Rapid Shortening of Telomere Length in Response to Ceramide Involves the Inhibition of Telomere Binding Activity of Nuclear Glyceraldehyde-3-phosphate Dehydrogenase*

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Ceramide has been demonstrated as one of the upstream regulators of telomerase activity. However, the role for ceramide in the control of telomere length remains unknown. It is shown here that treatment of the A549 human lung adenocarcinoma cells with C6-ceramide results in rapid shortening of telomere length. During the examination of ceramide-regulated telomere-binding proteins, nuclear glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified to associate with both single- and double-stranded telomeric DNA with high specificity in vitro. The association of nuclear GAPDH with telomeres in interphase nuclei was also demonstrated by co-fluorescence in situ hybridization and chromatin immunoprecipitation analysis. Further data demonstrated that the nuclear localization of GAPDH is regulated by ceramide in a cell cycle-dependent manner parallel with the inhibition of its telomere binding activity in response to ceramide. In addition, the results revealed that nuclear GAPDH is distinct from its cytoplasmic isoform and that telomere binding function of nuclear GAPDH is strikingly higher than the cytoplasmic isoform. More importantly, the functional role for nuclear GAPDH in the maintenance and/or protection of telomeric DNA was identified by partial inhibition of the expression of GAPDH using small interfering RNA, which resulted in rapid shortening of telomeres. In contrast, overexpression of nuclear GAPDH resulted in the protection of telomeric DNA in response to exogenous ceramide as well as in response to anticancer drugs, which have been shown to induce endogenous ceramide levels. Therefore, these results demonstrate a novel function for nuclear GAPDH in the maintenance and/or protection of telomeres and also show that mechanisms of the rapid degradation of telomeres in response to ceramide involve the inhibition of the telomere binding activity of nuclear GAPDH.

Telomeres, which are the DNA-protein complexes found at the end of chromosomes, play important roles in the protection of chromosome termini against degradation and fusion with other chromosome ends (for a review, see Ref. 1). Telomeres also function for the complete replication of chromosome ends, which can be extended by a specific reverse transcriptase, telomerase (2, 3). The telomeric ends of chromosomes comprise short tandem repeats of nucleotide sequences. Human telomeres have 5'-d(TTAGGG)-3' repeats in the 5'-3' orientation at the chromosome terminus, known as the G-strand, and this sequence is conserved in all vertebrates as well as in Trypanosoma, filamentous fungi, and slime molds. It has been demonstrated that the 3'-end of the G-strand is terminated with an unpaired overhang (4, 5).

It has been postulated that progressive shortening of telomere length in somatic cells leads to cellular senescence, whereas maintenance of telomeres at a critical length is associated with immortality in germ line and cancer cells (1). Therefore, it is believed that there are control mechanisms that monitor and regulate telomere length in these cells. It is well documented that telomeres are extended and maintained mainly by the activity of telomerase in the germ line and immortalized cells. However, telomerase activity is not present in various somatic and dividing primary cells, demonstrating that telomerase is not the sole determinant of telomere length. Numerous proteins that are associated with telomeric DNA have been identified to play roles in the regulation of telomere length in mammalian cells (6–9). Human telomeres have been shown to interact with two distinct proteins, namely TRF-1 and TRF-2, both of which are most closely related to their Mfy-type DNA binding domains, and both proteins have been shown to bind selectively to the duplex telomeric DNA in vitro and in vivo (6–9). TRF-1 and TRF-2 proteins have been localized to the ends of chromosomes (6–9). Specifically TRF-1 has been associated with the negative regulation of telomeric ends (7), and TRF-2 has been shown to be required for the assembly of 1-loop telomere structures (10, 11) and has been demonstrated to be important for the protection of chromosome ends (12).

Recently Pot1, which has been identified as a single-stranded telomeric DNA-binding protein in Schizosaccharomyces pombe, has been demonstrated to play a critical role in the protection of telomeric ends of chromosomes (13). The human homologue of Pot1 has recently been shown to interact with telomere-binding proteins TRF-2 and hRap1 in the interphase

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1 The abbreviations used are: TRF, telomere repeat binding factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; hPot, human protection of telomere protein; GMZ, gemcitabine; DOX, doxorubicin; ss, single-stranded; ds, double-stranded; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CHIP, chromatin immunoprecipitation.

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nuclei of human cells (14) and also shown to elongate telomeres by telomerase (15).

The sphingolipid ceramide has been demonstrated to be a bioeector molecule that plays roles in the signaling of biological stress responses such as apoptosis, cell cycle arrest, and senescence in various human cells (16). Recent findings from our laboratory have revealed that ceramide is an upstream regulator of telomerase activity in human lung cancer cells (17) and that inactivation of telomerase involves rapid proteolysis of c-Myc transcription factor, which is shown to up-regulate hTERT promoter activity, via increased ubiquitination in response to ceramide (18) in the A549 human lung adenocarcinoma cells. However, the role of ceramide in the regulation of telomere length was not determined previously.

In this study, during the investigation of ceramide-regulated single-stranded (ss)-telomere-binding proteins, nuclear glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was identified to associate with telomeric DNA both in vitro and in situ. Further analysis showed that the nuclear localization of GAPDH is regulated by cell cycle-dependent mechanisms and that ceramide controls the telomere binding activity of GAPDH by inhibiting its nuclear localization. In addition, we report here that the nuclear isoform of GAPDH, which is responsible for telomere binding, is distinct from its cytoplasmic isoform. More importantly, the data presented here show the functional involvement of nuclear GAPDH in the maintenance and protection of telomeres against ceramide and chemotherapeutic agents. Therefore, these results demonstrate that one of the mechanisms of ceramide-mediated rapid shortening of telomeres involves the inhibition of the unexpected and newly identified telomere binding activity of nuclear GAPDH.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture Conditions**—The A549 human lung carcinoma cells were obtained from Dr. Alice Boylan (Medical University of South Carolina, Charleston, SC). Cells were maintained in growth medium containing 10% fetal calf serum, 100 ng/ml penicillin, and 100 ng/ml streptomycin (Invitrogen) at 37 °C in 5% CO₂. Cell-permeable and biologically active short chain ceramide (C₆-ceramide) and its newly developed water-soluble analog LCL-124 were obtained from the Synthetic Lipid Core at the Department of Biochemistry and Molecular Biology, Medical University of South Carolina. Doxorubicin was purchased from Sigma, and gemcitabine was purchased from Eli Lilly.

**Telomere Length Assay**—The measurements of telomere lengths were performed using a telomere restriction fragment length measurement kit (Roche Applied Science) by Southern blotting as described by the manufacturer.

**Determination of Telomerase Activity**—Telomerase activity in cell extracts was measured by the PCR-based telomere repeat amplification protocol (TRAP) using the TRAPeze kit (Intergen, Gaithersburg, MD), which includes a 36-bp internal control to allow quantification of activity as described previously (17, 18).

**Gel Shift Assays and Affinity Column Chromatography**—The gel shift assays were performed as described previously (18). The ss-telomere-binding proteins were partially purified from nuclear extracts (19) by affinity chromatography using cyanogen bromide (CNBr)-activated Sepharose beads coupled with ss-TTAGGG, oligonucleotide as described previously (20). The UV cross-linking studies were performed as described elsewhere (20).

**Co-fluorescence in Situ Hybridization Analysis**—Co-localization of nuclear GAPDH with telomeric DNA in interphase nuclei of A549 cells was performed by SeeDNA Biotech Inc. (Ontario, Canada). Nuclear and chromosome preparations were prepared as described previously (21, 22). After hypotonic treatment, the nuclear/chromosomal suspension was fixed with methanol/acetic acid (3:1) and dropped onto the slides. Following rehydration in azide solution (10 mM Na₃HPO₄, 0.15 M NaCl, 1 mM EDTA, and 0.01% NaN₃) for 15 min, slides were incubated in TEEN buffer (1 mM triethanolamine, pH 8.5, 0.2 mM NaEDTA, pH 8.0, 25 mM NaCl, 0.05% Tween 20, 0.1% bovine serum albumin) for 20 min. Nuclear GAPDH was then visualized after incubation with the primary antibody (1:200) at 25 °C for 4 h, secondary antibody for 2 h, and extensive washes with the antibody detection buffer (10% goat serum, 3% bovine serum albumin, 0.05% Triton X-100). Slides were then counterstained with 4,6-diamidino-2-phenylindole. Fluorescence in situ hybridization detection was performed using fluorescently-labeled peptide nucleic acid-telomere probe (Applied Biosystems) as described by the manufacturer.

**Chromatin Immunoprecipitation (CHIP) Analysis**—The association of endogenous GAPDH with telomeric DNA in A549 cells was confirmed using a CHIP assay kit (Upstate Biotechnology) as described by the manufacturer. Cells (about 10⁶) were treated with formaldehyde (final concentration of 1%) for 60 min at 25 °C. Then cross-linked proteins (GAPDH, TRF-1, and TRF-2) were immunoprecipitated using anti-GAPDH (Chemicon) and -TRF-1 and -TRF-2 (Santa Cruz Biotechnology) antibodies. After they were pulled down with salmon sperm DNA-protein A-agarose beads, cross-links were reversed, and the protein-DNA complexes were separated from the DNA by chloroform extraction. The remaining DNA samples were loaded onto membranes by slot-blotting, and telomeric DNA was detected using digoxigenin-labeled telomeric probe by Southern blotting using a telomere restriction fragment length measurement kit (Roche Applied Science), and the signals were visualized using anti-digoxigenin antibody as described by the manufacturer. Normal IgGs were used as a negative control.

**Immunofluorescence and Flow Cytometry**—Cells were fixed in 4% paraformaldehyde at 25 °C for 10 min and permeabilized in 0.1% Triton X-100, 4% paraformaldehyde at 25 °C for 10 min, and protein localizations were visualized by immunofluorescence/confocal microscopy using mouse monoclonal anti-GAPDH (Chemicon, MAB 374) or anti-His antibodies at 1:500 and fluorescein isothiocyanate- or rhodamine-conjugated goat anti-mouse secondary antibodies (Jackson Immunoresearch Laboratories). The cell cycle profiles were determined using flow cytometry as described previously (17).

**Two-dimensional Gel Electrophoresis and Western Blotting**—The different isoforms of GAPDH in nucleus versus cytoplasm were examined by two-dimensional gel electrophoresis utilizing the Protean isoelectric focusing system (Bio-Rad) with the first dimension isoelectric ready gel strips (Bio-Rad) containing a pH gradient from pH 3.0 to 10.0 over 8 cm as described by the manufacturer. Then proteins on the strips were separated in 4–20% SDS-polyacrylamide second dimension gels, and different isoforms of GAPDH were detected by Western blotting.

**Plasmids, Small Interfering RNA (siRNA) (siRNAs), and Transfections**—The complete cDNA for human GAPDH was kindly provided by Dr. S. M. Lemon (University of Texas Medical Branch, Galveston, TX). The GAPDH cDNA was then cloned into pcDNA3(+) with 3′-His₅ tag, which was then used for transfections of A549 cells as described previously (17). The siRNAs against GAPDH and hexokinase II were synthesized and high pressure liquid chromatography-purified by Qiagen Biotechnology, and transfections of cells with siRNA molecules were performed using the Oligofectamine transfection kit (Invitrogen) as described by the manufacturer.

**RESULTS**

**Rapid Shortening of Telomere Length and the Inhibition of Telomere Binding Activity of C1 Complex in Response to Ceramide**—To determine whether ceramide is involved in the regulation of telomere length, A549 cells were treated with C₆-ceramide, and then the length of telomere restriction fragments was measured by Southern blotting. Treatment with 20 μM C₆-ceramide for 24 (Fig. 1A) and 96 h resulted in shortening of telomere restriction fragment length by around 300 ± 50 and 650 ± 80 bp, respectively, compared with that of untreated controls (Fig. 1, A and B). Since it has been previously demonstrated that ss-telomere-binding proteins, such as Pot1 in S. Pombe and Cdc13 in Saccharomyces cerevisiae, are involved in the protection of telomeres and that their deletion results in rapid loss of telomeres (13, 23), we investigated the effect of ceramide on ss-telomere-binding protein(s) in human A549 cells. Nuclear extracts were isolated from A549 cells grown in the absence or presence of 20 μM C₆-ceramide for 24–96 h, and then gel shift assays were performed using the end-labeled G-rich strand of telomeric sequence (TTAGGG₃) as a probe. Interestingly, one major telomere-protein complex (C1) was detected whose binding activity was inhibited about 75–80% in response to ceramide at 24–96 h in A549 cells when compared with untreated controls (Fig. 1C). UV Cross-linking following gel shift assay revealed that the molecular mass of the C1 protein is around 36–40 kDa (Fig. 1D).
Telomere Binding Characteristics and Identification of Nuclear GAPDH in the C1 Complex—To determine the binding characteristics of the C1 protein to the ss-telomere probe (TTAGGG)$_3$, gel shift assays were performed in the absence or presence of 250-fold unlabeled 18-mer competitors that contained either single-stranded TTAGGG, GGGATT, AATCCC, and CCCTAA or double-stranded TTAGGG and AATCCC (used as a nonspecific control) DNA sequences (Fig. 2A). The results showed that C1 binds to both ss-TTAGGG and ss-CCCTAA oligomers and also interacts with the ds-TTAGGG oligomer (Fig. 2A, lanes 2, 5, and 7, respectively), whereas non-telomeric oligomers did not compete with the binding of C1 to the ss-TTAGGG probe (lanes 3, 4, and 6). Dose-response studies showed that C1 binds to both ss-TTAGGG and ss-CCCTAA oligomers with high affinity; concentrations of oligomers that inhibited binding by 50% were around 2 and <1 ng/µl, respectively (Fig. 2B, lanes 1–5 and 6–10, respectively). The ds-TTAGGG oligomer showed an IC$_{50}$ of about 7.5 ng/µl (data not shown). These results demonstrate that the C1 complex interacts with both the G- and C-strand and also with the ds-telomeric DNA sequences but not with the non-telomeric oligomers, revealing that the C1 complex shows more specificity for binding to telomeric DNA sequences.

To identify the C1 protein, the C1 complex was partially purified from A549 nuclear extracts (about 100 µg) by affinity chromatography using CNBr-activated Sepharose 4B columns conjugated with the (TTAGGG)$_3$, oligomer. The bound proteins were eluted in the presence of 0.2–0.8 M NaCl and then subjected to SDS-PAGE and silver staining (Fig. 2C, lanes 1–5). The results showed that a single protein at 36–40 kDa was eluted with 0.8 M NaCl (Fig. 2C, lane 5). Other ss-telomere-binding proteins were also detected when higher amounts of nuclear extracts (1–10 mg) were used in similar experiments (data not shown); however, since the C1 appeared to be the most abundant complex, we focused our attention on this complex. Then fractions from 0.6 and 0.8 M NaCl were used in UV cross-linking studies, and the results showed that the protein eluted with 0.8 M NaCl bound the ss-telomere probe in vitro (Fig. 2C, lane 7). Afterwards, the affinity-purified C1 protein was subjected to amino-terminal peptide sequencing by Edman degradation. Four trypsin-digested C1 peptides were identical to GAPDH (Fig. 2D). These results were also confirmed by matrix-assisted laser desorption/ionization/nanospray mass spectrometry of the trypsin-digested peptides, which showed that the purified C1 complex was identical to GAPDH (data not shown). After identification of GAPDH as a candidate telomere-binding protein, its presence in the C1 complex was further confirmed by excising the gel fragment containing this complex and subjecting it to SDS-PAGE followed by Western blotting. Anti-GAPDH antibody detected a protein at 36–40
kDa, whereas no signal was detectable when the same membrane was blotted using normal mouse IgG (Fig. 2E). These results demonstrate that the C1 protein band contains predominantly GAPDH. Although the presence of other minor telomere-binding proteins in C1 complex might also be possible, there were no such proteins, other than GAPDH, detectable in this complex in our experiments.

To determine whether authentic GAPDH can bind telomeric sequences, purified GAPDH from human erythrocytes was used in UV cross-linking studies directly in vitro. The results showed that purified GAPDH also binds ss-telomeric DNA probe; however, the amount of purified protein (0.75–1.0 µg) needed for telomere binding was relatively high (the possible reason for this is shown and discussed in Fig. 6). Nevertheless binding characteristics of the purified GAPDH were similar to the C1 complex, which interacted with the oligomer containing human ss-telomeric sequence (TTAGGG)_3 but not with oligomers containing ss-telomeric sequences from *Tetrahymena* (TTGGGG)_3 or *Dictyostelium* (GGGGAGGGAGGAGAGGAGGGAGGA) (Fig. 3A, lanes 2–4 and 6–8, respectively).
which were used as unlabeled competitors in UV cross-linking studies. In addition, the presence of increasing concentrations of NAD$^+$ (2–4 mM) inhibited the telomere binding activity of purified GAPDH and the C1-GAPDH complex in nuclear extracts (lanes 2–4 and 6–8, respectively) at 250-fold excess was examined by UV cross-linking studies. Lanes 1 and 5 contain binding with the probe in the absence of unlabeled oligomers. B, the involvement of NAD$^+$-binding site with the association of purified GAPDH or in the C1 complex of nuclear extracts from A549 cells was determined using 0, 0.5, 1, and 2 mM NAD$^+$ (lanes 1–4) or 0, 1, 2, and 4 mM NAD$^+$ (lanes 5–8), respectively, as competitors in UV cross-linking studies. C, the requirements of a single, double, or triple TTAGGG repeat for the association of purified GAPDH was determined using oligomers (at 250-fold excess) containing (TTAGGG)$_3$, (TTAGGG)$_2$, or (TTAGGG)$_1$ (lanes 1–3, respectively) in UV cross-linking studies as described in A. D, the association of purified hexokinase II (lane 2) with telomeres was examined as in A and compared with that of purified GAPDH (lane 1).

The Association of Nuclear GAPDH with Telomeres in Situ—Although GAPDH is primarily known for its glycolytic function, several studies over the past decade have demonstrated several non-glycolytic functions, including membrane fusion, microtubule bundling, phosphotransferase activity, nuclear
Mechanisms of Regulation of Telomere Length by Ceramide

The association of GAPDH with telomeres in situ. A, the association of nuclear GAPDH protein with telomeric DNA in interphase nuclei of A549 cells was determined using co-fluorescence in situ hybridization (FISH) studies performed by SeeDNA Biotech Inc. using mouse monoclonal anti-GAPDH antibody and fluorescein isothiocyanate-labeled telomere-peptide nucleic acid probe (Applied Biosystems) as described under “Experimental Procedures.” The GAPDH protein was detected in the interphase nuclei of A549 cells using anti-GAPDH antibody (panel 1), telomeric DNA was hybridized with telomere-peptide nucleic acid probe (panel 2), and GAPDH and telomere signals in these nuclei were merged (panel 3) as described under “Experimental Procedures.” B, the interaction of GAPDH with telomeric DNA on chromosomes was also confirmed using CHIP analysis as described under “Experimental Procedures.” Lanes 2, 4, 6, and 8 contain DNA immunoprecipitated using TRF-1, GAPDH, TRF-2, and hexokinase antibodies. Lanes 1, 3, 5, and 7 contain input DNA before immunoprecipitations in each sample. Telomeric DNAs were detected by Southern blotting using the digoxigenin-labeled telomere probe supplied in the telomere restriction fragment detection kit (Roche Applied Science) as described by the manufacturer.

tRNA transport, and DNA replication and repair (for a review, see Ref. 24). It is also known that GAPDH may be detected in the nucleus in HeLa cells by immunofluorescence using a mouse monoclonal antibody that recognizes GAPDH (25). However, its precise localization in interphase nuclei has not been determined. Therefore, localization of endogenous GAPDH and its association with telomeres in situ were studied by immunofluorescence using an anti-GAPDH antibody (Fig. 4A, panel 1) and with fluorescence in situ hybridization using fluorescein-labeled telomeric DNA in interphase nuclei of A549 cells. The results showed that GAPDH was localized in the nucleus in about 30–40% of the cell population, whereas in the remaining cells it was primarily cytosolic and not detected in the nucleus. More importantly, the dual labeling studies demonstrated a speckled distribution of GAPDH, which coincided with telomere signals (Fig. 4A, panel 3). These results were also confirmed by CHIP analysis, which showed that endogenous GAPDH is associated with telomeric DNA in situ (Fig. 4B, lane 4). The telomere-binding proteins TRF-1 and TRF-2 were used as positive controls, whereas hexokinase was used as a negative control in CHIP analysis (Fig. 4B, lanes 2, 6, and 8, respectively), and input DNAs before immunoprecipitations were used as loading controls (lanes 1, 3, 5, and 7). Thus, these results demonstrate that GAPDH exhibits a clear nuclear localization, and nuclear GAPDH is enriched at human telomeres in chromosomes.

Regulation of Nuclear Localization of GAPDH by Cell Cycle-dependent Mechanisms in Response to Ceramide—Localization of GAPDH primarily in the nuclei of about 30–40% of A549 cell population suggested that its nuclear localization might be cell cycle-dependent. To investigate this possibility, we synchronized A549 cells by blocking them in G1/S boundary (see Fig. 5A, 0 h time point) using thymidine and then examined cell cycle profiles by flow cytometry (17) and subcellular localization of GAPDH by immunofluorescence using mouse monoclonal anti-GAPDH antibody after releasing the cells for various time points (Fig. 5A). The data presented in Fig. 5A show that GAPDH was localized mainly in the nucleus in about 83% of the population when the majority of the cells (about 88%) were in S phase after 4-h release. As cells progressed through G2/M (about 70%) after 8-h release, the number of cells that contained nuclear GAPDH slightly decreased (about 67%). Interestingly GAPDH was almost completely excluded from the nucleus and mostly localized in the perinuclear ring and cytosol when cells (about 74%) entered G1/G0 after 24-h release. These results show that nuclear localization of GAPDH is regulated by a cell cycle-dependent mechanism, which appears to be maximum at G1 phase, and can no longer be detected in the nucleus when the majority of the cells are in G1/G0.

The human genome appears to contain only one functional GAPDH gene although a number of pseudogenes exist (26, 27). Nevertheless the above results could not rule out the presence of distinct GAPDHs in nucleus and cytosol whose expression/ degradation is regulated in a cell cycle-specific manner as opposed to translocation of the same gene product. Therefore, to establish that it is the same gene product present in both locations, localization of exogenously overexpressed 3′-His tagged GAPDH in A549 cells was also examined in similar experiments (mouse monoclonal anti-His antibody was used in immunofluorescence). Results showed that exogenous GAPDH was detected mainly in the nucleus (in about 62%) when the cells were in S phase after 4-h release, whereas it was entirely in the cytoplasm (100%) when cells progressed through G2/M after 24-h release (Fig. 5B). The nuclear localization of His tagged GAPDH was also confirmed using anti-TRF-1 antibody, which is known to interact with telomeres in the nucleus (Fig. 5B, lower panel). Taken together, these data demonstrate that the nuclear localization of both endogenous and exogenous GAPDH is regulated in a cell cycle-dependent manner and that it is the same gene product that exhibits this behavior.

Ceramide has been shown to regulate the nuclear localization of CCAAT-binding protein β (28). Therefore, it became of great interest to determine the effects of ceramide on localization of GAPDH. The localization of endogenous GAPDH was examined by immunofluorescence, and the results showed that nuclear localization of GAPDH (nuclear in about 45% of the
untreated cells) was inhibited by 20 μM C6-ceramide at 24 h (nuclear in 10% of the cells), which correlated with the G0/G1 arrest of the cell population in response to ceramide (Fig. 5C).

These results, therefore, demonstrate that the subcellular trafficking of GAPDH is regulated in a cell cycle-dependent manner and that ceramide is involved in the regulation of nuclear localization of GAPDH in A549 cells. These data also suggest that the regulation of nuclear localization of GAPDH might be one of the mechanisms for the control of its telomere binding function by ceramide.

Biochemical Characterization of Nuclear and Cytoplasmic Isoforms of GAPDH and Their Distinct Roles in Telomere Binding—Given the dual localization of GAPDH, we next examined telomere binding activity of GAPDH in cytoplasmic versus nuclear fractions of A549 cells. Western blotting and UV cross-linking studies using ss-TTAGGG probe were performed, and the results showed that although the protein levels of GAPDH in the nuclear extracts were about 40–50% lower than cytoplasmic fractions as determined by Western blotting (Fig. 6A, lanes 1 and 2), telomere binding activity of GAPDH in the nucleus was around 15–20-fold higher than in cytoplasm (Fig. 6A, lanes 3 and 4). Interestingly two-dimensional gel electrophoresis followed by Western blotting demonstrated that nuclear fractions contained predominantly GAPDH with a basic PI of around 8.3–8.7, whereas cytoplasmic fractions (Fig. 6B) as well as commercially available GAPDH (Fig. 6C) purified from human erythrocytes (which lack nuclei) contained GAPDH with a more acidic PI of around 7.0–7.5 (Fig 6B). The purified GAPDH contains a very small amount of isoform with the basic PI (8.3), whereas its isoform with more acidic PI (around 7.0–7.5) was shown to be responsible for its conventional dehydrogenase activity (29). These data therefore suggest that the telomere binding function of GAPDH is carried out by its distinct nuclear isoform with the basic PI of 8.3–8.7.

The Functional Role of Nuclear GAPDH in the Maintenance and Protection of Telomere Length in Response to Chemotherapeutic Agents and Ceramide—The above results raised the possibility that GAPDH might be involved in the maintenance of telomere length in A549 cells. To test this hypothesis, the expression of GAPDH was down-modulated using siRNA in A549 cells, and its role in the regulation of telomeric length was evaluated by telomere restriction fragment analysis. The data showed that treatment of cells with 200 and 400 nM GAPDH-specific siRNA resulted in 15 and 80% decrease, respectively, in telomere length.
and/or protection of telomeres, we examined the effects of over-expression of His-tagged GAPDH (Fig. 8A) on telomere restriction fragment length in response to chemotherapeutic agents gemcitabine (GMZ) at 245 nM and doxorubicin (DOX) at 150 nM for 96 h; both are known inducers of endogenous ceramide in A549 cells (16, 30). As seen in Fig. 8B, treatment of A549/pcDNA3 cells with GMZ, DOX, and GMZ plus DOX in combination resulted in a significant decrease in telomere restriction fragment length by about 250, 400, and 800 bp, respectively (Fig. 8B, lanes 3–5). Therefore, these results are in agreement with previous data showing that chemotherapeutic agents are capable of inducing relatively acute shortening of telomeres (31). Moreover in this study overexpression of GAPDH in the A549/GAPDH cells abrogated the effects of GMZ, DOX, and GMZ plus DOX on telomere restriction fragment length (Fig. 8B, lanes 7–9). Similar results were also obtained in which overexpression of GAPDH completely blocked rapid shortening of telomere restriction fragment length in response to the newly developed C6-ceramide analog (LCL-124) at 100 and 250 nM for 48 h when compared with controls (Fig. 8C, lanes 5–7 and 2–4, respectively). It should also be noted that the overexpression of GAPDH results in a slight increase (about 200 bp) in telomere length when compared with vector-transfected controls in A549 cells (Fig. 8, B, lanes 2 and 6, and C, lanes 2 and 5, respectively). These results demonstrate that GAPDH is involved in the protection of telomeric DNA against damage caused by chemotherapeutic agents and ceramide and that the mechanisms by which ceramide mediates rapid shortening of telomeres involve the inhibition of telomere binding activity of nuclear GAPDH.

**DISCUSSION**

The data presented here demonstrate that the nuclear and basic isoform of GAPDH associates with telomeric DNA *in vitro* and *in situ* in a cell cycle-dependent manner. The nuclear localization of GAPDH and its cell cycle regulation may be an important mechanism in segregating the glycolytic function of GAPDH from its telomere binding functions. Further the data define a role for nuclear GAPDH in the maintenance and protection of telomere length against ceramide and chemotherapeutic drugs. More importantly, these results show for the first time that the rapid shortening of telomeres in response to ceramide mechanistically involves the inhibition of the telomere binding function of nuclear GAPDH. The binding of nuclear GAPDH to telomeric DNA in nuclear extracts of various different cell lines (such as HeLa, Wi-38, and SCC-UM22A human head and neck cancer cells) was also detected using UV cross-linking studies (data not shown).

The association of nuclear GAPDH with telomeric DNA and its functional role in maintenance and protection of telomeres have not been detected previously. Although it may sound unusual for an abundant glycolytic protein to be involved in such function, there are now growing number of studies in the literature that show various non-glycolytic nuclear functions of GAPDH, including tRNA transport and DNA repair and replication (23). In parallel with our data, which showed that NAD+ binding domain of GAPDH is involved in the association with telomeric DNA *in vitro*, it has been demonstrated by a recent study that the NAD+-binding site of GAPDH plays an important role in RNA binding (32). These studies demonstrate clearly that GAPDH is involved in diverse biological activities in cells, including maintenance and protection of telomeres. The recent finding that identified mitochondrial glucokinase (hexokinase IV) in a protein complex containing Bad1 and PP1 as having an important role in apoptosis (33) supports the idea that proteins involved in glucose metabolism might have diverse functions. In any case, using antibodies to endogenous

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**Fig. 6. Detection of nuclear and cytoplasmic isoforms of GAPDH with hTERT protein and telomerase activity.** A, nuclear (Nucl) and cytoplasmic (Cyt) fractions of A549 cells (1–2 μg) were separated by 4–20% SDS-PAGE, and the expression levels of GAPDH (lanes 1 and 2) or binding activity to labeled ss-(TTAGGG)3 probes (lanes 3 and 4) were determined by Western blotting or gel shift assays, respectively. Nuclear and cytoplasmic fractions of A549 cells (50 μg) (B) and purified total GAPDH from human erythrocytes (63 μg) (C) were examined by two-dimensional electrophoresis utilizing the Protean isoelectric focusing system (Bio-Rad) with the first dimension isoelectric ready gel strips (Bio-Rad) containing a pH gradient from pH 3.0 to 10.0 over 8 cm as described by the manufacturer. Then proteins on the strips were separated in 4–20% SDS-polyacrylamide second dimension gels, and different isoforms of GAPDH were detected by Western blotting.

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the expression of GAPDH in the nucleus (Fig. 7A, lanes 2 and 3, respectively). The expression of GAPDH in total cell lysates was decreased by 67% in response to 400 nM GAPDH siRNA as compared with controls (Fig. 7A, lanes 5 and 4, respectively). The down-modulation of GAPDH by 200 and 400 nM siRNA also caused about 120 ± 50- and 500 ± 140-bp decrease in telomere restriction fragment length, respectively, when compared with controls, which were transfected with scrambled siRNA (Fig. 7B, lanes 3, 4, and 2, respectively). The data therefore show that endogenous GAPDH plays a role in the maintenance of telomere length in these cells. Since GAPDH is one of the major enzymes of glycolysis, we also examined whether the inhibition of another glycolytic enzyme, hexokinase II, affects telomere restriction fragment length in these cells. The inhibition of hexokinase expression by siRNA in A549 cells had no significant effect on telomere restriction fragment length as compared with controls (Fig. 7B, lanes 9 and 8, respectively).

To examine the role of nuclear GAPDH in the maintenance
GAPDH as well as exogenous GAPDH with the His tag clearly indicate nuclear localization, a site devoid of glycolysis. This finding thus supports non-glycolytic functions of GAPDH.

The unexpected functional role of nuclear GAPDH in the positive regulation of telomere length raises the possibility that the regulation of GAPDH might be important in development, aging, and cancer. In parallel with this notion, it has been demonstrated previously that GAPDH expression is decreased in muscles of older rats when compared with their young counterparts, indicating a role for GAPDH in aging (34). Also there are number of studies showing increased expression levels of GAPDH in many cancer tissues especially in tissues obtained from lung (35) and prostate cancer patients (36), demonstrating a role for GAPDH in pathogenesis. Moreover a role for GAPDH in neurodegenerative disorders and interaction with hepatitis A virus RNA have been described previously (37–40).

Therefore, since ceramide has been shown to play a role in aging and also shown to be involved in antiproliferative responses, the results presented in this study, the regulation of telomere length by ceramide via the inhibition of telomere binding activity of GAPDH, might present an important and novel mechanistic link with its involvement in aging and cancer.

In several studies, various proteins with different functions were identified to be associated with telomeres in human cells, including nucleolar heterogeneous nuclear ribonucleoproteins, which specifically interact with the ss- but not with the ds- telomeric DNA (41–43), and Ku, which is conventionally involved in DNA repair (44–46). Moreover as-telomere-binding protein hPot1 has been reported to be ubiquitously expressed in almost all human tissues, suggesting that it is a housekeeping protein (14). These results therefore indicate that there might be other as yet unidentified factors with known physiological or biochemical functions that might also be involved in telomere homeostasis.

The results described in this report also show that telomere binding activity of GAPDH is regulated by the sphingolipid ceramide in a cell cycle-dependent manner. The nuclear localization of GAPDH is primarily detectable in S phase, and it is almost completely excluded from the nucleus when the majority of cells are in G1/G0. It is interesting to note that telomerase activity has also been reported to be associated with the S phase of the cell cycle (47–48), indicating that the maintenance of telomeres is mainly regulated in S phase. Importantly results of this study demonstrate that the rapid decrease in telomere length in response to ceramide mechanistically in-
volves the inhibition of telomere binding activity of GAPDH via G0/G1 arrest by ceramide. The mechanisms of nuclear localization of GAPDH are, however, unknown and need to be determined. Our biochemical studies using Amicon columns to separate proteins based on their molecular mass showed that the telomere binding activity of GAPDH can be recovered primarily in 100-kDa fractions (data not shown). These results suggest that the tetrameric form of GAPDH is mainly responsible for telomere binding. It is still unknown, however, whether GAPDH is imported to the nucleus as a tetramer or imported as a monomer, which then forms tetramers in the nucleus. Clearly the two-dimensional electrophoresis followed by Western blotting showed that the nuclear isoform of GAPDH has a more basic PI than its cytoplasmic isoform. The reasons for these biochemical differences in the two isoforms of GAPDH and the roles of the basic PI versus more acidic PI in the nuclear localization of GAPDH are not known and need to be determined.

Recently it has been shown that hPot1 associates with TRF-1, and binding of hPot1 to ss-telomeric DNA is regulated by TRF-1 in response to telomere length (49). It has also been shown that hPot1 facilitates telomere elongation by a telomerase-dependent mechanism (15). Since ceramide has also been reported to be involved in the regulation of telomerase activity, it will be of great interest to identify the molecular mechanisms by which nuclear GAPDH mediates the maintenance and/or protection of telomeres and whether these mechanisms involve ceramide-regulated association of nuclear GAPDH with the TRF-1 complex and/or telomerase.

In conclusion, the results presented here demonstrate that the protection and maintenance of telomeres can be regulated not only by specialized proteins but also by proteins with diverse functions in mammalian cells.

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Fig. 8. The role of overexpression of GAPDH in the protection of telomere length in response to chemotherapeutic agents and ceramide. A, A549 cells were transfected with 3'-His-tagged GAPDH, and nuclear GAPDH levels in transfected cells as compared with vector controls were examined by Western blotting (lanes 2 and 1, respectively). B, telomere restriction fragment length measurements were performed following treatment of A549/pDNA3 and A549/GAPDH-His in the absence or presence of GMZ (G, 245 nM), DOX (D, 150 nM), and GMZ plus DOX (G+D) in combination (lanes 2–5 and 6–9, respectively) for 96 h. Lane 1 contains molecular weight markers. C, same as in B except that the A549/pDNA3 and A549/GAPDH-His cells were treated with the Cc-ceramide analog (LCL-124) at 0, 100, and 250 nM for 48 h (lanes 2–4 and 5–7, respectively). C, control.
