Akt2 Kinase Suppresses Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)-mediated Apoptosis in Ovarian Cancer Cells via Phosphorylating GAPDH at Threonine 237 and Decreasing Its Nuclear Translocation*1

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Abstract

Protein kinase B (Akt) plays important roles in regulation of cell growth and survival, but while many aspects of its mechanism of action are known, there are potentially additional regulatory events that remain to be discovered. Here we detected a 36-kDa protein that was co-immunoprecipitated with protein kinase Bβ (Akt2) in OVCAR-3 ovarian cancer cells. The protein was identified to be glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by MALDI-TOF/TOF MS, and the interaction of Akt2 and GAPDH was verified by reverse immunoprecipitation. Our further study showed that Akt2 may suppress GAPDH-mediated apoptosis in ovarian cancer cells. Overexpression of GAPDH increased ovarian cancer cell apoptosis induced by H2O2, which was inhibited by Akt2 overexpression and restored by the PI3K/Akt inhibitor wortmannin or Akt2 siRNA. Akt2 phosphorylated Thr-237 of GAPDH and decreased its nuclear translocation, an essential step for GAPDH-mediated apoptosis. The interaction between Akt2 and GAPDH may be important in ovarian cancer as immunohistochemical analysis of 10 normal and 30 cancerous ovarian tissues revealed that decreased nuclear expression of GAPDH correlated with activation (phosphorylation) of Akt2. In conclusion, our study suggests that activated Akt2 may increase ovarian cancer cell survival via inhibition of GAPDH-induced apoptosis. This effect of Akt2 is partly mediated by its phosphorylation of GAPDH at Thr-237, which results in the inhibition of GAPDH nuclear translocation.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a major enzyme involved in glycolysis (1). A number of studies have demonstrated that besides its function in converting glyceraldehyde-3-phosphate to 1, 3-biphosphoglycerate during glycolysis, GAPDH is implicated in several non-metabolic processes, including transcriptional activation and cell apoptosis (1–4), which are related to cancer cell growth and progression. The ability of protein kinase B (Akt/PKB) to promote aerobic glycolysis including the up-regulation of GAPDH through hypoxia-inducible factor-1 (HIF-1) has been confirmed (1, 5–7). Whether Akt may affect the non-metabolic functions of GAPDH is of current interest.

Akt is a serine/threonine protein kinase belonging to a family of protein kinase A, G, and C (AGC kinases) (8). Until now, three Akt homologs have been identified, Akt1/PKBα, Akt2/ PKβB, and Akt3/PKBγ, that are activated by phosphorylation on serine residues 473 of AKT1, 474 of AKT2, and 472 of AKT3 or on threonine residues 308 of AKT1, 309 of AKT2, and 305 of AKT3 (9).

Akt is a direct target of phosphatidylinositol 3-kinase (PI3K) and plays an essential role in the PI3K/Akt signaling pathway, and its activation has been observed in a wide variety of cancers. This pathway is not only closely associated with the development of various human cancers but also with survival, migration, invasion, and metastasis of cancer cells (9–11). Therefore, the PI3K/Akt signaling pathways have been regarded as potentially beneficial treatment targets of cancer (12, 13). Although in vivo studies have shown that Akt1 and Akt2 share similar substrates (9, 14), several findings have suggested that they do not have the completely same physiological functions. Unlike Akt1, which is required for proliferation and is involved with cellular growth (15), Akt2 is mainly involved in cancer cell survival, apoptosis inhibition, migration, and invasion (11, 16). Human ovarian cancer is a highly malignant tumor that often shows overexpression of Akt proteins. With the aim of understanding whether Akt may play a role in non-metabolic functions of GAPDH (i.e. cancer cell apoptosis), human ovarian cancer cell lines were investigated in this study. Through co-immunoprecipitation and mass spectrometry (MS) anal-

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yses, we identified the interaction between Akt and GAPDH in ovarian cancer cells. We also explored the effects of Akt activation on GAPDH phosphorylation and nuclear localization in relation to oxidative stress-induced apoptosis of cancer cells. The correlation between nuclear GAPDH and Akt2 activation was also investigated in primary ovarian cancer tissues. This study provides further evidence to support Akt2 as a viable target for ovarian cancer treatments.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and SDS-PAGE—OVCAR-3 cells (American Type Culture Collection, Manassas, VA) were cultured in a serum-free medium for 16 h and then stimulated with 10% fetal bovine serum at 37 °C in an incubator containing 5% CO₂. Cells were transiently transfected with different plasmid using Lipofectamine 2000 or Lipofectamine LTX transfection reagent (Invitrogen) according to the manufacturer’s instructions. For stable transfection, at 36 h post transfection, the transiently transfected cells were cultured in McCoy’s 5A medium containing neomycin for about 4 weeks. Clones resistant to neo-mycin (stable transfectants) were examined for the presence of GAPDH or Akt2 protein by Western blots. Two stable transfectants of each pcDNA3.1-Myc-His(−) A empty vector, pcDNA3.1-Myc-His(−) A GAPDH, pcDNA3.1-Myc-His(−) A GAPDH T237A mutant, pcDNA3.1-Myc-His(−) A-Akt2 (wild type), and DN-Akt2 were generated and maintained in culture for further studies. The untransfected OVCAR-3 and SKOV3 cell lines were named parent OVCAR and SKOV3 cells.

Western Blot Analysis of Co-immunoprecipitated Proteins—12% SDS-PAGE was used to separate co-immunoprecipitated proteins. After electrophoresis and transferring of proteins from gel to membrane, Western blot (18) was performed with phospho-(Ser/Thr)Akt substrate antibody (Cell Signal Technology Inc) or other antibodies as primary antibodies.

RNA Interference—GAPDH siRNA targeting sequences 5′-GUAPUGACAACGCCCUCAGTT-3′ (forward) and 5′-CUUGAGGCUGUUGUCUAUACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China). Akt2 siRNA was designed by a GeneScript siRNA design tool with targeting sequences of 5′-GCUACUUCCUCAAGAUGAATT-3′ (forward) and 5′-UGACUUCGAGUCUGAGAACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China). Akt2 siRNA was designed by a GeneScript siRNA design tool with targeting sequences of 5′-GCUACUUCCUCAAGAUGAATT-3′ (forward) and 5′-UGACUUCGAGUCUGAGAACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China). Akt2 siRNA was designed by a GeneScript siRNA design tool with targeting sequences of 5′-GCUACUUCCUCAAGAUGAATT-3′ (forward) and 5′-UGACUUCGAGUCUGAGAACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China). Akt2 siRNA was designed by a GeneScript siRNA design tool with targeting sequences of 5′-GCUACUUCCUCAAGAUGAATT-3′ (forward) and 5′-UGACUUCGAGUCUGAGAACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China). Akt2 siRNA was designed by a GeneScript siRNA design tool with targeting sequences of 5′-GCUACUUCCUCAAGAUGAATT-3′ (forward) and 5′-UGACUUCGAGUCUGAGAACTT-3′ (reverse), corresponding to the cDNA sequence from 417 to 435, and a negative control (scrambled sequence) 5′-UUCUCCGAACGUCCGAGU-3′ (forward) and 5′-ACGGUGACGGACGAGAACTT-3′ (reverse) were purchased from GenePharma Co. (Shanghai, China).
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300 μM H₂O₂ (4 h in SKOV3 cells) in the presence or absence of wortmannin (50 or 100 nm) (Sigma). Apoptotic cells were detected with both the TUNEL assay and DNA ladder assay. Briefly, cells were fixed in 4% paraformaldehyde for 30 min following with treatment in Triton X-100 for 15 min. After the TdT Enzyme was mixed with fluorescence-labeled solution, the mixture was added to cells for 60 min. Apoptotic cells positive for green fluorescence were visualized and counted under fluorescence microscope. The apoptotic index (AI) was calculated as the ratio of apoptotic cells/total cell number × 100%. DNA ladder assay was carried out exactly according to the instructions of kit purchased from Applygen Technologies Inc, Beijing, China. Extracted DNA was loaded onto a 1.5% agarose gel containing ethidium bromide (EB) and then visualized under UV light after electrophoresis.

Cytoplasmic and Nuclear Localization of GAPDH—SKOV3 ovarian cancer cells were first cultured in a serum-free medium for 16 h, then some cells were treated with 300 μM H₂O₂ for 30 min or 1 h. Intracellular distribution of GAPDH was examined under confocal microscopy. Briefly, the cells were rinsed with PBS, fixed with 100% methanol for 30 min, and then permeabilized with 0.1% Triton X-100 in PBS for 20 min. After incubating cells with 3% BSA for 1 h, cells were reacted with a rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at 37 °C. Finally, cells were counterstained with DAPI and examined under Fluoview FV1000 laser-scanning confocal microscope (Olympus, Tokyo, Japan). Cytoplasmic and nuclear localization of GAPDH were further determined by isolating nuclear and cytoplasmic proteins from cells treated with or without 300 μM H₂O₂ (SKOV3 cells) or with 2 mM H₂O₂ (OVCAR-3 cells). Western blots were performed to measure the relative amount of GAPDH in nucleus versus cytoplasm. Anti-histone H2B (Bioworld Technology, Inc. Louis Park, MN) and anti-β-actin antibodies (Sigma) were used as loading controls for nuclear and cytoplasmic proteins, respectively.

Immunostaining for Akt2 and GAPDH in Ovarian Cancer Tissues—Akt2 and GAPDH immunostaining in ovarian cancers (n = 30) and normal ovarian tissues (n = 10) were performed using method as previously described (18). An anti-phosphorylated-Akt2 (Bioworld Technology) and an anti-GAPDH antibody (Cell Signal) were used as loading controls for nuclear and cytoplasmic proteins, respectively.

Statistical Analysis—Values are expressed as means ± S.D. Independent sample t tests were performed to determine the difference among the groups. The Spearman correlation test was used to analyze the correlation between the intensity of phosphor-Akt2 staining and cytoplasmic/nuclear localization of GAPDH. p values <0.05 were considered statistically significant.

RESULTS

Akt2 Interacts with GAPDH in Ovarian Cancer Cells—Ovarian cancer cells have been shown to overexpress Akt2 (9, 12, 21), and Akt2 likely plays a role in cancer cell survival. We first set out to identify Akt2 interacting proteins in ovarian cancer cells. H₂O₂ was used to treat cells in this study as it was expected to increase Akt2 activity via oxidative stress, which would help to amplify signaling pathways for the detection of Akt2 interacting protein(s). As expected, H₂O₂-induced Akt activation was demonstrated by the detection of phosphorylated Akt (Fig. 1A). Coomassie Blue staining showed that a 36-kDa protein was pulled down by the Akt2 antibody in H₂O₂-stimulated OVCAR-3 cells (Fig. 1B). This anti-Akt2-immunoprecipitated product was then analyzed by mass spectrometry (MS). Peptide mass fingerprinting and the amino acid sequence obtained from MALDI-TOF/TOF and MS-MS revealed this 36-kDa protein as GAPDH.

FIGURE 1. Co-immunoprecipitation of GAPDH and Akt2 from ovarian cancer cells. A, increased Akt activation (phosphorylation) was detected in OVCAR-3 ovarian cancer cells after H₂O₂ treatment. WB, Western blot. B, a representative SDS-polyacrylamide electrophoresis gel showed a 36-kDa protein immunoprecipitated (IP) by an anti-Akt2 antibody from H₂O₂-stimulated OVCAR-3 cells. Control, untreated cells. Bands corresponding to IgG and Akt2 were shown in immunoprecipitates of cells treated with or without H₂O₂. With or without wortmannin pretreatment, OVCAR-3 (C) and SKOV3 (D) cells were treated with H₂O₂ and then immunoprecipitated using a GAPDH antibody followed by Western blotting with an Akt2 antibody. H₂O₂ treatment increased the association of Akt2 with GAPDH. Co-immunoprecipitation of the 60-kDa Akt2 protein decreased after wortmannin pretreatment. Pre-Imm, immunoprecipitation with pre-immune IgG.

2 The abbreviation used is: TRITC, tetramethylrhodamine isothiocyanate.
of gi31645, a molecular mass of 36,031.4 (≈36 kDa), and isoelectric point (pI) of 8.26. To further confirm the results from MALDI-TOF and MS-MS, a reverse immunoprecipitation with a GAPDH antibody was performed. Samples immunoprecipitated from H$_2$O$_2$-stimulated OVCAR-3 cells with an GAPDH antibody showed the presence of a 60-kDa protein corresponding to Akt2 as detected by Western blot with an Akt2 antibody (Fig. 1C). The interaction of Akt2 with GAPDH was also detected in another ovarian cancer cell line, SKOV3, although this cell line expressed a lower level of Akt2 compared with that of OVCAR-3 cells (Fig. 1D). The 60-kDa Akt2 protein was visibly decreased when the cells had been pretreated with the PI3K/Akt inhibitor wortmannin, indicating that the interaction of Akt2 with GAPDH was dependent on this signaling pathway (Fig. 1, C and D).

**Akt2 May Interact with GAPDH at Thr-237**—Akt is known to phosphorylate proteins at the consensus sequence RXRXY(S/T) (22–25), but several investigations have found that it may also phosphorylate a similar consensus sequence lacking the arginine at position 5 in proteins, including β-actin, insulin-response element-binding protein 1, and cAMP-response element-binding protein (22, 26, 27). The amino acid sequence of GAPDH does not contain a typical Akt phosphorylation site. However, a bioinformatic search for an Akt phosphorylation site in GAPDH using the methods described by Vandermoere et al. (22, 28) yielded a non-typical Akt phosphorylation sequence GMAFRVP$^{237}$ similar to that found in β-actin and other proteins mentioned above. To determine whether GAPDH is indeed phosphorylated by Akt2, OVCAR-3 and SKOV3 cells were treated with H$_2$O$_2$ in the presence or absence of wortmannin or Akt2 siRNA. The resultant cell lysates were immunoprecipitated with a GAPDH antibody followed by Western blot using the phospho-Akt substrate antibody. Overexpression of Akt2 resulted in increased GAPDH phosphorylation in cells transfected with wild type GAPDH but not in cells transfected with GAPDH$^{237A}$ mutant. GAPDH and Akt2 inputs were shown.

FIGURE 2. Akt2 interaction with GAPDH may involve phosphorylation of GAPDH Thr-237. A, using a GAPDH antibody for immunoprecipitation (IP) and phospho-Akt substrate (PAS) antibody for Western blot (WB), a 36-kDa band corresponding to GAPDH was detected in lysates of OVCAR-3 and SKOV3 cells after stimulation with H$_2$O$_2$, indicating the interaction between Akt2 and GAPDH may involve the Akt phosphorylation of GAPDH. The phosphorylation was inhibited by wortmannin and Akt2 siRNA. Pre-Imm, lysate immunoprecipitated with pre-immune IgG instead of GAPDH antibody. B, transfection of Akt2 expression vector also caused increased GAPDH phosphorylation. GAPDH and Akt2 inputs from cell lysates before immunoprecipitation were shown. C, GAPDH wild type plasmid or GAPDH Thr-237 mutant plasmid was first co-transfected with empty vector or Akt2 plasmid into OVCAR-3 cells, and then immunoprecipitation was performed by using a GAPDH antibody followed by Western blot using the phospho-Akt substrate antibody. Overexpression of Akt2 resulted in increased GAPDH phosphorylation in cells transfected with wild type GAPDH but not in cells transfected with GAPDH$^{237A}$ mutant. GAPDH and Akt2 inputs were shown.

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Association of Akt2 with GAPDH Results in Increased Cell Survival—Because Akt is known as a survival factor in causing cancer cell resistance to oxidative stress-induced cell death (29–32), we examined if Akt2 had a similar role in ovarian cancer cells. We found that H$_2$O$_2$ stimulation caused an increase in phosphorylated Akt2, an indication of Akt2 activation (Fig. 3A).

GAPDH has been shown to play a role in apoptosis caused by oxidative stress in several cell types including cancer cells (4, 33, 34). We first examined the contribution of GAPDH to oxidative stress-induced ovarian cancer cell death. OVCAR-3 cells were stably transfected with a GAPDH expression plasmid, empty vector, or GAPDH siRNA (Fig. 3, B and C). A higher level of H$_2$O$_2$-induced apoptotic cell death was found in GAPDH stable cells compared with empty vector stable cells (Fig. 3, D and E).

Moreover, H$_2$O$_2$-induced OVCAR-3 cell death was significantly attenuated when GAPDH expression was decreased by siRNA, and knocking down Akt2 with different dose of siRNA (20–160 nM) caused a dose-dependent increase in cell death. Pro-apoptotic effect of GAPDH on ovarian cancer cells was strengthened in the presence of Akt inhibition of wortmannin or by decreasing Akt2 with siRNA. However, knockdown of Akt1 by siRNA had not effect on ovarian cancer cell death. E, H$_2$O$_2$-induced ovarian cancer cell death was also determined by TUNEL assay. **p < 0.01 versus cells transfected with empty vector and treated with H$_2$O$_2$. △△, p < 0.01 versus cells transfected with control siRNA and treated with H$_2$O$_2$. ◊ ◊, p < 0.01 versus cells transfected with Akt2 siRNA and treated with H$_2$O$_2$. □ □, p < 0.01 versus cells transfected with wild type GAPDH alone.
effect (Fig. 3, B, D, and E). Similarly, siRNA knockdown of Akt2 expression resulted in a greater apoptotic response to H$_2$O$_2$ (Fig. 3, C, D, and E), and the PI3K/Akt inhibitor wortmannin also significantly increased H$_2$O$_2$-induced OVCA-3 cell death (Fig. 3, D and E). Together these data support a protective role of Akt2 in oxidative stress-induced cell death in OVCA-3 cells.

Because Akt1, Akt2, and Akt3 may all have similar effects on cancer cell survival, we examined the expression of these three proteins in OVCA-3 and SKOV3 cells. Although both Akt1 and Akt2 were found to be present in these two cell lines, Akt3 levels were undetectable (Fig. 3A and data not shown). Thus, we knocked down Akt1 by siRNA to examine if the decrease in Akt1 expression (Fig. 3C) would also cause more oxidative stress-induced cell death. Unlike with Akt2 knockdown, Akt1 knockdown did not increase H$_2$O$_2$-stimulated cell death in ovarian cancer cells (Fig. 3, D and E). Thus, we focused our subsequent analysis on Akt2. To further examine the contribution of Akt2 to GAPDH-mediated cell death, GAPDH-overexpressing OVCA-3 cells were transfected to express wild type Akt2, and a 100% decrease in H$_2$O$_2$-induced apoptosis in GAPDH-overexpressing cells was observed (Fig. 3, D and E). When Akt2 function was suppressed by dominant negative AKT2 or Akt2 siRNA, there was a significant

FIGURE 4. Akt2 suppressed GAPDH-mediated apoptosis by inhibiting nuclear translocation of GAPDH. A, confocal microscopy showed that GAPDH was mostly located in the cytosol of SKOV3 cells without H$_2$O$_2$ treatment. B, phospho-Akt2 and interaction of phospho-Akt2 with GAPDH was detected after H$_2$O$_2$ treatment for 30 min. C, nuclear translocation and apoptosis were increased (some apoptotic cell characteristics by cell shrinkage were present in some cells), and phospho-Akt2 was decreased in cells pretreated with wortmannin and then stimulated with H$_2$O$_2$. D, decreased nuclear translocation and apoptosis and increased phospho-Akt2 were found in cells after transfection with the Akt2 plasmid and then treated with H$_2$O$_2$. Increased phospho-Akt2 co-localized with GAPDH in the cytosol of cells and was shown as yellow. E, GAPDH nuclear translocation was restored after Akt2-transfected SKOV3 cells was pretreated with wortmannin and then treated with H$_2$O$_2$. F, average percentages of apoptotic cells were detected by TUNEL assay. G, nuclear and cytosolic fractions were isolated from SKOV3 cells treated with H$_2$O$_2$ for 1 and 4 h (0, untreated; 1, treated for 1 h 2, treated for 4 h). A Western blot revealed that the cytosolic GAPDH (Ga) was decreased, whereas the nuclear GAPDH (Gb) was increased after H$_2$O$_2$ treatment. Overexpression of Akt2 blocked nuclear translocation of GAPDH induced by H$_2$O$_2$. Thus, GAPDH remained at cytosolic fraction. H, Western blot analysis of GAPDH expression in cytosolic (Ha) or nuclear (Hb) fractions of OVCAR-3 cells treated with H$_2$O$_2$ for 4 h in the presence of different amounts of Akt2 siRNA. The successful isolation of cytoplasmic and nuclear fractions of proteins was confirmed by the presence of β-actin in cytoplasmic fraction and H2B in nuclear fraction (G and H), ν ν ν < 0.01 versus unstimulated SKOV3 cells, the cells pretreated with wortmannin and then treated with H$_2$O$_2$, and the cells transfected with Akt2 plasmid and then treated with H$_2$O$_2$.  

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increase in GAPDH-mediated cell apoptosis induced by H$_2$O$_2$ (Fig. 3, D and E). Furthermore, direct inhibition of PI3K/Akt by wortmannin also significantly increased H$_2$O$_2$-induced cell death in GAPDH-overexpressing cells. Because a similar effect of Akt2 on GAPDH-mediated cell death was observed in SKOV3 cells, these data support an important role of Akt2 in GAPDH-mediated apoptosis induced by oxidative stress in ovarian cancer cells. To examine if the phosphorylation of GAPDH at Thr-237 is required for its nuclear translocation (34, 35). Therefore, nuclear translocation of GAPDH may be critical for its apoptotic function in ovarian cancer cells. To examine this possibility and a likely involvement of Akt2 in this process, SKOV3 cells were treated with or without H$_2$O$_2$. The cytoplasmic and nuclear localization of GAPDH were monitored by confocal microscopy with a GAPDH antibody (green fluorescently labeled). The activation of Akt2 was examined by a phospho-Akt antibody (red fluorescently labeled). GAPDH was mostly located in the cytosol of SKOV3 cells in the absence of H$_2$O$_2$ treatment, and there was a base-line activity of Akt2 (Fig. 4A). Thirty minutes after the addition of H$_2$O$_2$, the intensity of phospho-Akt2 staining visibly increased (Fig. 4B). Nuclear accumulation of GAPDH was obviously detected in cells pretreated with the PI3K/Akt inhibitor wortmannin and then stimulated with H$_2$O$_2$ for 1 h (Fig. 4C). Some cells showed some apoptotic cell morphology characteristics such as cell shrinkage at this time (Fig. 4C), and this was associated with a higher level of apoptotic cell death (Fig. 4F). Transfecting cells with wild type Akt2 prevented H$_2$O$_2$-induced GAPDH nuclear translocation and decreased apoptosis (Fig. 4, D and F). This effect of Akt2 was largely negated by wortmannin treatment.

To further confirm the results of confocal microscopy, GAPDH levels were determined by Western blot in nuclear and cytoplasmic fractions separated from SKOV3 cells transfected with Akt2 or not and treated with or without H$_2$O$_2$. As shown in Fig. 4, Ga and Gb, GAPDH in the cytoplasm decreased, whereas its presence in the nucleus increased after H$_2$O$_2$ stimulation. Akt2 overexpression completely prevented H$_2$O$_2$-induced nuclear translocation of GAPDH, and this effect was blocked by wortmannin. To further confirm the physiological relevance of Akt2 inhibition of GAPDH nuclear translocation, cytosolic (Fig. 4Ha) and nuclear (Fig. 4Hb) GAPDH levels in OVCAR-3 cells treated with H$_2$O$_2$ for 4 h in the presence of different amounts of Akt2 siRNA were analyzed by Western blot. A dose-dependent relationship of transfected Akt2 siRNA and increased GAPDH nuclear translocation was observed (Fig. 4, Ha and Hb).

Akt2 Activation in Ovarian Cancer Tissues Is Associated with Decreased GAPDH Nuclear Localization—Immunohistochemical examination of 30 cases of ovarian cancer and 10 normal ovarian tissues was performed to determine the potential correlation between Akt2 activation (positive phospho-Akt2 staining) and nuclear localization of GAPDH in ovarian cancer tissues. GAPDH staining was usually present in both the cytoplasm and nucleus of normal ovarian epithelial cells (Fig. 5A). No phospho-Akt2 staining was found in normal tissues. Although 19 of 30 (63.33%) ovarian cancers were also negative for phospho-Akt2 (Fig. 5B), 11 (36.67%) ovarian cancers had visible phospho-Akt2 staining in the cytoplasm (Fig. 5D). In phospho-Akt2 negative cancer tissues, GAPDH staining was visibly increased in the nucleus (Fig. 5C). By contrast, phospho-Akt2 positive ovarian cancers showed accumulation of GAPDH in the cytoplasm (Fig. 5E). The intensity of phospho-Akt2 staining was negatively correlated with nuclear localization of GAPDH ($r = -0.87; p < 0.001$).
DISCUSSION

In this study we demonstrated for the first time that Akt2 may be involved in GAPDH-mediated apoptosis in ovarian cancer cells. Akt2 has been identified as an important regulator in cancer cell survival via the PI3K/Akt signaling pathway (9–12). However, the molecular basis of this function of Akt2 is still unclear. In this study an interaction between Akt2 and GAPDH in ovarian cancer cells was found by immunoprecipitation assays and mass spectrometry. Baba et al. (36) also previously described the Akt and GAPDH interaction in cardiomyocytes. One of the consequences of this interaction is likely the alteration in glucose metabolism, as Akt is known to be critically involved in insulin signaling, and GAPDH is an important component in glycolysis. Interestingly, recent studies have shown that GAPDH may also participate in oxidative stress-induced apoptotic cell death (1, 37, 38). This function of GAPDH seems to require its translocation from the cytoplasm to the nucleus (34, 35). It is plausible for the PI3K/Akt signal transduction pathway to regulate GAPDH nuclear translocation, as it has been shown to mediate export of GAPDH from the nucleus to cytoplasm when activated by serum or growth factors in human diploid fibroblasts or NIH 3T3 cells (23, 39).

We reported here that Akt2 may regulate GAPDH-mediated apoptosis. Although overexpression of GAPDH increased H₂O₂-induced ovarian cancer cell apoptosis, up-regulation of Akt2 nearly completely blocked this effect. Moreover, inhibition of Akt2 by a dominant negative mutant or siRNA increased GAPDH-mediated apoptosis. Similarly, inhibition of PI3K/Akt by wortmannin exaggerated GAPDH-induced ovarian cancer cell death. The clinical relevance of this phenomenon was suggested by the finding that 11 of 30 cases of ovarian cancers had elevated Akt2 activation levels. One molecular mechanism by which Akt2 suppresses GAPDH-induced cell death may be related to its regulation of GAPDH nuclear translocation. Akt2 overexpression resulted in cytoplasmic accumulation of GAPDH, whereas Akt2 inhibition led to GAPDH nuclear accumulation. Importantly, nuclear localization of GAPDH was required for its pro-apoptotic effect.

Akt is well characterized as a serine/threonine protein kinase that phosphorylates the RXRXX(S/T) signature sequence, but...
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GAPDH does not contain this typical consensus site. However, Akt has also been shown to phosphorylate the sequence KERCQS in insulin-response element-binding protein 1 (26), LSRPSY in cAMP-response element-binding protein (27), and AATNRPNS and RFARRSVS in the valosin-containing protein (28). Based on the bioinformatics search for putative Akt phosphorylation sites, we identified GMAFRVPT237 as a potential Akt2 phosphorylation site in human GAPDH. Indeed, although wild type GAPDH was phosphorylated by Akt2, the phosphorylation did not occur when threonine 237 in GAPDH was mutated.

Hara et al. (40) found that nuclear translocation of rat GAPDH-mediated by Siah1 is essential for S-nitrosylated GAPDH-initiated apoptotic cell death, which is dependent upon a single amino acid Lys-225 in a critical 19-amino acid sequence (220–238) in rat GAPDH. The molecular models of rat and human GAPDH show that they are similar to a large extent (Fig. 6, A and B), with a difference in only one residue (236 in rat and 238 in human) between the critical 19-amino acid rat (220–238) and human (222–240) GAPDH sequences (Fig. 6C). Interestingly, Thr-237 identified by us is within the key 19-amino acid sequences. By molecular modeling, we can see the positions of the critical 19 residues in rat and human GAPDH are also similar, and they are both distributed over a wide area. Thus, we speculate that a change in the shape of these 19 residues is required to form a conformation-dependent binding interface with the Siah1 protein. The Thr-237 (Thr-235 in rat GAPDH) residue within this critical sequence after change is close to Lys-227 (Lys 225 in rat) and potentially through interaction with Lys-227 contributes to Lys-227 (Lys-225 in rat) with proper conformation during binding with Siah1. Therefore, phosphorylation at Thr-237 may interfere with the binding interactions of Siah1 and Lys-227, in turn blocking the translocation of GAPDH into the nucleus and ultimately leading to decreased cell apoptosis and death.

In summary, we have identified for the first time that Akt2 interacted with GAPDH in ovarian cancer cells, and this interaction could be inhibited by the PI3K/Akt signaling pathway inhibitor wortmannin. Akt2 was functionally involved in the inhibition of GAPDH-associated apoptosis by interacting with GAPDH in the cytoplasm and inhibiting its translocation into the nucleus. Activated Akt2 was correlated with decreased nuclear GAPDH in primary ovarian tumor tissues. As cancer development is closely related to decreased apoptosis and increased survival of cells, our study not only suggests that Akt2 has a novel role in apoptosis induced by H2O2 but also further supports Akt2 as a useful therapeutic target for ovarian cancer.

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