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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 ι/λ 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 Pro^{234} to Ser ($Pro^{234}Ser$) rendered CHO cells defective in endocytosis. To determine if GAPDH ($Pro^{234}Ser$) 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 ($Pro^{234}Ser$) 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.

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 multifunctional 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 (aPKC) 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 Lys^{160} [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 GADPH ($Pro^{234}Ser$) 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

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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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 ($Pro^{234}Ser$) 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 ($Pro^{234}Ser$) 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 ($Pro^{234}Ser$) 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 oligonucleotide primer containing an *Eco*RI 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 ($Pro^{234}Ser$) 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 ($Pro^{234}Ser$) or pGEX-2T GAPDH/GAPDH ($Pro^{234}Ser$) 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 4300*g* 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 Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) equilibrated in Buffer A (10 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 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 Centriprep 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 μCi Expre^{35}S^{35}S (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 μl 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 $CaCl_2$, 1 mM N-acetylglucosamine, an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate and 0.2 IU rabbit muscle creatine phosphokinase), 5 μl rat liver cytosol, (~20 μg of total protein), and 5 μl of semi-intact cells (~5×10^7 cells/ml, ~25–30 μg 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 ($Pro^{234}Ser$), 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 μg/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 ($Pro^{234}Ser$), 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 (5×10^5 cells /75 mm dish), and then transiently transfected with pcDNA4/HisMax-TOPO-GAPDH or pcDNA4/