in membrane trafficking.

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Abbreviations

aPKC	Atypical PKC
endo H	endoglycosidase H
ER	endoplasmic reticulum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
NGS	normal goat serum
NRK	normal rat kidney
pAkt	phosphoAkt
VSV-G	vesicular stomatitis virus G-protein
VTCs	vesicular tubular clusters

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Fig. 1. GAPDH (Pro²³⁴Ser) blocks ts045 VSV-G transport from the ER to the Golgi complex. (A) Semi-intact ts045 VSV-G infected NRK cells were incubated with the indicated concentration of recombinant wild-type GAPDH (□) or GAPDH (Pro²³⁴Ser) (◇) for 40 min at 32 °C. The fraction of ts045 VSV-G processed to the endo H resistant form (R₂) was determined after analysis by SDS-PAGE and quantified using a Phosphorimager. A representative autoradiograph from one of four independent experiments is shown. The results are the mean ± SD of four independent experiments. (B) ts045 VSV-G infected NRK cells were permeabilized with digitonin, and then incubated in transport cocktail (cont) and supplemented with recombinant wild-type GAPDH or GAPDH (Pro²³⁴Ser) (100 ng) for 30 min at 32 °C. The distribution of ts045 VSV-G was examined by indirect immunofluorescence. Images are representative of 3 independent experiments. Scale bar, 5 μm.

Fig. 2. GAPDH (Pro²³⁴Ser) interferes with an early event in ts045 VSV-G transport from VTCs. (A) NRK cells were permeabilized with digitonin, and then incubated in transport cocktail supplemented with recombinant GAPDH (Pro²³⁴Ser) (100 ng) for 30 min at 32 °C. The distribution of GAPDH (Pro²³⁴Ser) and the VTC marker protein p58 was examined by indirect immunofluorescence. Boxed area was analyzed using the Manders overlap co-localization, as described under “Materials and methods”. (B) ts045 VSV-G infected semi-intact NRK cells were preincubated for 60 min at 15 °C to accumulate VSV-G in VTCs, and then shifted to 32 °C. At the indicated time, either wild type GAPDH or GAPDH (Pro²³⁴Ser) (150 ng) was added and incubated for 25 min. The fraction of ts045 VSV-G processed to the endo H resistant form (R₂) was determined after analysis by SDS-PAGE and quantified using a Phosphorimager. A representative autoradiograph from one of three independent experiments is shown.

Fig. 3.

Biochemical properties of GAPDH (Pro²³⁴Ser) are comparable to wild type GAPDH. (A) The catalytic reduction of NAD to NADH by recombinant GAPDH (•) or recombinant GAPDH Pro²³⁶Ser (□) was monitored at times indicated by an increase in absorbance at 340 nm. Results are representative of 3 independent experiments. Data are given as mean +/– SE. (B) Purified recombinant GAPDH (a) and GAPDH (Pro²³⁴Ser) (b) were separated on a 4–16% NativePage Bis-Tris gel. Protein standards correspond to NativeMark™ unstained protein standards.

Fig. 4.

GAPDH (Pro²³⁴Ser) causes Golgi fragmentation without disrupting the MT network (A) Purified recombinant GAPDH or GAPDH (Pro²³⁴Ser) were incubated with paclitaxel assembled MTs for 20 min at 37 °C, layered on a 20% sucrose cushion, centrifuged 30 min at 100 K $\times$ g, and the pellet and supernatant analyzed by SDS-PAGE and western blot. P: pellet S: supernatant. (B) NRK cells were transfected with GAPDH cDNA or GAPDH (Pro²³⁶Ser) cDNA for 36 h, fixed, and co-immunolabeled with the indicated primary antibodies. Boxed area: high magnification image labeled with GM130. Shown are representative images from 4 independent experiments.

Fig. 5.

Akt associates with GAPDH but not with GAPDH (Pro²³⁴Ser). (A) NRK cells were transfected with empty vector (cont), GAPDH cDNA or GAPDH (Phe²³⁴Ser) cDNA for 48 h. Post-nuclear cell lysates were immunoprecipitated with anti-Xpress monoclonal antibody and the total immune complexes were separated on SDS-PAGE and immuno-blotted with the indicated primary antibody. (B) Rat liver cytosol was depleted of GAPDH [29]. Equal volume of rat liver cytosol (RLC) and depleted rat liver cytosol (GAPDH-) were analyzed by SDS-PAGE and Western blot. (C) Purified recombinant GST-GAPDH, GST-GAPDH (Phe²³⁶Ser), or GST (cont) were preincubated with glutathione-Sepharose B agarose for 2 h at room temperature, and then rat liver cytosol (GAPDH depleted) added and incubated for 2 h. The beads were washed extensively, and the bound proteins, separated by SDS-PAGE, and then transferred to nitrocellulose. The blot was probed with the indicated primary antibodies. The protein band detected with anti-GST and with anti-GAPDH co-aligned.

Fig. 6.

Rab1 stimulates Akt membrane association (A). The membrane binding assay was performed with salt-washed HeLa membranes and incubated with purified recombinant His6-tagged wild-type GAPDH in the presence or absence of Rab1 or Rab2 for 15 min at 37 °C. The membrane pellet with associated proteins was subjected to SDS-PAGE and the western blot probed with the indicated primary antibodies. The blot was quantified by densitometry and the arbitrary units graphed relative to the respective primary antibody. (B) The membrane binding assay was performed as above with His6-tagged GAPDH (Phe²³⁶Ser) in the presence or absence of Rab1 or Rab2 for 15 min at 37 °C, and analyzed as above.

Fig. 7.

Schematic model: GAPDH and the associated kinase play a role in regulating the directionality of membrane transport from VTCs. (A) Rab1 bound to a VTC subdomain stimulates recruitment of downstream effectors including GAPDH-pAkt. (1) pAkt phosphorylates GAPDH on the VTC subdomain. (2) pGAPDH recruits 14-3-3 protein to suppress COPI-dependent activity. (3) Release of anterograde-directed carriers containing Rab1-effectors that fuse with the cis Golgi. (B) Rab2 bound to a different VTC subdomain stimulates recruitment of GAPDH and subsequently Src. (1) Src phosphorylates GAPDH to recruit aPKC. (2) aPKC phosphorylates GAPDH. (3) Rab2-ppGAPDH-Src-aPKC stimulates recruitment of β -COP. (4) Formation and release of COPI retrograde-directed vesicle that traffics to the ER.