A Novel CRM1-mediated Nuclear Export Signal Governs Nuclear Accumulation of Glyceraldehyde-3-phosphate Dehydrogenase following Genotoxic Stress*

Received for publication, July 2, 2003, and in revised form, October 23, 2003
Published, JBC Papers in Press, November 14, 2003, DOI 10.1074/jbc.M307071200

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional protein with glycolytic and non-glycolytic functions, including pro-apoptotic activity. GAPDH accumulates in the nucleus after cells are treated with genotoxic drugs, and it is present in a protein complex that binds DNA modified by thio guanine incorporation. We identified a novel CRM1-dependent nuclear export signal (NES) comprising 13 amino acids (KKVVKQASEGPLK) in the C-terminal domain of GAPDH, truncation or mutation of which abrogated CRM1 binding and caused nuclear accumulation of GAPDH. Alanine scanning of the sequence encompassing the putative NES demonstrated at least two regions important for nuclear export. Site mutagenesis of Lys209 did not affect oligomerization but impaired nuclear efflux of GAPDH, indicating that this amino acid residue is essential for proper functioning of this NES. This novel NES does not contain multiple leucine residues unlike other CRM1-interacting NES, is conserved in GAPDH from multiple species, and has sequence similarities to the export signal found in feline immunodeficiency virus Rev protein. Similar sequences (KKV*–7–13PLK) were found in two other human proteins, U5 small nuclear ribonucleoprotein, and transcription factor BT3.

In addition to its integral role in glycolysis, converting glyceraldehyde-3-phosphate (GAPDH) into 1,3-bisphosphoglycerate, GAPDH has been shown to have diverse biological functions, including as a protein that signals apoptosis (1). GAPDH also participates in membrane, cytoplasmic, and nuclear functions for endocytosis, mRNA regulation, tRNA export, DNA replication, and DNA repair (2, 3). Some species, including humans and mouse, contain more than one functional GAPDH gene and a diversity of pseudogenes (4). In its monomeric form, GAPDH has a molecular mass of ~37 kDa, however, within cells, it exists mainly as a tetramer comprising four identical 37-kDa subunits (3, 5). GAPDH is located in multiple cellular compartments, including the plasma membrane, nucleus, and cytosol (6).

GAPDH plays an important role in stress response leading to apoptosis (5, 7), with the cytoplasmic to nuclear translocation of GAPDH preceding the onset of apoptosis (8, 9). K+ depolarization (7, 9), serum withdrawal (6), aging of cultures (10), or treatment with anticancer agents such as mercaptopurine or cytosine arabinoside (8, 10–12) cause nuclear accumulation of GAPDH. An increase in nuclear GAPDH is required for its apoptotic effects, which appear to be upstream from events that mediate apoptotic degradation (13), and the nuclear accumulation of GAPDH precedes chromatin condensation, nuclear fragmentation, and a decline in mitochondrial membrane protein (14). This is consistent with the reported involvement of GAPDH in apoptosis of primary cultures of cerebellar neurons following nuclear translocation (9) and the induction of intranuclear translocation of GAPDH by treatment of cells with thiopurines (12). Moreover, significant correlation has been shown between basal intranuclear GAPDH in acute lymphoblastic leukemia (ALL) cell lines and sensitivity to thiopurine treatment (12).

Recently, GAPDH was identified as a component of a nuclear protein complex that recognizes duplex DNA into which fraudulent nucleosides (e.g. thio guanosine, cytosine arabinoside, or 5-fluorouridine) have been incorporated (15). In vitro treatment of the complex with monoclonal anti-GAPDH antibody (anti-GAPDH mAb) resulted in its dissociation (12). This observation led us to hypothesize that the corresponding epitope recognized by anti-GAPDH mAb is localized at or near the surface involved in protein-protein interactions and provided the basis for the present study to characterize the region(s) of the GAPDH polypeptide chain involved in protein-protein interactions. We identified the region of GAPDH, which constitutes the anti-GAPDH mAb binding site, and demonstrated involvement of this region in binding with other nuclear proteins. Finally, we demonstrated that this region interacts with components of the nuclear export system and defines intracellular localization of GAPDH. Elucidating the mechanism of nuclear targeting of GAPDH has identified a novel nuclear export sig-
nal and provided new insights into disease pathogenesis and drug-induced apoptosis.

MATERIALS AND METHODS

Cell Cultures and Nuclear Extract Precipitation—Colon adenocarcinoma cell lines SW620 and DLD1 were obtained from ATCC (Manassas, VA). Cell lines were grown in RPMI 1640 (BioWhittaker, Walkersville, MD) medium supplemented with 10% fetal bovine serum (Invitrogen, Palo Alto, CA) and 1.0% l-glutamine. Cytotoxic effects of thiopurines (Sigma, St. Louis MO) were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (16) after incubation of SW620 and DLD1 cells with mercaptopurine (MP, 0.001–180 μM) or thioguanine (TG, 0.001–100 μM) for 3–6 days. The concentrations of MP or TG were determined by spectrophotometry at 320 and 340 nm, respectively. The 96-well plates were read by a microplate spectrophotometer (Bio-Rad, Hercules, CA). The IC50 values were obtained by fitting a sigmoid IC50 model to the cell viability (%) versus concentration of drug (micromolar), determined in triplicate. Experiments were performed using an initial concentration of 1 × 105 cells/ml of media before the addition of either MP or TG. In subsequent experiments, thiopurines were added to the media in a single dose to achieve a concentration of 10 μM MP or 10 μM TG in the growth medium.

The human T-lineage leukemia cell line Molt4 was obtained from ATCC (Manassas, VA); the human T-lineage leukemia P12 and B-lineage Nalm6 cell lines were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cell number and viability were determined in duplicate in a Burker-Turk chamber using trypan blue exclusion. All experiments were started with an initial concentration of 0.25 × 106 cells/ml. Nuclear extracts from human acute lymphoblastic leukemia cells (Molt4, CEM, or Nalm6) were prepared according to Dignam et al. (17). Protein concentration was determined by Bradford dye-binding procedure using Bio-Rad protein assay.

Fast Protein Liquid Chromatography—2.0 mg of GAPDH from human erythrocytes (control; Sigma, St. Louis, MO), K29BN mutant GAPDH, a polypeptide containing the epitope and total nuclear protein from ALL cells were analyzed by FPLC on Superdex 200 HR 10/30 columns (Amersham Biosciences, Piscataway, NJ) in elution buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl) at 0.3–0.4 ml/min at 4 °C. Composition of eluate was monitored by spectrophotometer at 280 nm. Fractions (0.25 ml) were collected during the FPLC separation and concentrated to 25 μl using an Ultrafree 0.5 centrifugal filter and tube (Millipore, Bedford, MA) and analyzed by Western blot. Membranes were developed with anti-GAPDH monoclonal antibody (mAb; Chemicon, Temecula, CA) (18).

Glycolytic Assay—GAPDH glycolytic activity was measured by spectrophotometric assay at 340 nm. Briefly, the assay was carried out in 0.015 M sodium pyrophosphate, 0.03 M sodium arsenate (Sigma), pH 8.5, in the presence of 3.5 mM DT, 0.26 mM NAD+ and 0.51 mM glyceraldehyde 3-phosphate (Sigma) using human GAPDH (Sigma) or lysates or nuclear extracts. The reactions were performed for 10 min at 25 °C.

Identification of mAb Epitope Using Pin-bound Peptides—Pin-bound peptides representing the entire GAPDH polypeptide sequence were synthesized using Multipin Peptide Synthesis kit (Chiron Technologies, San Diego, CA) in the Hartwell Center for Biotechnology (St. Jude Children’s Research Hospital) using standard peptide synthesis and Sepharose Coupling. Identification of epitopes was performed according to manufacturer’s instructions, using monoclonal antibody (clone 6C5; Chemicon, Temecula, CA) at a concentration of 500 ng/ml, and secondary antibody-labeled pin-bound peptides (Santa Cruz Biotechnology, CA) at a concentration of 50 ng/ml. A series of overlapping pin-bound 15-mer peptides was incubated with anti-GAPDH monoclonal antibody (Sigma) or lysates or nuclear extracts. The reactions were performed for 10 min at 25 °C. The putative NES is shown in white, and the corresponding mutated sequences are shown under each construct. Numbers above each construct indicate positions of nucleotide, and the letters below each represent the amino acid residues within GAPDH sequence. In the T3 construct, the broken line designates the missing section. In K29BN mutant construct, the mutated amino acid is underlined. In the alanine-mutated variants of GAPDH, the amino acids were sequentially mutated to alanine; each construct contained four mutated amino acids.

Affinity Chromatography—Cellular or nuclear extract from 1 × 106 cells (200 μl) was incubated with peptide 52 (DDIKVKQGASEGPL) or peptide 52 (KPAKDYDIDIKVQGKQAK) immobilized on Sepharose in 20 mM Tris-HCl, pH 8.5, 5 mM NaCl, 0.3 mM MgCl2, and 0.1 mM DTT (Buffer 1) in a 2-ml column for 30 min at room temperature. Column was washed with 15 ml of Buffer 1 and then eluted with 10 ml of Buffer 1 containing 0.1 M NaCl. Fractions were collected, concentrated, and analyzed by SDS gel electrophoresis and silver staining.

Mass Spectroscopy of Proteins—Proteins localized by silver staining following SDS-polyacrylamide gel electrophoresis were excised from the gel, and digested with trypsin. The peptides thus released from the gel plug were subjected to analysis by combined liquid chromatography/tandem mass spectrometry using an LCQ-Deca ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA) coupled with a capillary high-performance liquid chromatography system (Waters, Milford MA). Peptides were separated by reversed-phase chromatography using a 320-μM i.d. column packed with Waters Delta-Pak C18 stationary phase by Microtech Scientific (Santa Clara, CA). Peptides were assigned to known proteins on the basis of searches of the NCBI non-redundant protein data base performed on product ion spectra using the SEQUEST algorithm.

Plasmid Preparation—GAPDH cDNA was prepared by RT-PCR from total RNA isolated from 697 human pre-B leukemia cells. The forward primer, 5'-TGTATACATGGCGGCGGGCTGGAGGATGGGAGTTAAGTGCG-3', contained a NotI site, a 15-mer encoding (Gly)n link, and the first 20 nucleotides (including the initiation codon) of the coding sequence of the GAPDH cDNA. The reverse primer, 5'-GCCGCGGCCGT-TACTCTTGGAGGGAGGACACC-3', contained a BarG1 binding site and the last 20 nucleotides containing the termination codon of the coding sequence of the GAPDH cDNA. The modified GAPDH cDNA was cloned into pcDNA3.1/EGFP to obtain the pcDNA3.1/GFP-(Gly)n-GAPDH fusion construct (6, 19). The portion of the pEGFP vector (Clontech, Palo Alto, CA) containing the coding region for EGFP was previously cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) using conventional methods

FIG. 1. GFP-GAPDH fusion constructs. GFP polypeptide is depicted in green, the Gly linker is black, and the polypeptide chain of GAPDH is gray. The putative NES is shown in white, and the corresponding mutated sequences are shown under each construct. Numbers above each construct indicate positions of nucleotide, and the letters below each represent the amino acid residues within GAPDH sequence. The T3 construct, the broken line designates the missing section. In K29BN mutant construct, the mutated amino acid is underlined. In the alanine-mutated variants of GFP-GAPDH, the amino acids were sequentially mutated to alanine; each construct contained four mutated amino acids.
Truncated and mutated hybrids of the fusion constructs were prepared by site mutagenesis (Fig. 1). Sequences of all constructs were verified by DNA sequencing at the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children’s Research Hospital.

**Functional Characterization of the Putative NES**—Fig. 1 depicts the truncated (T1, T2, and T3) or mutated (K259N and Ala mutants M1–M6) GAPDH constructs. The GFP-tagged truncated GAPDH T1 contains nucleotides 1–774, which encodes the N terminus, however, it lacks any part of the putative NES. The truncated construct, T2, contains nucleotides 1–786, which encodes the N terminus and the first four amino acids, KKVV, of the putative NES. The second truncated construct, T2, contains nucleotides 969–1088, which encodes the last three amino acids, PLK, of the putative NES and the rest of the C terminus of GAPDH. In the GFP-tagged mutated fusion constructs 1–6, amino acids, including those within the putative NES, were sequentially replaced with four alanine residues. Additionally, a mutant hybrid of the fusion construct was generated in which lysine 259 was mutated to asparagine (K259N).

**Transfection Assay**—Transient transfection was done using LipofectAMINE Reagent 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Expression of the GFP fusion construct in the cell lines was documented by fluorescence microscopy. Flow cytometry was used to separate transfected from non-transfected cells, and the cell lines was documented by fluorescence microscopy. Flow cytometry was used to separate transfected from non-transfected cells, and the cell lines was documented by fluorescence microscopy.

**Immunoprecipitation**—Total cell lysate was prepared as recommended by Santa Cruz Biotechnology (Santa Cruz, CA), and protein concentration was determined using the PlusOne 2-D Quant kit (Amersham Biosciences, Piscataway, NJ). Immunoprecipitation was performed using a modification of the method suggested by Santa Cruz Biotechnology. Briefly, 80 μl of radioimmuno precipitation assay buffer was added to 5–10 μg (in 20 μl) of total cell lysate, and the mixture was precleared by adding 0.25 μg (in 5 μl) of normal rabbit IgG (Santa Cruz Biotechnology) and 20 μl of Protein G Plus-agarose beads (Santa Cruz Biotechnology). The mixture was incubated at 4 °C for 30 min. After the incubation, the beads were pelleted by centrifugation at 2500 rpm for 5 min. The supernatant was transferred to a fresh 1-ml microcentrifuge tube at 4 °C. 0.19 μg/μl (in 20 μl) of rabbit anti-GFP polyclonal antibody (Clontech) was added to the supernatant, and the mixture was incubated at 4 °C for 1 h. After incubation, 20 μl of Protein G Plus-agarose beads (Santa Cruz Biotechnology) were added to the mixture, followed by incubation overnight at 4 °C with constant rotation. The pellet was collected by centrifugation at 2500 rpm for 5 min, washed with radioimmuno precipitation assay buffer four times, and then resuspended in 10 μl of Nu-PAGE LDS 4× sample buffer (Invitrogen, Carlsbad, CA), vortexed, and boiled at 100 °C for 5 min. After cooling, 10 μl of running buffer and 1 μl of 1% DTT was added to the boiled sample, and the mixture was separated on 10–12% Nu-PAGE gel (Invitrogen).

**Western Analysis**—After immunoprecipitation and SDS-electrophoresis, the proteins were transferred to a Hybond-P membrane (Amersham Biosciences) by electrotransfer. The membrane was then incubated with mouse anti-CRM1 mAb antibody (BD Transduction Laboratories, San Diego, CA). Bound antibodies were detected using goat anti-mouse IgG HRP conjugate (Santa Cruz Biotechnology) and ECL-Plus kit (Amersham Pharmacia Biotech). The membrane was washed and re-developed with Rabbit anti-GFP polyclonal antibody (Clontech) and goat anti-rabbit IgG HRP conjugate as secondary antibody conjugate (Santa Cruz Biotechnology).

**Confocal Microscopy**—Samples were examined using a Leica TCS NT SP confocal laser scanning microscope equipped with argon (488 nm) and krypton (568 nm) lasers. Endogenous GAPDH was stained using mouse anti-GAPDH (Chemicon) as primary antibody and goat anti-mouse IgG fluorescein isothiocyanate conjugate (Santa Cruz Biotechnology) as secondary antibody, and the nuclei were stained with propidium iodide (PI). GFP-positive cells were stained with PI. Samples were imaged with detector slit widths of 500–548 nm in the green, or fluorescein isothiocyanate channel; and 580–609 nm in the red, or PI channel, using a 100× plan apochromatic 1.4 numerical aperture oil immersion objective. Overlay images combining the green and red channels were produced using the Leica software, and the images were re-scaled and gamma-corrected in Adobe Photoshop. The nuclear and cytoplasmic fluorescence intensities were quantified using a published procedure. Single section images containing 20–40 cells were used in the analysis to determine the nuclear and cytoplasmic fluorescence (single fixed intensity × number of pixels per nucleus or cytoplasm). The nuclear area is defined by the red fluorescence due to propidium iodide.

**RESULTS**

**Intracellular Localization of Endogenous GAPDH and GFP-GAPDH**—In untreated cells, endogenous GAPDH was localized mainly in the cytosol of colon adenocarcinoma cells (SW620 and DLD1), as revealed by immunostaining and confocal microscopy (Fig. 2A). However, following 24–48 h of thiopurine treatment (either MP or TG), GAPDH accumulated predominantly in the nucleus, with exclusion from the nucleoli. Fig. 2B shows the distribution of endogenous GAPDH in SW620 cells after 24 h of treatment with 10 μM MP.

GAPDH was localized predominantly in the cytoplasm of DLD1 cells expressing the GFP-GAPDH fusion construct, similar to endogenous GAPDH, as illustrated in Fig. 2C (compared with Fig. 2A). Treatment of DLD1 cells expressing the GFP-GAPDH fusion construct with 10 μM thiopurine resulted in subcellular redistribution of GAPDH, with accumulation in the nucleus and exclusion from the nucleoli, after 48 h of thiopu-
Fig. 3. Identification of the epitope containing peptides bound by anti-GAPDH monoclonal antibody. A, ELISA assay scan of the Multipin peptide array of GAPDH 15-mer peptides using monoclonal antibody 6C5. B, SDS-gel electrophoresis analysis of the proteins isolated from Molt4 cellular lysate by affinity chromatography. Molt4 cellular lysate (lane 1) was loaded onto the Sepharose column with immobilized peptide 52, washed (lanes 2–4), and the bound proteins were eluted with 1 M NaCl (lane 5). After electrophoretic separation, the major band (indicated by an arrow) was excised and identified by mass spectrometry, as described under “Materials and Methods.” C, comparison of the identified peptide sequences within GAPDH across different species (human, H. sapiens; rabbit, O. cuniculus; lobster, H. americanus; and lobster, P. versicolor). Asterisks designate invariant amino acid residues, colons and dots designate conservative substitutions, and non-conservative changes are designated by gaps. The amino acid sequence of the FIV Rev atypical nuclear export signal is shown in the bottom line.

rime treatment (Fig. 2, E and D, compared with C). This was similar to that observed for endogenous GAPDH (Fig. 2B). Therefore, GFP-GAPDH fusion construct (Fig. 1) was used as a model to assess the effect of truncation or mutation on the localization of GAPDH. In control experiments with SW620 and DLD1 cells expressing EGFP vector, GFP was uniformly present in both cytoplasm and nucleus (data not shown). Thiorune treatment of SW620 and DLD1 cells expressing EGFP vector had no effect on the cellular distribution of EGFP (data not shown).

Identification of the Anti-GAPDH-bound Epitope within the GAPDH Polypeptide Chain—Pepscan technology was used to delineate the amino acid sequences of peptides that constitute the epitope of GAPDH recognized by anti-GAPDH mAb. Peptides 51 and 52, corresponding to overlapping segments 258KPAGDYIKKVQKA264 and 258DDIKKVKQASEGPI269, respectively, gave the highest absorption at 405 nm (>2 optical units/ml) (Fig. 3A), and reaction with the other peptides resulted in negligible absorption (0.038–0.1 optical units/ml). Treatment with monoclonal anti-PCNA antibody (a negative control) did not result in any signal, indicating the lack of nonspecific interaction with pin-bound peptides (data not shown). N-terminal sequencing verified the sequences of pin-bound peptides 51 and 52. Unbound peptides 51 and 52 inhibited immunostaining of GAPDH in Western analysis, thus confirming specific binding of anti-GAPDH MAb with epitopes encompassing amino acids 250–269 of GAPDH (data not shown).

Putative NES Involved in Protein-Protein Interaction—Incubation of cellular lysate from Molt4 cells with 258DDIKKVKQASEGPI269 covalently bound to Sepharose resulted in specific binding of GAPDH, as confirmed by SDS gel electrophoresis and mass-spectroscopy (Fig. 3B). One major and at least four minor bands were detected by silver staining of the gel. Electrospray mass spectrometry of the major band identified it as GAPDH (Fig. 3B). The identified peptide sequence within GAPDH is conserved across different species (human, Homo sapiens; rabbit, Oryctolagus cuniculus; lobster, Homarus americanus; and lobster, Palinurus versicolor) and contains invariant amino acid residues, conservative substitutions, and non-conservative changes as illustrated in Fig. 3C. This GAPDH peptide sequence is comparable to FIV Rev protein that has an atypical nuclear export signal (Fig. 3C).

Oligomeric Forms and Glycolytic Activity of Nuclear GAPDH—Gel filtration FPLC chromatography depicts a typical profile of purified K259N mutant and wild-type GAPDH extracted from human erythrocytes used as control. The FPLC profile revealed that both wild-type and mutant GAPDH were eluted as tetramers with a molecular mass of 144 kDa (Fig. 3A). No peaks with molecular mass higher than 144 kDa were detected. The chromatography of nuclear extracts from ALL cells (P12, Molt4, and Nalm6) in non-denaturing conditions and subsequent Western blot analysis of the collected fractions revealed that GAPDH was present in several oligomeric forms or high molecular weight complexes in the nuclear compartment of untreated lymphoblast cells. Fig. 4B (analysis of nuclear protein from Molt4 cells) depicts a typical FPLC profile of nuclear protein distribution for ALL cells. Fractions 24–25 contained a high molecular weight protein complex exceeding the exclusion volume of the column (i.e. more than 2 × 106 Da), and fractions 35–43 contained different oligomeric forms of GAPDH, including the monomeric, dimeric, and tetrameric forms (Fig. 4C). Measurement of enzymatic activity in the nuclear extracts and cellular lysates from P12, Molt4, and Nalm6 cell lines revealed that GAPDH activity in the nucleus of untreated Molt 4, P12, and Nalm6 cells was lower when compared with cellular lysates (Fig. 4D).

Modification of the Putative NES Changes Intracellular Distribution of GAPDH—We performed alanine scanning using
Ala tetrapeptide sequence (AAAA) across the entire length of the tested region (Fig. 1). GFP-GAPDH mutant proteins bearing Ala4 substitution in different positions of the putative NES revealed different distribution between cellular compartments. DLD1 cells stably expressing the GFP-tagged Ala mutants M1 and M4 demonstrated cytoplasmic localization of the mutant GFP-GAPDH as depicted by green GFP fluorescence (Fig. 5, A, B, and H). In these panels, green denotes cytoplasmic localization, whereas the yellow/orange color denotes the nuclear localization of the protein. Results of the quantitative fluorescence analysis are shown in B, D, F, H, J, and L. Panels A and G demonstrate predominant localization of Ala mutants GFP-GAPDH M1 and M4 in the cytoplasm. By contrast, fusion proteins with Ala-mutated GAPDH NES (mutant GFP-GAPDH M3, M5, and M6, panels E, I, and K) and Ala-mutated GAPDH NES M2 (C and D) are localized predominantly in the nucleus (yellow/orange) compared with untreated wild-type GFP-GAPDH (Fig. 2C).

Fig. 5. Confocal microscopic analysis of intracellular localization of alanine mutated variants of GFP-GAPDH fusion protein expressed in untreated DLD1 colorectal adenocarcinoma cells. The intracellular localization of Ala-mutated GFP-GAPDH constructs in DLD1 cells is shown by the merged image (A, C, E, G, I, and K). In these panels, green denotes cytoplasmic localization, whereas the yellow/orange color denotes the nuclear localization of the protein. Results of the quantitative fluorescence analysis are shown in B, D, F, H, J, and L. Panels A and G demonstrate predominant localization of Ala mutants GFP-GAPDH M1 and M4 in the cytoplasm. By contrast, fusion proteins with Ala-mutated GAPDH NES (mutant GFP-GAPDH M3, M5, and M6, panels E, I, and K) and Ala-mutated GAPDH NES M2 (C and D) are localized predominantly in the nucleus (yellow/orange) compared with untreated wild-type GFP-GAPDH (Fig. 2C).

Fig. 4. Characterization of nuclear GAPDH in human ALL cells. Chromatography conditions are described under “Materials and Methods.” A, FPLC analysis of K259N mutant GAPDH. B, gel filtration chromatography of nuclear extract from Molt4 cells. Arrows indicate the retention volumes of the corresponding molecular markers. C, Western blot analysis of the fractions collected, as indicated in B, developed with anti-GAPDH monoclonal antibody. D, GAPDH glycolytic activity in cellular extracts (normalized per micrograms of cellular GAPDH protein, open bars) and nuclear extracts (normalized per microgram of nuclear GAPDH protein, black bars) from untreated human ALL cell lines P12, Molt4, and Nalm6. Experiments were performed in triplicate (mean ± S.E.).
DLD1 cells stably expressing the truncated GFP-tagged fusion construct T1 exhibited predominantly nuclear accumulation of the truncated GAPDH fusion construct (Fig. 6, A and B). Cells stably expressing the GFP-tagged fusion construct T2 exhibited mainly nuclear localization of the T2 GAPDH fusion construct (Fig. 6, C and D). Similar distribution of GAPDH was observed in cells stably expressing the GFP-tagged T3 (data not shown). Stable expression of the K259N mutant fusion construct in DLD1 cells resulted in nuclear accumulation of GAPDH in the absence of thiopurine treatment (Fig. 6, G and H). The localization of K259N was similar to that exhibited by DLD1 cells stably expressing Ala-mutated GFP-tagged fusion constructs M3 (Fig. 5, E and F), M5 (Fig. 5, I and J), and M6 (Fig. 5, K and L).

Effect of Thiopurine Treatment on Nuclear Accumulation of Modified GAPDH—Truncated or mutated forms of GAPDH, which accumulated in the nucleus, did not change nuclear localization of fluorescence after thiopurine treatment. Interestingly, the treatment of cells expressing the truncated fusion protein T2 with 10 μM thiopurine (TG or MP) resulted in nuclear rim association (Fig. 6, E compared with C). No further intracellular accumulation of GAPDH was observed in cells expressing the K259N mutant construct following thiopurine treatment. Similar observations were made for both SW620 and DLD1 cell lines.

Leptomycin B Prevents Export of GFP-GAPDH from the Nucleus—Treatment for 2 h with 2.5 ng/ml leptomycin B (LMB), an inhibitor of CRM1-mediated nuclear export, resulted in subcellular redistribution of GAPDH from the cytoplasm to the nucleus in DLD1 cells (Fig. 7, compare A and B with C and D). Similar results were obtained when SW620 cells were treated with LMB (data not shown).

Co-immunoprecipitation of GAPDH and CRM1—Western analysis using anti-GFP antibody indicated that GFP-GAPDH fusion protein was present in the cell lysate and in the anti-GFP antibody immunoprecipitate from DLD1 cells transfected with the GFP-GAPDH expression vector (Fig. 8A, lane 3, lower panel). Western analysis with anti-CRM1 antibody revealed that CRM1 was present in the immunoprecipitate, indicating that CRM1 was associated with the GFP-GAPDH fusion protein (Fig. 8A, lane 4, upper panel). In control experiments, the immunoblot of membranes obtained from DLD1 cells expressing the EGFP vector, using anti-GFP and anti-CRM1 antibodies, indicated that GFP and CRM1 were present in the cell lysate (Fig. 8A, lane 1). However, when EGFP was immunoprecipitated with anti-GFP, CRM1 was not co-immunoprecipitated (Fig. 8A, lane 2, upper panel). The same was true for SW620 cells (data not shown).
GAPDH is a member of a protein complex that recognizes modified DNA (15), and this complex was destroyed by an anti-GAPDH monoclonal antibody (15). Although protein-protein interacting domains within GAPDH, other than those involved in oligomerization, have not been defined, GAPDH is known to form a variety of protein-protein complexes (15, 22). Selective binding of GAPDH to β-amyloid precursor protein, huntingtin, α-synuclein, parkin, atrophin, ataxin-1, and androgen receptor indicate a potential role of GAPDH in neurodegenerative diseases associated with expansion of CAG repeats (4, 22). This proclivity to form multiprotein complexes led us to speculate that its partner proteins may play an important role in determining multiple biological functions of GAPDH within the cell. In particular, localization of GAPDH in distinct intracellular compartments likely plays an important role in determining its biological function(s).

To identify the binding site within GAPDH that interacts with a monoclonal antibody, which disrupts the multiprotein complex, we used pin-bound peptides overlapping the entire length of the GAPDH polypeptide chain. This identified two overlapping peptides, 251KPAKDKVKKQAS266 and 259DD-IKKVQASEGPG270 (Fig. 3) located in the C-terminal region of the protein. Furthermore, we prepared synthetic peptides with the same sequence and demonstrated by Western analysis that these peptides inhibited binding between GAPDH and antibody, supporting this as a bona fide protein-antibody interaction domain (data not shown). Therefore, we concluded that this region of the GAPDH molecule is involved in protein-protein interactions and could be a critical region of a multiprotein complex. The crystal structure of GAPDH (Protein Data Bank, PDB, code 3GPD) confirms that this peptide is outside the area where monomer subunits interact within the GAPDH tetramer (Fig. 9).

To further elucidate the role of the identified peptide in determining the intranuclear localization of GAPDH, we prepared truncated and mutated variants in which the amino acids constituting this peptide sequence were either modified or eliminated (Fig. 1) and generated stably transfected cell lines. Alterations introduced into this sequence by site-directed mutagenesis caused nuclear accumulation of GAPDH in the absence of genotoxic stress, suggesting that this peptide performs the function of a nuclear export signal (NES), rather than being involved in nuclear import (Figs. 5 and 6). In the GFP-tagged truncated fusion construct T1, which lacked the C-terminal part of the polypeptide chain that included the whole putative NES, GAPDH was localized predominantly in the nucleus (Fig 6A). Similarly, the GFP-tagged truncated fusion protein T2, which contained only the first four amino acids (KVVV) of the putative NES, also exhibited intranuclear localization in the absence of treatment (Fig. 6, C and D). The truncated fusion protein T3, which contained only C-terminal part of the GAPDH polypeptide, including the last three amino acids (PLK) of this peptide sequence, accumulated in the nucleus of both SW620 and DLD1 cell lines in the absence of treatment. Interestingly, thioptamine treatment caused nuclear rim association of the GFP-tagged truncated fusion construct T2 (Fig. 6E), whereas it had no effect on the distribution of the other mutated or truncated proteins (data not shown). The mechanism and biological significance of nuclear rim association of the T2 protein remain to be elucidated.

Alanine scanning of the putative NES by sequentially substituting adjacent amino acid residues by tetra-alanine blocks (Ala₄, AAAA) demonstrated the complex structure of this NES. Substitution of the sequence 251KPAK254 and 259DD-IKKVQASEGPG270 with Ala₄ in the sequence 251PKAYDDIKKVKKQASEGPG274 did not alter intracellular distribution of either GFP-tagged Ala

**DISCUSSION**

The intranuclear localization of GAPDH after cytotoxic treatment and its involvement in DNA repair have been previously demonstrated (21), but little is currently known about the mechanisms involved in nuclear localization of GAPDH. Nuclear accumulation of GAPDH becomes prominent after 24–48 h of treatment with the genotoxic agents cytarabine or thiopurine (2, 12, 21) or following other types of stress (14) and is accompanied by apoptotic cell death (13). Increased expression of GAPDH is essential for induction of apoptosis of cerebellar granule cells (5), and the level of nuclear GAPDH has been linked to the sensitivity of human leukemia cells to thiopurine treatment (12). Following treatment with MP or TG, we documented subcellular redistribution of endogenous GAPDH or GFP-GAPDH (with full-length nuclear export signal, NES) from the cytoplasm to the nucleus in ALL and carcinoma cell lines (Fig. 2).

In our experiments, we detected the presence of tetrameric, dimeric, and monomeric forms of GAPDH, along with high molecular weight complexes, in the nuclear fraction of acute lymphoblastic cells (Fig. 4, B and C). Intranuclear GAPDH exhibited decreased glycolytic activity when compared with cytosolic GAPDH (Fig. 4D), consistent with alternative functions of nuclear GAPDH. We previously demonstrated that
CRM1-mediated Export of GAPDH

A.

B.

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**Fig. 9. Molecular modeling of the GAPDH K259N mutant.** A, the energy-minimized model of the Lys259 → Asn mutant (red) shows a difference from the structure of wild-type GAPDH (gray) in the helical portion of the helix loop formed by the NES sequence. B, close-up view of K259N interactions; colors as in panel A. Side chains of Tyr255, Tyr276, Ala297, and Asp256 predicted to be altered by mutation of Lys259 to Asn are represented as ball-and-stick. The K259N mutation is also predicted to alter the structural elements, and arrows indicate their predicted movement. The solid line indicates a hydrogen-bond interaction between Lys259 and Asp256.

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GAPDH has a degenerate motif, KKVV*7 with Ala4 resulting in intranuclear accumulation of mutated GAPDH, evidently due to abrogation of nuclear export (Fig. 5). This observation indicates that, within the peptide 251KPAK-255YDDIKKVVQASEPGPLKGIL274, two blocks of amino acids are important for interaction with nuclear export machinery (italicized and underlined), whereas the sequence of amino acids located between these two stretches is not critical. This NES bears clear sequence resemblance to bi-partite nuclear localization signals; therefore, to further characterize the role of positive charges in the NES, we introduced a K259N mutation located in the center of the first block within the NES. In accordance with our results with alanine scanning, this substitution resulted in nuclear accumulation of K259N GAPDH (Fig. 6, G and H) (7, 23–26).

A computer search across databases containing known nuclear localization and nuclear export signals did not reveal sequences with close homology to the GAPDH export signal (i.e. sequence 251–270). A search of the Swiss-Prot database with a degenerate motif, KKV*7–13*PLK, identified four proteins outside the family of GAPDH from different species. These were all nuclear proteins: U5 small nuclear ribonucleoprotein (human), transcription factor BT3 (human), β-subunit of transcription initiation factor IIF (yeast), and yemanucin-α (Drosophila). Whether the corresponding sequences play the role of an NES for these proteins remains to be determined, but each has putative functions that would require nuclear localization.

GAPDH is a highly conserved protein, which changed relatively little during evolution; therefore, phylogenetic analysis of GAPDH from distant species does not reveal elements related to the nuclear functions of GAPDH. Comparison of GAPDH sequences from four organisms for which the three-dimensional structure has been solved demonstrated similarities in the overall structure and distribution of charged and neutral amino acid residues (Fig. 3C). This suggests that the structure of this fragment (α helix) and distribution of charges along the helix may provide a recognition signal for the interacting proteins.

Our finding that LMB causes nuclear accumulation of GAPDH (Fig. 7) indicates that nuclear export of GAPDH is CRM1-dependent. This was evident in both SW620 and DLD1 cells expressing the GFP-GAPDH construct containing a wild-type NES. There are several classes of NES, with typical NES containing hydrophobic amino acids, usually a set of leucine residues (26). Direct interaction with the importin-β-related export factor CRM1 (exportin 1) is typically essential for export of proteins containing a leucine rich NES (27–29). Leptomycin B (LMB) is a specific inhibitor of CRM1-mediated nuclear export, inactivating CRM1 by covalent modification of a cysteine residue in the central domain of the polypeptide (30), thereby altering the three-dimensional structure of the CRM1 protein (30, 31). Examples of proteins that contain a leucine-rich NES include HIV-1 Rev protein (31, 32), human T-cell leukemia virus type 1 (33), and protein kinase inhibitor PKI (34). A second type of NES comprises mainly acidic amino acids and is found in the C-terminal domain; transport (nucleus to cytoplasm) of proteins containing such signals is not dependent on CRM1 and is independent of a nuclear localization signal (35). A third class involves bi-directional nucleocytoplasmic shuttling signals, which transport proteins in and out of the nucleus (27) and are insensitive to LMB. The NES we identified in GAPDH fits into none of these typical classes of NES. There are also atypical CRM1-dependent export signals found in the C-terminal region of proteins, such as the NES of the feline immunodeficiency virus (FIV) and equine infectious anemia virus Rev proteins (25, 26). Like GAPDH NES, the atypical FIV Rev NES involves a CRM1/exportin nuclear transport mechanism and is sensitive to LMB inhibition (26), but neither of these NES are leucine-rich. The present finding with GAPDH represents the first human protein shown to have this type of NES.

Co-immunoprecipitation of CRM1 and GFP-GAPDH by anti-GFP antibody from cellular lysates of GFP-GAPDH-expressing cells, but not GFP-expressing cells or cells in which the GAPDH NES had been mutated, established that GAPDH interacts with CRM1 (Fig. 8). Moreover, CRM1 did not co-immunoprecipitate with the GFP-tagged R259N mutant fusion protein, indicating the critical role of Lys259 in CRM1-mediated export of GAPDH.

The available structure of human GAPDH provides insights into the location of the NES sequence, 259KKVQASEPGPLKGIL274, and the possible involvement of this conserved sequence in protein-protein interactions. The putative NES sequence forms a helix and a loop in the structure of human GAPDH, and this helix loop is located away from the oligomerization interface (Fig. 8A), suggesting that the helix loop is likely to be involved in direct binding of CRM1. To assess the
role of the peptide 259KKVKQASEGPLK271, K259N mutated GAPDH was synthesized, and its effect on oligomerization was assessed using FPLC. This revealed a peak at 144 kDa corresponding to the tetrameric form of the protein; similar to that obtained with wild-type GAPDH, indicating that mutation within the putative NES does not affect oligomerization of GAPDH, and pure GAPDH did not form high molecular weight complexes. FPLC analyses supported by computer modeling of K259N mutated GAPDH indicates that the tetrameric form of GAPDH is not affected by the presence of mutation within the putative signal, 259KKVKQASEGPLK271 yet the K259N mutation prevents nuclear efflux of GAPDH protein. These findings and LMB inhibition indicate that distortion of nuclear export signal should reveal new insights into the regulation of proteins that require nuclear accumulation to exert their biological activity.

Acknowledgments—We gratefully acknowledge the valuable help and expertise provided by Robert Cassell of the Hartwell Center, Kenneth Barnes and Donna Davis of Scientific Imaging Department, Richard Heath of the Protein Production Facility, and Leigh Hankins of the Department of Pharmaceutical Sciences at St. Jude Children’s Research Hospital for their excellent technical assistance. We thank Dr. Gerard Grosveld for his helpful comments in the preparation of the manuscript.

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