Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has diverse biological functions including its nuclear translocation in response to oxidative stress. We show that GAPDH physically associates with APE1, an essential enzyme involved in the repair of abasic sites in damaged DNA, as well as in the redox regulation of several transcription factors. This interaction allows GAPDH to convert the oxidized species of APE1 to the reduced form, thereby reactivating its endonuclease activity to cleave abasic sites. The GAPDH variants C152G and C156G retain the ability to interact with but are unable to reactivate APE1, implicating these cysteines in catalyzing the reduction of APE1. Interestingly, GAPDH-small interfering RNA knockdown sensitized the cells to methyl methane sulfonate and bleomycin, which generate lesions that are repaired by APE1, but showed normal sensitivity to 254-nm UV. Moreover, the GAPDH knockdown cells exhibited an increased level of spontaneous abasic sites in the genomic DNA as a result of diminished APE1 endonuclease activity. Thus, the nuclear translocation of GAPDH during oxidative stress constitutes a protective mechanism to safeguard the genome by preventing structural inactivation of APE1.

The evolutionary conserved enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) exists as a tetramer that catalyzes a critical reaction in the second stage of the glycolytic pathway. It uses the oxidized form of nicotinamide adenine dinucleotide (NAD\(^+\)) and converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate with the concomitant release of NADH in an oxido-reduction reaction. GAPDH is also a key redox-sensitive protein that possesses an active site cysteine sulfhydryl that is susceptible to oxidation. Under oxidative stress, GAPDH rapidly undergoes disulfide bond formation leading to reduction in its enzymatic activity. GAPDH has the propensity to interact with several proteins that are vulnerable to aggregation and are associated with neurodegenerative disorders such as in the case of the pro-oxidant amyloid \(\beta\) peptide involved in Alzheimer disease. Recent studies have documented that GAPDH is also involved in several other nuclear processes that include histone H2B gene expression, nuclear RNA export, apoptosis, and cellular response to DNA damage.

Several lines of evidence support a role for GAPDH in DNA damage and repair. For example, GAPDH can translocate from the cytoplasm to the nucleus when cells are challenged with the potent chemical oxidant and DNA-damaging agent \(H_2O_2\), although it is not clear what is the function executed by GAPDH under this stress condition. However, a more recent study documented that nitric oxide can also induce nuclear localization of GAPDH where it is acetylated by the acetyltransferase p300/CBP via direct protein interaction, which in turn causes stimulation of the catalytic activity of p300/CBP, resulting in the activation of downstream targets such as p53. Other studies have shown that GAPDH is associated with DNA-containing genotoxic lesions such as thioguanylated DNA generated by the chemotherapeutic agent mercaptopurine used for treating acute lymphoblastic leukemia. Thus, it seems that GAPDH response to oxidative stress might be linked to a role in maintaining the integrity of the genome.

In this study, we report that human GAPDH directly interacts with APE1, an essential enzyme that functions in the base excision repair pathway to process spontaneous and drug-induced abasic or apurinic/apyrimidinic (AP) sites, as well as to regulate the redox state of a number of transcriptional factors such as p53, AP-1, c-Jun, c-Fos, and NF-\(\kappa\)B. Using recombinant proteins, we show that the active site cysteine 152 of GAPDH is not required for the interaction with APE1 but indispensable for converting the oxidized forms of APE1 to its reduced form. The catalytic action of GAPDH on oxidized APE1 re-establishes a key biological function of the protein, that is, the ability to cleave AP sites, indicating that the redox-sensitive cysteine of GAPDH can function to reduce APE1. Concomitantly, this reduction process greatly enhanced the detection of APE1 by anti-APE1 antibodies consistent with the oxidized form of the protein undergoing a structural change. We further demonstrate that siRNA knockdown of GAPDH in HCT116 cells caused sensitivity to agents.
known to create DNA lesions that are processed by APE1, including the alkylating DNA-damaging agent methyl methane sulfonate (MMS), which produces AP sites, but not to 254 nm of UVC (19, 20). Importantly, the GAPDH knockdown cells accumulated a higher level of spontaneous AP sites in the genomic DNA. We propose that GAPDH serves a critical role in maintaining genomic stability by protecting APE1 from aberrant structural changes caused by oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Bacteria Strains**—The *Escherichia coli* strains used in this work were BW528 [Δ(xth, pnc), nfs1-kan] (kindly provided by B. Weiss, Emory University, Atlanta, GA) and BL21(DE3)pLysS [dcn ompT hsdS (r}_{gal} m_{(-)}]) galA(DE3) (pLysS Cam’)] (Stratagene).

**Cell Culture**—Primary human diploid lung fibroblast LF1 (low passage) and human colon carcinoma HCT116 cell lines were kindly provided by Dr. Drobetsky (University of Montreal, Montreal, Canada), and the human colon carcinoma DLD1 cell lines kindly provided by Dr. Drobetsky (University of Montreal, Montreal, Canada). The HDLFL, HCT116, and DLD1 cells were maintained in Ham’s F10 nutrient medium (Sigma-Aldrich), McCoy5A medium (Invitrogen), and RPMI 1640 medium (BioWhittaker Inc., Walkersville, MD), respectively, supplemented with 10% fetal bovine serum, 0.1 mg/ml penicillin, and 100 IU/ml streptomycin.

**Plasmids and Site-directed Mutagenesis**—PCR was used to amplify the entire GAPDH cDNA from the plasmid template (ATCC no. 817954R, accession number M33197, NCBI), digested with BamHI and EcoRI, and cloned into the *E. coli* expression vector pGEX-4T-1 (Amersham Biosciences) to produce the plasmid pGST-GAPDH. Site-directed mutagenesis (QuickChange kit, Stratagene, La Jolla, CA) was used to mutate the three cysteines to glycine in GAPDH using pGST-GAPDH as the template and the following primers C152G-F1 (5’-CATCAGCAATGCTTCCGGAACCAACACTGTCCTAGG-3’), GAPDH C156G-F2 (5’-GCCTCCTGACACCCAGCCTTAGCACCCTTGCGG-3’), and GAPDH C247G-F3 (5’-GTGCTGTAACCTGACCCGCCGTCAGAAAAACCTGCC-3’). The resulting plasmids were pGST-GAPDH C152G, pGST-GAPDH C156G, and pGST-GAPDH C247G, and the mutations were verified by DNA sequence analysis.

**Purification of GST and His-tagged Proteins**—GST-fusion proteins were overexpressed in *E. coli* BW528 strain and purified by glutathione-Sepharose 4B mini columns as previously described (23), and the His-AP1 was purified using Talon affinity beads according to the manufacturer’s protocol (Clontech).

**APE1 Pulldown Assay**—One ml of matrix slurry (Talon beads) was placed in a plastic disposable 10-ml gravity-flow column (Bio-Rad). The beads were equilibrated with 10 ml of buffer B (50 mM sodium phosphate, pH 7.0, and 300 mM NaCl). At least 10 μg of purified His-APE1 protein was then incubated with the beads for 30 min at room temperature with gentle shaking. The beads were washed with 30 ml of buffer B, and 100-μl aliquots with the equivalent of ~400 ng of bound His-APE1 were mixed with either 2 μg of purified GST-PNKP, GST-GAPDH, GST-GAPDH C152G, GST-GAPDH C156G, or GST-GAPDH C247G and incubated for 1 h at room temperature. The beads were washed three times with 3 ml of buffer B, and the aliquots were analyzed directly for bound proteins by Western blot.

**GST Pulldown Assays**—Glutathione-Sepharose 4B beads (100 μl) alone or bound to 10 μg of the indicated GST-tagged proteins were mixed with purified N-terminal His-tagged APE1 (1 μg) in 0.5 ml of buffer B and incubated for 30 min at room temperature with gentle rotation. The beads were washed three times with buffer B, and aliquots were analyzed for bound pro-
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teins by Western blots probed with either anti-His or anti-GST
monoclonal antibody.

Immunoprecipitation—Immunoprecipitation was performed
on whole cell lysates prepared in radioimmune precipitation
assay buffer (24). Following preclearing with 30 μl of Aminolink
beads plus coupling gel (Pierce), 1.5 mg of total protein was
incubated with anti-APE1 antibodies overnight at 4 °C and then
incubated with 50 μl of Aminolink beads for another 1 h at 4 °C.
The beads were washed four times with radioimmune pre-
cipitation assay buffer and then boiled in 2× SDS sample
buffer. The immunoprecipitates were then detected by
immunoblotting with the indicated antibodies.

Extract Preparation and Immunodetection—Total cells
extracts were prepared and analyzed by Western blots as pre-
viously described (24). The antibodies used in this study were
monoclonal anti-His (Santa Cruz), anti-GAPDH (Chemicon),
anti-APE1 (R & D Systems), polyclonal anti-GST (Sigma), and
anti-APE1 (kindly provided by Dr. Bruce Demple, Harvard Un-
iversity and subsequently purchased from Santa Cruz).

Oxidation of APE1 Protein with H2O2.—The His-APE1 pro-
tein bound to the TALON metal affinity column (1 ml; BD
Biosciences) was treated with H2O2 (5 μM for 5 min), washed
three times with buffer B (50 mM sodium phosphate, pH 7.0,
and 300 mM NaCl), and then eluted using buffer B containing
150 mM imidazole. The eluted proteins were dialyzed in a
Spectra/Por 1 dialysis tube (molecular weight cut-off of
6,000–8,000; Spectrum) by repeated concentration and
dilution with 20 mM Tris-HCl (pH 7.5) and 50 mM NaCl for
five times. The concentrations of the proteins were esti-
mated on a Coomassie Blue-stained 12% SDS-PAGE gel.

siRNA-mediated GAPDH Silencing—siRNA (Ambion) was
used to knock down the expression of GAPDH in DLD1 and
HCT116 cells. The cells were seeded on Petri plates and trans-
fected with 75 nM of GAPDH siRNA using Lipofectamine 2000
(Invitrogen) in Opti-MEM (Invitrogen) according to the man-
ufacturer’s protocol. Negative control (75 nM) that has no sig-
nificant homology to any known gene sequences from mouse
or human was also included. The plates were incubated at 37 °C
in 5% CO2 for 4 h, after which the complete growth medium was
added, and the cells were processed as indicated.

Drug Exposure and Clonogenic Assay—The siRNA trans-
fected cells (1 × 10⁶) were plated in 60-mm × 15-mm Petri
plates and incubated for 24 h before exposure to different drugs.
The adherent cells were incubated with the indicated concen-
trations of either bleomycin for 1 h or methyl methane
sulfonate for 30 min in fetal bovine serum-free incomplete
medium. The colony-forming unit assay was performed as
described previously (24).

UV Irradiation—The cells were irradiated with monochro-
matic 254-nm UV (UVc from G25T8 germicidal lamp (Sankyo
Denki) at a dose ranging from 0–20 J/m² in 1× phosphate-
buffered saline. UVC 254-nm UV fluorescence was measured
with a Spectroline DRC 100× digital radiometer (Spectronics,
Westbury, NY) equipped with 254 sensors (25).

DNA Extraction and DNA Damage Quantification—Briefly,
~5.0 × 10⁶ of the indicated cells transfected with either the
control or GAPDH-siRNA were subjected to genomic DNA
extraction as previously described (25). Formation of AP sites
was measured by using the DNA damage quantification kit
according to the manufacturer’s protocol (Biovison Inc.).

RESULTS

A Minor AP Endonuclease Activity Is Associated with the Gly-
colytic Enzyme GAPDH—We initially set out to examine
whether mammalian cells contained a Mg²⁺-independent AP
endonuclease(s) belonging to the E. coli endonuclease IV fam-
ily, because such an activity has not been previously identified.

Using total cell extracts derived from cultured human lung
fibroblast, we detected a weak Mg²⁺-independent AP endonuclease activity detected in total cell extracts derived from lung fibroblast cells was sub-
ected to several purification steps followed by a discontinuous NaCl gradient
onto MonoS. The activity was monitored by nicking of the AP site 5'-32P-labeled 42-mer substrate (80 ng), and the final active fractions were analyzed by SDS-
PAGE stained with Coomassie Blue. Lane 1, molecular mass standards; lanes
2–7, MonoS fractions from NaCl step gradient. 8, increasing concentrations of
the 0.4 M NaCl MonoS fraction was incubated with the AP site substrate and
monitored for the formation of the 20-mer product. Lane 1, substrate alone;
lane 2, purified Endo IV (10 ng); lanes 3–7, increasing concentrations (25–300
ng) of the purified 0.4 M NaCl MonoS fraction. The arrows indicate the posi-
tions of the 42-mer substrate and the 20-mer product.

![FIGURE 1. Copurification of a Mg²⁺-independent AP endonuclease activity with a 37-kDa protein. A, the Mg²⁺-independent AP endonuclease activity detected in total cell extracts derived from lung fibroblast cells was subjected to several purification steps followed by a discontinuous NaCl gradient on MonoS. The activity was monitored by nicking of the AP site 5'-32P-labeled 42-mer substrate (80 ng), and the final active fractions were analyzed by SDS-PAGE stained with Coomassie Blue. Lane 1, molecular mass standards; lanes 2–7, MonoS fractions from NaCl step gradient. 8, increasing concentrations of the 0.4 M NaCl MonoS fraction was incubated with the AP site substrate and monitored for the formation of the 20-mer product. Lane 1, substrate alone; lane 2, purified Endo IV (10 ng); lanes 3–7, increasing concentrations (25–300 ng) of the purified 0.4 M NaCl MonoS fraction. The arrows indicate the positions of the 42-mer substrate and the 20-mer product.](image-url)
stranded oligonucleotide containing the U-G mismatch, unless the uracil is removed, consistent with the notion that the purified protein fraction has associated AP endonuclease and no hint of uracil-DNA glycosylase activity nor trace amounts of other enzymes that belong to the base-excision DNA repair pathway such as AP lyases, DNA polymerase, and DNA ligase (data not shown).

We next determined the identity of the 37-kDa purified protein by excising the Coomassie-stained polypeptide from the SDS-PAGE and subjecting it to sequencing using microcapillary reverse-phase high pressure liquid chromatography coupled to a nano-electrospray ionization source of an ion trap mass spectrometer. Unexpectedly, of 59 individual sequences analyzed, all showed 95–100% identity to the human GAPDH enzyme. This finding suggests that the Mg2+-independent AP endonuclease activity is likely the result of a minor protein that copurified with GAPDH. To ascertain that the purified protein preparation indeed contained GAPDH, we tested for the enzyme activity by monitoring the reduction of NAD+.

GAPDH and Its Variants Interact with APE1—To directly test whether GAPDH is associated with APE1, we used Talon affinity beads consisting of functionally active APE1 tagged at the N-terminal end with the His tag and examined for retention of the purified GST-GAPDH (see “Experimental Procedures”). When purified GST-GAPDH (Fig. 2A, lane 4) was mixed with the His-APE1 affinity beads (Fig. 2B, lane 4), followed by extensive washing, a significant amount of the protein was pulled down by the beads as detected by Western blot probing with anti-GST monoclonal antibody (Fig. 2C, lane 4). Likewise, three cysteine variants of GST-GAPDH: the presumed active site cysteine 152 (C152G), the adjacent cysteine 156 (C156G), and cysteine 247 (C247G) with no documented role in the redox function of GAPDH (Fig. 2A), were also retained by the His-APE1 affinity beads (Fig. 2C). In controls, the purified DNA repair protein GST-PNKP (Fig. 2A, lane 2) was not retained by the His-APE1 affinity beads (Fig. 2C). In these experiments, a very minute amount of either GST-GAPDH or the variants (Fig. 2A) was pulled down by the empty beads (Fig. 2B), suggesting a possible weak and nonspecific interaction of the fusion proteins with the beads. Of note, the fragmented forms of GST-GAPDH may contain a possible interacting region with His-APE1. Several additional control experiments revealed that GST-GAPDH was not pulled down by beads carrying a different protein His-protein phosphatase 2A activator, a peptidyl prolyl cis/trans isomerase (data not shown). Moreover, the glycolytic enzymes pyruvate dehydrogenase and 6-phosphofructokinase obtained from a commercial source were not retained by the His-APE1 affinity beads (data not shown). Thus, these combined data support the notion that GAPDH may interact with APE1 and that none of the three cysteine residues of GAPDH is essential for the association, contrasting the interaction of GAPDH with inositol 1,4,5-trisphosphate receptors requiring the cysteine residues of both proteins (26).
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To further confirm the GAPDH-APE1 interaction, we conducted the reciprocal experiment by preparing GST-GAPDH affinity beads (Fig. 2D, lane 3) and examined for the retention of His-APE1. As shown in Fig. 2D, purified His-APE1 was pulled down by the GST-GAPDH affinity beads as detected by Western blot probed with anti-His monoclonal antibodies (lane 6). In contrast, neither GST-PNKP nor GST beads (Fig. 2D, lanes 1 and 2) pulled down the His-APE1 protein (Fig. 2D, lanes 4 and 5). Thus, the serendipitous isolation of GAPDH with AP endonuclease activity is due to the interaction of GAPDH with APE1. It is therefore not surprising that commercial preparations of GAPDH, but not pyruvate dehydrogenase and 6-phosphofructokinase obtained from mammalian sources, contained AP endonuclease activity (data not shown).

To assess whether GAPDH is associated with APE1 in vivo, an immunoprecipitation reaction was performed with total cell extracts derived from HCT116 cells using anti-APE1 polyclonal antibodies. After eliminating the nonspecific interaction by washing, the immunoprecipitated complexes were analyzed by Western blot using anti-GAPDH monoclonal antibody. As shown in Fig. 2E, the endogenous GAPDH was coimmunoprecipitated with anti-APE1 antibodies (lane 4), but not by the mock reaction lacking anti-APE1 antibodies (lane 3). We note that anti-GAPDH antibody did not cross-react with APE1, nor did anti-APE1 antibody cross-reacted with GAPDH (supplemental Fig. S3). Because GAPDH is also localized to the mitochondria and can interact with many proteins, for example, the voltage-dependent anion channel of the mitochondrial membrane and the inositol 1,4,5-triphosphate receptor involved in the release of calcium from endoplasmic reticulum (26, 27), it raises the possibility that GAPDH interaction with APE1 could be the result of a “sticky” nature. As such, we checked whether GAPDH could be coimmunoprecipitated with a subunit COX IV of the membrane-associated cytochrome c oxidase complex of the mitochondria. Anti-COX IV antibodies did not coimmunoprecipitate GAPDH (Fig. 2E, lane 4), dismissing the possibility of a generalized sticky feature of this protein and underscoring a relevant physiological interaction with APE1, as reported recently for the GAPDH-p300/CBP interaction (11). In other controls, neither PNKP nor protein phosphatase 2A activator (with cytoplasmic and nuclear distribution) (24) was coimmunoprecipitated with GAPDH (data not shown). Thus, we conclude that the data from whole cell extracts is consistent with the in vitro observation with purified proteins that GAPDH interacts with APE1.

Native GAPDH, and Not the Variant C152G or C156G, Activates the AP Endonuclease Activity of APE1—It has been shown that APE1 can exist in the oxidized and reduced forms and that the reduced form is necessary to activate transcription factors including p53, NF-κB, and c-Jun and c-Fos, subunits of AP-1 (16, 18, 28). Moreover, thioredoxin is required to physically interact with APE1, and this association potentiates AP-1 transcriptional activity (29). We therefore explored whether GAPDH could function to activate the AP endonuclease activity of APE1. In this experiment, we first determined the minimal amount of purified His-APE1 that showed no detectable AP endonuclease activity. Purified His-APE1 in the range of 0–0.4 pmol showed no measurable AP endonuclease activity (Fig. 3A, lane 3), whereas the activity was detected if the protein was in excess of 2.7 pmol (Fig. 3A, lane 4). Interestingly, preincubation of a fixed amount (0.4 pmol) of His-APE1 with increasing amounts of GST-GAPDH resulted in the detection of the AP endonuclease activity (Fig. 3A, lanes 13–16). In control experiments, neither purified GST nor GST-PNKP activated the AP endonuclease activity of His-APE1 (lanes 9–12).
and data not shown). Because the purified GST-GAPDH from *E. coli* is free of contaminating AP endonuclease activity (Fig. 3A, lanes 7 and 8), the above data therefore suggest that GAPDH may play a distinct role by reactivating an inactive form of the purified His-APE1.

We next checked whether the cysteine variants would interfere with the ability of GAPDH to activate APE1. The purified variants C152G and C156G showed nearly 60% reduction in the glycolytic activity, whereas C247G retained full parental GST-GAPDH activity (supplemental Fig. S1). Increasing amounts of either the variant C152G or C156G did not activate the AP endonuclease activity of His-APE1 (Fig. 3B, lanes 6–9 and 10–13), as compared with the native GST-GAPDH (Fig. 3B, lanes 2–5). Moreover, H$_2$O$_2$-oxidized GAPDH did not stimulate the AP endonuclease activity of His-APE1 (supplemental Fig. S4). Unlike C152G and C156G, the variant C247G possessed full ability to reactivating the AP endonuclease activity of His-APE1 (Fig. 3B, lanes 14–17). Because Cys$^{152}$ is the active site cysteine that corresponds to the rabbit GAPDH Cys$^{149}$, recently shown to play an essential role in oxidative stress-induced disulfide bridge formation and aggregation of the protein (30), we reasoned that the cysteines Cys$^{152}$ and Cys$^{156}$, but not Cys$^{247}$, of human GAPDH may participate in a redox reaction to reduce the oxidized form of APE1.

GAPDH, but Not C152G, Functions to Reduce the Oxidized Form of APE1—APE1 composed of oxidized and reduced forms can be visualized on nonreducing SDS-PAGE gels as a set of heterogeneous polypeptide species (29). We tested whether these species of APE1 are affected by GST-GAPDH. In this experiment, the purified His-APE1 was incubated with and without purified GST-GAPDH and examined for mobility changes under nonreducing conditions. When purified His-APE1 (8.11 pmol) was incubated with a 3-fold excess of GST-GAPDH, the heterogeneous species became more intensely stained with silver nitrate (Fig. 4A, lane 4). This phenomenon was not observed when His-APE1 was incubated with either glutathione or purified GST (Fig. 4A, lanes 2 and 3). Under reducing gel migration conditions, i.e. with 2-mercaptoethanol, the same amount of His-APE1 was intensely stained by silver nitrate and independently of GST-GAPDH (Fig. 4A, lanes 6 versus lane 9). This observation strongly suggests that GAPDH might act to reduce APE1 leading to its enhanced staining with silver nitrate.

In separate experiments, quantitative analysis revealed that at least 1 pmol of GAPDH was required to increase the silver staining intensity of 2.23 pmol of His-APE1 by 50% during 5 min of incubation at room temperature (Fig. 4B), indicating that GAPDH is not needed in excess to rapidly reduce His-APE1. Interestingly, the incubation of GST-GAPDH with His-APE1 also significantly enhanced its detection with the anti-APE1 polyclonal antibodies (Fig. 4B, lane 5 versus lane 1). For these experiments, 2-mercaptoethanol treatment was used for generating maximal reduction of APE1 to be detected by silver stain-
GAPDH Reactivates Oxidized APE1

FIGURE 5. GAPDH reactivates the AP endonuclease activity of oxidized APE1. A fixed amount (2.7 pmol) of oxidized His-APE1oxd was incubated with increasing concentrations of either purified GST (lanes 10–12) or purified GST-GAPDH (lanes 13–16) for 5 min at room temperature and then assayed for AP endonuclease activity. Shown are the controls, substrate (80 ng) alone (lane 1) and the following purified proteins Endo IV (0.3 pmol, lane 2), His-APE1 (lanes 3–5), oxidized His-APE1oxd (lanes 6 and 7), GST (lane 8), and GST-GAPDH (lane 9).

We next examined whether the variant C152G could act on His-APE1 to enhance its detection by the anti-APE1 antibodies. As shown in Fig. 4C, the C152G variant lacked the ability to induce detection of a fixed amount of His-APE1 by the anti-APE1 antibodies, as compared with the same amount of the native GST-GAPDH (Fig. 4C, lanes 3 and 4 versus lanes 5 and 6). Similarly, the C156G variant also was defective in enhancing detection of His-APE1 by anti-APE1 antibodies, but not the C247G variant (data not shown). These data are consistent with the notion that the cysteine residues Cys152 and Cys156 of GAPDH might be required to catalyze the reduction of oxidized species of APE1, thereby causing a conformational change in the protein.

GAPDH Reactivates Chemically Oxidized APE1—We next assessed whether GST-GAPDH could reactivate the AP endonuclease activity of His-APE1 that was inactivated by pretreatment with the chemical oxidant H2O2. Following H2O2 treatment of His-APE1, the resulting His-APE1oxd (2.7 pmol) showed no detectable AP endonuclease activity when compared with the same amount of untreated His-APE1 (2.7 pmol) that exhibited a measurable level of AP endonuclease activity (Fig. 5, lane 6 versus lane 4). The conditions of H2O2 treatment did not completely inactivate the His-APE1oxd, because a 10-fold higher concentration of the protein (27 pmol) displayed a significant level of AP endonuclease activity (Fig. 5, lane 7 versus lane 6). When a fixed amount of the H2O2-inactivated His-APE1oxd (2.7 pmol) was incubated with increasing concentrations of GST-GAPDH for 5 min, the AP endonuclease activity was rapidly regenerated (Fig. 5, lanes 13–16). In contrast, increasing amounts of either N-acetylcycteine, glutathione, di-thiothreitol, or purified GST did not reactivate the AP endonuclease activity of His-APE1oxd (supplemental Fig. S5 and data not shown), indicating that GAPDH performs a specific role in the reactivation of His-APE1oxd. In other control experiments, the addition of various metal ions including MgCl2 to the His-APE1oxd did not reactivate the AP endonuclease activity, excluding the possibility that GAPDH is acting as a donor of metal ions (supplemental Fig. S6). On the basis of these observations, we conclude that GAPDH is endowed with the ability to reduce the oxidized species of APE1, thereby maintaining the enzyme in its active state.

Cellular Sensitivity to a Subset of DNA-damaging Agents Is Dependent upon GAPDH Endogenous Levels—Because APE1 is an essential enzyme that plays a key role in processing several genotoxic DNA lesions such as AP sites, we reasoned that altering the endogenous level of GAPDH would interfere with the DNA repair functions of APE1. To assess this, we examined whether siRNA knockdown of GAPDH would sensitize cells to DNA-damaging agents that produce lesions repairable by APE1. When HCT116 cells were challenged with siRNA against GAPDH for 48 h, the level of the GAPDH protein (monitored by anti-GAPDH monoclonal antibody) present in total cell extract was sharply reduced by nearly 80%, as compared with the same amount of total cell extract derived from cells exposed to the scrambled control siRNA (Fig. 6A, upper panel, lanes 3 versus lane 1). Similar reduction in GAPDH level was also observed in the DLD1 cells challenged with the GAPDH siRNA (Fig. 6A, lanes 7 versus lane 5). Under this treatment condition, neither the control nor the siRNA against GAPDH altered the endogenous level of APE1 as determined by Western blot probed with anti-APE1 antibodies (Fig. 6A, middle panel, lanes 3 versus lane 1), suggesting that GAPDH does not influence the expression level of APE1. However, the GAPDH siRNA decreased the survival of a fraction (~15–20%) of the cells, as compared with cells transfected with the control siRNA (data not shown). Interestingly, these GAPDH knockdown cells were hypersensitive to the alkylating agent MMS and the oxidant bleomycin (BLM), but not to 254-nm UV or cisplatin (Fig. 6, B–D, and supplemental Fig. S7). The GAPDH knockdown cells exhibited 55 and 40% decreased survival by colony assay when challenged with MMS (0.25 mM for 30 min) and BLM (0.1 μg/ml for 1 h), as compared with cells treated with the control siRNA which showed 30 and 15% decreased survival, respectively (Fig. 6, B and C). Thus, the sensitivity of the GAPDH knockdown cells to MMS and BLM could be related to an inability to process MMS-induced AP sites and BLM-induced DNA strand breaks with blocked 3’-termini. Both of these lesions are substrates for the APE1 DNA repair activities.

Accumulation of Spontaneous AP Sites in GAPDH Knockdown Cells Is Associated with Decreased AP Endonuclease Activity—If indeed GAPDH governs the DNA repair function of APE1, we anticipate that this would have a direct consequence on the repair of endogenous AP sites. To test this, we measured the spontaneous level of AP sites in the genomic DNA isolated from...
HCT116 and DLD1 cells treated with either the control siRNA or siRNA against GAPDH. Briefly, AP sites were quantified using an aldehyde-reactive probe that binds to AP sites followed by detection with biotin coupled streptavidin. As shown in Fig. 7A, GAPDH siRNA knockdown caused nearly a 1.5-fold increase in the level of spontaneous AP sites in the genomic DNA derived from either cell lines, as compared with the control siRNA treatment. Consistent with this observation, total extracts prepared from the GAPDH depleted cells showed a decrease in AP endonuclease activity, as compared with the control siRNA treatment. We believe that a possible physiological relevance of the GAPDH-APE1 interaction is to promote the reactivation of oxidized species of APE1 that occur during normal aerobic metabolism. This role seems logical from several standpoints: GAPDH (i) possesses a redox active cysteine in its catalytic center, (ii) translocates from the cytoplasm to the nucleus in response to stress causing oxidative damage to the DNA, and (iii) binds to damaged DNA. Moreover, siRNA knockdown of GAPDH (i) sensitizes cells to agents that produce genotoxic lesions that are processed by APE1 and, more importantly, (ii) engenders relatively higher levels of spontaneous AP site formation (5, 10). This latter finding correlates with the GAPDH knockdown cells displaying an inability to proliferate, as well as exhibiting a profound defect in cell cycle progression, largely arresting in the G1 phase, where these AP site lesions would need to be repaired (31). Because AP sites are mutagenic, we postulate that GAPDH could serve an essential role in protecting cells against genomic instability that may arise as a result of oxidative stress by maintaining APE1 in its reduced state. Alternatively, GAPDH may function to prevent cell death by ensuring the efficient repair of spontaneous AP sites, which if left unprocessed are also known to trigger cell death (14). Furthermore, the observation that GAPDH knockdown cells exhibit an ~15–20% reduction in viability in the absence of exogenous treatment may be accounted for by the concomitant 2-fold increase in spontaneous AP site formation. In this respect it remains to be verified whether the diminished survival of GAPDH knockdown cells can be avoided by ectopic expression of a redox-insensitive AP endonuclease such as the yeast Apn1 (14).

**FIGURE 6.** GAPDH depletion influences survival of mammalian cells following exposure to genotoxic agents. A, cells were incubated with 75 nm of either control siRNA or GAPDH siRNA and total cell extracts analyzed for GAPDH and APE1 levels by Western blot analysis. B–D, control and siRNA-treated cells were challenged with increasing doses of either MMS (B), bleomycin (C), or UVC (D) and monitored for survival by clonogenic assay. The means and standard deviations of at least three independent experiments are shown.

DISCUSSION

In this study, we show for the first time that GAPDH interacts with a key DNA repair enzyme, APE1, which functions in the base excision repair pathway. We believe that a possible physiological relevance of the GAPDH-APE1 interaction is to promote the reactivation of oxidized species of APE1 that occur during normal aerobic metabolism. This role seems logical from several standpoints: GAPDH (i) possesses a redox active cysteine in its catalytic center, (ii) translocates from the cytoplasm to the nucleus in response to stress causing oxidative damage to the DNA, and (iii) binds to damaged DNA. Moreover, siRNA knockdown of GAPDH (i) sensitizes cells to agents that produce genotoxic lesions that are processed by APE1 and, more importantly, (ii) engenders relatively higher levels of spontaneous AP site formation (5, 10). This latter finding correlates with the GAPDH knockdown cells displaying an inability to proliferate, as well as exhibiting a profound defect in cell cycle progression, largely arresting in the G1 phase, where these AP site lesions would need to be repaired (31). Because AP sites are mutagenic, we postulate that GAPDH could serve an essential role in protecting cells against genomic instability that may arise as a result of oxidative stress by maintaining APE1 in its reduced state. Alternatively, GAPDH may function to prevent cell death by

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GAPDH Reactivates Oxidized APE1

The mechanism by which GAPDH executes its redox function could involve the active site cysteine of GAPDH, because C152G, but not C247G, was incapable of reactivating the AP endonuclease activity of oxidized APE1. In a similar manner, the GAPDH variant C156G lacks the ability to reactivate the AP endonuclease activity of oxidized APE1, suggesting that Cys156 also might be involved in the mechanism by which GAPDH reduces APE1. The specific role performed by Cys152 and Cys156 in reducing oxidized APE1 warrants further study, because the functions of these residues cannot be substituted by any of these including Cys65 to generate a population of ubiquitin-protein isopeptide ligase SIAH1 (33). The GAPDH-SIAH1 complex translocates to the nucleus and degrades SIAH1 substrates. It is not clear how GAPDH stabilizes SIAH1, but it is noteworthy that SIAH1 is a cysteine-rich protein. Whether GAPDH uses Cys156 to reduce SIAH1 and protect it from structural deformation caused by oxidation remains to be investigated.

APE1 has an active site cysteine residue (Cys65) that is required for the regulation of c-Jun protein, which possesses a redox sensitive cysteine (Cys252) that can be readily oxidized leading to the inhibition of its DNA binding ability (16, 34). It has been proposed that oxidized APE1 contains a disulfide bridge between Cys65 and Cys93, thereby preventing Cys65 from participating in the redox reactivation of the DNA binding activity of oxidized Jun. Because the APE1 variant C65A displayed the equivalent level of AP endonuclease activity as the native enzyme, it is unlikely that GAPDH exerts a redox function on Cys65 (28). In fact, oxidation of Cys310 has been shown to interfere with APE1 repair activities because it is located adjacent to His309, which is required for catalytic function of the enzyme (28). Because APE1 contains five additional cysteine residues (Cys93, Cys99, Cys138, Cys208, and Cys296), we reason that Cys310 may form various intramolecular disulfide bridges with any of these including Cys65 to generate a population of residues in the aggregation process. It is noteworthy that the nearby Cys156 (corresponding to Cys152 of human) does not appear to perform a similar role in forming GAPDH aggregates (30, 32). It is possible that GAPDH could form aggregates following incubation with oxidized APE1, although this reaction could be limited by several factors.

To date, GAPDH is known to interact with a myriad of proteins with nuclear function. For example, GAPDH is one of seven polypeptides that constitute the OCA-S complex, a coactivator of the DNA binding transcription factor gel (8). GAPDH interacts directly with the POU transactivation domain of OCT1 to play an essential role during the S phase-dependent transcription of histone H2B. Moreover, the interaction between GAPDH and OCT1 is stimulated by NAD+, but inhibited by NADH, suggesting that GAPDH serves a redox sensing role in the H2B gene transcription (8). A more recent study documented that in response to nitric oxide, rat GAPDH becomes S-nitrosylated at the catalytic cysteine (Cys150, corresponding to Cys152 in human) causing the modified GAPDH to bind and stabilize the GAPDH-SIAH1 complex translocates to the nucleus and degrades SIAH1 substrates. It is not clear how GAPDH stabilizes SIAH1, but it is noteworthy that SIAH1 is a cysteine-rich protein. Whether GAPDH uses Cys156 to reduce SIAH1 and protect it from structural deformation caused by oxidation remains to be investigated.

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heterogeneous oxidized molecules compromised for AP endonuclease activity. Thus, creating cysteine to alanine variants of APE1 is likely to reveal the key cysteine(s) that participate in the disulfide bridge formation leading to aberrant molecules with altered AP endonuclease activity. Such APE1 variants are expected to resist oxidation and remain fully active as an AP endonuclease independently of GAPDH (28). Nonetheless, it is clear that oxidized species of APE1 that are reactivated by GAPDH possess distinct physical properties from the native enzyme with respect to mobility on SDS-PAGE and lack of requirement for the metal ion Mg^{2+}. Whether oxidized forms of APE1 bind more tightly to Mg^{2+} or lose the metal ion requirement warrants further studies (35). This is especially important because it is well established that the substrate specificity and protein conformation of APE1 can be modulated by the concentration of Mg^{2+} (36–38).

In addition to GAPDH, thioredoxin (TRX), a pleiotropic cellular factor possessing thiol-mediated redox activity, can also interact with APE1 (29). Like GAPDH, TRX can translocate to the nucleus upon phorbol 12-myristate 13 acetate treatment and associates with APE1 to promote activation of the AP-1 transcriptional factor (29). This activation process requires the cysteine residues in the catalytic center of TRX. However, it is not known whether TRX specifically reduces the redox active cysteine (Cys^{60}) of APE1 that is required to maintain AP-1 in the reduced state. Herein, we did not examine whether GAPDH association with APE1 might potentiate the DNA binding activity of c-Jun, although it remains possible that TRX and GAPDH may perform distinct redox functions on oxidized APE1.

In short, we postulate that GAPDH may serve to maintain the redox state of numerous proteins that are susceptible to oxidative stress-induced structural changes. Perhaps this might explain the diverse activities of GAPDH, unrelated to glycolysis, including its implication in some oxidative stress related neurodegenerative diseases such as Alzheimer and Parkinson diseases (39). Our key observation that GAPDH is responsible for the preserving activity of APE1 may suggest that one of the essential roles of the former protein is to maintain genetic stability.

Acknowledgments—We thank Drs. E. Drobeta and J. Filep, and E. B. Affar for helpful comments.

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