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Human Cytomegalovirus Major Immediate-Early Proteins and Simian Virus 40 Large T Antigen Can Inhibit Apoptosis through Activation of the Phosphatidylinositide 3′-OH Kinase Pathway and the Cellular Kinase Akt

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The temperature-sensitive cell line *ts*13 is mutated in CCG1, the gene encoding TAF_{II}250, the largest of the TATA-binding protein-associated factors (TAFs) in TFIID. At the nonpermissive temperature, the temperature-sensitive phenotypes are (i) transcription defects, (ii) cell cycle arrest in G_1, and (iii) apoptosis. We previously demonstrated that the human cytomegalovirus (HCMV) major immediate-early proteins (MIEPs) can rescue the transcription defects and inhibit apoptosis at the nonpermissive temperature. In the work presented, we show that activation of the cellular kinase Akt alone can inhibit apoptosis in *ts*13 cells grown at the nonpermissive temperature. More significantly, we show that the HCMV MIEPs can activate Akt, resulting in the inhibition of apoptosis. In parallel experiments, we found that simian virus 40 (SV40) large T antigen can mediate the same function. These experiments were done by transfecting the HCMV major immediate-early gene or a cDNA encoding T antigen into *ts*13 cells, and thus neither viral attachment to receptors, viral tegument proteins, nor any other viral protein is required for Akt activation. Akt is activated by the phosphatidylinositide 3′-OH (PI3) kinase pathway. Using a specific inhibitor of PI3 kinase, we show that the ability of the MIEPs and T antigen to activate Akt and inhibit apoptosis is eliminated, suggesting that the viral proteins utilize the PI3 kinase pathway for Akt activation. Transfection of plasmids which express the individual 86-kDa (IEP86; $IE2_{579aa}$) and 72-kDa (IEP72; $IE1_{491aa}$) MIEPs indicate that each MIEP could inhibit apoptosis via activation of the PI3 kinase pathway.

We have previously used the temperature-sensitive BHK-21 cell line *ts*13 for studies of transcriptional activation by viral proteins (26, 27). The temperature-sensitive defect is a single base change causing a Gly to Asp substitution in CCG1, the gene encoding TAF_{II}250, the largest of the TATA-binding protein-associated factors (TAFs) in TFIID (34). When *ts*13 cells are cultured at the nonpermissive temperature, three distinct temperature-sensitive phenotypes result. First, there are promoter- and activator-specific effects on cellular gene transcription. There is no global alteration in transcription, but specific genes are affected; for example, activities of the cyclin A, D1, and D3 promoters are decreased and activity of the p21/*WAF1*/*CIP1* promoter is increased (40, 45, 46). However, transcription from many genes (e.g., *fos*, *myc*, cyclin B, and TAF_{II}250) is unaffected (40, 45, 46). Second, the cell cycle is arrested in G_1 (16, 41), indicating a link between TAF_{II}250 and the cell cycle. Third, the cell cycle block leads to apoptosis (16, 32, 35). Expression of wild-type human TAF_{II}250 ($hTAF_{II}$250) rescues all of these defects, allowing the cells to grow at the nonpermissive temperature (16, 26, 32, 35, 36, 40, 46).

The *ts*13 cell line has provided a unique system to address questions of the effects of the human cytomegalovirus (HCMV) major immediate-early proteins (MIEPs) on transcription, the cell cycle, and apoptosis. Transcription of the major immediate-early gene of HCMV results in a primary transcript which can be alternatively spliced and polyadenylated to form several mRNAs encoding different MIEPs (30, 37, 38). Two of these proteins, IEP72 (72 kDa; also called IE1, $IE1_{491aa}$, and ppUL123) and IEP86 (86 kDa; also called IE2, $IE2_{579aa}$, and ppUL122a), appear in greater abundance in the lytic infection and have been extensively examined because many studies have shown that they affect RNA polymerase II transcription in a cell- and promoter-specific manner.

We have previously suggested that IEP72 and IEP86 perform a function similar to the TATA-binding protein-associated factors (TAFs) as components of the basal transcription factor TFIID (27). Similar experiments have yielded the same conclusions for simian virus 40 (SV40) large T antigen (9). One of the supporting pieces of evidence for these conclusions came from the use of *ts*13 cells; we found that T antigen and the MIEPs could rescue the temperature-sensitive transcriptional defect in TAF_{II}250 (9, 27). However, while T antigen can also rescue the cell cycle defect at the nonpermissive temperature (T. G. Maguire and J. C. Alwine, unpublished data), the MIEPs cannot (26). In fact, numerous analyses indicate that the MIEPs slightly enhance the G_1 block caused by the *ts*13 temperature-sensitive mutation. This is consistent with previous reports of a G_1 block during HCMV infection of primary foreskin fibroblasts (25) and an inability of the MIEPs to overcome G_1 arrest in retinoblastoma protein (Rb)-transfected Saos-2 cells (13) and in fibroblasts treated with actinomycin D (2). Thus, the failure to rescue the cell cycle defect in *ts*13 cells is consistent with a model in which a function of

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wild-type MIEPs is to establish and/or maintain a cell cycle block that inhibits the G_1 to S transition. However, other data have suggested that the MIEPs may exert blocks at other points in the cell cycle (5, 31).

Although the MIEPs maintained the cell cycle block in *ts*13 cells, we previously reported that they inhibit the onset of apoptosis at the nonpermissive temperature (26). Interestingly, MIEP mutants which failed to rescue the temperature-sensitive transcriptional defect maintained the ability to inhibit apoptosis, suggesting that the MIEP functions which mediate transcription are separate from the functions which affect apoptosis (26). In the present study, we set out to determine the mechanism by which the MIEPs can inhibit apoptosis.

The cellular kinase c-Akt (also known as protein kinase B) is the cellular homolog of the oncoprotein of the AKT8 retrovirus; it has been reviewed by Datta et al. (10). Akt is activated by phosphatidylinositide 3′-OH (PI3) kinase in response to many factors, including insulin and insulin-like growth factor (IGF) (10). The Akt signaling pathway was originally studied as an important regulator of glucose uptake and metabolism which is stimulated by insulin, IGF, and other growth factors. Akt is an essential gene in the maintenance of normal glucose homeostasis (4, 7, 18, 42). Interestingly, the Akt pathway is also a general mediator of cell survival. Once Akt is activated it can phosphorylate a number of factors to ensure cell survival, for example, Bad, caspase 9, the forkhead family of transcription factors (causing them to be sequestered in the cytoplasm), and IκB kinase, causing it to phosphorylate IκB, releasing NF-κB for nuclear entry (10). The combined data on Akt suggest that it plays a central role in the regulation of cell survival.

Recently it has been shown that HCMV infection can activate the PI3 kinase pathway (19), suggesting that viral structural components and/or viral proteins produced in the infected cells may affect the activity of Akt. In the data below we establish that activation of the PI3/Akt kinase pathway, by insulin, IGF-1, SV40 large T antigen, or the HCMV MIEPs, leads to inhibition of apoptosis in *ts*13 cells at the nonpermissive temperature. A specific inhibitor of PI3 kinase eliminated the ability of large T antigen and the MIEPs to inhibit apoptosis, suggesting that the viral activators utilize PI3 kinase for Akt activation. These studies were done by transfection of the major immediate-early gene for production of the MIEPs or a cDNA for production of large T antigen; thus, activation of the PI3/Akt kinase pathway and the promotion of cell survival occur in the absence viral contact with receptors, tegument proteins, and other virally encoded proteins. Our studies suggest that this mechanism of apoptotic inhibition can be mediated by either of the predominant MIEPs, IEP72 or IEP86.

MATERIALS AND METHODS

Plasmids. All plasmids were propagated in *Escherichia coli* HB101. Plasmid DNA for transfections was prepared using Qiagen plasmid maxi kits. Plasmid pRL43a, a gift of Gary Hayward, contains the HCMV major immediate-early gene and can potentially express all of the HCMV MIEPs from the major immediate-early promoter. Plasmid pSG5-T contains the cDNA of wild-type T antigen in the Stratagene pSG5 vector. Plasmids pRSV72 and pRSV86 contain cDNAs encoding IEP72 and IEP86, respectively (1). Plasmids pWT-Akt and pDN-Akt were gifts from James Woodgett (47) and express wild-type and dominant negative forms of the cellular kinase Akt, respectively. Plasmid pMyr-Akt was a gift of Michael Greenberg (11) and expresses a constitutively active form of Akt. The NF-κB-responsive luciferase reporter plasmid, p3xκB-Luc, was a gift of Albert Baldwin, Jr. (29). pDsRed1-C1 was obtained from Clontech (Palo Alto, Calif.); it expresses red fluorescent protein and was used to mark transfected cells. pCDNA3 (Invitrogen), pCMV B/Neo, and pRSV3/Bgl are control plasmids and were used as filler when needed.

Cell culture and transfection. The *ts*13 cell line (41) was propagated and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 32°C in 5% CO_2. From 2×10^5 to 3×10^5 cells were seeded in six-well plates and grown overnight. Cells were transfected with 1.0 μg of DNA using 6.0 μl of FuGENE6 (Roche Molecular Biochemicals, Indianapolis, Ind.). After the addition of DNA, cells were maintained at 32°C for 6 to 8 h, and then, depending on the experiment conditions, maintained at 32°C or changed to 39°C for additional time (up to 28 h). For luciferase analysis, cells were processed using the luciferase assay system (Promega) and procedures supplied by the manufacturer.

Apoptosis detection by ELISA. Cell death was measured using the cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals, Indianapolis, Ind.). A total of 1.0×10^5 *ts*13 cells were seeded on 12-well plates and grown overnight at 32°C in 10% FCS–DMEM. The medium was then changed to 0.5% FCS–DMEM and placed at either 32°C or 39°C. For growth factor treatment, the medium was supplemented with 100 ng of insulin or IGF-1 per ml. To inhibit PI3 kinase, cells were pretreated for 1 h with medium containing 20 μM LY294002 (44), a specific PI3 kinase inhibitor, and then refed with medium containing both 100 ng of growth factor per ml and 20 μM LY294002. After 20 h at 39°C, the cells were washed with chilled phosphate-buffered saline (PBS), and cell lysates were prepared and analyzed by ELISA according to the manufacturer's protocol: the absorbance at 405 nm was measured with a reference wavelength of 490 nm (Roche Molecular Biochemicals, Indianapolis, Ind.).

Apoptosis detection by TUNEL. *ts*13 cells grown on glass cover slips in six-well plates were transfected with 1.0 μg of the following plasmids: pCDNA3, pMyr-Akt, pWT-Akt, pDN-Akt, pCMV B/Neo, or pRL43a. Then 0.1 μg of pDsRed1-C1 was added to each transfection to mark the transfected cells. The cells were analyzed after 24 to 28 h at 39°C. For transfections containing the Akt-expressing plasmids, the medium was changed to 0.5% FCS–DMEM shortly before transfection. The cells on cover slips were washed, fixed, permeabilized and analyzed by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay as described previously (26). Transfected cells were identified by Ds-Red fluorescence and scored for apoptosis by TUNEL fluorescence (green). For each coverslip, 200 to 400 transfected cells were counted. Multiple coverslips were counted for each assay.

Western blot analysis. Protein from total cell lysates (50 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) and transferred to nitrocellulose. Specific proteins were detected by incubation with appropriate antibody, followed by visualization using the Lumi-Light Western blotting substrate kit (Roche Molecular Biochemicals, Indianapolis, Ind.). Goat anti-Akt1 (SC-1618) and monoclonal anti-GSK-3β (SC-7291) were both from Santa Cruz Biotechnologies, Santa Cruz, Calif. All phosphorylation (phospho)-specific antibodies were purchased from Cell Signaling Technologies, Beverly, Mass.; these include rabbit anti-phospho-Akt (Ser473) and anti-phospho-GSK-3α/β (Ser21/9). Monoclonal antihemagglutinin (HA) antibody (Roche Molecular Biochemicals, Indianapolis, Ind.) and monoclonal anti-actin antibody (Chemicon International, Inc., Temecula, Calif.) were also used. Rabbit antiserum directed against the amino terminus common to both IEP72 and IEP86 was prepared commercially for this laboratory by Cocalico Biologicals, Inc. (15). Detection of large T antigen was performed using monoclonal antibody PAB419.

Akt kinase assay. Akt kinase activity assays were performed using an Akt kinase assay kit (Cell Signaling Technologies, Beverly, Mass.) with modifications. Cells were lysed in the supplied cell lysis buffer, and 400 μg of the cell lysate was mixed with 2.0 μg of anti-Akt1 antibody in a final volume of 400 μl for 2 h at 4°C. This was followed by the addition of 20 μl of protein G-Sepharose (Life Technologies, Rockville, Md.) and further incubation for 1 h. The beads were then washed twice in 0.5 ml of cell lysis buffer and once in the supplied kinase buffer. Akt kinase activity was determined by suspending the beads in 40 μl of kinase buffer containing 10 μCi of [γ-^{32}P]ATP, 200 μM ATP, and 1.0 μg of GSK-3 fusion protein and incubating the mixture for 30 min at 30°C. The reactions were stopped by the addition of 20 μl of 3× SDS sample buffer. The ^{32}P-labeled proteins were separated by SDS-PAGE (12% polyacrylamide) and detected by autoradiography. The relative intensity of labeling was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

The final Akt activity data were calculated to compare the activities only in the transfected cells. Specifically, the transfection efficiency of *ts*13 cells was determined in parallel cultures transfected with pDsRed1-C1 producing red fluores-

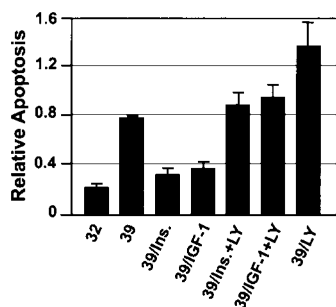

FIG. 1. Effects of insulin and IGF-1 on apoptosis in *ts*13. A total of 10^5 *ts*13 cells/well were grown overnight in 12-well plates at 32°C. The medium was then changed to 0.5% FCS–DMEM and placed at either 32°C or 39°C. For growth factor treatment, the medium was supplemented with 100 ng of insulin or IGF-1 per ml. To inhibit PI3 kinase, 20 μM LY294002 (LY) was used as described in Materials and Methods. After 20 h at 39°C, the cells were harvested, and apoptosis was analyzed using the cell death detection ELISA as described in Materials and Methods.

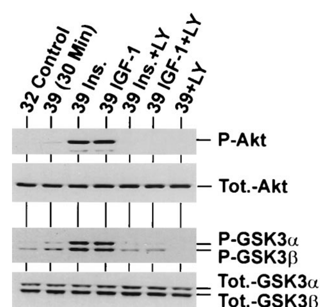

FIG. 2. Phosphorylation of Akt and GSK-3 in *ts*13 cells in response to insulin or IGF-1 treatment. *ts*13 cells were serum starved for 24 h in 0.25% FCS–DMEM and then treated with 100 ng of insulin or IGF-1 per ml for 30 min at 39°C. Then 20 μM LY294002 (LY) was used to inhibit PI3 kinase as described in Materials and Methods. A total of 50 μg of protein from total cell extracts was analyzed by Western blotting for total (Tot.) Akt and GSK levels as well as phosphorylated (P-) Akt and GSK levels. Samples were probed with anti-phospho-Akt (Ser473), anti-Akt1, anti-phospho-GSK-3α/β, and anti-GSK-3β.

cent protein and quantitated by fluorescence-activated cell sorting analysis. The average transfection efficiency was 25% ± 1%. The Akt kinase activity measured in extracts reflects the activity in the entire cell population (transfected plus untransfected cells). The effects of the viral activators on Akt activity derives from the transfected cells and equals ΔAkt, the difference between the total activity from cells transfected with the viral activators minus the total activity from vector control-transfected cells. All quantitative comparisons must therefore be made beginning with the Akt activity in 25% of the vector control-transfected cells, i.e., 25% of the total activity from the vector control-transfected cell extracts where cell numbers are constant. This amount of Akt activity (25% of the total activity from vector control-transfected cell extracts) is also added to ΔAkt, since it represents the normal level of Akt activity in the cells.

RESULTS

Activation of the PI3 kinase pathway reduces apoptosis in *ts*13 cells at the nonpermissive temperature. In order to establish that activation of the PI3 kinase pathway could inhibit apoptosis of *ts*13 cells at the nonpermissive temperature, we cultured cells in normal medium overnight at the permissive temperature. The cells were then refed with low-serum medium (0.5% FCS, warmed to the appropriate temperature) plus or minus insulin or IGF-1, and placed at either the permissive or nonpermissive temperature for 20 h. Cell lysates were then prepared and tested for apoptosis using cell death detection ELISA.

Figure 1 shows the increased apoptosis when cells were grown at the nonpermissive temperature (39°C) compared to the permissive temperature (32°C). Treatment of the cells at 39°C with insulin or IGF-1 reduced apoptosis to nearly the level seen at the permissive temperature, suggesting that activation of the PI3 kinase pathway is involved. To further establish the involvement of the PI3 kinase pathway, the same stimulation by insulin or IGF-1 was performed in the presence of the specific inhibitor of PI3 kinase LY294002 (LY) (44). Figure 1 shows that under these conditions, apoptosis was not rescued, confirming that the antiapoptotic effects of insulin and IGF-1 were mediated through the PI3 kinase pathway.

Activation of the PI3 kinase pathway in *ts*13 cells at the nonpermissive temperature results in phosphorylation of Akt and glycogen synthetase kinase 3 (GSK-3). We next asked whether the activation of the PI3 kinase pathway in *ts*13 cells at the nonpermissive temperature resulted in the phosphorylation of expected kinases in the pathway, particularly Akt. In the first four lanes of Fig. 2, cells were incubated for 24 h in DMEM containing 0.25% FCS, then shifted to 39°C, and the medium was changed to DMEM containing 0.25% FCS plus or minus growth factor and preheated to 39°C. Cellular extracts were prepared after 30 min. In the top two panels, the extracts were used for the Western analysis of total Akt and phosphorylated Akt. The levels of total Akt protein were very similar in all the samples; however, phosphorylated Akt is only seen in the insulin- and IGF-1-treated samples, as expected due to the PI3 kinase pathway's being activated. To confirm that Akt phosphorylation is due to the activation of the PI3 kinase pathway, similar activation experiments were done in the presence of the PI3 kinase inhibitor LY. The last three lanes of Fig. 2 show that in the presence of the inhibitor, Akt phosphorylation was completely inhibited.

In the lower two panels, we asked whether the activation of Akt by insulin and IGF-1 results in the phosphorylation of GSK-3, one of the targets of activated Akt (14, 33). The levels of total GSK-3α and -3β are similar in all the extracts; however, significant levels of the phosphorylated forms appear only in the samples where Akt had been activated and not in the samples where the PI3 kinase pathway was inhibited.

Constitutively active Akt can inhibit apoptosis of *ts*13 cells at the nonpermissive temperature. The above data suggest that activation of the PI3 kinase pathway, leading to the activation of Akt, results in the inhibition of apoptosis of *ts*13 cells at the nonpermissive temperature. To further demonstrate that the activation of Akt leads directly to the inhibition of apoptosis, we transfected *ts*13 cells with vector control plasmids or various plasmids expressing different forms of Akt. In addition, the cells were cotransfected with pDsRed1-C1, a plasmid which constitutively expresses the red fluorescent protein so that the transfected cells could be identified microscopically. Transfected cells were maintained at 32 or 39°C for 24 to 28 h after transfection, and then apoptosis among the transfected cells was determined microscopically using the TUNEL assay (see Materials and Methods).

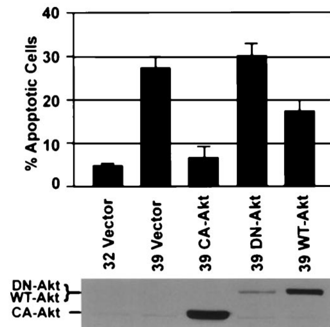

FIG. 3. Effects of Akt on apoptosis in *ts*13 cells at nonpermissive temperature. Cells were cotransfected with pDsRed1-C1 and various plasmids which express constitutively active Akt (CA-Akt); dominant negative Akt (DN-Akt); wild-type Akt (WT-Akt), or a vector control plasmid. The transfected cells were maintained at 32°C for 6 h and then shifted to 39°C for 24 h or maintained at 32°C for 24 h. Apoptosis among the transfected cells (red fluorescent protein-expressing cells) was determined microscopically using the TUNEL assay. Data presented are the means and standard deviations of results of three independent experiments. Typically, about 200 cells from each coverslip were counted. In the lower panel, equal amounts of cell lysates were analyzed by Western analysis using an anti-HA antibody to detect exogenous Akt expression (see Materials and Methods).

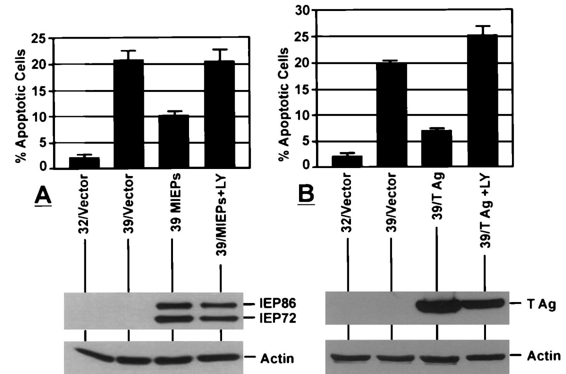

FIG. 4. Effects of HCMV MIEPs and SV40 T antigen on apoptosis in *ts*13 cells at the nonpermissive temperature. *ts*13 cells were cotransfected with pDsRed1-C1 and either a plasmid which expressed the MIEPs (A), a plasmid which expressed SV40 large T antigen (Ag) (B), or appropriate vector control plasmids. The cells were otherwise treated and apoptosis was quantitated as described for Fig. 3. Below the panel, the levels of the MIEPs (A) or T antigen (B) in equal amounts of cell lysate were determined by Western analysis probed with rabbit anti-MIEP or monoclonal anti-T antigen antibodies (see Materials and Methods).

Figure 3 shows that apoptosis increased nearly sixfold upon growth of vector-transfected cells at the nonpermissive temperature. However, this increased apoptosis was eliminated by transfection of the plasmid expressing the constitutively active form of Akt (CA-Akt). Likewise, the expression of wild-type Akt reduced apoptosis by approximately one half. In contrast, the expression of a dominant negative form of Akt (DN-Akt) had no effect on apoptosis. These data indicate that activation of Akt alone results in inhibition of apoptosis in *ts*13 cells at the nonpermissive temperature.

Each of the Akt constructions used contains a fused HA tag. The panel at the bottom shows the results of a Western analysis probed with anti-HA antibody to detect Akt from transfected cell extracts. Constitutively active and wild-type Akt express very well. The constitutively active Akt migrates faster due to a deletion at the N terminus (11). Expression of DN-Akt was low; this was a consistent result, as we found that DN-Akt is very deleterious to *ts*13 cell growth.

HCMV MIEPs and SV40 T antigen rescue apoptosis through activation of the PI3 kinase pathway. All the data thus far confirm that activation of the PI3 kinase pathway in *ts*13 cells leads to the expected activation of Akt, which inhibits apoptosis at the nonpermissive temperature. We next asked whether T antigen or the HCMV MIEPs inhibit apoptosis in *ts*13 cells by activation of the PI3 kinase/Akt pathway. Cultures of *ts*13 cells were transfected with vector control plasmids or plasmids capable of expressing either the MIEPs from the major immediate-early gene or SV40 T antigen from a cDNA. As described above, the cells were cotransfected with pDsRed1-C1 to identify the transfected cells microscopically. After 24 to 28 h at 32 or 39°C, apoptosis was determined among the transfected cells using the TUNEL assay. It should be kept in mind that in counting the apoptotic transfected cells, we could only consider cells nonapoptotic if they showed no TUNEL staining. Cells in which the viral proteins incompletely inhibited apoptosis would be counted as apoptotic, and thus the assay would tend to underestimate the effects of the viral proteins.

In Fig. 4A, the vector-transfected controls show that apoptosis increases eight- to ninefold after the shift from 32 to 39°C. However, as previously determined (26), in the presence of the MIEPs the level of apoptosis at 39°C was significantly reduced. However, the MIEP-mediated reduction was eliminated by the addition of the PI3 kinase inhibitor (LY). In the lower panel of Fig. 4A, the expression of the MIEPs in the transfected cells was determined by Western analysis using an antibody which detects the common N-terminal end of both IEP72 and IEP86. Both proteins were well expressed in the cells transfected with the plasmid encoding the MIEPs; in addition, MIEP production was not affected by the presence of the PI3 kinase inhibitor. These data suggest that one mechanism by which the MIEPs can inhibit apoptosis is through activation of the PI3 kinase pathway, which leads to the activation of Akt.

Similarly, the expression of SV40 large T antigen inhibited apoptosis of *ts*13 cells at the nonpermissive temperature (Fig. 4B), and this was reversed by the addition of the PI3 kinase inhibitor, suggesting that large T antigen also activates the PI3 kinase pathway. The Western analysis in the lower panel of Fig. 4B shows T antigen production in *ts*13 cells. This level of production was not significantly affected by the PI3 kinase inhibitor.

The finding that the HCMV MIEPs activate the PI3 kinase pathway led us to ask which of the MIEPs perform the function. Thus, similar experiments were performed in which plasmids containing cDNAs encoding the individual MIEPs IEP72 and IEP86 were transfected into the *ts*13 cells at the nonpermissive temperature and the effect on apoptosis was determined. The data in Fig. 5 show that both IEP72 and IEP86 can rescue apoptosis and that this is eliminated by the PI3 kinase inhibitor. The levels of production of the individual MIEPs are

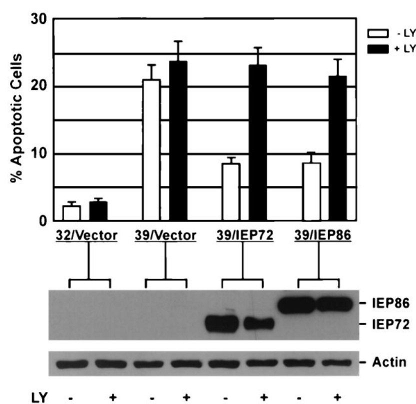

FIG. 5. Effects of IEP72 and IEP86 on apoptosis in *ts*13 cells at the nonpermissive temperature. The experiment was done exactly as that shown in Fig. 4 except the *ts*13 cells were cotransfected with pDsRed1-C1 and plasmids expressing IEP72 or IEP86 individually or appropriate vector control plasmids. Below the panel, the levels of IEP72 and IEP86 produced in each transfection were determined by Western analysis probed with rabbit anti-MIEP (see Materials and Methods).

shown in the Western analysis in the lower panel. Since the two proteins have the identical 85 amino acids at the N terminus, it is possible that this region mediates the apoptosis inhibition function.

HCMV MIEPs and SV40 T antigen increase Akt kinase activity. To further support the finding that the MIEPs and T antigen function through the PI3 kinase/Akt pathway, we asked whether we could detect activation of Akt kinase activity in *ts*13 cells expressing the MIEPs or T antigen at the nonpermissive temperature. In this experiment, cells were transfected with vector control plasmids or the plasmids encoding the MIEPs or T antigen for 24 h at 39°C.

As shown in Fig. 6, when the *ts*13 cells were grown at the nonpermissive temperature, the activity of Akt was reduced by 50% compared to the levels at the permissive temperature (100%). In considering the significance of the 50% reduction, it is important to recall that the temperature-sensitive phenotypes in the *ts*13 cells occur over a relatively long time; the entire population is not affected apoptotically at the same time. Thus, the 50% reduction in Akt activity may reflect reduction of Akt activity in only a part of the population. However, the important observation is that the expression of the MIEPs or T

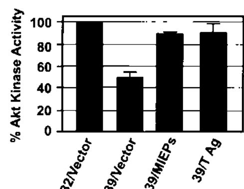

FIG. 6. Activation of Akt kinase by the MIEPs and T antigen in *ts*13 cells. Cells were transfected with vector control plasmids or plasmids expressing the MIEPs or SV40 T antigen. After 24 h at 32 or 39°C the cells were extracted and Akt kinase activity was measured (see Materials and Methods). The Akt kinase activity at 32°C was considered 100%, and all other activities were expressed relative to it. Data were averaged from three independent experiments and are corrected to reflect the activity in the transfected cells (see Materials and Methods).

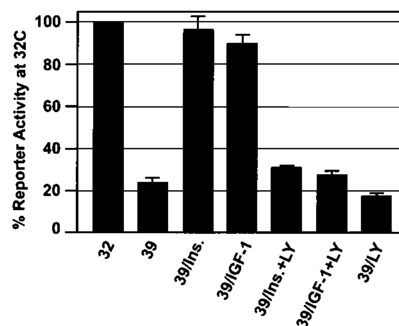

FIG. 7. NF-κB-dependent transcriptional activity in *ts*13 cells. *ts*13 cells were cultured in 0.25% FCS–DMEM and transfected with 1.0 μg of p3xκB-Luc for 16 h at 32°C. Then the cells were treated as that shown in Fig. 1 with insulin (Ins.) or IGF-1 with or without LY294002 (LY). Cell lysates were harvested after an additional 16 h at 32 or 39°C, and luciferase reporter activity was assayed. The luciferase activity at 32°C was set as 100%, and the activities at 39°C were expressed as percentages of the activity at 32°C.

antigen restored activity to levels comparable to those seen at the permissive temperature (Fig. 6). As explained in Materials and Methods the data were calculated to reflect only the activity for the proportion of cells which were transfected; this may underestimate the effect of the viral activators.

Activation of NF-κB responsive promoters. One of the substrates of Akt kinase is IκB kinase (IκK), which phosphorylates IκB and results in the nuclear localization of NF-κB, allowing the activation of antiapoptotic genes (10, 20). In order to show that this aspect of the pathway functions in *ts*13 cells, we transfected cells for 16 h at the permissive temperature with a luciferase reporter plasmid run by a promoter containing 3 NF-κB sites and a TATA element (p3xκB-Luc) (29). The cells were then incubated an additional 16 h at either the permissive or nonpermissive temperature. The cells at the nonpermissive temperature were treated as in Fig. 1 with insulin or IGF-1 in the presence or absence of the PI3 kinase inhibitor (LY).

Figure 7 shows the results; in this experiment, the expression of the reporter at the permissive temperature (32°C) is considered 100% expression, and reporter activities at the nonpermissive temperature (39°C) are shown as a percentage of the activity at the permissive temperature. Thus, it can be seen that after 16 h at the nonpermissive temperature, the activity of the NF-κB promoter is only 24% of its activity at the permissive temperature. The addition of insulin or IGF-1 reverses this effect and returns the activity of the promoter at the nonpermissive temperature to levels comparable to the permissive temperature. As expected for a PI3 kinase-mediated event, the addition of the PI3 kinase inhibitor (LY) eliminated the effects of insulin and IGF-1. Thus, the activation of PI3 kinase by insulin and IGF-1, leading to activation of Akt, results in the expected activation of NF-κB-responsive promoters.

In the experiment shown in Fig. 8, *ts*13 cells were cotransfected with a fixed amount of p3xκB-Luc and increasing amounts of a plasmid expressing the HCMV MIEPs; the difference in plasmid amounts was made up with the vector control plasmid. Again the promoter activity at the nonpermissive temperature is expressed as the percentage of its activity at the permissive temperature (100%). As expected, the activity of the 3xNF-κB/Luc promoter alone (0 μg of wild-type MIEP

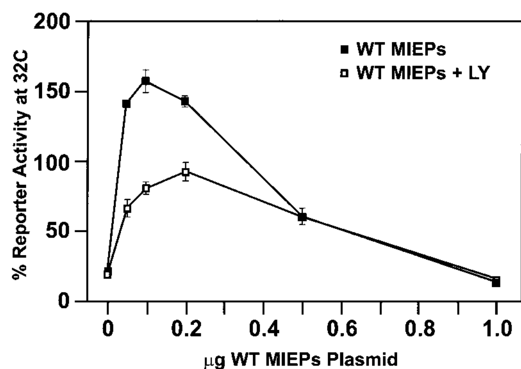

FIG. 8. Effect of HCMV MIEPs on NF-κB-dependent transcriptional activity in *ts*13 cells at the nonpermissive temperature. Cells were transfected with 0.05 μg of p3xκB-Luc plus vector control plasmid (pCMVB/Neo) or increasing amounts of a plasmid encoding the HCMV MIEPs. Vector control plasmid was used to normalize the total amount of DNA transfected. The reporter activity was assayed as described for Fig. 6. The transfection experiment was also done in the presence of 20 μM LY294002 (LY) to inhibit PI3 kinase.

plasmid) decreases at the nonpermissive temperature. However, the addition of as little as 0.05 to 0.2 μg of wild-type MIEP-expressing plasmid significantly activated the promoter to levels in excess of the maximal activity at the permissive temperature. This activation fell off at higher input amounts of the MIEP-expressing plasmid. Since the MIEPs are known to interact with a number of transcription factors (27), this reduction most likely occurs due to a squelching mechanism. It is known that during HCMV infection NF-κB is activated in multiple ways (21, 48, 49). In order to determine how much of the activation of the 3xNF-κB promoter resulted from activation of the PI3 kinase pathway, we did the same activation experiment in the presence of the PI3 kinase inhibitor (LY). We see that with 0.05 to 0.2 μg of input wild-type MIEP-expressing plasmid, the level of activation of the promoter is decreased by at least 50%. These data suggest that MIEP activation of the PI3 kinase pathway is a major source of increased NF-κB in the nucleus, resulting in increased transcription of antiapoptotic genes.

DISCUSSION

The data presented show that activation of Akt by the PI3 kinase pathway can inhibit apoptosis in *ts*13 cells grown at the nonpermissive temperature. The activation of Akt was shown to be critical for the inhibition of apoptosis by expression of a constitutively active form of Akt in *ts*13 cells at the nonpermissive temperature; this alone was sufficient to inhibit apoptosis. Having established the central role of Akt, we asked whether the activation of Akt may be central to the ability of the HCMV MIEPs to inhibit apoptosis in *ts*13 cells at the nonpermissive temperature. We show that a specific inhibitor of PI3 kinase eliminates the ability of the MIEPs and SV40 T antigen to inhibit apoptosis, suggesting that the MIEPs and T antigen utilize the PI3 kinase pathway to activate Akt. To confirm this, we show that the MIEPs and SV40 T antigen cause the activation of Akt kinase. These experiments were done by transfecting only the cloned HCMV major immediate-early gene or a cDNA encoding T antigen, and thus neither viral attachment to receptors, viral tegument proteins, nor any other viral proteins are required for activation of the PI3 kinase pathway and the activation of Akt.

Previous data (19) suggested that HCMV infection activates the PI3 kinase pathway. This activation was biphasic, being seen first as a rapid activation, 15 to 30 min postinfection, most likely due to viral attachment. This initial burst declined by 2 h postinfection; however, the second phase of activation was noted at 4 h postinfection, after which PI3 kinase activity remained elevated. Our data suggest that the second phase of PI3 kinase activation is due to the effects of the MIEPs.

Exactly how the MIEPs can mediate activation of the PI3 kinase pathway remains to be determined. The activation of a kinase associated with the plasma membrane seems incongruous with the established nuclear location of the MIEPs. A transcriptional mechanism might be expected, given that the MIEPs have well-established functions in transcription and can rescue the temperature-sensitive transcriptional defects of $TAF_{II}250$ in *ts*13 cells at the nonpermissive temperature (26–28). However, the finding that both IEP72 and IEP86 can, individually, activate the PI3 kinase pathway and rescue apoptosis suggests the possibility of a nontranscriptional mechanism, since IEP72 by itself shows little ability to rescue the temperature-sensitive transcriptional defect in *ts*13 cells (27).

The observation that T antigen also activated the PI3 kinase pathway suggests a potential mechanism for future consideration. Although T antigen is a well-characterized nuclear protein, small amounts of it are detected on the cell surface (surface T) (23, 24). T antigen localized to the cell surface may have a passive role in SV40 tumor rejection by cytotoxic T cells as a component of SV40 tumor-specific transplantation antigen (3, 6). Previous work has suggested that surface T and a minor subset of large T antigen have a high cell surface binding affinity in vitro and behave like integral membrane proteins (17, 24). A subset of large T antigen is known to be fatty acylated, suggesting a means for it to anchor in the plasma membrane (17). It is possible that this form of T antigen interacts with receptors which activate PI3 kinase. Whether or not the MIEPs have fatty-acylated forms or any type of surface form is unknown.

The significance of the activation of the PI3 kinase/Akt pathway must be considered in the larger scope of host-virus interactions. A starting point would be to consider the three vital cellular processes which make the critical determination of cell size (and the mass of an organ): (i) cell metabolism and growth, e.g., glucose uptake, energy production and synthetic activity; (ii) cell division, i.e., the cell cycle; and (iii) apoptosis. Much evidence suggests that the maintenance of these three processes is so critical that their control is coordinated, utilizing a common mechanism (8, 43).

Interestingly, each of these processes is also of great significance to a productive viral infection. The cell cycle and apoptosis are well-characterized targets for viral regulatory proteins, since the virus needs to maintain the cell cycle at an optimal point while keeping the cell from dying from damage caused by the presence of the virus. The virus must also target cellular metabolism and growth, given that the goal of the productive infection is to synthesize a large quantity of viral structural proteins and viral DNA. Thus, it is necessary for the

virus to alter the cell in ways that would prepare it, both energetically and synthetically, for maximum production of viral particles. In fact, several older studies have suggested that metabolic alterations occur very early in both SV40- and HCMV-infected cells.

Studies done over 25 years ago showed that glucose transport increases significantly at early times in the lytic cycle of many viruses and in virally transformed cells (references 12 and 22 and references therein). Specifically, Eckert and Weber (12) showed that infection with polyomavirus or SV40 increased the rate of glucose transport 10-fold in resting cells and more than 3-fold in actively growing cells. In a lytic infection, glucose uptake began to increase during the early phase of infection, suggesting that T antigen was responsible; this was confirmed using temperature-sensitive mutants of T antigen, which failed to increase glucose transport at the nonpermissive temperature. Since glucose transport began to increase at 8 h postinfection and DNA synthesis does not start until 12 to 14 h postinfection, the timing of the increased glucose uptake is consistent with the need to prepare the cell for the increased energy and metabolic activity necessary for virus production.

In similar studies (22), permissive cells infected with HCMV were evaluated for effects on glucose uptake. In these studies it was found that glucose transport began to increase at 12 h postinfection and increased for at least 48 h. This timing suggested that an immediate-early function was involved. This conclusion was supported by the findings that increased glucose transport required de novo protein synthesis and thus tegument or virion-associated proteins were not directly responsible; immediate-early antigens were detected (although the identity of these immediate-early antigens was unclear); and glucose transport was not increased when the cells were infected with UV-irradiated virus, arguing that the mechanism of activation did not involve virus attachment to receptors, resulting in signal transduction. All of these data suggest the need for immediate-early gene expression for alteration of glucose uptake.

These data suggest that a very early function of SV40 T antigen and HCMV MIEPs is to alter cellular metabolism. This is mediated, at least in part, by the alteration of glucose uptake, increasing the intracellular glucose level for energy production and increased synthetic capability. If we assume, as discussed above, that metabolism and growth, the cell cycle, and apoptosis are coordinately linked, then it seems most efficient for viruses to target a cellular factor common to the control of each of the processes. Akt provides such a factor, in that it functions in regulating glucose uptake and metabolism (4, 18, 39, 42) and is a general mediator of the cell cycle and cell survival (10). Clearly the activation of Akt by the MIEPs may not account for the entirety of the effects an HCMV infection has on metabolism, the cell cycle, and apoptosis. However, the activation of Akt by the MIEPs and T antigen would stand as a major early event providing a coordinate alteration in key cellular processes which would be beneficial to the viral life cycle.

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