Overexpression of Interferon-γ-inducible GTPase Inhibits Coxsackievirus B3-induced Apoptosis through the Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway and Inhibition of Viral Replication*

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Our previous studies using differential mRNA display have shown that interferon-γ-inducible GTPase (IGTP), was up-regulated in coxsackievirus B3 (CVB3)-infected mouse hearts. In order to explore the effect of IGTP expression on CVB3-induced pathogenesis, we have established a doxycycline-inducible Tet-On HeLa cell line overexpressing IGTP and have analyzed activation of several signaling molecules that are involved in cell survival and death pathways. We found that following IGTP overexpression, protein kinase B/Akt was strongly activated through phosphorylation, which leads to phosphorylation of glycogen synthase kinase-3 (GSK-3). Furthermore, in the presence of CVB3 infection, the intensity of the phosphorylation of Akt was further enhanced and associated with a delayed activation of caspase-9 and caspase-3. These data indicate that IGTP expression appears to confer cell survival in CVB3-infected cells, which was confirmed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt cell viability assay. However, the ability of IGTP to induce phosphorylation of Akt and to promote cell survival was attenuated by the phosphotyrosylinducible 3-kinase (PI3-K) inhibitor LY294002. Transient transfection of the cells with a dominant negative Akt construct followed by doxycycline induction and CVB3 infection reversed Akt phosphorylation to basal levels and returned caspase-3 activity to levels similar to those when the PI3-K inhibitor LY294002 was added. Moreover, IGTP expression inhibited viral replication and delayed CVB3-induced cleavage of eukaryotic translation initiation factor 4G, indicating that IGTP-mediated cell survival relies on not only the activation of PI3-K/Akt, inactivation of GSK-3 and suppression of caspase-9 and caspase-3 but also the inhibition of viral replication.

Interferon-γ (IFN-γ) is an important cytokine for the control of the regulation of the host immune response to a variety of infectious agents. This cytokine is known to exert its effects largely by activation of IFN-γ-responsive genes (1-3). One such gene has been identified recently as IFN-γ-inducible GTPase (IGTP). This gene is representative of a growing family of at least six genes encoding 47-48-kDa GTP-binding proteins localizing predominantly to the endoplasmic reticulum and is probably involved in processing and trafficking of immunologically relevant proteins (4-10). IGTP has been demonstrated to be an active GTPase that accumulates in response to interferon-γ in macrophages as well as non-immune cells (4, 6, 8, 10). Recently, it has been found that IGTP-deficient mice display a loss of host resistance to acute infection by the protozoan Toxoplasma gondii (6). However, the mechanism by which IGTP mediates an antimicrobial response is unknown. Our previous study by differential mRNA display has demonstrated that IGTP is up-regulated in coxsackievirus B3 (CVB3)-infected mouse hearts (11), suggesting the involvement of IGTP in host cell defense to viral infection. However, the role of IGTP in such a defense mechanism has not yet been defined. Our previous unpublished data also showed that IGTP overexpression delayed CVB3-induced cleavage of pro-caspase-3, implicating that IGTP may be involved in cellular survival signaling pathways. This speculation was supported by reports indicating the involvement of GTPases in pleiotropic-signaling events ranging from cell adhesion, spreading, transcription activation, and cell cycle (reviewed in Ref. 12). It has also been reported that Ras (p21Ras), a GTP-binding protein in another GTPase family, once activated, may interact directly with the catalytic subunit of phosphatidylinositol 3-kinase (PI3-K) in a GTP-dependent manner (12) and that PI3-K has been shown to up-regulate Ras-GTPase levels in myelomonocytic U937 cell lines (13). These data imply that IGTP-mediated inhibition of apoptosis may occur via activation of PI3-K/Akt survival pathway. Therefore, in this study, we focused our investigation on the IGTP-mediated PI3-K/Akt signaling pathway in the

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Experimental Procedures

Cells, Virus, Antibodies, Reagents, and Enzymes—CvB3 was routinely propagated in HeLa cells (ATCC). The virus supernatant was obtained by three cycles of freeze-thaw and centrifugation to remove cell debris and stored at −80 °C. Virus titers were determined by plaque assay prior to infection as described previously (11). Tet-ON HeLa cells (Clontech) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM HEPS buffer, and 10% Clontech approved fetal bovine serum specially prepared for Tet-On system. Antibodies against phosphorylated Akt (p-Akt), total Akt, phosphorylated ERK (p-ERK), and phosphorylated GSK3 protein kinase were purchased from Cell Sciences. The total ERK, Akt non-radioactive immunoprecipitation-kinase assay kit (Upstate Biotechnology, Inc.) was purchased from Cell Sciences. Cell lines transfected with empty vectors, pTRE and pTK-Hyg, were also screened by hygromycin and G418 in parallel to be used as a control in later experiments. The reaction was stopped by adding 20 μl of 4× SDS gel loading buffer. Akt kinase activity was determined by Western blot analysis using phospho-GSK3β/8 antibody.

Cell Viability Assay—To determine the effect of Igtp expression on cell viability, Tet-On/IGTP cells were cultured in the presence or absence of Dox and harvested at different time points post-induction. To isolate total protein, cells from a 100-mm dish were washed with cold PBS and resuspended in 700 μl of lysis buffer (0.025 M Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA). A 1% Triton X-100, 1 mM Na3VO4, 1 mM β-glycerol phosphate, 1 μg/ml aprotinin). After incubation for 20 min on ice, the supernatant was recovered by centrifugation at 15,000 rpm for 20 min at 4 °C. The proteins were analyzed by Western blot analysis. Briefly, proteins were separated on 11% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with PBS containing 0.1% Tween 20 and 5% non-fat dry milk for at least 1 h at room temperature. Membranes were incubated with primary and secondary antibodies in the above solution on an orbit shaker at 4 °C overnight and at room temperature for 45 min, respectively. Membranes were further developed by the ECL method as per the manufacturer’s instructions (Amersham Bioscience).

Akt Kinase Assay—Akt kinase assay was conducted using the Akt kinase assay kit (Upstate Biotechnology, Inc.) as per the manufacturer’s instructions. Briefly, Tet-On/IGTP cells or empty vector plasmid-transfected Tet-On/IGTP cells were serum-starved and induced with Dox for 3 h after pretreatment with or without LY294002. Cells were washed twice with cold PBS and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 10 μg/ml aprotinin). After incubation for 20 min on ice, the supernatant was recovered by centrifugation at 15,000 rpm for 20 min at 4 °C. The proteins were analyzed by Western blot analysis. Briefly, proteins were separated on 11% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with PBS containing 0.1% Tween 20 and 5% non-fat dry milk for at least 1 h at room temperature. Membranes were incubated with primary and secondary antibodies in the above solution on an orbit shaker at 4 °C overnight and at room temperature for 45 min, respectively. Membranes were further developed by the ECL method as per the manufacturer’s instructions (Amersham Bioscience).

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Construction of pTRE/IGTP Expression Plasmid—cDNA clone of mouse Igtp (pcDNA3/IGTP, a generous gift from Dr. Gregory A. Taylor) was digested with MluI and NotI to isolate the IGTP fragment containing the entire open reading frame. The DNA fragment was ligated with the plasmid vector pTRE (Clontech) pre-linearized with MluI and NotI at the multiple clone site. The recombinant expression plasmid was checked for correct size and orientation of the IGTP gene insertion by restriction enzyme digestion and agarose gel electrophoresis.

Establishment of the Double-stable Tet-On/IGTP HeLa Cell Line—Tet-On HeLa cells were cultured in DMEM supplemented with 10% Clontech fetal bovine serum and 100 μg/ml G418. The double-stable Tet-On/IGTP cell line, which can grow well in the presence of both G418 and hygromycin, was established by plasmid transfection as per the manufacturer’s instructions (Clontech). Briefly, 105 HeLa cells were co-transfected with 2.5 μg of pTRE/IGTP and 125 ng of pTK-Hyg plasmid encoding the hygromycin-resistant gene by the LipofectAMINE method. After treatment with 4 μg of LipofectAMINE and plasmids for 8 h, the mixture was replaced with DMEM supplemented with 10% serum, and incubation was continued for 2 days before initiating selection. Double-resistant clones were picked up 3 weeks later and further screened for IGTP protein expression by Western blot using anti-IGTP antibody (BD Biosciences). Cell lines transfected with empty vectors, pTRE and pTK-Hyg, were also screened by hygromycin and G418 in parallel to be used as a control in later experiments. To induce the expression of IGTP, cells were cultured in medium containing 10% fetal bovine serum and 1 μg/ml of doxycycline (Dox). Cell cultures prepared for detection of phosphorylation signals were serum-starved overnight before performing the experiments.

Western Blot Analysis—Tet-On/IGTP cells were cultured in the presence or absence of Dox and harvested at different time points post-induction. To isolate total protein, cells from a 100-mm dish were washed with cold PBS and resuspended in 700 μl of lysis buffer (0.025 M Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 2.5 mM sodium pyrophosphate, 10 μg/ml aprotinin). After incubation for 20 min on ice, the supernatant was recovered by centrifugation at 15,000 rpm for 20 min at 4 °C. The proteins were analyzed by Western blot analysis. Briefly, proteins were separated on 11% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with PBS containing 0.1% Tween 20 and 5% non-fat dry milk for at least 1 h at room temperature. Membranes were incubated with primary and secondary antibodies in the above solution on an orbit shaker at 4 °C overnight and at room temperature for 45 min, respectively. Membranes were further developed by the ECL method as per the manufacturer’s instructions (Amersham Bioscience).

Akt Kinase Assay—Akt kinase assay was conducted using the Akt kinase assay kit (Upstate Biotechnology, Inc.) as per the manufacturer’s instructions. Briefly, Tet-On/IGTP cells or empty vector plasmid-transfected Tet-On/IGTP cells were serum-starved and induced with Dox for 3 h after pretreatment with or without LY294002. Cells were washed twice with cold PBS and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 2 mM β-glycerol phosphate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Total cellular proteins were extracted, and Akt was selectively immunoprecipitated by gentle rocking incubation of the sample with anti-Akt antibodies at 4 °C for 3 h. The pellets were washed twice with 500 μl of lysis buffer and twice with 500 μl of kinase buffer. Kinase reaction was done in 40 μl of kinase buffer containing 200 μM ATP and 1 μg of GSK-3 fusion protein for 30 min at 30 °C. The reaction was stopped by adding 20 μl of 4× SDS gel loading buffer. Akt kinase activity was determined by Western blot analysis using phospho-GSK3β/8 antibody.

Cell Viability Assay—To determine the effect of Igtp expression on cell viability, Tet-On/IGTP cells were cultured in the presence or absence of Dox and infected with CvB3. To test whether Igtp-mediated cell survival occurs through activation of the PI3-K/Akt/GSK-3, suppression of caspase-9 and caspase-3, and inhibition of viral protein synthesis.
FIG. 1. IGTP is inducibly expressed in a Tet-On HeLa cell line. Hygromycin- and G418-resistant cells were cultured in DMEM containing 1 μg/ml Dox, and cells were harvested and lysed at the indicated time points. Immunoblotting was performed using anti-mouse IGTP antibody. Equal loading was assessed with an anti-α-tubulin antibody, which detects total cytoskeleton protein. Results were quantitated by densitometric analysis of the bands using NIH Image (version 1.61) and normalized to the control level (no Dox added), which was arbitrarily set to 1.0. Fold increase is the ratio of the density of each Dox-induced sample to that of non-induced sample. These data are representative of two independent experiments.

cells was determined according to the absorbance corrected to a background reading. Cell viability was expressed relative to the sham-infected Dox-induced and non-induced control cells, respectively.

Cell Morphological Observation—The Tet-On/IGTP cells grown in 6-well plates were serum-starved overnight and then divided into five groups. Two groups were treated with PI3-K inhibitor LY294002 for 30 min and then induced or not induced with Dox and infected with CVB3 at an m.o.i. of 10. A third group was not treated with either Dox or LY294002 but infected with CVB3. To confirm that LY294002 itself cannot induce cell death, two additional groups were induced with Dox but pretreated or not pretreated with LY294002. These two groups were sham-infected with PBS. Cell morphological changes were observed by phase contrast microscopy 8 h post-infection.

Viral Plaque Assay—The Tet-On/IGTP cells grown in 100-mm dishes at ~80% confluence were divided into three groups. These groups of cells were induced or non-induced with Dox or Dox-induced after pretreatment with LY294002 for 30 min. All cells were infected with CVB3 at an m.o.i. of 10. Twenty four hours post-infection, the supernatants from the three groups of cell cultures were used to perform viral plaque assay on HeLa cell monolayers to determine viral titers as described previously (33). The experiment was repeated three times.

RESULTS

IGTP Was Inducibly Expressed in a Tet-On HeLa Cell Line—To study the role of IGTP on CVB3-induced pathogenesis, a double-stable HeLa cell line inducibly overexpressing IGTP was established by co-transfection of Tet-On HeLa cells with pTRE/IGTP and pTK-Hyg. In the selection of transfecants, 105 hygromycin- and G418-resistant colonies were obtained, and 15 positive colonies that inducibly overexpress IGTP upon Dox induction were further confirmed by Western blot analysis using an anti-mouse IGTP antibody. These cells were named Tet-On/IGTP. These clones display highly regulated expression of the IGTP protein as shown in Fig. 1. We chose colony 12 for the subsequent experiments to analyze the function of IGTP in regulating CVB3-induced cell survival and death.

IGTP Expression Induces Specific Akt Phosphorylation—Our preliminary experiments done in the present work have shown that IGTP overexpression inhibits CVB3-induced activation of pro-caspase-3. This implies that IGTP expression may be involved in promoting cell survival during CVB3-induced apoptosis. As the mitogen-activated protein kinase (MAPK) and the PI3-K/Akt pathways are well studied major cell survival mechanisms, we focused our investigation on the role of IGTP expression in these two pathways. Tet-On/IGTP and control cells were induced with Dox at 1 μg/ml for IGTP overexpression and harvested at the indicated time points. Immunoblotting for phospho-ERK and phospho-Akt was achieved by densitometric analysis as described in Fig. 1. B, the Akt kinase assay was performed by immunoprecipitation of the Akt from the cell lysates and detection of phosphorylation of downstream target protein of Akt using GSK-3 fusion protein as a substrate. The immunoblot was probing using a p-glycogen synthase kinase-3 (Ser-21/9) antibody, and the negative (lanes 1–3) and positive (lane 7) control cells are indicated. They were all induced with Dox for 3 h before harvesting except lane 3. Lanes 4–6 represent samples harvested 0.5, 1, and 3 h post-induction, respectively. Equal loading was controlled by re-probing the membrane with an antibody to total Akt.

FIG. 2. IGTP expression induces specific activation of Akt kinase by phosphorylation. A, Tet-On/IGTP cells at ~80% confluence were serum-starved overnight and cultured in the medium containing 1 μg/ml Dox. Cells were harvested and lysed at the indicated time points. Cell extracts were separated by 11% SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibody against phosphorylated ERK44/42 (p-ERK). The membrane was stripped and re-probed for p-Akt using an antibody against p-Akt which recognizes phosphosierine 473. Equal loading was assessed by re-probing the membrane with anti-ERK44/42 and anti-Akt antibodies, respectively, which detects total ERK and Akt regardless of their phosphorylation status. Vector-alone transfected cell line was used as a control to confirm that the p-Akt is induced by IGTP overexpression. Quanitation of p-ERK and p-Akt was achieved by densitometric analysis as described in Fig. 1. B, Akt kinase assay was performed by immunoprecipitation of the Akt from the cell lysates and detection of phosphorylation of downstream target protein of Akt using GSK-3 fusion protein as a substrate. The immunoblot was probing using a p-glycogen synthase kinase-3α/β (Ser-21/9) antibody, and the negative (lanes 1–3) and positive (lane 7) control cells are indicated. They were all induced with Dox for 3 h before harvesting except lane 3. Lanes 4–6 represent samples harvested 0.5, 1, and 3 h post-induction, respectively. Equal loading was controlled by re-probing the membrane with an antibody to total Akt.
IGTP inhibits CVB3-induced apoptosis via PI3-K/Akt pathway

In order to determine whether Akt phosphorylation was affected by other proteins presented in the media during Dox induction, vector-alone transfected cells were induced with Dox, and Western blot analyses were performed by probing the membrane sequentially with antibody to p-Akt and to Akt. No apparent increase in Akt phosphorylation was observed as compared with basal levels (Fig. 2). This suggests that Akt phosphorylation is IGTP-dependent.

To determine further whether the Akt has kinase activity, Akt kinase activity assay was performed using nonradioactive immunoprecipitation kinase assay kit to measure the phosphorylation of GSK-3 which is catalyzed by p-Akt. As shown in Fig. 2B, upper panel, the only sample that was induced with Dox for 3 h but not treated with LY294002 and not transfected with DN/Akt construct (lane 6) shows strong Akt kinase activity as compared with the negative and positive controls, which are not induced with Dox (lane 3) and grown in the presence of the serum and treated with Dox (lane 7), respectively. This suggests that the IGTP-induced Akt has kinase activity.

**IGTP-induced Akt Phosphorylation Occurs through the PI3-K Pathway and Promotes Cell Survival**—To determine whether IGTP-induced Akt phosphorylation is catalyzed by PI3-kinase, Tet-On/IGTP cells were preincubated in medium containing different concentrations of LY294002 for 30 min followed by (i) Dox induction, and (ii) Dox induction followed by CVB3 infection. Western blot analyses were performed for detection of p-Akt upon Dox induction. As shown in Fig. 3A, Akt phosphorylation was diminished by the addition of LY294002 to the culture medium in a concentration-dependent manner. Five or 10 μM LY294002 partially inhibited IGTP-induced phosphorylation of Akt, whereas 20 μM LY294002 almost completely restored Akt phosphorylation to the basal level, indicating that IGTP-induced phosphorylation of Akt occurs through the PI3-kinase pathway. This concentration was used for subsequent experiments.

To determine IGTP expression on cell viability, Tet-On/IGTP cells were treated or non-treated with LY294002 inhibitor and then incubated in the presence and absence of Dox for 3 h before CVB3 infection. Cell viability was determined by the MTS assay 12 h post-CVB3 infection. Fig. 3B shows a concentration-dependent inhibition of IGTP-mediated cell survival by LY294002. When cells were exposed to 10 μM LY294002, the cell viability was partially reduced as compared with the culture in the absence of inhibitor, whereas with 20 μM LY294002 in the medium, cell viability was totally restored to levels when Dox was absent in the medium. This was further confirmed by morphological assessment on five cell cultures infected or not infected with CVB3. As shown in Fig. 3C, in the first three CVB3-infected cultures, the IGTP cells induced with Dox but not treated with LY294002 contain many more viable cells than the other two control cultures treated or not treated with Dox and LY294002, in which the majority of the cells are rounded and floating. These data demonstrate that IGTP inhibits CVB3-induced apoptotic cell death by promoting cell survival via activation of the PI3-K/Akt pathway. To test whether LY294002 itself can induce apoptosis, two additional
were serum-starved overnight and then induced with Dox for 3 h before Procedures.

antibody.

expression dramatically reduces Akt phosphorylation on serine 473 cells were used for detection of Akt phosphorylation. As shown, DN/Akt/Tet-On/IGTP cells were grown in 6-well plates for 18 phosphorylation in transient transfected Tet-On/IGTP cells.

one of the two independent experiments. Total Akt or non-infected cell cultures were induced with Dox but either transfected with the CA/Akt construct and the vector PUSE amp(—) only, respectively. As shown in Fig. 4A, Western blot analysis, using anti-Myc tag antibody, revealed that CA/Akt-transfected cells regardless of induction status. To determine the functional significance of this dominant negative construct, we performed Western blot assays that no apparent induction of Bad phosphorylation and Bcl-2 infection, different concentrations of the inhibitor were used to treat the HeLa cells prior phosphorylation of Akt catalyzed by IGTP-activated PI3-kinase. To confirm that LY294002 inhibitor itself cannot induce activation phosphorylation was enhanced by IGTP expression. However, addition of LY294002 completely blocked Akt phosphorylation.

Caspase activation is an important event in CVB3-induced apoptosis, which leads to cleavage of downstream substrates and DNA fragmentation (15). Thus we examined the consequence of enhanced Akt phosphorylation in the regulation of CVB3-induced apoptosis. Immunoblotting for activation of caspase-9 and caspase-3 and cleavage of the downstream substrate, DFF-45/ICAD, was conducted. As shown in Fig. 5B, the majority of the cleaved pro-caspase-9 product appeared in non-induced IGTP cells at 7 h post-infection, and complete cleavage occurs at 10–11 h post-infection. In contrast, in Dox-induced IGTP cells, the majority of pro-caspase-9 cleavage product appeared 8 h post-infection, and complete cleavage was not observed even 12 h post-infection. This suggests that IGTP overexpression delays activation of caspase-9. Similar results were observed in the activation of caspase-3 (Fig. 5C), in which the nearly complete cleavage of the 32-kDa pro-caspase-3 was delayed by 2 h in Dox-induced cells as compared with the non-induced cells. Furthermore, pro-caspase-3 was still detectable 10 h post-infection in Dox-induced cells. This delayed activation was restored when LY294002 was added into the medium or DN/Akt plasmid was transfected into the cells, suggesting that the delayed activation of caspase-3 is mediated by phosphorylation of Akt catalyzed by IGTP-activated PI3-kinase. To confirm that LY294002 inhibitor itself cannot induce activation of caspase-3 in the absence of CVB3 infection, different concentrations of the inhibitor were used to treat the HeLa cells prior to Dox induction. Fig. 5C demonstrates that LY294002 did not induce cleavage of pro-caspase-3 at 20 μM concentration, which is the concentration used in all the experiments in this study. The delayed activation of caspase-3 is further verified by the delayed cleavage of its substrate, DFF-45/ICAD. As shown in Fig. 5D, DFF-45/ICAD was almost completely cleaved 6 h post-infection in non-induced cells, whereas in Dox-induced cells DFF-45/ICAD was completely cleaved 8 h post-infection. All these data indicate that activation of Akt by IGTP overexpression results in the attenuation of apoptotic signal and therefore contributes to the IGTP-mediated cell survival during CVB3 infection.

IGTP Expression Strongly Promotes Expression of Phospho-GSK-3 but Not Phospho-Bad, Phospho-FKHR, and Bcl-2—To determine the downstream target proteins that may be phosphorylated by Akt kinase, we performed Western blot assays using antibodies against p-Bad, p-GSK3α/β, and p-FKHR, respectively. We also determined whether Bcl-2, a pro-survival protein, is up-regulated by activated Akt. Fig. 6A demonstrates that no apparent induction of Bad phosphorylation and Bcl-2

**Fig. 4.** Dominant negative Akt expression suppresses Akt phosphorylation in transient transfected Tet-On/IGTP cells. Tet-On/IGTP cells were grown in 6-well plates for 18–24 h and then transiently transfected with Myc-tagged plasmid encoding CA/Akt or DN/Akt by LipofectAMINE method as outlined under "Experimental Procedures." A, after 48 h of incubation following transfection, cells were serum-starved overnight and then induced with Dox for 3 h before harvesting for detecting the Akt expression using an anti-Myc Tag antibody. B, cell lysates from DN/Akt-transfected and non-transfected cells were used for detection of Akt phosphorylation. As shown, DN/Akt expression dramatically reduces Akt phosphorylation on serine 473 (lane 3) as compared with two controls (lanes 1 and 2). Data represent one of the two independent experiments. Total Akt or α-tubulin expression was used as a loading control.

**Inhibition of Akt Phosphorylation in Tet-On/IGTP Cells by Dominant Negative Akt—**To characterize the functional significance of IGTP-induced Akt in cell survival, the DN/Akt construct was transiently transfected into the Tet-On/IGTP cells and followed by Dox induction. Control cells were transfected with the CA/Akt construct and the vector PUSE amp(+) only, respectively. As shown in Fig. 4A, Western blot analysis, using anti-Myc tag antibody, revealed that CA/Akt-transfected cells and DN/Akt-transfected cells expressed comparable levels of Akt protein, but no Akt protein was detected in vector-only-transfected cells regardless of induction status. To determine the functional significance of this dominant negative construct, immunoblotting for p-Akt was performed using DN/Akt-transfected and non-transfected Tet-On/IGTP cell lysates. As shown in Fig. 4B, a significant decrease in Akt phosphorylation on serine 473 was observed in cells transfected with DN/Akt in the presence of Dox induction as compared with two other cultures that are Dox-induced or non-induced but all not DN/Akt-transfected. This suggests that the ability of IGTP to induce Akt phosphorylation was impaired by the DN/Akt plasmid.
expression were detected as compared with the non-induced control cells. The FHRK phosphorylation was only slightly up-regulated following induction of Akt activity by IGTP as compared with the control. However, the phosphorylation of GSK-3 was strongly induced by the activated Akt. Furthermore, this phosphorylation was significantly inhibited by adding LY294002 into the cell culture as shown in Fig. 6B, suggesting that IGTP induces anti-apoptosis effect through PI3-K/Akt/GSK-3 signaling, which is independent of phosphorylation of Bad and up-regulation of Bel-2 expression.

IGTP Expression Prevents CVB3-induced Akt Degradation and Delays Cleavage of eIF4G—Because Akt protein was reported to be cleaved by caspasas during apoptosis (34, 35), we next examined whether IGTP overexpression is responsible for the integrity of total cellular Akt during CVB3 infection. Western blot was conducted using stripped membranes and reprobed for total Akt protein. As demonstrated in Fig. 7A, the Akt protein levels started to decrease 6 h post-infection and became undetectable 10 h post-infection in non-induced Tet-On/IGTP cells. However, it was still detectable at this latter time point in Dox-induced cells, suggesting that IGTP overexpression protects Akt protein from cleavage by CVB3-activated caspasas. Interestingly, ERK levels in both cell lines infected with CVB3 are unaffected by Dox induction (data not shown), suggesting that the IGTP-mediated anti-apoptotic protection is specifically through PI3-K/Akt activation.

The eukaryotic translation initiation factor 4G, a host protein responsible for eukaryotic mRNA translation initiation, is cleaved by virus protease 2A during picornavirus infection (46, 47). Cleavage of this factor results in the shut off of the majority of host mRNA translation and thus contributes to the virus-induced apoptosis (20, 46, 47). To determine the effect of IGTP on the CVB3-induced cleavage of this important translation initiation factor, Western blot was performed using Dox-induced and non-induced cells as well as cells treated with LY294002 prior to induction with Dox. The data in Fig. 7B demonstrate that cleavage of eIF4G in Dox-induced Tet-On/IGTP cells occurs 4 h later than that in non-induced cells. In addition, cells treated with LY294002 prior to Dox induction shows a 2-h further delay of eIF4G cleavage as compared with cells induced with Dox but not treated with LY294002, suggesting that IGTP overexpression inhibits viral replication and, in turn, suppresses proteolytic cleavage of eIF4G.
IGTP-mediated anti-apoptosis may occur not only by promoting cell survival but also by inhibition of viral replication. This hypothesis was tested by performing Western blot to detect CVB3 capsid protein VP1 and viral plaque assay to determine viral titer in the cells. Fig. 8A shows that VP1 expression started 5 h post-infection in non-induced cells but 7 h post-infection in Dox-induced cells, suggesting that IGTP expression delays the VP1 expression. VP1 expression was further reduced and delayed until 9 h post-infection in Dox-induced cells pre-treated with LY294002. This suggests that inhibition of PI3-K activity leads to a further down-regulation of viral protein synthesis and implies that PI3-K/Akt activation negatively regulates the antiviral activity of IGTP. Similar results were obtained with viral plaque assay. As seen in Fig. 8B, 24 h post-infection, supernatants from cultures of non-induced, Dox-induced, and Dox-induced but also pre-treated with LY294002 were collected, and viral titers were determined by plaque assays on HeLa cell monolayers. The viral titer in cells pre-treated with LY294002 and then induced with Dox was significantly reduced as compared with the titers in cells induced or non-induced with Dox but all not pre-treated with inhibitor.

**DISCUSSION**

In this study, we demonstrated that IGTP overexpression activates the PI3-K/Akt pathway under normal conditions and inhibits host cell apoptosis during CVB3 infection. As shown in our previous study by differential mRNA display, IGTP expression was up-regulated in CVB3-infected murine hearts (11). This up-regulation of IGTP in response to CVB3 infection is likely part of an innate host defense mechanism against virus-induced apoptosis (9). Thus, it is of interest to characterize the IGTP expression in more detail and to determine its biological functions in the regulation of cell survival and death during CVB3 infection.

To achieve this goal, an inducible Tet-On HeLa cell line that overexpresses IGTP was established. By using this cell line, we initially found that IGTP overexpression significantly delayed activation of caspase-3. This raised the possibility that IGTP-
mediated anti-apoptosis may occur through induction of anti-apoptotic gene expression or promotion of cell survival pathways. We first determined the expression of Bel-2 in response to IGTP induction by Western blot, and we found no changes of the expression of this anti-apoptotic gene. We then turned our attention to the survival pathways. As two major survival pathways, MAPKs and PI3-K, may be related to this anti-apoptotic effect (26, 27, 36), we examined the MAPK/ERK pathway but only determined the transient and modest activation of ERK by IGTP expression. In contrast, we found that IGTP expression could induce sustained and intensive activation of Akt kinase activity. Thus, it is likely that the PI3-K/Akt pathway may be responsible, at least in part, for the transduction of survival signals initiated by IGTP expression. This hypothesis is supported by the following findings. First, Akt activation by phosphorylation is IGTP-dependent. This conclusion is drawn according to the data showing that the empty vector-transfected cell line did not induce notable changes in the amount of p-Akt as compared with the non-induced control and the induced Tet-On/IGTP cells, suggesting that the increased Akt phosphorylation in Dox-induced Tet-On/IGTP cells is due to the IGTP stimulation. Second, IGTP activation of Akt occurs through a PI3-K-dependent pathway. This conclusion is supported by our evidence that IGTP-induced phosphorylation of Akt, one of the downstream effectors of PI3-K, is significantly diminished by pretreatment of the culture with a specific PI3-K inhibitor LY294002. In addition, this specific activation of PI3-K/Akt by IGTP expression is further confirmed by the data showing the suppression of the biological functions of Akt by LY294002 inhibitor. These include promoting the Dox-induced pro-caspase-3 cleavage, enhancing CVB3-induced apoptotic cell death, decreasing Dox-induced host cell viability, and inhibiting CVB3 capsid protein translation and production of viral particles. The functional significance of Akt in promoting cell survival was further supported by transient transfection of Tet-On/IGTP cells with a dominant negative Akt mutant plasmid, which blocked phosphorylation of Akt induced by IGTP overexpression and in turn reversed IGTP-induced delayed activation of caspase-3. Moreover, IGTP-induced activation of Akt and phosphorylation of downstream target protein, GSK-3, provides evidence that IGTP-mediated cell survival likely occurs through activation of the PI3-K/Akt/GSK-3 pathway.

Akt is a recognized mediator of cell survival in response to many growth factors (37–40). Once activated, Akt has several important molecular targets that may serve to allow Akt to promote cell survival, including the Bel-2 family member Bad (30), the apoptosis-inducing protease caspase-9 (29), and transcriptional factors including the Forkhead factor FKHR1 and NF-κB (32, 41). However, some of these observations are dependent on the experimental system used. It appears that survival signaling of the PI3-K/Akt cascade in anti-apoptosis effect may employ different antagonistic mechanisms in different cell types and in response to different stimuli. In cardiomyocytes, survival signals stimulated by insulin-like growth factor-I did not induce phosphorylation of BAD but suppressed activation of caspase-3 (42). In Chang liver cells, hepatitis B virus-X protein promoted PI3-K/Akt survival signals through phosphorylation of BAD and inactivation of caspase-3 (43). Furthermore, interferon α/β-induced cell survival signals in human Daudi cells were mediated by the activation of NF-κB factor (44). In our study, to determine the potential downstream targets of Akt in a Tet-On/IGTP HeLa cell system, we examined the protein levels of phosphorylated Bad following Dox induction for IGTP expression. No detectable increase of Bad phosphorylation was observed. These data, along with the observation that IGTP overexpression did not up-regulate Bel-2 protein expression, suggest that IGTP confers cell survival by activating the PI3-K/Akt cascade, which is likely to be independent of Bad and Bel-2. In further probing for targets of activated Akt, we found that pro-caspase-9 cleavage was delayed in Dox-induced cells during CVB3 infection. It is known that functional caspase-9 is activated mainly by cleavage of its pro-form by cytochrome c released from mitochondria in the presence of Apaf-1 and dATP or ATP (45). Once activated, caspase-9 can activate caspase-3 and induce apoptotic cell death. We further verified the delay of caspase-9 activation by detecting the downstream activation of caspase-3, and we demonstrated a corresponding delay of pro-caspase-3 cleavage. Notably, when inhibitor LY294002 was employed or dominant negative Akt mutant plasmid was transfected into the cells, IGTP-induced delayed cleavage of pro-caspase-3 was restored. These data suggest that the IGTP-induced cell survival pathway is, at least in part, through activation of PI3-K/Akt/GSK-3 and suppression of caspase-9/caspase-3. This suppression of caspase activity by IGTP expression is further supported by the data demonstrating that IGTP overexpression protected Akt protein from degradation by caspases.

More interestingly, unlike other survival signals, IGTP-induced survival effects in CVB3-infected cells may rely on not only the activation of PI3-K survival pathway but also the inhibition of viral replication. Previous reports have suggested that IGTP has antiviral activity, but the molecular mechanisms are not known. IGTP, being a GTPase, does not likely directly inhibit CVB3 replication but may function via induction of downstream gene expression. Here we show that Dox-induced cells could delay the cleavage of eIF4G upon IGTP overexpression as compared with non-induced cells. These data suggest the following two possible explanations for the IGTP-mediated antiviral activity. First, IGTP expression may induce downstream gene expression, leading to inhibition of viral replication at either transcriptional or translational levels and thus decrease the synthesis of viral protein 2A, a viral protease responsible for cleavage of eIF4G (47, 48). Second, IGTP expression may activate signaling pathways, leading to the direct inactivation of CVB3 2A protease activity by phosphorylation or dephosphorylation or other unknown mechanism, thus causing delayed cleavage of eIF4G. The eukaryotic translation initiation factors play important roles in translation initiation of cellular mRNAs that employ a cap-dependent ribosome-scanning mechanism (46). CVB3 and other picornaviruses employ a cap-independent mechanism for translation initiation, and their infections can shut off cellular mRNA translation by cleavage of eIF4G and induce apoptotic cell death. This cleavage event is not caspase-dependent but depends on viral protease 2A activity (47, 48). Thus, the delay of eIF4G cleavage implies that IGTP expression may participate in suppression of viral protease activity. On the other hand, as mentioned above most cellular mRNA and CVB3 RNA use different mechanisms for translation initiation, and the cleavage of eIF4G will be detrimental to host mRNA translation but beneficial to viral replication. Therefore, the delayed cleavage of eIF4G may cause an extension of cell survival and inhibition of viral RNA translation. These data were further supported by the ability of LY294002 to delay the cleavage of eIF4G and reduce the viral VP1 synthesis as well as the production of infectious virus particles as measured by their plaque-forming ability. This may be due to the fact that LY294002 can counteract IGTP-induced suppression of caspase-3 activation and sensitize cells to CVB3-induced cell death. As in other viral infection, premature host cell death limits viral replication and reinfection of surrounding cells. Here it is worth stressing that although the activated PI3-K/Akt survival pathway is beneficial to CVB3
replication, this enhancement occurs only transiently. With the overexpression of IGTP, this interferon-inducible protein has a dominant influence on the balance between cell survival and CVB3-induced apoptosis, which means that IGTP can strongly reduce CVB3 replication by its overwhelming negative effect on viral RNA translation. Taken together, these data show that IGTP-induced activation of PI3-K/Akt is essential but not sufficient for IGTP-mediated cell survival effect during CVB3 infection and that the strong inhibition of viral replication by IGTP overexpression also likely contributes to the cell survival.

In summary, this study has shown that IGTP-mediated inhibition of CVB3-induced cell apoptosis occurs through induction of the PI3-K/Akt/GSK3-3 survival pathway and inhibition of viral cap-independent translation. Further characterization of the PI3-K/Akt/GSK-3 survival pathway and inhibition of CVB3-induced cell apoptosis occurs through induc-

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