Human Gene Profiling in Response to the Active Protein Kinase, Interferon-induced Serine/threonine Protein Kinase (PKR), in Infected Cells

INVOVLEMENT OF THE TRANSCRIPTION FACTOR ATF-3 IN PKR-INDUCED APOPTOSIS

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The interferon-induced serine/threonine protein kinase (PKR) has an essential role in cell survival and cell death after viral infection and under stress conditions, but the host genes involved in these processes are not well defined. We used human cDNA microarrays to identify, in infected cells, genes differentially expressed after PKR expression and analyzed the requirement of catalytic activity of the enzyme. To express PKR, we used vaccinia virus (VV) recombinants producing wild type PKR (VV-PKR) and the catalytically inactive mutant K296R (VV-PKR-K296R). Most regulated genes were classified according to biological function, including apoptosis, stress, defense, and immune response. Transcriptional changes detected by microarray analysis were confirmed for selected genes by quantitative real time reverse transcription PCR. A total of 111 genes were regulated specifically by PKR catalytic activity. Of these, 97 were up-regulated, and 14 were down-regulated. The ATF-3 transcription factor, involved in stress-in-duced β-cell apoptosis, was up-regulated. Activation of endogenous PKR with a VV mutant lacking the viral protein E3L (VVΔE3L), a PKR inhibitor, triggered an increase in ATF-3 expression that was not observed in PKR−/− cells. Using null cells for ATF-3 and for the p65 subunit of NF-κB, we showed that induction of apoptosis by PKR at late times of infection was dependent on ATF-3 expression and regulated by NF-κB activation. Here, we identified human genes selectively induced by expression of active PKR in infected cells and linked ATF-3 to a novel mechanism used by PKR to induce apoptosis.

The double-stranded RNA (dsRNA)2-dependent protein kinase (PKR) is a key mediator in the antiviral effects of interferon (IFN) and a dynamic participant in apoptosis induced by various stimuli (1) (reviewed in Ref. 2). PKR controls cell processes, such as growth (3) differentiation (4), apoptosis (5, 6), stress response (7), anti-tumor activity (8, 9), and antiviral functions (10, 11). After PKR activation, the initiation of protein synthesis is inhibited by phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF-2α) by PKR (12). This kinase regulates the activation of several transcription factors involved in virus-induced apoptosis (13), including p53 (14), IRF-1 (15), and IRF-3, and activation of c-Jun (16). By regulating the expression of genes involved in cell proliferation, NF-κB induction is important in mediating PKR function (16). NF-κB activation by PKR also triggers production of IFN-β (16, 17), an essential component in PKR functionality.

The genes involved in PKR-induced apoptosis in infected human cells have not been defined. We previously described an isopropyl-β-D-thiogalactopyranoside-inducible VV system in which PKR expression triggers apoptosis (5) (for a review, see Ref. 18). When PKR was expressed, the antiviral action against VV and vesicular stomatitis virus provoked a translational block by phosphorylation of eIF-2α and triggered NF-κB activation through the IκB kinase complex (5, 10, 11, 19, 20). Results using this inducible virus-cell system have been validated in transfected cells or cells derived from PKR gene knock-out mice (6, 18). The VV inducible system was also used to identify apoptotic viral genes (21), as well as cellular and viral inhibitors of apoptosis (18, 19, 22). To identify host genes involved in PKR-induced apoptosis of cells infected with VV recombinants, we used cDNA microarray high-throughput screening of over 15,000 human genes. With RNAs obtained from VV-PKR-infected HeLa cells at late but not at early times postinfection, we found transcriptional alteration in defined gene subsets. Comparison of VV-PKR with the catalytically inactive VV-PKR-K296R mutant showed that PKR catalytic activity was required for regulation of these genes. When grouped into functional categories, a significant proportion of altered transcripts consisted of genes involved in cell cycle, apoptosis, stress, defense, and immune response.

The genes up-regulated by PKR activity included ATF-3, a stress-inducible gene that encodes a member of the ATF/cAMP-response element-binding protein family of transcription factors (23, 24). ATF-3 is induced by PERK and GCN4 (25), but this is the first report of ATF-3 up-regulation by PKR. Here, we provided evidence that ATF-3 is specifically induced by PKR and is involved in PKR-induced apoptosis and that induction of apoptosis by PKR is regulated by NF-κB. These findings add the transcription factor ATF-3 to the specific mechanisms used by PKR to induce apoptosis.

EXPERIMENTAL PROCEDURES

Cells and Viruses—HeLa cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn bovine serum and antibiotics. Mouse 3T3-like fibroblasts derived from...
homzygous PKR-deficient mice (PKR−/−) (26) were obtained from C. Weissmann (University of Zurich, Switzerland) and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. ATF-3−/− cells and their wild type counterparts were kindly provided by T. Hai (Ohio State University, Columbus, OH). Briefly, MEF cells were isolated by trypsinization of embryos from ATF-3−/− mice and immortalized by infection with a recombinant retrovirus expressing simian virus 40 large T antigen (25). p65−/− cells and wild type counterparts were a gift from A. Martin (Centro de Investigación Príncipe Felipe, Valencia, Spain). p65−/− MEF cells were also isolated from embryos of p65−/− mice and immortalized by several passages (27). MEF cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. VV recombinants expressing PKR (wild-type enzyme, VV-PKR), VV-PKR-K296R (the catalytically inactive point mutant K296R), and VV-Hsp70 (VV expressing the human heat shock protein Hsp-70) were grown on monkey BSC-40 cells, purified by sucrose gradient banding, and titrated on BSC-40 cells by plaque assay. Unless otherwise indicated, VV infections were performed at a multiplicity of infection of 5 pfu/cell.

Microarray Production—To generate cDNA arrays, we used the Research Genetics 40K sequence-verified clone human cDNA library (available on the World Wide Web at www.resgen.com/products/SVHcDNA.php3), as described (28). Slides contained 15,360 cDNAs, of which 13,295 corresponded to known genes and 2,257 corresponded to control genes. DNA was printed on CMT-GAPS II slides (Corning) with a Microgrid II (BioRobotics) at 22 °C and 40–45% relative humidity.

Microarray Hybridization—Total RNA was isolated from VV-PKR-, VV-, or VV-PKR-K296R-infected HeLa cells cultured in 10-cm plates with Ultraspect-II RNA (Biotech) following the manufacturer’s instructions. At 6 and 16 h postinfection (hpi), two RNA samples (1 µg each) were processed for analysis from each infected HeLa cell culture; each sample was used for two distinct hybridizations (dye swapping). Two different microarray approaches were used. In the first approach, we hybridized cDNA from VV-PKR-infected cells against VV-infected HeLa cells; in the second, we hybridized cDNA from VV-PKR-infected cells against VV-PKR-K296R-infected cells. In both cases, we performed four hybridizations for each postinfection time. Labeling and hybridization conditions were as described (28, 29). Slides were dried by centrifugation and scanned on a ScanArray 4000 (Packard Biosciences) using ScanArray 3.1 software. Raw data were obtained from Cy5 and Cy3 images using QuantArray 3.0 software (Packard Biosciences) and processed using SOLAR software (BioALMA, Spain). Briefly, background was subtracted from the signal, and the log 10 (signal) was plotted versus log 2 (ratio) and Lowess-normalized to adjust most spots to proportion to the log of the template copy number. The correlation values of standard curves were always >0.99.

Immunoblotting—HeLa cells were infected in 6-well plates with VV-PKR, VV, or VV-PKR-K296R (5 pfu/cell) and collected at 6 and 16 hpi in lysis buffer (50 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% Nonidet P-40, 1% SDS). Protein lysates were resuspended in 2× Laemmli buffer, and equal amounts of protein (100 µg) were separated by 14 or 8% SDS-PAGE, transferred to nitrocellulose membranes, and incubated with primary anti-PKR (31), anti-PKR-P (Calbiochem), actin, eIF-2α-P phosphorylated at Ser-51, and total eIF-2α levels were measured by immunoblot analysis. Based on protein standards, molecular mass (in kDa) is indicated. Uninfected cells (M) served as control. Postinfection times are noted above the gels.

Quantitative Real Time Reverse Transcription-PCR (RT-PCR)—RNA (1 µg) was reverse-transcribed using the Superscript first-strand synthesis system for RT-PCR (Invitrogen). A 1:40 dilution of the RT reaction mixture was used for quantitative PCR. Primers and probe sets used to amplify H2BFB, caspase-9, ATF-3, NFkBIA, NFkB-2, interleukin-6, and IFN-γ were purchased from Applied Biosystems. RT-PCRs were performed according to Assay-on-Demand, optimized to work with TaqMan Universal PCR MasterMix, No AmpErase UNG, as described (29). All samples were assayed in duplicate. Threshold cycle (Ct) values were used to plot a standard curve in which Ct decreased in linear proportion to the log of the template copy number. The correlation values of standard curves were always >0.99.

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FIGURE 1. Protein synthesis pattern of HeLa cells infected with vaccinia virus VV-PKR or VV-PKR-K296R. HeLa cells were infected with 5 pfu/cell as described under “Experimental Procedures” and labeled with [35S]methionine (50 µCi, 30 min) at the times indicated. Cellular lysates were analyzed by 12% SDS-PAGE, transferred, and visualized by autoradiography. PKR, autopsyphosphorylated Ser-PKR, actin, eIF-2α-P phosphorylated at Ser-51, and total eIF-2α levels were measured by immunoblot analysis. Based on protein standards, molecular mass (in kDa) is indicated. Uninfected cells (M) served as control. Postinfection times are noted above the gels.
At the postinfection times indicated, cells were washed with methionine-free medium and incubated in methionine-free medium containing [35S]methionine (50 μCi/well, 30 min, 37 °C). Proteins from cell extracts prepared in lysis buffer were fractionated by 12% SDS-PAGE and developed by autoradiography.

**Immunofluorescence**—HeLa or PKR−/− cells cultured on coverslips were infected with the recombinant viruses indicated. At 16 hpi, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, and permeabilized (10 min, room temperature) with 0.1% Triton X-100 in PBS, washed, and blocked with 20% bovine serum albumin in PBS. Cells were incubated (1 h, 37 °C) with anti-PKR, anti-Hsp-70, or anti-ATF-3 antibody; coverslips were washed extensively with PBS and were further incubated (1 h, 37 °C) with ToPro (Molecular Probes, Inc.) and appropriate fluorescein- or Texas Red-conjugated isotype-specific secondary antibodies. After washing with PBS, coverslips were mounted on microscope slides using Mowiol (Calbiochem). Images were obtained using a Bio-Rad Radiance 2100 confocal laser microscope.

**Apoptosis Measurement by ELISA**—HeLa, ATF-3−/−, and p65−/− cells and their wild type counterparts were grown in 12-well plates, infected (5 pfu/cell) with the viruses indicated, and harvested at 24 hpi. We used the cell death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science) to determine the absorbance at 405 nm. This assay is based on the quantitative sandwich enzyme immunoassay principle and uses mouse monoclonal antibodies against DNA and histones to estimate the amount of cytoplasmic histone-associated DNA. Duplicate samples were measured in two independent experiments.

**Measurement of Apoptotic Cell Death by Cell Cycle Analysis**—Cell cycle stages and the percentage of cells with sub-G0 DNA content were assayed by flow cytometry.

| Gene product | VV-PKR versus VV | VV-PKR-K296R versus VV-PKR |
|--------------|------------------|---------------------------|
| Microarray   | RT-PCR           | Microarray                | RT-PCR         |
| H2BFB        | 3.48             | 4.21                      | 4.78           |
| NFκBIA       | 2.92             | 3.21                      | 3.10           |
| Interleukin  | 9.3              | 12.6                      | 10.5           |
| IFN-γ        | 1.2              | 1.13                      | 1.03           |
| NFκB-2       | 4.52             | 3.69                      | 4.03           |
| Caspase-9    | 2.59             | 1.25                      | 1.35           |
| ATF-3        | 7.01             | 3.69                      | 4.82           |

**FIGURE 2. PKR regulates the levels of caspase-9 and Hsp70.** A, activation of caspase-9 and PARP-1 cleavage were measured in mock-, or VV-PKR-, VV-PKR-K296R-, or VV-infected HeLa cells at 16 hpi. Total proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-caspase-9. Caspase-9 presented a pro form (46 kDa) that it is cleaved into a heterodimer of a 35- and 10-kDa chains. An 85-kDa cleavage product of PARP-1 is observed in VV-PKR-infected cells. The activation of caspase-9 corresponded with a decrease in the levels of hsp70 measured by Western blot in HeLa cells infected with VV or VV-PKR. Actin and total eIF-2α levels indicate that the same amount of protein was loaded into each lane of the gel. On the right, the molecular mass of the proteins in kDa is indicated. B, PKR and Hsp70 co-localize in HeLa cells. HeLa cells were infected with 3 pfu/cell of VV-PKR and 3 pfu/cell of VV-Hsp70, fixed at 16 hpi, and processed for immunofluorescence analysis by confocal microscopy using antibodies directed against PKR (red), Hsp70 (green), and TOPRO for staining nuclei (blue). PKR antibody does not recognize PKR activity. C, coexpression of Hsp70 inhibits induction of apoptosis triggered by PKR. HeLa cells grown in 12-well plates were infected at a multiplicity of infection of 6 with the viruses indicated and harvested at 24 hpi for the determination of the absorbance at 405 nm. Mean values of triplicate experiments with S.D. values are given. Lanes 1, uninfected cells; lane 2, 3 pfu of VV-PKR and 3 pfu of VV per cell; lane 3, 3 pfu of VV-PKR-K296R and 3 pfu of VV per cell; lane 4, 3 pfu of VV-Hsp70 and 3 pfu of VV per cell; lane 5, 3 pfu of VV per cell; lane 6, 3 pfu of VV-PKR and 3 pfu of VV-Hsp70 per cell; lane 7, 3 pfu of VV-PKR-K296R and 3 pfu of VV-Hsp70 per cell.
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were analyzed by propidium iodide staining (32). ATF-3−/− and p65−/− cells and their wild type counterparts were uninfected or infected at 5 pfu/cell with VV or VV-PKR strains in the presence or absence of benzylxoycarbonyl-VAD-fluoromethyl ketone, a general caspase inhibitor (40 µM; Calbiochem), or staurosporine, an apoptosis activator (0.5 µM; Sigma). Cells were permeabilized at 24 hpi with 70% ethanol in PBS (4 °C, 30 min). After three washes with PBS, cells were incubated with RNase A (0.1 mg/ml, 37 °C, 45 min) and stained with propidium iodide (10 µg/ml). The percentage of cells with hypodiploid DNA content was determined by flow cytometry. Data were acquired on 6000 cells/sample and analyzed as described (32).

**RESULTS**

**Gene Profiling of HeLa Cells Infected with VV Recombinants Expressing Wild Type PKR or the Catalytically Inactive PKR Mutant K296R—Isopropyl-β-D-thiogalactopyranoside-inducible VV recombinants that express wild type (VV-PKR) or the K296R mutant PKR (VV-PKR-K296R) were used to study the cell transcriptional response after PKR expression in HeLa cells. In this virus-cell system, we previously reported that PKR is produced between 2 and 4 hpi, it is autophosphorylated by 6–9 hpi (10, 35), and its substrate eIF-2α is phosphorylated from 6 to 10 hpi, leading to severe protein synthesis inhibition by 16 hpi (10) (reviewed in Ref. 19). As shown in Fig. 1, in HeLa cells infected with VV-PKR, protein synthesis is strongly inhibited at late times postinfection, and this translational block correlated with phosphorylation of PKR and of eIF-2α. The reduced protein synthesis was not due to a decrease in protein load in the gels, since the levels of actin and total eIF-2α were similar in all lanes of Fig. 1. A clear phosphorylation of wild type PKR was shown from 6 hpi. However, the absence of catalytic activity of the mutant PKR correlates with the lack of autophosphorylation (Fig. 1).

We used cDNA microarray technology to compare the relative abundance of specific PKR-induced mRNAs in VV-PKR- versus VV-infected HeLa cells at 6 and 16 hpi. At 6 hpi, we observed, with the exception of PKR mRNA, very few altered genes after PKR expression, validating the virus-cell system (not shown). At 16 hpi, analysis of the list of cellular genes up-regulated by PKR expression indicated several gene families with distinct biological functions (supplemental Table 1); these included genes involved in cell cycle, apoptosis, stress, defense, and
immortalized PKR-induced cell death. VV-PKR infection induced apoptosis.

We chose selected regulated genes identified in microarray analysis for target verification by quantitative RT-PCR. The RNA preparation used was the same as that used in the microarray. We analyzed five up-regulated genes (NFκBIA, NFκB-2, interleukin-6, ATF-3, caspase-9, and H2BFB) and one unaltered gene (IFN-γ); HPRT was used as internal control. The RT-PCR data confirmed microarray results, showing the same relative transcription regulation of the selected genes (Table 1) and validating microarray analysis. Absolute values are not identical when comparing microarray and RT-PCR data, probably due to differences intrinsic to the techniques.

Although changes in mRNA levels do not necessarily represent changes in protein expression, we analyzed whether changes in gene expression detected in microarray analysis correlated with expression and activation of selected gene products. To confirm the microarray data, we analyzed two proteins, one that is up-regulated (caspase-9) and another that is down-regulated (Hsp70). Transcriptional activation of caspase-9 detected by microarray does not correlate with an apparent increase in the caspase-9 protein level, as measured by Western blot. However, in VV-PKR-infected HeLa cells, there is enhanced caspase-9 activation, as indicated by cleavage of the pro-caspase-9 (Fig. 2A). Activation of caspase-9 was not observed in cells infected with VV-PKR-K296R or VV or in uninfected HeLa cells (Fig. 2A). Apoptosis is mediated by activation of caspase-8 or -9, leading to induction of effector caspase-3 and -7, which cleave specific substrates, including PARP-1. This enzyme catalyzes formation of poly(ADP-ribose) polymers on acceptor proteins involved in the maintenance of chromatin structure, indicating activation of the apoptotic cascade (36). Since PKR expression induced changes in caspase-9 activation, we analyzed whether PKR activation correlated with PARP-1 cleavage using an antibody that recognizes only the cleaved protein. After VV-PKR infection, we found the 89-kDa PARP-1 cleavage product, indicating that effector-caspases were activated (Fig. 2A). To confirm catalytic activation of PKR, we measured phosphorylation of eIF-2α as a PKR substrate. Phosphorylation of eIF-2α was observed only in VV-PKR-infected cells (Fig. 2A).

In contrast to caspase-9 activation, Hsp70 protein levels decreased after PKR expression, concuring with the Hsp70 transcript down-regulation observed in microarray analysis (Fig. 2A). Since Hsp70 suppresses PKR activity in hematopoietic cells (37), we analyzed the subcellular localization of Hsp70 and PKR in HeLa cells by triple confocal microscopy, using anti-PKR and anti-Hsp70 antibodies and ToPro to detect DNA (Fig. 2B). Hsp70 expression by VV resulted in characteristic punctate cytosolic localization of endogenous protein (not shown). PKR expression was mainly cytosolic, with strong perinuclear localization. When PKR and Hsp70 were coexpressed, we detected PKR and Hsp70 colocalization in the merged images (Fig. 2B). To evaluate the functional effect of Hsp70 on PKR activity, we coexpressed these proteins and measured PKR-induced cell death. VV-PKR infection induced apoptosis in HeLa cells, whereas in the presence of Hsp70, the percentage of PKR-triggered apoptotic cells was reduced by more than 70%. Apoptosis was not detected following expression of Hsp70 alone (Fig. 2C). These data indicate that Hsp70 inhibits the proapoptotic effect of PKR. Since PKR down-regulates Hsp70, reduced Hsp70 synthesis would promote more active PKR in the cells.

PKR Induces ATF-3 Protein Expression—ATF-3, a 181-amino acid protein, is a member of the ATF/cAMP-response element-binding protein family of transcription factors and is maintained at low levels in quiescent cells (23). To confirm the increase in the ATF-3 messenger after PKR expression, we used Western blot to analyze the effect of PKR on ATF-3 protein levels in PKR−/− cells. Whereas overall protein levels were reduced, ATF-3 was increased after VV-PKR infection in comparison to mock−, VV−, or VV-PKR-K296R-infected PKR−/− cells (Fig. 3A). Results were similar in HeLa, the cell line used for microarray analysis (not shown). Since PKR catalytic activity is required for the increase in ATF-3 levels, we studied ATF-3 levels and subcellular localization by comparative immunofluorescence microscopy in uninfected or VV-PKR-infected PKR−/− cells. Whereas ATF-3 was nearly undetectable in uninfected cells, there was a clear increase in
ATF-3 staining intensity after PKR activation, with a mainly nuclear and perinuclear localization (Fig. 3B). ATF-3 and PKR showed distinct subcellular distribution patterns, and no physical interaction between these proteins was observed (Fig. 3B).

Phosphorylation of eIF-2α enhances the transcription of genes involved in stress-sensing pathways, such as ATF-4 and GADD34 (38). To validate the up-regulation of GADD34 expression observed in the microarray profile, we measured GADD34 protein levels in PKR−/− cells infected with VV-PKR in comparison with mock-, VV-, or VV-PKR-K296R-infected cells. GADD34 signal was higher in VV-PKR than in mock-, VV-, or VV-PKR-K296R-infected cells, validating the microarray data (Fig. 3A). Under conditions of an overall reduction in protein synthesis, ATF-4 levels were also increased after VV-PKR infection (Fig. 3A), in agreement with ATF-4 behavior in previously described work (38).

Since protein synthesis was severely reduced at 16 h after infection with VV-PKR (Fig. 1), whereas levels of ATF-3 protein were prominently increased, we then analyzed in more detail the mechanism that regulates ATF-3 induction. Thus, we compared ATF-3 levels after 16 h of infection with VV-PKR-infected or mock-treated cells, followed by a 10-h chase in the presence of actinomycin D, a general inhibitor of transcription, or cycloheximide, an inhibitor of protein synthesis. At 16 hpi (10 h after the chase) the levels of ATF-3 and of PKR were measured by Western blot. As shown in Fig. 4A, in VV-PKR-infected cells, ATF-3 levels were about 3-fold higher compared with those in mock-infected cells. The increase in ATF-3 protein levels required RNA transcription, as observed by chasing the infecting cells in the presence of actinomycin D. In VV-PKR + actinomycin D, the levels of ATF-3 were lower than in VV-PKR but higher than in VV-PKR + cycloheximide and in mock + actinomycin D. Moreover, the increase in ATF-3 levels required de novo protein synthesis, since cycloheximide blocked its accumulation. The findings represented in Fig. 4A discard the possibility that the increase in ATF-3 levels in VV-PKR-infected cells is due to an increase in protein stability. Moreover, these findings reveal that ATF-3 mRNA is able to be translated under conditions of phosphorylated eIF-2α, and hence diminished amounts of the initiation factor should be available for translation. Whereas phosphorylation of eIF-2α is generally thought to inhibit translation of most mRNAs, translation of mRNAs encoding proapoptotic functions under conditions of phosphorylatable eIF-2α has been described (39). In yeast, translation of GCN4 mRNA despite increased phosphorylation has been shown. This process occurs through the unusual 5′-untranslated region of GCN4 mRNA, which has four upstream open reading frames (40). When we analyzed the nucleotide sequence of ATF-3, we observed that the 5′-untranslated region of ATF-3 also contains an upstream open reading frame, which could be subjected to the same type of translational control as GCN4 mRNA (Fig. 4B).

**Up-regulation of ATF-3 Is PKR-dependent**—To analyze the effect of endogenous PKR activation on ATF-3 protein levels, we infected PKR−/− and PKR+/+ cells with the prototypic rhabdovirus vesicular...
stomatitis virus and the mutant VV-ΔE3L. VV-ΔE3L is a VV mutant lacking the viral protein E3L, a PKR inhibitor (5, 41–43) that is sensitive to the antiviral effects of IFN (44) and induces apoptosis in HeLa cells (5). In cells infected with VV-ΔE3L, overall protein synthesis is severely inhibited at late times postinfection (45). ATF-3 levels were also measured in cells transfected with pIC (a synthetic dsRNA polymer). We
detected large increases in ATF-3 levels following VV-ΔE3L infection and after pIC treatment of PKR−/− cells, as compared with uninfected cells. In the absence of PKR, we found up-regulation only in pIC-treated cells, since ATF-3 levels after VV-ΔE3L infection were reduced in PKR−/− compared with PKR+/+ cell lines (Fig. 5A). There was no effect on ATF3 levels after vesicular stomatitis virus infection in either cell line. These results suggest that distinct mechanisms are used to up-regulate ATF-3; VV-ΔE3L up-regulation of ATF3 is PKR-dependent, whereas the treatment of 16 h with pIC is PKR-independent.

We analyzed eIF-2α phosphorylation in response to these stimuli. As predicted, phosphorylation was observed only in PKR+/+ cells (Fig. 5A). Immunofluorescence showed a clear increase in ATF-3 signal in VV-ΔE3L-infected PKR+/+ cells (not shown).

We studied the kinetics of ATF-3 induction and eIF-2α phosphorylation during VV-ΔE3L infection in PKR+/+ and PKR−/− cells. In VV-ΔE3L-infected PKR+/+ cells, eIF-2α phosphorylation is detected at 4–6 hpi, whereas ATF-3 induction is at 10–16 hpi, when eIF-2α phosphorylation had occurred. In VV-ΔE3L-infected PKR−/− cells, there was no induction of ATF-3 or eIF-2α phosphorylation, although we observed low eIF-2α phosphorylation levels late in infection, probably due to other kinases (Fig. 5B). This result indicated that endogenous PKR was necessary for ATF-3 regulation, which occurs after eIF-2α phosphorylation.

**ATF-3 Is Involved in PKR-induced Apoptosis**—Since PKR activation ultimately leads to apoptosis, immediate up-regulation of the proapoptotic factor ATF-3 by PKR suggested a role for ATF-3 in PKR-mediated apoptosis. Apoptotic cells are characterized by the presence of fragmented DNA in the nucleus; propidium iodide-stained apoptotic cells show reduced fluorescence compared with normal cells, and cells appear in the sub-G₀/G₁ peak in flow cytometry (46). To study the role of ATF-3 in PKR-dependent apoptosis, we quantified the percentage of cell death by flow cytometry in mock-, VV-ΔE3L-, and PKR- and PKR-infected ATF-3−/− and ATF-3+/− cell lines. PKR expression induced apoptosis in wild-type ATF-3 cells, whereas the percentage of ATF-3−/− apoptotic cells was reduced by ~60% compared with VV-infected control cells (Fig. 6, A and B). These results were supported by an ELISA-based assay that detects cytoplasmic histone-associated DNA fragments; the amount of cytoplasmic histone-associated DNA produced after PKR activation decreased in the absence of ATF-3 (Fig. 6D). Moreover, by Western blot, we found the 89-kDa PARP-1 cleavage product generated after PKR activation in the ATF-3−/− cells, whereas greatly diminished in ATF-3+/− cells, indicating reduced effector caspase activation in the absence of ATF-3 (Fig. 6C). These effects correlated with phosphorylation of PKR and of eIF-2α. Loss of ATF-3 decreased PKR proapoptotic function, implying a role for ATF-3 in PKR-triggered apoptosis.

Recently, it has been described that a spliced variant of ATF-3 but not of the full-length ATF-3 sensitizes cells to apoptosis, partly by suppressing expression of NF-κB-dependent antiapoptotic genes (47). Thus, we next defined by EMSA the NF-κB activation in ATF3−/− and ATF-3+/+ cells during the course of infection with VV-PKR. As observed in Fig. 6E, in ATF-3−/− cells, the NF-κB activation is observed by 6 hpi, with a peak at 16 hpi and then decline by 24 hpi. However, in ATF3−/− cells, the NF-κB activation was maintained at 16 and 24 hpi. These findings indicate that the decrease in PKR proapoptotic function previously observed in ATF-3−/− cells could be due to enhanced NF-κB activation by VV-PKR or a process independent of NF-κB activation. To define which ATF-3 splice isoforms are induced in the PKR expression system, we performed an RT-PCR assay. Total RNA was obtained from HeLa cells infected with VV-PKR or from VV-, VV-PKR-K296R-, or mock-infected cells, and the levels of full-length and spliced variant ATF-3 mRNAs were analyzed by RT-PCR. Based on previous findings (34), we chose a pair of primers that allow discrimination between the full-length ATF3 (546 bp band) and the spliced form ATF-3 (858 bp band). As shown in Fig. 7, in VV-PKR-infected cells, but not in mock-, VV-, or VV-PKR-K296R-infected cells, the isoform ATF-3Zip2a was present. Thus, a correlation was found between the inhibition of NF-κB and the presence of the ATF-3Zip2a spliced form.

**The PKR-induced Apoptosis Is Regulated by NF-κB Activation**—Next we wanted to test if the apoptotic phenotype observed after VV-PKR infection correlated with the presence or absence of NF-κB activity. To this end, we quantified the percentage of cell death by flow cytometry in mock-, VV-ΔE3L-, and VV-PKR-infected p65−/− cells, which lack the 65-kDa subunit of the NF-κB complex, and in the wild-type counterpart. As determined by flow cytometry, VV-PKR- and VV-ΔE3L-infected cells induced apoptosis in p65−/− and in p65−/− cells with levels slightly higher in the absence of NF-κB activity, indicating that apoptosis through PKR was regulated by NF-κB (Fig. 8, A and B). These results were further supported by an ELISA-based assay (Fig. 8D). These cells respond to PKR activation as observed by the 89-kDa PARP-1 cleavage product generated after PKR activation in p65−/− cells and in p65−/− cells (Fig. 8C). Loss of NF-κB activity determined by EMSA (Fig. 8E) increased PKR proapoptotic function (Fig. 8D), implying an NF-κB-regulated role for ATF-3 in PKR-induced apoptosis. Additional support was provided by Western blot analysis, revealing that in the absence of active NF-κB, there is induction of ATF-3 by catalytically active PKR, but not by its inactive mutant, in both p65−/− and p65−/− cells (Fig. 8F).
PKR-induced apoptosis is independent of NF-κB activation. A, p65+/+ or p65−/− MEFs were infected with 5 pfu/cell VV-PKR or VV-ΔE3L and collected at 24 hpi, and the percentage of apoptotic cells defined as sub-G0/G1 cell was determined by fluorescence-activated cell sorting analysis. As a control, we used staurosporine, an apoptosis activator. B, graphic representation of the quantitative data obtained in A. C, cell lysates were subjected to Western blotting, and PARP levels were detected. p65+/+ or p65−/− MEFs were infected with VV, VV-ΔE3L, VV-PKR, or VV-PKR-K296R. Total proteins (100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies specific for PKR, p65, ATF-3, the phosphorylated form of eIF-2α, and total eIF-2α. Total eIF-2α levels indicated that the same amount of protein was loaded into each lane of the gel. On the right, the molecular masses of the proteins in kilodaltons are indicated. An asterisk indicates a 15-kDa ATF-3 spliced form. D, apoptosis assays after mock treatment or VV-, VV-ΔE3, or
**DISCUSSION**

The IFN system forms a first line of defense to virus infection in mammalian cells. Following virus infection, IFN is produced and released from cells, and after binding to its receptor, IFN triggers through the Jak/Stat pathway expression of several genes, some of which are direct inhibitors of viral replication (48). One of the best characterized IFN-induced gene products is PKR, a ubiquitously expressed 65–68-kDa serine/threonine protein kinase that initiates antiviral responses by binding to the dsRNA virus replication product (7, 48, 49). Activated PKR phosphorylates eIF-2α, which sequesters eIF-2B in an inactive eIF-2(2P)-eIF-2B complex, inhibiting the formation of eIF-2-GTP, and inhibits protein synthesis initiation in the infected cell (12). PKR activates two opposite, sequential antiviral strategies: a survival pathway characterized by production of proinflammatory cytokines, such as interferons and interleukins (8, 50, 51), and a cell death pathway (5, 35, 52–54).

As a critical regulatory component of the transcriptional activation of proinflammatory and antiviral genes, NF-xB is a downstream target of PKR that mediates antiviral activities (15, 35). The mechanism by which PKR induces apoptosis in infected cells is not resolved. Here we provided evidence for a new role of ATF-3 in apoptosis triggered by PKR in infected cells. Microarray technology has been used to identify cellular genes induced by PKR expression. Donze et al. (52) used murine PKR-expressing cells in a tetracycline-inducible manner to identify NF-xB-dependent genes induced by PKR. Similar studies were performed by Kazemi et al. (56) that used human cells expressing PKR as a fusion with gyrase subunit B, where the presence of coumermycin produced the dimerization and the activation of PKR, as was described (55). Both studies (52, 56) used cells that expressed PKR in the absence of the activator dsRNA, and it is thus unclear whether the spectrum of genes triggered by PKR in the course of virus infection as a result of its activation by the natural product of virus replication, dsRNA, is similar to and/or different from in uninfected cells. Our studies reported here with active PKR in infected cells reveal differences and similarities with previous findings (52, 56). In particular, in agreement with previous studies in noninfected cells (52, 56), we observed induction of EGR-1, JUN-, JUNB-, NF-xBIA-, and NF-xB-dependent genes. In addition, we found regulation of specific genes belonging to family members also described in previous analysis (52, 56); examples of up-regulated gene families are DUSP9, ISG20, and CASP3, previously described (52, 56), but here we show up-regulation of genes from the same families, like DUSP1, ISG15, and CASP9 (supplemental Tables 1 and 2). In addition, our studies identified new PKR-induced genes in virus-infected human cells and defined the role of some PKR-induced genes within an apoptotic pathway. To identify human genes that are induced in response to catalytically active PKR in the context of virus infection and which may have a role in the induction of apoptosis, we used VV recombinants expressing wild type PKR or the inactive mutant (K296R) in an isopropyl-β-D-thiogalactopyranoside-inducible manner. In this virus-cell system, we observed apoptosis following wild type PKR expression but not when mutant PKR was induced.

To define genes that are specifically regulated by PKR, we extracted RNAs from human VV-PKR- to VV-infected HeLa cells. We hybridized cDNA from VV-PKR and cDNA from VV-infected cells in DNA chips containing >15,000 genes to produce a pattern of differentially expressed PKR-dependent genes. To show that PKR activity was a requirement for selected gene expression profiling, we performed microarray analysis with cDNA from VV-PKR and cDNA obtained from VV-PKR-K296R-infected cells. These approaches enabled us to define PKR catalytically induced genes.

A total of 111 genes were regulated specifically by PKR catalytic activity; of these, 97 genes were up-regulated and 14 were down-regulated (supplemental Table 2). Nine of the genes activated encoded cytoskeletal proteins involved in cell adhesion, three have a role in immune modulation, and 19 have metabolism and signaling functions. In addition, 21 of the up-regulated genes were implicated in transcription, and 11 were implicated in translation functions. Two proteosome subunits, PSMD8 and PSMA7, both involved in antigen peptide production, were up-regulated by PKR. These proteosome proteins form a chain that produces antigen peptides in the proteosome and then transports them to the major histocompatibility complex molecules for presentation on the cell surface (57). PKR also induces major histocompatibility complex class I (human leukocyte antigen subtypes) and class II molecules, such as the coactivator of the immune complex, β2-macroglobulin. This protein coordinates the activation of immune effector cells and is important for a robust, long lasting immune response against infectious agents (58).

Although Hsp70 induction was reported following vaccinia virus infection (59, 60), after VV-PKR infection, we found down-regulation of several heat-shock protein family genes, including hsp10, hsp40, and hsp70. Hsp70 is an antiapoptotic chaperone protein (61) that inhibits mitochondrial release of cytochrome c and blocks procaspase-9 recruit-
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ment to the apoptosome complex (62). Previous studies showed PKR activation of caspase-9 (31); we detected transcriptional activation of caspase-9 by microarray, in correlation with decreased expression of the antiapoptotic protein Hsp70. PKR thus induced apoptosis via different mechanisms, up-regulating apoptotic genes and down-regulating anti-apoptotic genes.

The identification of nine up-regulated genes with roles in apoptosis concurs with the implication of PKR in the host apoptotic response to viral infection. One of the genes is ATF-3, a transcription factor that is up-regulated in response to mechanical injury and stress stimulus (23, 24). ATF-3 is composed of 181 amino acids, and the bZip domain from amino acid 88 to 147 is required for dimer formation and specific DNA binding. A homodimer of ATF-3 represses transcription, whereas heterodimers with c-Jun or JunB activate the transcription. Various spliced isoforms of ATF-3 may further generate functional diversity in different cellular context (47). ATF-3 function is reported both to inhibit (63, 64) and to promote (65) p53-dependent apoptosis. Although ATF3 induction is associated with cell damage (23, 24), the physiological relevance of ATF-3 induction by stress signals is not understood. Protective and detrimental effects of ATF-3 expression have been reported for cell fate events (66, 67). ATF-3 induces apoptosis following curcumin treatment and participates in stress-induced β-cell apoptosis (67, 68). ATF-3 acts as a sensor that interacts with and activates p53 under various types of stress by blocking its ubiquitination (69). Furthermore, ATF-3 overexpression enhances caspase-3 activity (70). Overexpression of full-length ATF-3 protein in colorectal cancer has anti-tumorigenic properties, whereas ATF-3 antisense has the opposite effect (71).

Here, we show that ATF-3 levels are up-regulated following the inducible expression of PKR by VV-PKR but not by expressing the K296R mutant form. Since the VV system expresses PKR, we tested whether endogenous PKR, when activated, could trigger ATF-3 expression. Using the VV-ΔE3L mutant, we show that endogenous PKR up-regulated ATF-3 levels and that apoptosis produced after PKR activation is ATF-3-dependent (Figs. 5 and 8). Whereas in cells infected with either VV-PKR or VV-ΔE3L there is extensive inhibition of protein synthesis (Fig. 1) (45), with phosphorylation of eIF-2α (Figs. 5 and 8), a question must be asked as to how ATF-3 mRNA is translated under limited initiation factor availability. These observations suggest an alternative mechanism of translation. This mechanism could be similar to that used in eukaryotes by other genes, such as Bax, whose mRNA has three upstream AUGs, all in frame with the authentic initiation codon, the first and third of which are followed by a termination codon (72). It has been speculated by laguz et al. (73) that the same mechanism could be used with the Fas mRNA. As indicated in the mRNA sequence of ATF-3 in Fig. 4B, there is an upstream AUG followed by a termination codon and a downstream AUG. We would like to propose a similar mechanism for ATF-3 translation as described for GCN4 under starvation conditions (40). According to this model, we proposed that under stress conditions and with significant levels of eIF-2α phosphorylated (i.e. VV-PKR and VV-ΔE3L infections), there is only a fraction of eIF-2B inactivated, and ribosomes can scan and bypass the first AUG codon to reinstate translation at the canonical AUG. Reduction of the ternary initiation complex will have a greater impact on reinitiation, as proposed for GCN4 (40). Recently, it has been described that ATF-3ΔZip2 but not the full-length ATF-3 sensitizes cells to apoptotic death, partly by suppressing expression of NF-κB-dependent antiapoptotic genes (47). ATF-3ΔZip2 (15 kDa) with a nuclear localization (and not the full-length protein) binds directly to the p65 subunit of NF-κB (47). ATF-3ΔZip2, which is generated through stress-activated alternative splicing, represses NF-κB activity and in this way may play a proapoptotic role in stress response.

Using RT-PCR, we determined that the ATF-3ΔZip2 spliced form is up-regulated following the inducible expression of wild type PKR (Fig. 7). Taking into consideration that a 15-kDa form of ATF-3 was present after PKR activation (see Figs. 3A, 4A, 5A, and 8C) and has a nuclear localization (Fig. 3B), we propose that this isoform is equivalent to ATF-3ΔZip2. If so, the decrease in NF-κB activation in ATF-3ΔZip2/+/+ at late times postinfection (Fig. 6E) could be due to the presence of this spliced form.

Based on previous results (45, 52, 56) and results shown in this paper, we propose a model for the activation of survival and death programs by PKR (Fig. 9). Activation of PKR during infection will activate the two pathways, NF-κB and eIF-2α phosphorylation, leading to up-regulation and down-regulation of specific cellular genes as indicated in supplemental Tables 1 and 2. Among the genes up-regulated is ATF-3, whose translation will not be affected by eIF-2α phosphorylation due to the special 5’-end-untranslated region of the ATF-3 mRNA. Under those stress conditions, ATF-3ΔZip2 isoform protein is produced. This spliced variant goes to the nucleus; it competes for the binding with p65 and suppresses the NF-κB-dependent survival gene transcription, thereby sensitizing cells to apoptosis. Hence, the death program mediated by active PKR will involve up-regulation of ATF-3 and of its ATF-3ΔZip2 isoform. Modulation of the apoptotic response by ATF-3 represents a novel mechanism of PKR action. This mechanism together with others previously assigned to PKR highlights the diversity of effects that this kinase exerts on cells.

Overall, our findings demonstrate that PKR activation produces an increase in ATF-3 levels, independently of NF-κB activation, which correlates with PKR-induced apoptosis, in an NF-κB-regulated manner. The biological implication is that some of the anti-tumor activities of IFNs may be linked to PKR-induced ATF-3 expression. If so, induction of ATF-3 during IFN therapy could be used as a surrogate marker for clinical efficacy. Moreover, the use of microarrays to help understand the role of PKR activation may yield important information on pathways of inducible gene expression. As reported here, we have identified specific human genes that are up-regulated and down-regulated after activation of PKR in a process of virus infection. We identified ATF-3 as a gene involved in PKR-induced apoptosis. Our findings provide new insights into specific mechanisms used by PKR; we conclude that during infection, PKR produced transcriptional alteration in genes of various pathways, coordinating PKR-regulated apoptotic functions.

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