Translation Inhibition in Apoptosis
CASPASE-DEPENDENT PKR ACTIVATION AND eIF2-α PHOSPHORYLATION

Xavier Saelens‡, Michael Kalai‡, and Peter Vandenabeele§

From the Department of Molecular Biology, Unit of Molecular Signaling and Cell Death, Flanders Interuniversity Institute for Biotechnology and Ghent University, 9000 Ghent, Belgium

The protein kinase PKR is a major player in the cellular antiviral response, acting mainly by phosphorylation of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2-α) to block de novo protein synthesis. PKR activation requires binding of double-stranded RNA or PACT/RAX proteins to its regulatory domain. Since several reports have demonstrated that translation is inhibited in apoptosis, we investigated whether PKR and eIF2-α phosphorylation contribute to this process. We show that PKR is proteolysed and that eIF2-α is phosphorylated at the early stages of apoptosis induced by various stimuli. Both events coincide with the onset of caspase activity and are prevented by caspase inhibitors. Using site-directed mutagenesis we show that PKR is specifically proteolysed at Asp^{51} during cellular apoptosis. This site is cleaved in vitro by recombinant caspase-3, caspase-7, and caspase-8 and not by the proinflammatory caspase-1 and caspase-11. The released kinase domain efficiently phosphorylates eIF2-α at the cognate Ser^{51} residue, and its overexpression in mammalian cells impairs the translation of its own mRNA and of reporter mRNAs. Our results demonstrate a new and caspase-dependent activation mode for PKR, leading to eIF2-α phosphorylation and translation inhibition in apoptosis.

Protein synthesis is essential to sustain life. Inhibition of translation, for example by treatment with cycloheximide (CHX), often enhances the induction of apoptosis by different stimuli (1). This sensitization of apoptosis by CHX has been explained by the inhibition of translation-dependent ant apoptotic mechanisms (2). Another known cytotoxic synergy, both in vitro and in vivo, is the combined addition of tumor necrosis factor (TNF) and interferon (IFN) (3–5). IFNs are pleiotropic cytokines with antiviral and antiproliferative properties (6). One of the best characterized proteins induced by IFNs is the dsRNA-activated protein kinase (PKR). PKR is intimately linked with the cellular IFN-inducible antiviral response. Its activation results in host cell protein synthesis shutdown, a powerful countermeasure of the cell against viral infection (7–9). Not surprisingly, many viruses, which are a prime source of dsRNA, encode PKR-inhibiting molecules.

Down-regulation of PKR expression by antisense mRNA in U937 cells protects these cells against TNF-induced apoptosis (10), and overexpression of the cellular PKR inhibitor-p58ipk renders cells resistant to dsRNA- and TNF-induced apoptosis (11). Thus PKR^{0/0} mouse embryonic fibroblasts are resistant to TNF-induced apoptosis (12). Conversely, induction of apoptosis by TNF increases phosphorylation of the α-subunit of the heterotrimeric eukaryotic translation initiation factor (eIF) 2 (eIF2-α) (13). The mechanism linking PKR activation to the TNF-signaling pathway leading to programmed cell death is still unclear.

PKR is a serine-threonine kinase consisting of two functionally distinct domains: an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain (14). The regulatory domain consists of two dsRNA-binding motifs, followed by a spacer. Binding of dsRNA exposes the catalytic site and induces dimerization of PKR. Dimerization allows autophosphorylation, rendering PKR active (15–19). Once the enzyme is activated, dsRNA is dispensable for further substrate phosphorylation. Recently, also dsRNA-independent protein activators of PKR, such as PACT (human) and RAX (mouse), have been identified (20, 21).

Activated PKR can phosphorylate eIF2-α. eIF2 forms a ternary complex with the methionyl initiator tRNA (Met-tRNA_{Met}) and GTP. This complex is essential for recruiting Met-tRNA_{Met} to the 40 S ribosomal subunit and initiating mRNA translation. After delivery of Met-tRNA_{Met} at the proper AUG initiation codon of an mRNA, eIF2-bound GTP is hydrolyzed to GDP. Subsequent loading of eIF2 with Met-tRNA_{Met} requires exchange of eIF2-bound GDP by GTP, a reaction catalyzed by the guanine nucleotide exchange factor eIF2B. Phosphorylation of eIF2-α converts eIF2 to a competitive inhibitor of eIF2B, resulting in a general protein synthesis shutdown (22, 23). Phosphorylation of only a fraction of the cellular eIF2-α is sufficient to severely impair translation initiation, because eIF2B is a limiting component of the cellular translation machinery (24, 25).

Apoptosis involves ordered activation of cysteiny1 aspartate-specific proteinases (caspases), giving rise to cleavage of a particular subset of the cellular proteome (26). These cleavage...
events most often lead to loss of function of the target protein. In a number of cases, however, the substrate becomes activated after cleavage due to removal of regulatory domains (27).

Since PKR activation by an unknown mechanism has been described downstream of TNF-induced apoptosis (10, 12, 13), we searched for a possible mechanism of PKR activation and consequent phosphorylation of eIF2-α during extrinsic apoptosis by death domain receptor aggregation and during apoptosis by staurosporine (STS), a broad-spectrum kinase inhibitor. In both apoptotic pathways phosphorylation of eIF2-α, as well as proteolysis and activation of PKR, occur in a caspase-dependent manner, illustrating the existence of a novel, caspase-dependent mode of PKR activation.

MATERIALS AND METHODS

Cell Culture and Apoptosis Assays—Jurkat T cells and promonocytic U937 cells were cultured in RPMI supplemented with 10% fetal bovine serum, 1 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Jurkat T cells were transfected with DMRIE-C reagent according to the manufacturer’s instructions (Life Technologies, Paisley, UK). Apoptosis was induced in Jurkat T cells by treatment with 100 ng/ml CH-11 anti-Fas antibody (BioCheck, Burlingame, CA) or with STS (Sigma). In U937 cells, apoptosis was induced with 5,000 IU/ml TNF in the presence of 10 μg/ml CHX. Inhibition of caspases was obtained by preincubating cells for 30 min with 50 μM benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) or benzyloxycarbonyl-Asp-Glu-Val-Ala-Asp-fluoromethylketone (zDEVD-cmk) (Bachem, Bubendorf, Switzerland) before addition of an apoptotic stimulus. Cell death was monitored by trypan blue exclusion.

At different time points after addition of anti-Fas antibody, cells were collected by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS), and lysed on ice with cell-free system buffer containing 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH2PO4, 10 mM Hepes, pH 7.4, 1 mM aprotinin, 1 mM leupeptin, and 100 μM phenylmethanesulfonyl fluoride (PMSF), supplemented with 0.05% Nonidet-P40 and 1 mM oxidized glutathione. Supernatant (25 μg of 14,000 × g) was analyzed for caspase activity at 37 °C in the presence of 50 μM of acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Ac-DEVD-amec) (Peptide Institute, Osaka, Japan) as described previously (28). The relative caspase activity was defined as the ratio of maximal Ac-DEVD-ame cleavage activity in test samples versus blank samples.

Metabolic Labeling of Proteins—Jurkat T cells (5 × 10⁶/ml) were treated with anti-Fas antibody or were left untreated. At selected time points, 1.5 × 10⁶ cells were transferred to six-well plates and labeled for 30 min with 10 μCi of Translabel (ICN Biomedicals, Irvine, CA). Cells were washed twice with ice-cold PBS and lysed on ice with lysis buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 400 mM NaCl, 2 mM dithiothreitol, 20% glycerol, 1% Triton X-100, 1 mM aprotinin, 1 mM leupeptin, and 100 μM PMSF. Supernatant (30 μg of protein of 14,000 × g) was resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by Coomassie Brilliant Blue staining. The same gel was used for autoradiography.

Immunoblot Analysis—Protein extracts from cells (10⁶ cells/sample) were prepared by lysis with sample buffer containing 6% SDS, 1.4 M β-mercaptoethanol, 20% glycerol, 0.01% w/v bromphenol blue, and 125 mM Tris-HCl, pH 6.8. Samples were boiled for 10 min, separated in 12.5% SDS-PAGE gels, and transferred to nitrocellulose. Block, antibody incubation steps, and washing of the membrane were performed in Tris-buffered saline Tween containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20, supplemented with 3% skimmed milk. Antibodies specific for the regulatory and kinase domain of human PKR were purchased from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies against phosphorylated and unphosphorylated eIF2-α were obtained from Santa Cruz Biotechnology. Antibodies to human caspase-3, eIF4G, and poly(ADP-ribose) polymerase (PARP) were supplied by BIOSOURCE International (Camarillo, CA), Transduction Laboratories, and Biomol Research Laboratories (Plymouth Meeting, PA), respectively. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies to mouse and rabbit immunoglobulin (Amersham Pharmacia Biotech, Uppsala, Sweden), as well as to goat antibody (Santa Cruz Biotechnology). Proteins were visualized using a chemiluminescence substrate (Du Pont), and blots were exposed to film (Amersham Pharmacia Biotech). Signals were quantified by densitometric analysis (except for eIF2-α and captured by a Lumi-Imager work station (Roche Molecular Biochemicals, Basel, Switzerland).

Plasmid Constructs—Plasmid constructs were made using current molecular biology techniques. Human PKR cDNA cloned in-frame with NH₂-terminal His tag in pET15b was a kind gift from Dr. B. Williams (The Cleveland Clinic Foundation, Cleveland, OH). cDNA was transferred to a pCDM8 vector (Invitrogen, San Diego, CA) with and without NH₂-terminal His tag to obtain pCDM8-His-PKR and pCDM8-PKR, respectively. PKR-K296R, PKRD251N, and PKRD251NK296R mutants (Asp⁵⁵¹ to Asn⁵⁵¹) were generated by overlap polymerase chain reaction.
and cloned into pEF6/Myc-His C (Invitrogen) in-frame with a COOH-terminal Myc tag. pEF6A-E-tag-PKR-caspase-generated kinase domain (Kdcasp) and pEF6A-E-tag-PKR-Kdcasp R296R were obtained by transferring cDNA coding for PKR and PKR-K296R starting from Met231, in-frame with an NH2-terminal E-tag (amino acid sequence MGPVYPYDPPLEPRAA), and inserted into a pEF6A/Myc-His A vector (Invitrogen). Human eIF2-α-cDNA was obtained from HeLa cells total RNA by reverse transcription-polymerase chain reaction and was confirmed that it contributes to the shutdown of protein synthesis. Therefore, we monitored the degree of eIF2-α phosphorylation, which is considered to be the most critical step in the regulation of protein synthesis, using the modified phosphosphate assay described above. Treatment with anti-Fas significantly increased the phosphorylation of eIF2-α at Ser51, as evidenced by the reduced electrophoretic mobility of eIF2-α on SDS-PAGE gels (Fig. 2A). This result was consistent with the observation that anti-Fas treatment leads to the activation of caspase-3, which cleaves and inactivates eIF2-α kinase (PKR). The effect of PKR and PKR-KDcasp expression on translation of a reporter mRNA was assayed by co-transfecting 300 ng of the respective PKR constructs and 50 ng of a modified pEGFP-N1 plasmid (CLONTECH), encoding green fluorescent protein (GFP) with a nuclear localization signal. 48 h after transfection, GFP expression was monitored by Western blot analysis and was found to be significantly reduced in cells transfected with PKR or PKR-KDcasp. These results indicate that PKR and PKR-KDcasp expression inhibited translation at the level of initiation. The effect of PKR and PKR-KDcasp expression on the level of translation was further confirmed using in vitro transcription-translation assays. Cells were transfected with pCDM8-based PKR, amino-terminal His-tagged PKR, and PKR-K296R vectors (1 μg) were used as template for in vitro coupled transcription-translation in a reticulocyte lysate system (Promega Biotec, Madison, WI). Translation reaction (2 μl) was incubated with 200 nM recombinant-uridylated capsn (28) in a total volume of 25 μl of cell-free system buffer for 1.5 h at 37 °C. The resulting cleavage products were analyzed on SDS-PAGE gels and autoradiography.

**Kinase Assays**—HEK293T cells were transiently transfected, using calcium phosphate precipitation, in 100-mm culture dishes with 5 μg of different PKR- and eIF2-α-encoding constructs. PKR, PKR-K296R, eIF2-α, and eIF2-α-S51A constructs were Myc-tagged; PKR-KD and PKR-KDcasp R296R constructs were E-tagged. 36 h after transfection, cells were harvested and washed twice with ice-cold PBS. Cell lysis, immunoprecipitation, and kinase reaction were performed as described previously (20). Briefly, tagged proteins were isolated from 500 μg of cell lysate by overnight immunoprecipitation at 4 °C with anti-c-myc (Roche Molecular Biochemicals) or E-tag antibody (Amersham Pharmacia Biotech), followed by addition of protein A Trisacryl (Pierce). Immune complexes were washed twice with lysis buffer and twice with high salt buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 1% Triton X-100, 1 μM aprotinin, 1 mM leupeptin, and 100 μM PMSF. After the last wash, immune complexes were resuspended in 70 μl of kinase buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM MgCl2, 2 mM MnCl2, 5% glycerol, and 100 μM PMSF. Kinase reactions were set up by mixing 10 μl of the respective immune complexes with 5 μl of [γ-32P]ATP (3,000 Ci/mmol) in 25 μl total volume. Reactions were allowed to proceed for 15 minutes at 30 °C and were analyzed by SDS-PAGE and autoradiography. A reaction without ATP was added to set up in parallel and was analyzed by Western blot to estimate the input. Translation Inhibition Assays—Transfections of HEK293T cells were done using calcium phosphate precipitation in six-well plates with 300 ng of the respective pEFPKR vector. To assay for translation inhibition of PKR and PKR-KDcasp, cells from three independent transfections were pooled 36 h after transfection, washed in PBS, and divided in two. One half was used for Western blot analysis of expressed proteins, the other half was used for Northern blot analysis of total RNA isolated with an RNaseasy kit (Qiagen, Chatsworth, CA). 10 μg of total RNA was denatured in glyoxal and Me3SO (29), separated in a phosphate-buff ered agarose gel, and transferred onto a nylon membrane. Filters were hybridized with an [α-32P]CTP-labeled PKR-KDcasp cDNA fragment, stripped, and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GPD) probe. Western blot signals were quantitated with a Lumi-Imager, normalized for intensity of anti-β-actin. Northern blot signals were quantitated with a Lumi-Imager, normalized for intensity of GPD.

**RESULTS**

eIF2-α Is Phosphorylated during Apoptosis—Stimulation of the death domain-containing Fas receptor in Jurkat cells was used as a prototype apoptotic model to assess if inhibition of translation occurs during apoptosis. Light microscopy examination confirmed that anti-Fas-treated Jurkat cells rapidly died from apoptosis, as exemplified by cell shrinkage and membrane blebbing (Fig. 1A). Cytotoxicity was caspase-dependent, since zVAD-fmk and zDEVD-cmk significantly delayed cell death (Fig. 1B). Pulse labeling of cells with radiolabeled methionine and cysteine revealed that de novo protein synthesis was inhibited starting from the third hour of anti-Fas treatment (Fig. 1C).

In eukaryotic cells, protein synthesis is tightly controlled at the level of initiation (22). The heterotrimeric eukaryotic translation initiation factor eIF2 recruits the initiator Met-tRNA(Met) to the ribosome. This recruitment is severely impaired by phosphorylation of the regulatory α-subunit of eIF2, viz. eIF2-α. Therefore, we monitored the degree of eIF2-α phosphorylation in the course of Fas-mediated apoptosis by Western blotting using antibodies specific for phosphorylated and nonphosphorylated eIF2-α (31). Increased eIF2-α phosphorylation was detected 3 h after anti-Fas treatment (Fig. 1D). Other groups reported on the caspase-mediated cleavage of eIF2-α and suggested that it contributes to the shutdown of protein synthesis in apoptosis (32, 33). With both eIF2-α-specific antibodies we observed minor proteolysis of the 35-kDa eIF2-α into a 22-kDa fragment, coinciding with an increase in eIF2-α phosphorylation (Fig. 1D). These proteolytic and phosphorylation events occurred while the majority of the cells still had intact membranes, as demonstrated by trypan blue exclusion. This means that the events documented occur at least in the executioner
phase of the apoptotic process (Fig. 1B).

eIF2-α Phosphorylation Is Caspase-dependent—To explore the relationship between proteolytic activation of caspases and phosphorylation of eIF2-α, we compared the time kinetics of the enzymatic activity of caspases in cytosolic extracts of anti-Fas-triggered cells with the ratio of phosphorylated versus total eIF2-α phosphorylation (Fig. 2A). The data demonstrate that inhibition of new protein synthesis and eIF2-α phosphorylation are both co-linear with DEVDase activity (Figs. 1C and 2A). Furthermore, treatment of Jurkat cells with zVAD-fmk or zDEVD-cmk prior to anti-Fas exposure abolished phosphorylation of eIF2-α as well as caspase-3 maturation (Fig. 2B). These results suggest that phosphorylation of eIF2-α coincides with and depends on activation of caspases.

PKR Cleavage and eIF2-α Phosphorylation Concur during Apoptosis—A conceivable molecular mechanism to explain the caspase dependence of eIF2-α phosphorylation in apoptosis would imply a caspase-dependent proteolytic activation of an upstream kinase (27). Activation of PKR by an unknown mechanism and phosphorylation of its primary target eIF2-α were shown to be an integral part of TNF-induced apoptosis in NIH3T3 and U937 cells (10, 12, 13). Western blot analysis revealed that PKR is cleaved during Fas-mediated apoptosis in Jurkat cells, resulting in two fragments: the first corresponding with the NH2-terminal part and the second with the COOH-terminal part of the protein (Fig. 3A). The estimated size of these fragments agreed well with a physical separation of the regulatory NH2-terminal domain (38 kDa) and the kinase domain (43 kDa) of PKR. This proteolysis coincided in time with the increase in eIF2-α phosphorylation, the characteristic cleavage of the prototype caspase substrate PARP, and cleavage of eIF4G (Fig. 3A). The latter is a translation initia-
tion factor essential for mRNA recruitment to the ribosome. Its cleavage by caspases was suggested to be involved in the inhibition of cellular translation in apoptosis (34, 35). Treatment with the caspase inhibitors zVAD-fmk or zDEVD-cmk prior to anti-Fas exposure suppressed proteolysis of PKR and proteolytic maturation of caspase-3, suggesting that caspases led to cleavage of PKR (Fig. 3B). Similar results were obtained in apoptotic cell death mediated by STS, a potent Ser/Thr protein kinase inhibitor that does not affect the eIF2-β/α kinase activity of PKR (Fig. 3C) (36), as well as in promonocytic U937 cells treated with TNF (Fig. 3D). These results demonstrate that PKR is proteolysed in apoptosis induced by death domain receptor aggregation and in apoptotic cell death initiated by STS. PKR fragmentation in these apoptotic conditions coincides with phosphorylation of eIF2-β/α.

Identification of the Caspase Cleavage Site in PKR

To identify the nature of the caspase(s) implicated and to demonstrate that PKR is a direct substrate for caspases, a panel of purified recombinant caspases (28) was coincubated with [35S]methionine-labeled PKR. Among the six proteases tested, the apoptotic caspase-3, -7, and -8, and to some extent also caspase-6, could cleave PKR, whereas the inflammatory caspase-1 and -11 did not (Fig. 4A). Cleavage of PKR in apoptotic cells resulted in two peptide fragments of different sizes (~38 kDa for the NH2 terminus and 43 kDa for the COOH terminus). But the in vitro translation factor essential for mRNA recruitment to the ribosome. Its cleavage by caspases was suggested to be involved in the inhibition of cellular translation in apoptosis (34, 35). Treatment with the caspase inhibitors zVAD-fmk or zDEVD-cmk prior to anti-Fas exposure suppressed proteolysis of PKR and proteolytic maturation of caspase-3, suggesting that caspases led to cleavage of PKR (Fig. 3B). Similar results were obtained in apoptotic cell death mediated by STS, a potent Ser/Thr protein kinase inhibitor that does not affect the eIF2-β/α kinase activity of PKR (Fig. 3C) (36), as well as in promonocytic U937 cells treated with TNF (Fig. 3D). These results demonstrate that PKR is proteolysed in apoptosis induced by death domain receptor aggregation and in apoptotic cell death initiated by STS. PKR fragmentation in these apoptotic conditions coincides with phosphorylation of eIF2-α.

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cleavage assay seemingly yielded two closely running fragments of ~40 kDa. This discrepancy with apoptotic cell lysates can be attributed to the NH₂-terminal polyhistidine tag present in the in vitro translated PKR. Indeed, caspase-7 proteolysis performed on in vitro translated PKR without His tag revealed double bands, resembling the fragments observed in apoptotic cell lysates (Fig. 4B). Analysis of the primary amino acid sequence of PKR suggested Asp²⁵⁶-Leu-Pro-Asp²⁵¹ as a potential caspase cleavage site that would generate the fragments observed during apoptosis. Therefore, Asp²⁵¹ was mutated to Asn, and the resulting protein was tested for susceptibility to cleavage by caspase-3, −7, and −8 (Fig. 4, C and D). The PKR-D251N mutant was completely resistant to proteolysis by caspase-3 and −8, although some minor proteolysis still occurred, mostly with caspase-7, but with a different cleavage pattern. These in vitro data are consistent with the results obtained in apoptotic cells and show that PKR is a potential substrate for caspase-3, −7, and −8. To confirm that Asp²⁵¹ was the PKR cleavage site used in vivo during apoptosis, we transfected Jurkat T cells with PKRK296R, PKRK296K, and D251N double-mutant cDNAs. 4 h after anti-Fas treatment, the tagged COOH-terminal PKR fragment was detected in cells transfected with the PKR construct containing the wild-type (wt) caspase target site, but not in cells expressing the D251N mutant (Fig. 4E). This demonstrates that PKR is cleaved by caspases at Asp²⁵¹ during apoptotic cell death.

The Caspase-generated PKR Kinase Domain Is Active—Several structure-function studies have shown that the isolated kinase domain of PKR can phosphorylate eIF2-α and inhibit translation in vitro (17, 37, 38). Since these reports involved a kinase domain different from the one released by caspases (Fig. 5A), we ectopically expressed wt and catalytically inactive K296R mutants of PKR and PKR-KDcasp in HEK293T cells, after which the autophosphorylation and eIF2-α phosphorylation properties were assessed. Western blot analysis revealed that the expression of wt PKR and PKR-KDcasp in transient transfection experiments was lower than that of the PKRK296R and PKR-KDcasp-K296R mutants (Fig. 5B). Northern blot analysis demonstrated that the difference in protein expression level of wt versus K296R mutant PKR and PKR-KDcasp was due to a lower translation efficiency, suggesting that PKR and PKR-KDcasp can inhibit their own translation.

To further characterize the activity of PKR-KDcasp, an in vitro kinase assay was performed. Tagged PKR and mutant derivatives were immunopurified from transfected HEK293T lysates and incubated with [γ-³²P]ATP. Strong autophosphorylation was observed with full-size wt PKR, whereas autophosphorylation of PKR-KDcasp was almost below detection level (Fig. 5A). However, examination of the ability of PKR-KDcasp to phosphorylate eIF2-α demonstrated that caspase-generated PKR-KDcasp is fully capable to phosphorylate eIF2-α and to the same extent as full-size PKR (Fig. 5B). This kinase activity was dependent on the presence of Lys²⁹⁸, which is directly involved in phosphate transfer, since the PKR-KDcasp-K296R mutant did not phosphorylate eIF2-α (Fig. 5B). To verify that eIF2-α was phosphorylated at the orthodox phosphorylation site (59), Ser⁵¹ was substituted by Ala by site-specific mutagenesis, generating eIF2-α S51A. Indeed, PKR and PKR-KDcasp failed to phosphorylate the eIF2-α S51A mutant, demonstrating that Ser⁵¹ is the target for transphosphorylation by both PKR forms (Fig. 5C).

To assess whether the translation inhibition of overexpressed PKR-KDcasp could be extended to other mRNAs, we co-expressed PKR-KDcasp with GFP and assayed its expression in Western blot. Co-expression of PKR-KDcasp or PKR with GFP decreased the expression level of the latter at least 10-fold (Fig. 7A). Northern blot analysis of GFP mRNA levels revealed that the lower expression level was due to impaired translation. Similar results were obtained using influenza A virus hemagglutinin as a reporter and increasing amounts of PKR-KDcasp. These experiments demonstrate that an increase in relative amount of PKR-KDcasp inhibits hemagglutinin expression (Fig. 7B). We conclude that the PKR-KDcasp fragment released by caspases during apoptosis is active both in vitro and in vivo.

DISCUSSION

The antiviral protein kinase PKR is expressed in many cells, where it is present in a dormant state. The typical mode of
activation of PKR requires the presence of dsRNA, a metabolic intermediate of viral infection (40). In this paper we demonstrate that caspase-mediated cleavage of PKR, at the boundary between the regulatory and kinase domain of PKR, liberates an active eIF2-α kinase domain. This cleavage and the increased eIF2-α phosphorylation are general features of apoptosis, triggered by either extrinsic (TNF, anti-Fas) or intrinsic (STS) cell death pathways (Fig. 8).

Our results suggest that the released kinase domain of PKR is constitutively active, which is well supported by structure-function studies (17, 38). Is this cleavage-dependent mode of activation compatible with the current model of PKR control mechanisms? Given the detrimental impact of PKR activity on protein synthesis and normal cellular function, the kinase is kept in a latent state at two levels. The second dsRNA-binding motif of PKR interacts intramolecularly with the kinase domain, ensuring a “locked” inactive conformation, and probably masks the dimerization domain (17, 18, 41). Although the crystal structure of the entire PKR molecule has not been determined yet, it seems likely that Asp251, the residue in the PKR peptide sequence targeted by caspases, should be accessible in such a “bent-over” model. In addition, a number of cellular inhibitors have been described that either compete with PKR for dsRNA binding (42) or prevent its homodimerization (43, 44). Since PKR is a major component of the IFN-mediated antiviral response, it is not surprising that many viruses encode PKR-inhibitory molecules. Some of these prevent PKR activation by interfering with its dsRNA binding, e.g., vaccinia virus E3L and influenza virus NS1, or homo-dimerization and transphosphorylation, e.g., influenza virus and hepatitis C virus (45). The caspase-mediated activation of PKR releases the kinase domain from the control of the regulatory amino-terminal domain. This may provide the cell with a bypass mechanism to fight such virally and celluly encoded PKR inhibitors.

Our study highlights the rapid and ordered shut down of protein synthesis during apoptosis. In previous publications the recurrent feature responsible for the observed translation inhibition was cleavage of distinct translation initiation factors by caspases primarily targeting cap-dependent translation. However, the translation of certain mRNAs, coding for several survival factors, oncogenes and proteins involved in controlling apoptosis, is regulated by an internal ribosome entry site (IRES). Proteolysis of eIF4G will most probably lead to inhibition of translation of most mRNAs, but may still allow translation of IRES-containing transcripts (46–48). Phosphorylation of eIF2-α prevents recruitment of the methionyl-initiator tRNA to the start codon of small ribosomal subunit-associated mRNAs, a common step in initiation of translation of all mRNAs, including most known IRES-containing RNAs (49, 50).

We could not demonstrate complete cleavage of PKR, whereas eIF4G and PARP were eventually entirely processed. Proteolysis of PKR, however, liberates an active catalytical fragment and can be regarded as a gain of function. The final outcome of the enzyme’s action is removal from the circulation of eIF2B, a limiting component of the translation initiation pathway. Hence, it is conceivable that even a limited amount of active PKR would in due course have a strong inhibitory impact on translation. By contrast, the aim of cleavage of eIF4G and PARP is loss of a potentially life-saving function. Therefore, abolishment of their respective cap-mRNA recruiting and DNA repair functions would require their complete digestion.

Our results as well as other published data suggest that during rapid apoptosis shutdown of protein synthesis occurs at multiple levels and presumably leaves little possibility for any messenger to be translated (46, 51). The cellular in vitro cell systems used by us and others to study the effect of caspases on the translational apparatus may be affected by apoptotic conditions occurring during the development or depletion of activated immune cells. Under such conditions, a more gradual translational shutoff may take place, allowing cell recovery at
different levels of the cell death pathway. Such a rescue pathway could be mediated by an IRES-controlled expression of antia apoptotic molecules, e.g. XIAP, e-Myc, eIF4G, Hap70, and BiP (47). Translational impairment due to increased eIF2-α phosphorylation has been reported to preferentially allow translation of the proapoptotic molecules Bax and Fas (52). On the other hand, short-lived antiapoptotic proteins (such as cFLIP) were shown to be quickly lost by translation inhibition, unclosing the default cell death pathway (2). This suggests that control of translation plays a major role in the programmed death process.

In view of the paradigm that apoptosis is a clean way for a cell to die, inhibition of de novo death process. This suggests that antiapoptotic molecules, particularly antiapoptotic molecules, may be important to prevent inadvertent synthesis of proinflammatory molecules (and of any other proteins), once the dying cell has been phagocytosed (53, 54).

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