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Protein Kinase R Is Responsible for the Phosphorylation of eIF2α in Rotavirus Infection▽

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The eukaryotic initiation translation factor 2 (eIF2) represents a key point in the regulation of protein synthesis. This factor delivers the initiator Met-tRNA to the ribosome, a process that is conserved in all eukaryotic cells. Many types of stress reduce global translation by triggering the phosphorylation of the α subunit of eIF2, which reduces the formation of the preinitiation translation complexes. Early during rotavirus infection, eIF2α becomes phosphorylated, and even under these conditions viral protein synthesis is not affected, while most of the cell protein synthesis is blocked. Here, we found that the kinase responsible for the phosphorylation of eIF2α in rotavirus-infected cells is PKR, since in mouse embryonic fibroblasts deficient in the kinase domain of PKR, or in MA104 cells where the expression of PKR was knocked down by RNA interference, eIF2α was not phosphorylated upon rotavirus infection. The viral component responsible for the activation of PKR seems to be viral double-stranded RNA, which is found in the cytoplasm of infected cells, outside viroplasms. Taken together, these results suggest that rotaviruses induce the PKR branch of the interferon system and have evolved a mechanism to translate its proteins, surpassing the block imposed by eIF2α phosphorylation.

Protein translation is the final step in the flow of genetic information, and unlike transcriptional control, regulation at this step allows for an immediate and rapid response to changes in physiological conditions. While every step of the translation process is amenable to regulation, under most circumstances mRNA translation is regulated primarily at the level of initiation (5). The translation of eukaryotic mRNAs involves the recognition and recruitment of mRNAs by the translation initiation machinery and the assembly of the 80S ribosome on the mRNA; this process is mediated by the eukaryotic initiation factors (eIFs). Translation initiation is a complex process that begins with the recognition of the cap nucleotide structure (m7GpppN) at the 5′ end of mRNAs by the cap-binding protein eIF4E that is part of the cap-binding complex eIF4F. This complex is composed of eIF4E, eIF4A (an ATP-dependent RNA helicase), and the scaffolding protein eIF4G. The binding of Met-tRNA to the 40S ribosomal subunit is mediated by a ternary complex composed of eIF2-GTP-Met-tRNA. The binding of GTP to eIF2 is the rate-limiting step in the assembly of the ternary complex and is regulated by eIF2B. Once the 40S ribosomal subunit is bound to the mRNA, it is thought to scan the mRNA in the 5′ to 3′ direction (26). The joining of the 40S and 60S ribosomal subunits to form an 80S initiation complex then takes place. The release of eIFs is assisted by eIF5, which facilitates the hydrolysis of GTP carried out by eIF2. The GDP on eIF2 is exchanged for GTP by eIF2B in a regulated manner that is essential for ensuing rounds of initiation (25).

Many types of stresses reduce global translation by triggering the phosphorylation of the α subunit of eIF2 (eIF2α) at residue Ser51. This phosphorylation inhibits the exchange of GDP for GTP catalyzed by eIF2B, which then is sequestered in a complex with eIF2. Since the cellular level of eIF2B is 10 to 20 times lower than the level of eIF2, even small changes in the phosphorylation of eIF2α have a drastic effect on protein translation. Four protein kinases are known to phosphorylate eIF2α at residue Ser51: the heme-regulated inhibitor kinase (HRI), which is activated by heme deficiency, treatment with arsenite, or heat shock; protein kinase R (PKR), which is activated by double-stranded RNA (dsRNA); PKR-like ER kinase (PERK), which is activated in response to endoplasmic reticulum (ER) stress; and the general control nonderepressible-2 (GCN2) kinase, activated in response to amino acid starvation (29). These kinases serve to arrest translation upon different conditions that threaten cell survival, such as viral infection, nutrient deprivation, and misfolded proteins.

Rotaviruses are the leading etiologic agents of severe diarrheal disease in infants and young children, being responsible for an estimated incidence of 600,000 annual deaths globally and placing a significant economic burden on the global health care system (22). These viruses have a genome composed of 11 segments of dsRNA enclosed in a capsid formed by three concentric layers of protein. During or shortly after cell entry, the infecting virus uncoats, letting loose the two surface proteins and yielding a double-layered particle (DLP) that is transcriptionally active. The viral transcripts direct the synthesis of six structural (VP1 to VP4, VP6, and VP7) and six nonstructural (NSP1 to NSP6) proteins (6). Once a critical mass of viral proteins is synthesized, 3 to 4 h postinfection they start to accumulate into discrete, cytoplasmic inclusions termed viroplasms, where the replication of the virus genome (39) and the assembly of DLPs take place (6).

Early in the infection process the virus takes over the host

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translation machinery, causing a shut off of cell protein synthesis. The viral mRNAs contain 5′-methylated cap structures but lack the poly(A) tails characteristic of most cellular mRNAs. Instead, rotavirus mRNAs have at their 3′ end a consensus sequence (UGACC) that is conserved in all 11 viral genes (28). The rotavirus nonstructural protein NSP3 binds, through its amino-terminal domain, to this consensus sequence. NSP3 also binds, through its carboxy-terminal domain, to eIF4GI in the same region as that used by the poly(A) binding protein (PABP), but with higher affinity; thus, it was proposed that during rotavirus infection NSP3 evicts PABP from eIF4GI, impairing the translation of cellular mRNAs, while leading to the enhancement of the translation of rotaviral mRNAs (27, 28). We recently found that while NSP3 indeed inhibits the translation of cellular mRNAs, this nonstructural protein is not needed for the translation of viral mRNAs (17). The virus-induced inhibition of cell protein synthesis also seems to be mediated by a second mechanism, since eIF2α becomes phosphorylated early after infection and is maintained in this state throughout the virus replication cycle (18). The continuous phosphorylated status of eIF2α might be beneficial for the virus, since under these conditions the viral mRNAs are efficiently translated, while the synthesis of most, but not all, cellular proteins stops.

In this work, we found that during rotavirus infection there is a significant amount of viral dsRNA in the cytoplasm, which apparently is responsible for PKR activation, eIF2α phosphorylation, and the modification of the cellular translation machinery. Most probably, the cellular response to rotavirus dsRNA is aimed to block the infection, but this virus has evolved a mechanism that subverts this response, since viral proteins are efficiently synthesized under these conditions. Also, we found that in rotavirus-infected cells the phosphorylated form of eIF2α and PKR cosedimented with the 40S ribosomal subunits. These changes in the translation initiation complexes suggest that rotavirus induces a remodeling of the host translation machinery. These results were reproduced by transfecting MA104 cells with purified rotavirus dsRNA or synthetic dsRNA [poly(I:C)], suggesting that these changes form part of an integral cellular response to dsRNA.

MATERIALS AND METHODS

Cells, viruses, and antibodies. The rhesus monkey epithelial cell line MA104 was grown in advanced medium (Dulbecco's modified Eagle's medium [DMEM]) (Invitrogen, Carlsbad, CA) supplemented with 4% fetal bovine serum (FBS). Wild-type (wt) mouse embryonic fibroblasts (MEFs) and MEFs expressing S51A mutant eIF2α (S51A) were obtained from N. Sonenberg, McGill University, Montreal, Canada. Isogenic $PKR^{+/+}$ and catalytic $PKR^{-/-}$ MEFs, previously described (41), were obtained from A. E. Koromilas, McGill University, Montreal, Canada. Isogenic $PERK^{+/+}$ and $PERK^{-/-}$ MEFs (11) were obtained from D. Ron, New York University. MEFs were grown in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS and nonessential amino acids (Invitrogen, Carlsbad, CA). Rhesus rotavirus RRV was obtained from H. B. Greenberg, Stanford University. The virus was propagated in MA104 cells as described previously (21). Rotavirus lysates were activated with trypsin (10 μg/ml; Gibco BRL) for 30 min at 37°C. The antibodies used were rabbit anti-phospho-eIF2α, rabbit anti-eIF2α, mouse anti-S6 ribosomal protein (54D2), and rabbit anti-phospho-S6 ribosomal protein (Ser235/236) from Cell Signaling (Boston, MA); mouse anti-eIF2A (MO1) from Abnova (Taiwan); mouse anti-eIF2Bε (B-7) and mouse anti-PKR (B-10) from Santa Cruz (Santa Cruz, CA); and mouse anti-dsRNA (J2) from Scicons (Hungary). Rabbit polyclonal serum to NSP5 has been described previously (9). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Perkin Elmer), horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed, Carlsbad, CA), goat anti-mouse coupled to Alexa 568 or 488, and goat-anti-rabbit coupled to Alexa 488 or 568 (Molecular Probes, Eugene, OR).

Viral infection and titration of viral progeny. MEFs seeded in gelatin-coated 96-well plates were washed twice with minimal essential medium (MEM), and serial dilutions of the RRV viral lysate were adsorbed to cells for 60 min at 37°C. After the adsorption period, virus inoculum was removed, cells were washed twice with MEM, and the infection was left to proceed for 14 to 16 h at 37°C. The infectious titer was obtained by an immunoperoxidase focus assay as described previously (21). To determine the yield of viral progeny produced in the different cell lines used, MEFs in gelatin-coated 48-well plates were infected with RRV at a multiplicity of infection (MOI) of 3 as described above, 14 to 16 h postinfection (hpi) cells were lysed by two freeze-thaw cycles, and the lysates were treated with 10 μg trypsin/ml for 30 min at 37°C. The infectious titer of these viral lysates was obtained by infecting monolayers of MA104 cells as described above.

Rotavirus DLPs and dsRNA purification. MA104 cells grown in 150-cm^2 flasks were infected as described previously and were harvested until complete cytopathic effect was attained, the viral lysates were freeze-thawed twice, and viral particles were concentrated by centrifugation for 1 h at 30,000 rpm at 4°C in an SW40 rotor (Beckman, Fullerton, CA). The viral pellet was resuspended in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM $CaCl_2$), sonicated once for 20 s, extracted with Genetron (trichloro-monofluoro-ethane), and used to obtain genomic viral dsRNA or DLPs. To obtain the genomic dsRNA, the virus suspension was pelleted through a 40% sucrose cushion by centrifugation for 1 h at 30,000 rpm at 4°C in an SW40 rotor, and viral dsRNA was isolated from the pellet by two extractions with phenol-chloroform.

To prepare rotavirus DLPs, CsCl was added to the aqueous phase obtained from the Genetron extraction to obtain a density of 1.36 g/cm^3; the mixture was centrifuged for 18 h at 35,000 rpm in an SW40Ti rotor; and the opalescent bands corresponding to TLPs and DLPs were collected by punction, mixed, and concentrated by centrifugation for 1 h at 40,000 in an SW40 rotor. The pellet was resuspended in 10 mM Tris-HCl, pH 7.5, and treated with 3 mM EGTA for 15 min at 37°C to remove the outer layer of the particles, and the sample then was diluted with TNC buffer and pelleted as described above.

Transfections. The short interfering RNA (siRNA) for PKR was obtained from Ambion, Inc. (Austin, TX). The transfection mixture containing Oligofectamine (15 μl/ml) (Invitrogen, Carlsbad, CA) and 200 pmol/ml of the siRNA was added to wells of 48-well plates and incubated for 20 min at room temperature. After this period, cells (4×10^4 cells/well) were seeded over the transfection mixture and incubated for 72 h at 37°C. Cells then were infected at an MOI of 3.

For RNA transfections, MA104 cells seeded in 48-well plates or in 15-cm^2 plates were incubated for 1 h at 37°C with a mixture of Lipofectamine (40 μg/ml) (Invitrogen, Carlsbad, CA) and purified viral dsRNA (10 μg/ml), poly(I:C) (5 μg/ml) (Sigma Aldrich Co, St. Louis MO.), or rotavirus gene 10 mRNA (10 μg/ml), prepared as previously described (1). The transfection mixture was replaced by MEM, and cells were incubated for different times before being harvested. To lipofect DLPs, viral particles were diluted in MEM at 5 μg/ml, and Lipofectamine (40 μg/ml) was added. After 30 min of incubation at room temperature, 100 μl of the transfection mixture was added to cell monolayers for 1 h at 37°C. The lipofection mixture was removed and replaced by MEM, and cells were incubated for the indicated times.

Radiolabeling of proteins. For protein labeling, cells grown in 48-well plates were left uninfected or were infected with rotavirus at an MOI of 3. Before harvest, the medium was replaced by MEM without methionine, supplemented with 25 μCi/ml of Easy-tag express-[^{35}S] labeling mix (Dupont NEN, Boston, MA), and incubated for 30 min at 37°C. Cells were lysed as indicated below, and samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography.

Immunoblot analysis. Cells were incubated for 15 min at 4°C in lysis buffer (25 mM NaF, 1 mM sodium orthovanadate [Na_3VO_4], 50 mM Tris [pH 7.4], 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 10% glycerol, and 1% Triton X-100) and supplemented with a protease inhibitor cocktail (Complete EDTA-free; Roche, Indianapolis, IN). The lysates were centrifuged for 5 min at 13,000 rpm, and the supernatants were collected. Samples were diluted in Laemmli sample buffer, denatured by being boiled for 5 min, subjected to 10% SDS-PAGE, and transferred to Immobilon NC membranes (Millipore, Billerica, MA). After treatment with blocking solution (5% bovine serum albumin [BSA], 0.05% Tween 20 in phosphate-buffered saline [PBS], overnight at 4°C), the membranes were incubated with primary antibodies diluted in blocking solution for 12 to 24 h at 4°C. The membranes were rinsed with PBS-Tween 20, and

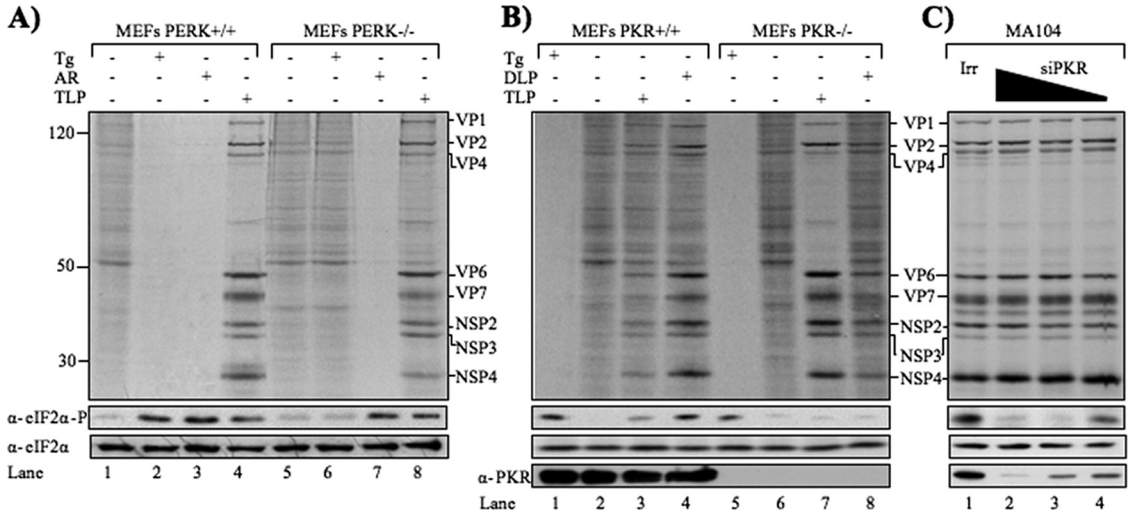

FIG. 1. Kinase PKR phosphorylates eIF2α during rotavirus infection. wt (+/+) or mutant (−/−) mouse embryonic fibroblasts (MEFs), lacking either PERK (A) or PKR (B) kinase activity, were infected with triple-layered particles (TLP) of rotavirus strain RRV at an MOI of 3 or were transfected with rotavirus double-layered particles (DLP) during 1 h, and they were harvested at 8 h postinfection (hpi) or posttransfection. As a control for eIF2α phosphorylation, the cells were treated with 1 μM thapsigargin (Tg) or 100 μM sodium arsenite (AR) for 1 h. (C) MA104 cells were transfected with an siRNA to GFP (Irr) or with increasing amounts (100 to 400 pm/ml) of an siRNA directed to PKR; 72 h posttransfection the cells were infected with RRV at an MOI of 3, and 8 hpi the cells were lysed. Thirty min before being harvested, the cells were metabolically labeled with 25 μCi/ml of Easy-tag express-[^{35}S] (maintaining the thapsigargin or sodium arsenite treatment during the labeling period). The labeled proteins were resolved by 10% SDS-PAGE and detected by autoradiography. In parallel, the same samples were analyzed by Western blotting using antibody against PKR (α-PKR), phospho-eIF2α (α-eIF2α-P), or total eIF2α (α-eIF2α).

bound antibodies were developed by incubation with a peroxidase-labeled secondary antibody and the Western Lightning system (Perkin Elmer, MA).

Immunofluorescence. Cells grown on coverslips were fixed with 3% paraformaldehyde for 30 min at room temperature and washed four times with washing buffer (50 mM NH_4Cl in PBS). Fixed cells were permeabilized by incubation in washing buffer supplemented with 0.5% Triton X-100 and 5% of BSA for 15 min and then were treated with blocking solution (5% BSA–50 mM NH_4Cl in PBS) at 4°C overnight. The coverslips then were incubated with primary antibodies diluted in blocking solution for 1 h at room temperature, followed by incubation with the corresponding secondary antibody for 1 h at room temperature. Coverslips were mounted on glass slides using Fluokeep (Argene, France). The slides were analyzed with an E600 epifluorescence microscope coupled to a DXM1200 digital still camera (Nikon).

RNase treatments. The RNase III coding sequence was amplified by PCR from *Escherichia coli* strain K-12 by following the protocol described by Yang et al. (44), and the PCR product was cloned in plasmid pGEX-4T1 using standard recombination techniques. The recombinant glutathione *S*-transferase (GST)-RNase III was purified by affinity chromatography (ÄKTA, GE Healthcare) as reported previously (10). RNase A was purchased from Sigma-Aldrich (St. Louis, MO). For *in situ* digestions, cells grown on coverslips and transfected or infected as indicated were fixed and permeabilized as described above and were incubated for 2 h at 37°C with RNase III (2 μg/ml) in reaction buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 140 mM NaCl, 2.7 mM KCl, and 5% glycerol) or with RNase A (1 μg/ml) in PBS. After the incubation period, the cells were washed 10 times with washing buffer and processed for immunofluorescence as described above. For *in vitro* digestions, CsCl-purified TLPs or DLPs or purified rotavirus dsRNA were incubated with RNase III (2 μg/ml) in reaction buffer or with RNase A (1 μg/ml) in PBS for 2 h at 37°C. The viral RNA was purified by two extractions with phenol-chloroform, ethanol precipitated, and analyzed by PAGE and silver staining as previously described (14).

Sucrose gradient polysome profiles. Cells seeded in 15-cm^2 plates were left uninfected or were infected with rotavirus or transfected with rotavirus genomic dsRNA or poly(I:C) for 1 h, and then they were incubated in serum-free medium at 37°C for 6 h. Three minutes before harvest, cycloheximide (100 μg/ml) was added to cells. The cells were washed three times with ice-cold buffer A (20 mM Tris, pH 7.8, 140 mM KCl, 5 mM $MgCl_2$, 100 μg/ml cycloheximide) and then lysed with buffer A supplemented with 1 mg/ml heparin, 0.5 mM DTT, 0.5% Triton X-100, 25 mM NaF, 1 mM Na_3VO_4, Complete EDTA-free (Roche, Indianapolis, IN), and RNasin (80 U/ml) (Promega, Madison, WI); the plates were kept on ice at all times. After clarification (10,000 × *g* for 15 min at 4°C), the supernatants were layered onto discontinuous sucrose gradients (10 to 50%) and subjected to centrifugation (36,000 rpm for 2 h in an SW-40 rotor). Gradients were fractionated (Amersham AktaPrime UV monitor at an optical density of 260 nm [OD_{260}]), collecting 22 fractions/gradient. RNA was isolated from each fraction by phenol-chloroform extraction, and the proteins in the selected fractions were concentrated by methanol-chloroform precipitation.

RESULTS

Phosphorylation of eIF2α during rotavirus infection is mediated by the kinase PKR. We have shown that during rotavirus infection, the initiation factor eIF2α becomes phosphorylated and remains as such throughout the replication cycle of the virus. However, the kinase responsible for this phosphorylation has not been identified (18). Since part of the morphogenesis of rotavirus takes place in the ER, and given the dsRNA nature of the rotaviral genome (6, 24), the two kinases that most likely become activated during rotavirus infection are PERK and PKR. To determine if either of these two kinases phosphorylates eIF2α during rotavirus infection, mouse embryonic fibroblasts (MEFs) from PERK or PKR knockout mice (PERK$^{-/-}$ or PKR$^{-/-}$), and wild-type MEFs (PERK$^{+/+}$ or PKR$^{+/+}$) with genetic backgrounds identical to those of their respective knockout MEFs (11, 41), were infected with simian rotavirus strain RRV, and the viral protein synthesis and the yield of infectious virus were analyzed in these cells.

Both PERK$^{+/+}$ and PERK$^{-/-}$ MEFs were susceptible to rotavirus infection, as judged by the similar rates of protein synthesis observed in both cell lines (Fig. 1A, lanes 4 and 8). When the phosphorylation of eIF2α was assessed by Western blotting, we found that the factor became phosphorylated in both types of MEFs when infected with rotavirus but not in

uninfected cells, suggesting that this kinase is not responsible for the phosphorylation of eIF2α during rotavirus infection. The eIF2α phosphorylation phenotype of these MEFs was demonstrated by treating cells with thapsigargin, a known PERK inducer, and with sodium arsenite, which mainly induces the HRI kinase. As expected, the treatment with sodium arsenite induced the phosphorylation of the factor in both $PERK^{+/+}$ and $PERK^{-/-}$ MEFs, which correlated with the shutting off of cellular protein synthesis, while when treated with thapsigargin, eIF2α was phosphorylated only in the $PERK^{+/+}$ MEFs but not in the $PERK^{-/-}$ MEFs, indicating the lack of PERK activity in the mutant cells (Fig. 1A).

Similarly, to establish whether PKR was the kinase responsible for the phosphorylation of eIF2α during rotavirus infection, $PKR^{+/+}$ or $PKR^{-/-}$ MEFs were left uninfected or were infected with rotavirus. In this case, the infection of mutant $PKR^{-/-}$ MEFs resulted in an effective infection, as judged by the amount of viral proteins synthesized (Fig. 1B, lane 7), as well as by the yield of viral progeny produced; however, in these cells eIF2α was not phosphorylated. Unexpectedly, the wild-type $PKR^{+/+}$ MEFs appeared to be less susceptible to the infection, since they produced smaller amounts of viral protein when infected with complete, triple-layered particles (TLPs), and consequently the level of eIF2α phosphorylation was reduced (Fig. 1B, lane 3). Since the infection in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs was very different, it was difficult to compare both wt and mutant cells directly. To overcome this problem, instead of infecting the cells with TLPs, RRV double-layered particles (DLPs) were transfected into both types of cells. Using this strategy, the $PKR^{+/+}$ MEFs were more efficiently infected, resulting in a larger production of viral proteins and in the phosphorylation of eIF2α (Fig. 1B, lane 4). In clear contrast, during the infection or transfection of rotavirus particles into $PKR^{-/-}$ MEFs, eIF2α was not phosphorylated (Fig. 1B, lanes 7 and 8). As in the case of the PERK MEFs, the phenotype of these cells was further confirmed by detecting the phosphorylation of eIF2α when the cells were treated with thapsigargin; in this case, eIF2α appeared phosphorylated in both MEFs. Also, the presence of PKR was detected by Western blotting. As expected, the complete protein was present only in the $PKR^{+/+}$ MEFs, since their mutant counterparts produce a truncated PKR protein that lacks the kinase domain (41). These data suggest that the phosphorylation of eIF2α during rotavirus infection is most likely the result of the activity of PKR. To further confirm this observation using an alternative approach, we silenced the expression of PKR using RNA interference (RNAi). In this case, MA104 cells (the cell line of choice to grow rotavirus) were transfected with different amounts of an siRNA directed to PKR or with a control, irrelevant siRNA directed against green fluorescent protein (GFP). The cells were infected 72 h after transfection with the siRNAs, and 8 hpi the phosphorylation status of eIF2α and the presence of PKR were evaluated by Western blotting, and the synthesis of viral proteins was estimated by autoradiography. A reduction in the amount of PKR present in the transfected cells was found, which correlated with the amount of siRNA transfected (Fig. 1C, lanes 2 to 4). The reduction of PKR also correlated with a decrease in the level of eIF2α phosphorylation, but it did not have an effect in the synthesis of viral proteins, confirming again that the phosphorylation of eIF2α during the infection with rotavirus is caused by PKR and is not required for viral protein synthesis.

Double-stranded RNA is the trigger that induces the phosphorylation of eIF2α in rotavirus-infected cells. To determine the viral component responsible for the activation of PKR during the replicative cycle of the virus, MA104 cells were infected or transfected with infectious or UV-psoralen-inactivated rotavirus (i-RRV), and the activation of PKR was indirectly analyzed through the phosphorylation of eIF2α. Since the transfection of viral particles overcomes the process of virus entry, we also were able to evaluate the role of this process in the induction of eIF2α phosphorylation. When the inactivated rotavirus particles either were used for infection or were transfected into the cells, there was no viral protein synthesis or induction of eIF2α phosphorylation, in contrast with cells infected or transfected with the infectious virus, where the phosphorylation of eIF2α was clear (Fig. 2A). These results suggest that the viral entry process does not have a role in the activation of PKR, and that a *de novo*-synthesized viral component might be responsible for triggering the phosphorylation of eIF2α.

Since the most likely candidate to activate PKR was the viral dsRNA produced during the infection, we evaluated this possibility by transfecting MA104 cells with purified rotavirus genomic dsRNA and analyzed the level of eIF2α phosphorylation and the cellular protein synthesis at different times posttransfection (Fig. 2B). In cells transfected with dsRNA, the phosphorylation of eIF2α was apparent by 2 h (Fig. 2B), and similarly to rotavirus infection (18), this factor remained phosphorylated up to 8 h posttransfection, which correlated with the almost-complete shut off of cellular protein synthesis, as judged by the poor synthesis of S^{35}-labeled proteins (Fig. 2B). To make sure that the observed effect was due to viral dsRNA and not to a possible contamination of the RNA preparation with a small amount of infectious virus, we looked for the synthesis of viral proteins by Western blotting in cells transfected with the dsRNA. We were not able to detect the presence of viral proteins under this condition (not shown). In addition, transfecting cells with poly(I:C), a well-characterized molecule known to induce PKR, reproduced the results obtained with the transfected viral dsRNA (Fig. 2C).

It is known that rotavirus RNA replication occurs concurrently with the packaging of the genome into newly formed viral cores (23), which suggests that there is no genomic dsRNA in the cell's cytoplasm, outside viroplasms, to activate PKR; however, our results indicate the presence of dsRNA in the cytoplasm during rotavirus infection that could be responsible for the activation of this kinase. To examine this possibility, MA104 cells were infected with RRV or transfected with poly(I:C) and immunostained with a monoclonal antibody (MAb), J2, that specifically recognizes dsRNA stretches that are >40 bp in length (36). As reported previously (43), poly(I:C)-transfected cells showed a strong immunofluorescent signal scattered in the cytoplasm that was not present in nontransfected cells (Fig. 2D). In cells infected with rotavirus, we also observed a strong signal indicative of the presence of dsRNA (Fig. 2D). Interestingly, some of the dsRNA fluorescent signal was found to colocalize with viroplasms, but most of it was found scattered in the cytoplasm. The dsRNA signal observed in rotavirus-infected cells increased with time after infection,

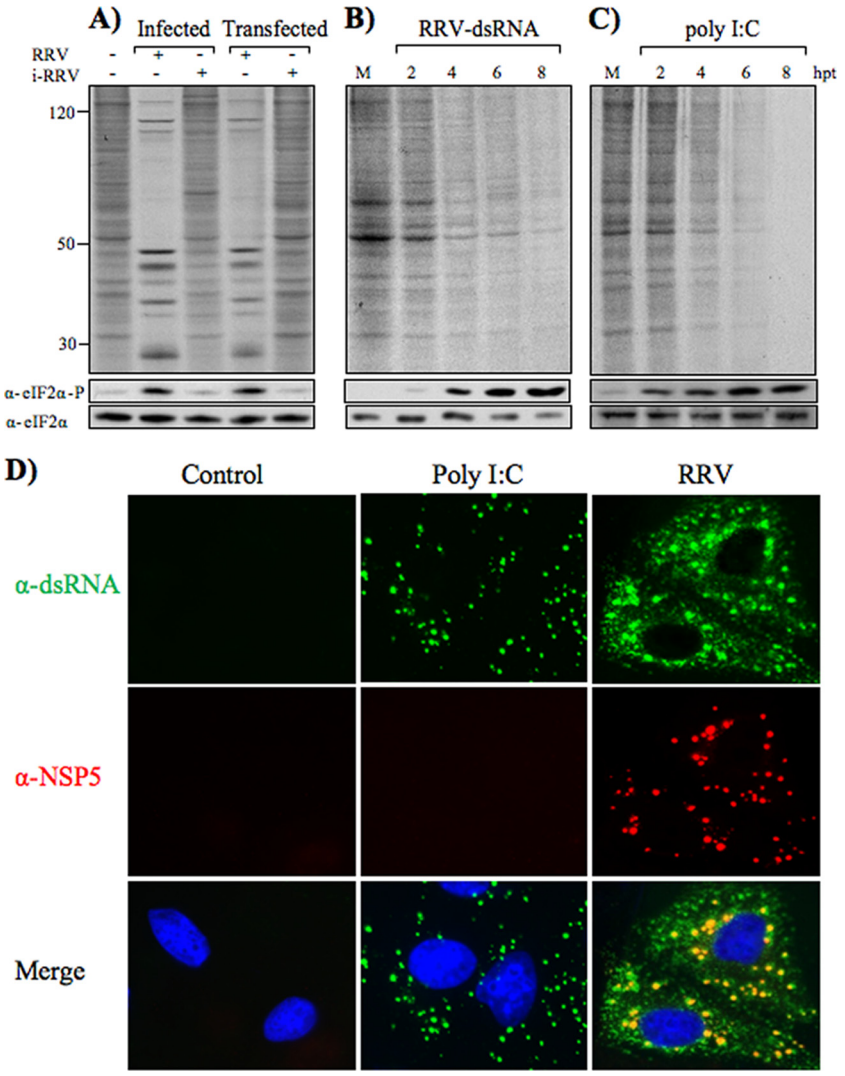

FIG. 2. Rotavirus dsRNA triggers eIF2α phosphorylation. (A) MA104 cells were infected or transfected with infectious (RRV) or UV psoralen-inactivated (i-RRV) rotavirus at an MOI of 3 during 1 h at 37°C. Eight hours postinfection or posttransfection (hpt), the cells were harvested. (B and C) MA104 cells were transfected or left untransfected (M) during 1 h with 10 μg/ml of purified rotavirus dsRNA or 5 μg/ml of poly(I:C), and they were harvested at the indicated hours posttransfection. Thirty min before being harvested, the cells were metabolically labeled with 25 μCi/ml of Easy-tag express-[^{35}S]. The labeled proteins were resolved by 10% SDS-PAGE and detected by autoradiography. In parallel, the same samples were analyzed by Western blotting using antibodies against phospho-eIF2α (α-eIF2α-P) or total eIF2α (α-eIF2α). (D) MA104 cells were transfected or left untransfected during 1 h with 5 μg/ml of poly(I:C) or were infected with rotavirus RRV at an MOI of 3. After an incubation period of 8 h, the cells were fixed and stained with the mouse monoclonal antibody J2 to dsRNA (α-dsRNA) (green) and the polyclonal antibody to NSP5 (α-NSP5) (red) as primary antibodies, followed by incubation with goat-anti-mouse immunoglobulin G coupled to Alexa 488 and goat-anti-rabbit immunoglobulin G coupled to Alexa 568 as secondary antibodies, respectively. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; in blue).

as opposed to the signal in poly(I:C)-transfected cells, which remained constant over time (results not shown). Taken together, these results suggest that dsRNA stretches longer than 40 bp in length produced during the infection cycle trigger the activation of PKR with the consequent phosphorylation of eIF2α.

RNase sensitivity data suggest that dsRNA is present in the cytoplasm of infected cells. The dsRNA detected by MAb J2 in the cytoplasm of rotavirus-infected cells but outside viroplasms could be either genomic dsRNA or structured viral mRNA, as has been described previously for positive RNA and DNA virus-infected cells (43). To determine the nature of the dsRNA recognized by MAb J2, cells were transfected with *in vitro*-transcribed rotavirus gene 10 mRNA or purified viral dsRNA or were infected with RRV. At 8 h posttransfection or infection, the cells were fixed, permeabilized, and treated with either *E. coli* RNase III, known to cleave dsRNA (19), or with pancreatic RNase A, which cleaves single-stranded RNA (31). After an incubation period of 2 h, the cells were extensively washed and processed for immunofluorescence staining with the J2 antibody or with an anti-NSP5 antibody, as indicated (Fig. 3). We found that cells transfected with either mRNA or dsRNA gave a positive signal with the J2 antibody, which disappeared when the cells were treated with RNase A or with

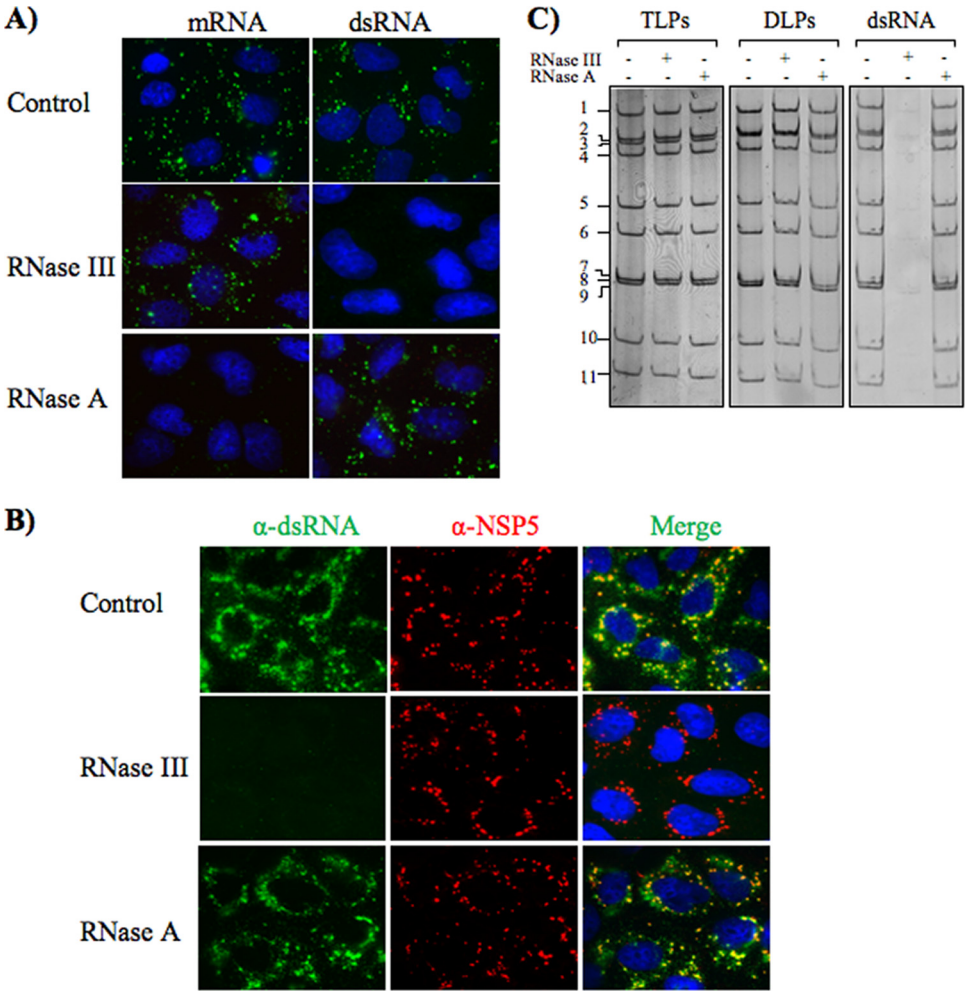

FIG. 3. RNA present in the cytoplasm of infected cells is viral dsRNA. (A) MA104 cells were transfected for 1 h with 10 μg/ml of purified rotavirus dsRNA or 10 μg/ml of rotavirus gene 10 mRNA or (B) were infected with rotavirus RRV at an MOI of 3. Eight hours posttransfection or postinfection, the cells were fixed, permeabilized, and treated with 2 μg/ml of RNase III or 1 μg/ml of RNase A for 2 h at 37°C. After extensive washes, the cells were stained with MAb J2 to dsRNA (α-dsRNA) (green) and with a polyclonal antibody to NSP5 (α-NSP5) (red) as primary antibodies, followed by incubation with goat anti-mouse immunoglobulin G coupled to Alexa 488 and goat anti-rabbit immunoglobulin G coupled to Alexa 568 as secondary antibodies, respectively. The cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; in blue). (C) CsCl-purified TLPs and DLPs or rotavirus dsRNA were incubated with RNase III or RNase A as indicated above for 2 h at 37°C. The genomic dsRNA was phenol extracted and analyzed by PAGE and silver staining as described in Materials and Methods.

RNase III, respectively (Fig. 3A). When cells infected with rotavirus were treated with these two RNases, we found that the signal of the J2 antibody disappeared only when the cells were treated with RNase III but not with RNase A (Fig. 3B). To discard the possibility that the dsRNA present in viral particles was accessible to RNase III digestion, we treated CsCl-purified DLPs or TLPs, or purified viral dsRNA, with both RNases, and after an incubation period of 2 h the RNA in each sample was phenol extracted and analyzed by PAGE and silver staining. Figure 3C shows that the RNA present in viral particles (TLPs or DLPs) was not susceptible to RNase treatment, while the purified dsRNA was degraded only by RNase III. These results suggest that the nature of the RNA stained by J2 in rotavirus-infected cells is dsRNA, most probably genomic viral dsRNA.

The synthesis of rotavirus proteins does not depend on the GADD34/PP1 phosphatase complex. As previously shown, during rotavirus infection eIF2α becomes phosphorylated, and it remains in this state during the infection, yet the synthesis of the viral proteins is not affected (18). Since the phosphorylation status of eIF2α at a given time is the result of the equilibrium between the activities of the kinase(s)/phosphatase(s) involved, one possibility to explain this observation is that during infection there are fast and/or localized events of eIF2α dephosphorylation that allow viral protein synthesis. Generally, under stress conditions mammalian cells express a protein known as growth arrest and DNA damage protein-34 (GADD34) that forms a complex with phosphatase PP1, which specifically promotes the dephosphorylation of eIF2α on Ser51 (20). To evaluate if the phosphatase activity of the GADD34/PP1 complex was important during rotavirus infection, we tested the effect of salubrinal, a selective inhibitor of the dephosphorylation of eIF2α by the GADD34/PP1 complex (4), with the idea that if during infection the phosphatase activity of

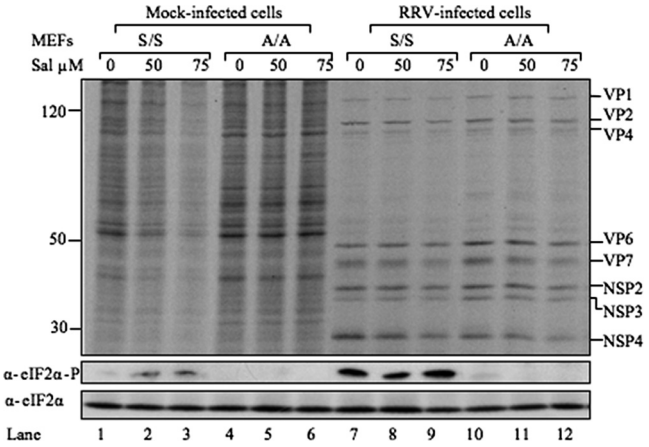

FIG. 4. Rotavirus protein synthesis is resistant to the inhibition of the GADD34/PP1 complex. Wild-type (S/S) or mutant (A/A) MEFs, which express a nonphosphorylatable form of eIF2α, were left uninfected or were infected with rotavirus RRV at an MOI of 3, and 1 h postinfection they were incubated with the indicated doses of salubrinal (sal) and harvested at 8 hpi. Thirty min before being harvested, the cells were metabolically labeled with 25 μC/ml Easy-tag express-[^{35}S] (maintaining the drug during the labeling period). The labeled proteins were resolved by 10% SDS-PAGE and detected by autoradiography, and in parallel the same samples were analyzed by Western blotting using antibodies against phospho-eIF2α (eIF2α-P) or total eIF2α.

the complex was necessary for the translation of viral proteins, its inhibition would result in a decreased viral protein synthesis. In these assays we used mouse embryonic fibroblasts with wild-type eIF2α (MEF eIF2α S/S) or a mutant in which serine 51 of eIF2α was changed to alanine (MEF eIF2α A/A), thus it cannot be phosphorylated. These cells were left uninfected or were infected with the rotavirus RRV, and after the adsorption period the cells were treated with different concentrations of salubrinal. Eight hours postinfection the synthesis of cellular and viral proteins was analyzed by autoradiography, and the phosphorylation level of eIF2α was analyzed by Western blotting (Fig. 4). In mock-infected eIF2α S/S MEFs, salubrinal caused the accumulation of eIF2α in its phosphorylated state in a dose-dependent manner, which correlated with a decrease in the synthesis of cellular protein. It has been shown previously that this phenomenon is due to the inhibition of GADD34/PP1 complex and not to the activation of the eIF2α kinases (4). As expected, the protein synthesis in eIF2α A/A MEFs was not affected by treatment with salubrinal (Fig. 4). On the other hand, when either wt or mutant MEFs were infected with rotavirus, we found that the inhibition of the GADD34/PP1 complex did not affect the synthesis of viral proteins either in the eIF2α A/A MEFs or in their wild-type counterparts, suggesting that viral mRNAs are translated through a mechanism that is less sensitive to the effects of salubrinal and that they overcome the blockage imposed by the phosphorylation of eIF2α.

Phosphorylated eIF2α and PKR are associated with 40S ribosomal subunits during rotavirus infection. Since viral protein synthesis proceeds even when eIF2α is phosphorylated, we decided to look for the presence of other initiation factors known to attenuate or substitute for the absence of a functional eIF2α in the 40S and 80S ribosomal complexes. To do this, the ribosomal complexes from mock-infected, RRV-infected, or thapsigargin-treated cells were purified by sucrose density gradients. These gradients were fractioned, and their OD_{260} profiles were obtained (Fig. 5A). Interestingly, the ri-

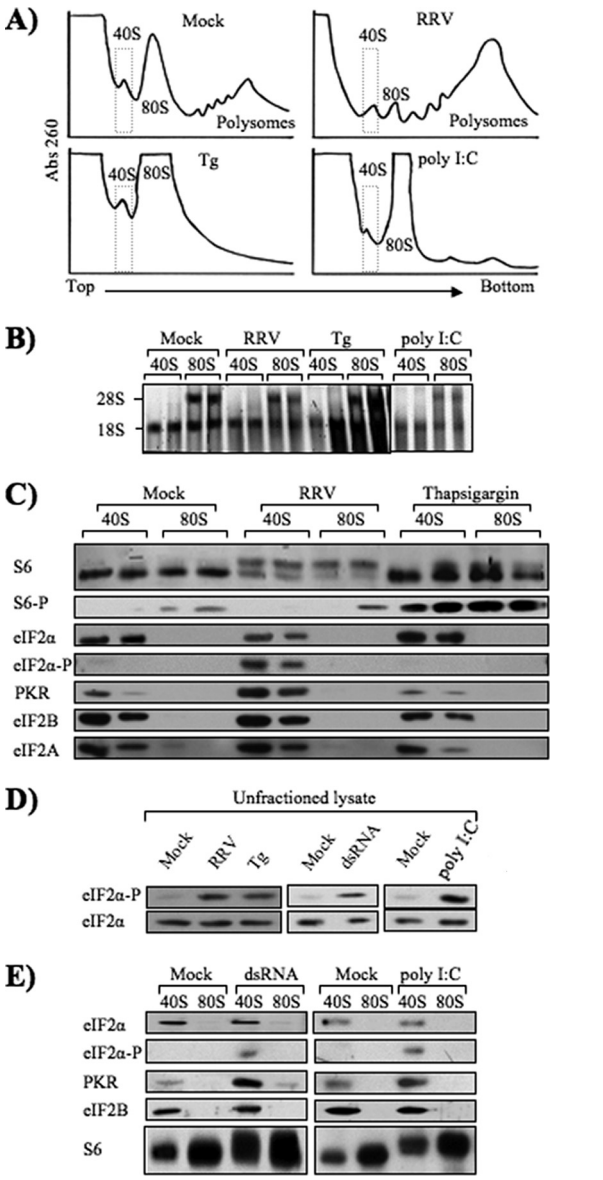

FIG. 5. Analysis of initiation translation complexes formed during rotavirus infection. MA104 cells were infected with rotavirus RRV at an MOI of 3, treated with thapsigargin (400 nM), or transfected for 1 h with 5 μg/ml poly(I:C) or purified rotavirus dsRNA. Six hours after the indicated treatment, cells were lysed by homogenization in the presence of 100 μg/ml cycloheximide and were loaded into discontinuous sucrose gradients (10 to 50%). (A) The sucrose gradients were fractionated using an absorbance monitor, and the OD_{260} profiles were obtained. (B) The presence of the indicated ribosomal complexes was confirmed by visualizing the 28S and 18S ribosomal RNAs by the ethidium bromide staining of agarose gels. (C and E) The indicated fractions or the total, nonfractionated lysates (D) were tested for the presence of several translation initiation factors by immunoblotting using antibodies against phospho-eIF2α (eIF2α-P), total eIF2α, PKR, eIF2Bε, eIF2A, S6, or phospho-S6 (S6-P) as indicated.

bosomal profiles were distinct for each condition; the peaks containing the 40S and 80S ribosomal complexes were more pronounced in mock-infected cells than in RRV-infected cells, whereas the polysomal profiles were more prominent in infected cells, suggesting that the initiation and, in general, the efficiency of the protein synthesis is increased in rotavirus-infected cells. In contrast, in cells treated with thapsigargin the polysomal profiles were not detectable, while the 80S fraction was very prominent (Fig. 5A), as previously reported (16). The presence of the small and large ribosomal subunits in the fractions indicated as 40S and 80S was confirmed by the presence of the 18S and 28S rRNAs, respectively (Fig. 5B). The protein contents present in the fractions containing the 40S and 80S ribosomal subunits from cells under different conditions were analyzed for the presence of several eIFs by Western blotting. When an antibody to the small ribosomal protein 6 (S6) was used, we found that this protein was present in all fractions (Fig. 5C). Interestingly, the pattern of S6 was different in cells infected with rotavirus (appearing as a double band) compared to that of mock-infected cells or cells treated with thapsigargin. It has been shown that in stress conditions S6 can be modified by phosphorylation, which promotes the selective translation of some mRNAs (15). When an antibody against the phosphorylated form of S6 was used, we found that this protein appeared phosphorylated in thapsigargin-treated cells (Fig. 5C), while in the cells infected with rotavirus the phosphorylation of S6 was scarce and did not correspond to the double band observed when probed with the anti-S6 antibody. These observations suggest that during rotavirus infection, there could be another modification of the S6 protein that is different from phosphorylation (Fig. 5C). When the presence of eIF2α was assessed, we found that it was present in the 40S fractions, but not in the 80S fractions, of all three conditions; however, we found that the phosphorylated factor was present only in the 40S fractions of rotavirus-infected cells and not in the corresponding fractions of thapsigargin-treated cells (Fig. 5C), even though when total cell lysates from each condition were probed with antibodies against the phosphorylated eIF2α, this factor was clearly phosphorylated in the cells treated with thapsigargin (Fig. 5D). Similarly, when the presence of PKR was analyzed, we found that this protein appeared in the 40S fractions of RRV-infected cells, and in smaller amounts in the corresponding fractions of the mock-infected or thapsigargin-treated cells. We then looked for the presence of initiation factors in the 40S fractions that could replace the function of eIF2α when this factor is phosphorylated. Initiation factor eIF2A has been shown to have the same activity as that of eIF2α, and it is functional during Sindbis and Semliki Forest viral infections (40). Factor eIF2Bε has been found to be overexpressed in vesicular stomatitis virus (VSV)-infected MEFs and to reduce the effect of eIF2α phosphorylation by increasing the rate of nucleotide exchange on eIF2 (2). By Western blot analysis, we found that both factors were present in the 40S fractions of all three conditions tested (Fig. 5C).

The changes in protein content of the 40S ribosomal subunits during rotavirus infection are caused primarily by dsRNA. To establish if the trigger that causes the association of phosphorylated eIF2α and PKR to the 40S fractions is particular to the infection with rotavirus or if it is part of a general antiviral response of the cell to the presence of dsRNA, MA104 cells were transfected with rotavirus-purified dsRNA or with poly(I:C), and 8 h posttransfection the cells were lysed and the ribosomal complexes were purified, as previously described. The polysomal profile obtained with these treatments was very similar to that obtained from thapsigargin-treated cells [shown in Fig. 5A for poly(I:C)], with an accumulation of 80S complexes and a near loss of the polysomal fractions. However, in contrast to our findings for thapsigargin-treated cells, the dsRNA or poly(I:C) treatments induced the accumulation of phosphorylated eIF2α and PKR in the 40S complexes, and the modification of S6, similarly to the changes observed during rotavirus infection (Fig. 5E). Taken together, these results indicate that the changes of protein content observed in the 40S fractions are not specifically induced by the viral infection, and more likely they form part of the antiviral cellular response that is activated by dsRNA.

DISCUSSION

Changes in physiological conditions, such as DNA damage, the accumulation of unfolded proteins, and viral infections, are examples of stress, which can result in eIF2α phosphorylation, the main checkpoint of translation initiation. The phosphorylation of this factor results in the shut off of almost all protein synthesis. In the case of viral infections, this inhibition of protein synthesis prevents viral spread. To overcome the inhibitory effects of eIF2α phosphorylation, a wide range of viruses encode gene products that either prevent the activation of the eIF2α kinases, like adenovirus and vaccinia virus (7, 34, 35), or increase the dephosphorylation rate of eIF2α, like herpes simplex 1 and coronavirus infectious bronchitis virus (13, 42). However, in some cases, viral protein synthesis can proceed under conditions of elevated eIF2α phosphorylation as has been observed for cricket paralysis virus, hepatitis C virus, and mouse hepatitis coronavirus (8, 30, 32, 33). Rotaviruses belong to this last group, since the synthesis of viral proteins proceeds robustly in the presence of a phosphorylated eIF2α (18), and the level of protein translation is not modified when the GADD34/PPI complex is inhibited (Fig. 4).

In this work, we found the same level of eIF2α phosphorylation in $PERK^{+/+}$ and $PERK^{-/-}$ MEFs infected with rotavirus, indicating that this kinase is not responsible for the phosphorylation of eIF2α during the viral replication cycle (Fig. 1A). On the other hand, eIF2α was strongly phosphorylated in rotavirus-infected $PKR^{+/+}$ MEFs, whereas its phosphorylation was almost undetectable in $PKR^{-/-}$ MEFs (Fig. 1B) or in MA104 cells in which PKR was silenced by RNAi, indicating that PKR is the main kinase involved in the phosphorylation of eIF2α during rotavirus infection. Many events during the infection could induce the activation of eIF2α kinases; these events include virus binding and the penetration of the cell membrane, viral protein synthesis, and the transcription and replication of the viral genome. The fact that UV-psoralen-inactivated particles did not induce the phosphorylation of eIF2α, together with the observation that transfected infectious virus promotes the phosphorylation of this factor, indicate that the virus entry process is not the event that triggers the activation of PKR during rotavirus infection. On the other hand, the transfection of MA104 cells with purified viral

dsRNA, or with synthetic poly(I:C), was able to induce the phosphorylation of eIF2α for up to 8 h (Fig. 2B and C). Moreover, besides the shut off of cell protein synthesis observed in cells infected with rotavirus or transfected with dsRNA, several of the proteins that were present in the 40S ribosomal fractions of rotavirus-infected cells, but not in mock-infected or thapsigargin-treated cells, also were present in dsRNA-treated cells, like the phosphorylated form of eIF2α and PKR. These results suggest that dsRNA is the trigger of PKR activation during rotavirus infection.

dsRNA originally was characterized as a key mediator of interferon (IFN) induction in response to virus infection; historically, dsRNA has been considered a by-product of viral replication in mammalian cells, which is exploited by the cell to restrict virus growth and limit virus spread. Once the cell sensors detect dsRNA, a cascade of events is activated promoting the shut off of cell protein synthesis, the transcriptional induction of interferon (IFN) and other cytokines, and finally cell death. This response to viral dsRNA is a key component of the IFN system, and it represents the first line of defense of the cell to limit viral replication. Traditionally it has been assumed that the rotaviral dsRNA is hidden from the IFN system by ensuring that genome replication takes place within replicative intermediate particles, such that the single-stranded RNA (ssRNA) is replicated as it enters these particles (23). To our surprise, staining with a MAb that recognizes dsRNA stretches longer than 40 bases detected the presence of dsRNA scattered in the cytoplasm (outside viroplasms) of rotavirus-infected cells (Fig. 2D). The dsRNA detected by the antibody is most likely the RNA sensed by PKR, with its consequent activation. The nature of the viral dsRNA present in the cytoplasm of infected cells is not clear; the possibility that it could be highly structured viral mRNA containing stretches of dsRNA of more than 40 bp was discarded, since bona fide mRNA that was stained with the anti-dsRNA MAb was degraded by RNase A, but the RNA detected in infected cells was degraded only by RNase III, which is specific for dsRNA (Fig. 3), suggesting that most likely the RNA in the cytoplasm of infected cells is viral dsRNA. This last possibility also is supported by the observation that when RRV VP1 and VP2 proteins are silenced by RNAi, there is a limited synthesis of viral mRNAs (more likely due to the transcriptional activity of the entering particle), and the production of dsRNA is almost completely abolished (1); we found that the signal detected by MAb J2 (i.e., the presence of dsRNA in the cytoplasm of the cells) was greatly reduced (data not shown). It remains to be determined how the viral dsRNA reaches the cytoplasm, outside viroplasms, which are the sites of rotaviral genome replication.

In conclusion, our results show that during rotavirus infection, viral dsRNA can be detected in the cytoplasm, inducing the activation of PKR, eIF2α phosphorylation, and the modification of the cellular translation machinery. Rotavirus seems to be immune to this response of the cell, since its mRNAs can be efficiently translated under these conditions, although the precise translation mechanism used by rotavirus remains to be uncovered. The viral response to the other branch elicited by dsRNA, the IFN system, seems to be elegantly controlled (in a cell type- and strain-specific manner) by NSP1 (3, 37, 38). Finally, besides PKR, dsRNA induces the activation of the 2′-5′-oligoadenylate synthetase/RNase L system, which catalyzes the degradation of most RNAs, contributing to a general shut off of protein synthesis (12). Whether or not this system is activated during rotavirus infection currently is under investigation; however, given the efficient translation of the viral mRNAs in infected cells, we believe that this virus has evolved a mechanism to overcome this system as well.

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