Rotavirus Infection Induces the Unfolded Protein Response of the Cell and Controls It through the Nonstructural Protein NSP3

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The unfolded protein response (UPR) is a cellular mechanism that is triggered in order to cope with the stress caused by the accumulation of misfolded proteins in the endoplasmic reticulum (ER). This response is initiated by the endoribonuclease inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and PKR-like ER kinase, which increase the expression of the genes involved in the folding and degradation processes and decrease the protein input into the ER by inhibiting translation. It has been shown that viruses both induce and manipulate the UPR in order to protect the host cells from an ER stress-mediated death, thus permitting the translation of viral proteins and the efficient replication of the virus. To understand the cellular events that occur during the rotavirus replication cycle, we examined the activation of the three UPR arms following infection, using luciferase reporters driven by promoters of the ER stress-responsive genes and real-time reverse transcription-PCR to determine the levels of the stress-induced mRNAs. Our findings indicated that during rotavirus infection two of the three arms of the UPR (IRE1 and ATF6) become activated; however, these pathways are interrupted at the translational level by the general inhibition of protein synthesis caused by NSP3. This response seems to be triggered by more than one viral protein synthesized during the replication of the virus, but not by the viral double-stranded RNA (dsRNA), since cells transfected with psoralen-inactivated virions, or with naked viral dsRNA, did not induce UPR.

Eukaryotic cells encounter a range of physiological and environmental stressful conditions that require adaptive responses in gene expression; these include temperature changes, nutrient limitation, chemical insults, oxidative stress, hypoxia, and virus infection, among others. Exposure of cells to stress elicits adaptive responses that require the coordinated expression of stress response genes which affect survival, cell cycle progression, differentiation, and apoptosis (63). The endoplasmic reticulum (ER) is an organelle that integrates signals from throughout the cell to orchestrate a coordinated response in these situations (33). It is the ER where the folding of proteins destined for both intracellular organelles and the cell surface takes place (5). Accumulation of misfolded proteins in the ER causes stress and leads to activation of a coordinated adaptive program called the unfolded protein response (UPR) (reviewed in references 17 and 26). The function of the UPR is to deal with unfolded proteins by upregulating the expression of chaperone proteins and degradation factors to refold or eliminate misfolded proteins and to reduce incoming protein traffic into the ER by attenuation of translation (50). An important function of the UPR is to reduce the demand on the protein-folding machinery to protect cells from stress. Failure to alleviate the ER stress leads to activation of apoptotic pathways and cell death (25). Three ER-resident transmembrane proteins are activated in response to ER stress: the PKR-like ER kinase (PERK), the activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). It is proposed that under normal conditions the ER chaperone GRP78/BiP is bound to the luminal domain of each sensor. When misfolded proteins accumulate in the ER, GRP78 binds these proteins and releases the sensors. Upon release, PERK and IRE1 homodimerize, causing autophosphorylation and activation, while released ATF6 relocalizes to the Golgi complex, where it is cleaved and activated. Once activated, PERK (a eukaryotic initiation factor 2 [eIF2] kinase) phosphorylates the alpha-subunit of eIF2 (eIF2α) at serine 51. Phosphorylated eIF2α inhibits global translation (48) and stimulates the translation of ATF4 (32), which in turn transcriptionally activates UPR-responsive genes encoding proteins that ameliorate the ER stress (19, 20). The CCAT/ enhancer binding protein (CHOP) is a target gene of ATF4 that, depending on the strength or duration of the stress, can function as a proapoptotic or prosurvival transcription factor (21, 34, 49). Both transcription factors, ATF4 and CHOP, can induce the transcription of GADD34, a protein that interacts with protein phosphatase 1 (PP1) to dephosphorylate eIF2α, resulting in a negative feedback loop that recovers protein synthesis and allows the translation of stress-induced transcripts (40). When ATF6 is cleaved in the Golgi apparatus, one of its cleavage products becomes an active transcription factor that promotes the transcription of chaperone genes. Finally, upon dimerization of IRE1, it autophosphorylates and mediates the removal of an intron from X-box binding protein 1 (XBP1) mRNA. The spliced form of XBP1 encodes a transcription factor that activates the transcription of genes encoding chap-
erones and proteins involved in ER stress-associated protein degradation (ERAD) (reviewed in references 17, 26, and 50).

Rotaviruses are one of the most important causes of viral diarrhea in infants and young children, accounting for approximately 500,000 deaths annually around the world (42). These nonenveloped viruses are formed by three concentric layers of proteins that enclose a genome composed of 11 segments of double-stranded RNA (dsRNA). Each genomic segment, with the exception of segment 11, encodes one viral protein, resulting in a total of six structural (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6). The nucleocapsid of the virion is formed by VP2 and also contains the RNA-dependent RNA polymerase VP1 and the guanylyltransferase/methylase VP3. The middle layer is composed of VP6, while the outer layer is formed by VP7 and VP4 (reviewed in reference 8).

Soon after cell entry, the virus loses the two outer surface proteins, yielding a double-layered particle (DLP) that is transcriptionally active. RNA replication and assembly of the new DLPs take place in structures known as viroplasms. Subsequently, DLPs bud into the ER through their interaction with NSP4, a viral transmembrane ER glycoprotein. During the budding process the immature virion acquires VP4, glycoprotein VP7, and a transient lipid envelope. Once inside the ER, the lipid envelope and NSP4 are lost by an ill-defined mechanism. Mature virions are then released from MA104 cells by cell lysis (8).

Several viruses have been shown to induce ER stress and UPR signaling (23, 43, 56). However, viruses that induce ER stress must face the consequences of activating the UPR. The general arrest of protein synthesis caused by phosphorylation of eIF2α must be dealt with in order to express viral and cellular proteins essential for the replication cycle of the virus, while the overexpression of chaperones and the regulation of the redox environment must be beneficial. Thus, it has been proposed that viruses induce mechanisms that modulate the UPR, keeping the beneficial aspects while suppressing the deleterious ones (23). We previously found that during rotavirus infection, the change in threshold cycle (ΔCT) of eIF2α was significantly modified in rotavirus-infected cells (data not shown).

MATERIALS AND METHODS

Cells, viruses, and antibodies. The rhesus monkey epithelial cell line MA104 was grown in advanced Dulbecco’s modified Eagle’s minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 4% fetal bovine serum (FBS) and was used for all experiments carried out in this work. Rhesus rotavirus was obtained from H. B. Greenberg, Stanford University, Stanford, CA, and was propagated in MA104 cells as described previously (13). For infection, RRV was activated with trypsin (10 μg/ml; Gibco, BRL) for 30 min at 37°C. To prepare UV-psoralen-inactivated virus, an RRV lysate was mixed with psoralen to a final concentration of 40 μg/ml and was exposed to a UV lamp for 1 h on ice. To verify the inactivation, the titer of the UV-psoralen-treated virus was determined in an immunoperoxidase focus assay, and the integrity of the viral particles was determined by a hemaggulation assay. Rabbit hyperimmune sera to rotavirus particles, and to NSP5, were produced in our laboratory and have been described previously (10). The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Perkin-Elmer, Waltham, MA).

Reverse transcription-PCR (RT-PCR) analysis of XBP1. ER stress-induced processing of XPB1 mRNA was evaluated as previously described (51). Total RNA was isolated from RRV-infected cells at different times postinfection by using TRIzol reagent (Invitrogen, Carlsbad, CA) as indicated by the manufacturer. cDNA was reverse transcribed from 1 μg of total RNA using the Moloney murine leukemia virus (M-MuLV) reverse transcriptase (New England Biolabs) and amplified with Vent polymerase (New England Biolabs) using the pair of specific primers for mRNA XBP1 (5'-CTGGAACAGCAAGTGGTAGA-3' and 5'-CTGGGTCTTCTGTTGTA-3'). Amplified fragments of 386 bp and 424 bp, representing the spliced (XBP1s) and unspliced (XBP1a) forms, respectively, were separated in 6% polyacrylamide–Tris-borate-EDTA (TBE); 20 mM Tris-borate, 0.5 mM EDTA; pH 8), visualized by ethidium bromide staining, and detected using a Typhoon Trio imager (Amersham Biosciences).

Real-time RT-PCR analysis. Confident MA104 cells in 48-well plates were infected with RRV and harvested at different time points with TRIzol reagent (Invitrogen). Total RNA was purified and treated with RNA-free DNase (Roche) to eliminate possible DNA contamination. The level of stress-responsive mRNAs was determined by one-step real-time quantitative RT-PCR (qRT-PCR) as previously reported (35). The primers used for the amplifications are shown in Table 1. Quantitative analysis of the data was performed as previously described (7) using the 7800 analysis software (Applied Biosystems). To quantify the changes in gene expression, the change in threshold cycle (ΔCT) was used to calculate relative fold changes normalized against the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control (31).

Plasmids. The GRP78-luc reporter construct was provided by K. Mori, Kyoto University, Kyoto, Japan. GRP78-luc contains the region between nucleotides (nt) –304 and +34 of the human GRP78 promoter. This promoter has multiple copies of the ER stress response element (ERSE) and is fused to the firefly luciferase coding region (66). The CHOP-luc reporter plasmid contains the region from nt –644 to +91 of the human CHOP promoter fused to the firefly luciferase coding region and was provided by N. S. Wong, University of Hong Kong (27). The 5′-ATF6-luc plasmid, which contains five copies in tandem of the ATF6 consensus binding site upstream of the firefly luciferase coding region, under the c-fos minimal promoter, was kindly provided by R. Prywes, Columbia University, New York, NY (61). Construct mUTR-ATF4-luc, which contains the 5′-untranslated region (UTR) of the mouse ATF4 mRNA fused to the coding region of firefly luciferase driven by a thymidine kinase promoter, was provided by D. Ron, University of Cambridge, Cambridge, United Kingdom (18). The pRL-TK plasmid (Promega), containing the Renilla luciferase coding region under the thymidine kinase promoter, was used as an internal control to normalize for transfection efficiency. The activity of the Renilla luciferase was not significantly modified in rotavirus-infected cells (data not shown).

Transfection and reporter assays. MA104 cells grown on a 48-well plate were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer-
er’s instructions. Reporter plasmids GRP78-luc and CHOP-luc were used at 250 ng/well, while mATF4-UTR-luc and 5xATF6-luc were used at 1 µg/well. All four constructs were cotransfected with the control plasmid pRL-TK (25 ng/well). At 8 h posttransfection (hpt), the medium was replaced with DMEM+2% FBS, and 24 h later the cells were infected with RRV at a multiplicity of infection (MOI) of 10 and harvested at different times postinfection. Cellular lysates were assayed in duplicate for firefly and Renilla luciferase activities using the dual-luciferase kit (Promega, Madison, WI) according to the manufacturer’s instructions. In each assay, the firefly luciferase activity was normalized to the Renilla luciferase activity obtained in the same well and was expressed as a percentage of the mock-infected or control-treated cells (which were set as 100% at each time point).

Cotransfection of siRNAs and plasmids. Small interfering RNAs (siRNAs) were obtained from Dharmacon Research (Lafayette, CO); the sequences of the siRNAs used in this work have been previously reported (38). As an irrelevant control, an siRNA directed to the green fluorescent protein was used. Cotransfection of siRNAs and reporter plasmids into MA104 cells was performed using a reverse transfection method previously described (15). Briefly, 15 µl of Oligo-lectamine (Invitrogen, Carlsbad, CA) was diluted in 1 ml of MEM and incubated for 10 min at room temperature. Ninety microliters of this mixture was added to a well of a 48-well plate containing a mix of 200 pmol/ml of the siRNA and the reporter plasmids. After an incubation of 20 min at room temperature, 200 µl of a single-cell suspension of 1.5 × 10^5 MA104 cells/ml was added to each well, and the cells were incubated at 37°C for 24 h. The transfection mixture was removed, and then the cells were washed twice with DMEM and infected with RRV at an MOI of 10 or treated with thapsigargin (400 nM; Sigma-Aldrich, St. Louis, MO). Nine hours postinfection (hpi) or posttreatment, cells were lysed and assayed for luciferase activity and immunoblotting. The relative induction levels were calculated based on the values obtained in mock-infected cells transfected with the irrelevant siRNA, which were set as 100% at each time point.

RNA transfection. To transfect dsRNA, the cells previously transfected for 24 h with the reporter plasmids were incubated for 1 h at 37°C with MEM containing Lipofectamine 2000 (40 µg/ml; Invitrogen, Carlsbad, CA) and 5 µg/ml of synthetic poly(I-C) (Sigma-Aldrich, St. Louis, MO) or 5 µg/ml purified viral dsRNA. After this time, the transfection mixture was replaced with DMEM supplemented with 4% FBS, and cells were incubated for different times before harvesting.

Radiolabeling of proteins. Cells grown in 48-well plates were mock transfected or transfected with synthetic poly(I-C) (5 µg/ml) or purified viral dsRNA (5 µg/ml) for 9 h as previously mentioned. Before harvesting, the medium was replaced with MEM without methionine, supplemented with 25 µCi/ml of Easy Tag Express 35S 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 the lysis buffer of the dual-lucerase kit (Promega, Madison, WI) supplemented with a protease inhibitor cocktail (Complete; Roche, Indianapolis, IN). The lysates were diluted in Laemml sample buffer, denatured by boiling for 5 min, subjected to 10% SDS-PAGE, and transferred to Immobilon NC membranes (Millipore, Billerica, MA). After incubation with blocking solution (5% nonfat milk, 0.05% Tween 20), the membranes were incubated with primary antibodies diluted in blocking solution for 1 h at room temperature or overnight at 4°C. The membranes were rinsed with PBS-Tween 20, and bound antibodies were developed by incubation with a peroxidase-labeled secondary antibody and the Western Lightning system (Perkin-Elmer, Waltham, MA).

Statistics. Significant differences between the experimental conditions were determined using an analysis of variance (ANOVA), and a P value of <0.05 was considered statistically significant.

RESULTS

Activation of the XBP1/IRE1 pathway in rotavirus-infected cells. IRE1 is a bifunctional enzyme with both serine/threonine kinase and endoribonuclease activities (57). Activated IRE1 cleaves a 26-nucleotide intron from the primary XBP1 transcript (58). This unconventional splicing occurs in the cytoplasm and causes a translational frameshift at XBP1 mRNA that stimulates its translation, and the spliced XBP1 mRNA encodes an active XBP1 factor that is involved in the transcriptional in-

duction of genes encoding ER chaperones, such as GRP78 and GRP94, as well as genes involved in the ER stress-associated protein degradation pathway, like EDEM (degradation-enhancing mannosidase-like protein) and P58, the negative regulator of kinases PERK and PKR (28).

To determine if during the replication cycle of rotavirus the XBP1/IRE1 arm of the UPR becomes activated, MA104 cells were infected with RRV, and at various times postinfection total mRNA was isolated. RT-PCR was performed using primers flanking the XBP1 mRNA splice site, and the PCR products were resolved by PAGE and visualized by ethidium bromide staining (Fig. 1A). A pharmacological inducer of ER stress, thapsigargin, was used as a positive control in all experiments. In mock-infected cells the predominant RT-PCR product found represented the unspliced form of the XBP1 transcript (424 nt; referred to as XBP1u), while in thapsigargin-treated cells the predominant form was the spliced transcript (398 nt; referred to as XBP1s). During the time course of infection, a gradual splicing of XBP1 mRNA could be observed (Fig. 1A), suggesting that the XBP1/IRE1 pathway became activated. The progression of rotavirus infection was followed at the same time by autoradiography of the cell lysates, which were labeled with 35S for 30 min prior to harvesting at the different times postinfection (Fig. 1B). To determine if the activation of this pathway was mediated by the early interaction of the virus with the host cell, rotavirus particles were inactivated by a psoralen-UV irradiation treatment, which blocks viral RNA transcription, genome replication, and protein synthesis but has no effect on receptor binding and subsequent entry of the virus into the cell (11). Cells were incubated for 9 h with inactivated RRV particles, and the splicing of XBP1 transcript was analyzed (Fig. 1A, lane iRRV). In this case, the predominant RT-PCR product found was the unspliced XBP1, suggesting that the activation of IRE1 that leads to the splicing of XBP1 mRNA depends on viral replication rather than virus infection.

Next, the mRNA levels of EDEM and P58, two genes whose transcription is activated by XBP1, were measured by qRT-PCR in rotavirus-infected cells. In contrast to the treatment with thapsigargin, which resulted in 8-fold and 5-fold increases in the levels of EDEM and P58 mRNAs, respectively, no significant changes in the level of these transcripts was detected at any time point postinfection (Fig. 1C). These results indicate that even though the mRNA of XBP1 was spliced in rotavirus-infected cells, either the transcriptional activity or the translation of XBP1 was blocked.

Analysis of the ATF6 pathway in rotavirus-infected cells. Upon ER stress, ATF6 dissociates from GRP78 and moves to the Golgi apparatus, where it is cleaved by the site 1 and site 2 proteases (S1P and S2P), thus releasing the active transcription factor p50ATF6, which is translocated to the nucleus and activates the transcription of mRNAs that code for the ER-resident chaperones GRP78 and GRP94 as well as for the transcription factors CHOP and XBP1, among others (52). To study if the ATF6 pathway is activated during rotavirus infection, the activities of two luciferase reporter constructs were determined in MA104 cells infected with RRV or treated with thapsigargin. The luciferase reporters used were 5xATF6-luc, a firefly luciferase reporter that contains five copies of the ATF6 consensus binding site upstream of the luciferase gene, which even though the mRNA of XBP1 was spliced in rotavirus-infected cells, either the transcriptional activity or the translation of XBP1 was blocked.
and plasmid GRP78-luc, a firefly luciferase reporter driven by the promoter of the human GRP78 gene. These two plasmids have been extensively used to monitor the activation of UPR (61, 66). Cells transfected with either of these two plasmids were infected with RRV, harvested at different times postinfection, and the luciferase activity was measured. We found that under these conditions there was not a significant increase in the luciferase activity driven from either of the two reporters employed, compared with the activity detected in mock-infected cells (Fig. 2A). In contrast, treatment with thapsigargin strongly induced the luciferase activity from both reporter constructs. In each case, pRL-TK plasmid encoding Renilla luciferase was cotransfected and used for normalization of transfection efficiency. Cells were then infected with rotavirus at an MOI of 10 or treated with thapsigargin (TG) for 9 h. (A) At the indicated time points, cell lysates were harvested for a dual-luciferase assay as described in Materials and Methods. The ratio of firefly to Renilla luciferase activities was expressed as a percentage relative to activity in uninfected cells, which was set as 100%. (B) Total RNA was extracted from cells cotransfected with plasmids, or from nontransfected cells, that were either infected or treated with TG for 9 h and used to quantify the level of firefly and Renilla luciferase mRNAs in the case of the plasmid-transfected cells or the level of endogenous GRP78 mRNA, by qRT-PCR analysis. The mRNA level of firefly luciferase was normalized to the level of Renilla luciferase mRNA, and the level of GRP78 mRNA was normalized to that of GAPDH mRNA. The fold increase shown is relative to the values obtained in mock-infected or mock-treated cells (set as 1.0). The results represent the means ± standard errors for three independent experiments. The asterisks indicate significant differences between mock- and thapsigargin-treated cells, determined by ANOVA ($P < 0.05$).

FIG. 1. Rotavirus infection induces the splicing of XBP1 mRNA but does not increase the transcription of XBP1 target genes. MA104 cells were either treated with thapsigargin (400 nM) for 9 h, infected with psoralen-inactivated virus (rrv) for 9 h, or infected with RRV at an MOI of 10 and harvested at the indicated time points. (A) Total RNA isolated from infected or thapsigargin (TG)-treated cells was used for the amplification of XBP1 transcripts by RT-PCR using primers flanking the XBP1 splice site. M, mock-infected cells. The PCR products were resolved by gel electrophoresis and visualized by ethidium bromide staining as described in Materials and Methods. The XBP1u and XBP1s forms are shown. The percent spliced refers to the total amount of XBP1 (XBP1u + XBP1s) present under each condition. GAPDH mRNA was amplified by RT-PCR as an RNA loading control under each condition. (B) MA104 cells were mock infected (M) or infected with RRV at an MOI of 10 for the indicated times and labeled with Easy Tag Express 35S labeling mix for 30 min before harvesting. Labeled proteins were resolved by SDS-PAGE and autoradiography. (C) Total RNA extracted from infected or TG-treated cells was used to quantitate the level of EDEM and P58 mRNA by qRT-PCR. Results are expressed as the fold increase relative to mock-infected controls (set at 1.0) at each time point. The arithmetic mean ± the standard error for three independent experiments performed in triplicate is shown. * $P < 0.05$ by ANOVA.

FIG. 2. Rotavirus infection activates the ATF6 pathway but prevents the expression of its target genes. MA104 cells were transfected for 24 h with either 5×ATF6-luc or GRP78-luc reporter plasmids. In each case, pRL-TK plasmid encoding Renilla luciferase was cotransfected and used for normalization of transfection efficiency. Cells were then infected with rotavirus at an MOI of 10 or treated with thapsigargin (TG) for 9 h. (A) At the indicated time points, cell lysates were harvested for a dual-luciferase assay as described in Materials and Methods. The ratio of firefly to Renilla luciferase activities was expressed as a percentage relative to activity in uninfected cells, which was set as 100%. (B) Total RNA was extracted from cells cotransfected with plasmids, or from nontransfected cells, that were either infected or treated with TG for 9 h and used to quantify the level of firefly and Renilla luciferase mRNAs in the case of the plasmid-transfected cells or the level of endogenous GRP78 mRNA, by qRT-PCR analysis. The mRNA level of firefly luciferase was normalized to the level of Renilla luciferase mRNA, and the level of GRP78 mRNA was normalized to that of GAPDH mRNA. The fold increase shown is relative to the values obtained in mock-infected or mock-treated cells (set as 1.0). The results represent the means ± standard errors for three independent experiments. The asterisks indicate significant differences between mock- and thapsigargin-treated cells, determined by ANOVA ($P < 0.05$).
treated cells. Taken together, these results suggest that rotavirus infection induces the activation of the ATF6 pathway, as indicated by the upregulation of transcription of the luciferase reporter gene placed under the control of ATF6 elements, as well as that of GRP78. However, there seems to have been a block in the translation of these mRNAs, as judged by the lack of the reporter luciferase activity in infected cells.

Translation of ATF4, CHOP, and GADD34 in rotavirus-infected cells. Another arm of the UPR is the phosphorylation of eIF2α by PERK, which results in a dramatic inhibition of protein translation. Under these restrictive conditions, in which there is little eIF2α available, the translation of a group of cellular proteins is stimulated. Such is the case for ATF4, CHOP, and GADD34 (30, 41, 59). Since eIF2α is phosphorylated in rotavirus-infected cells (38), we analyzed if these factors were selectively translated. For this, MA104 cells were transfected either with the construct mUTR-ATF4-luc, which contains the 5'UTR of the mouse ATF4 mRNA fused to the coding region of the firefly luciferase, or with the construct CHOP-luc, a luciferase reporter driven by the CHOP promoter. The cells were then infected with RRV and harvested at different times postinfection to measure the luciferase reporter activity. We found that, even though eIF2α is phosphorylated throughout the replication cycle of the virus (38, 47), the luciferase activities from ATF4-UTR-luc or CHOP-luc constructs did not change over the course of infection relative to the activity detected in mock-infected cells, whereas addition of thapsigargin did result in a significant increase in the luciferase activities of both reporters (Fig. 3A). These results suggest that in the rotavirus-infected cells neither ATF4 nor CHOP was translated.

To confirm the lack of ATF4 activity, the levels of GADD34 and CHOP mRNAs were quantitated by qRT-PCR. We found that, in contrast to the results obtained with the CHOP-luciferase reporter assay (Fig. 3A), rotavirus infection resulted in a gradual increase in the level of CHOP mRNA over the 9-h time course of infection. In the case of GADD34 mRNA, the increase became significant at 6 h postinfection, and at 9 h postinfection it attained a level equivalent to that seen in thapsigargin-treated cells (Fig. 3B), indicating the upregulation of these mRNAs in rotavirus-infected cells. Taken together, these results suggest that the infection of MA104 cells with rotavirus causes an ER stress, as indicated by the elevated levels of GRP78, CHOP, and GADD34 mRNAs observed in infected cells. However, apparently the UPR pathways activated in response to the infection were modulated at the translational level, since the expression of the luciferase reporters used was prevented.

Translation of the UPR genes in rotavirus-infected cells is prevented by NSP3. It has been previously shown that NSP3 prevents the translation of poly(A)-containing cellular genes by interacting with eIF4G at the same place where poly(A) binding protein binds (12, 44, 45), and in a previous work we showed that silencing the expression of NSP3 did not affect the translation of the viral proteins (38). To determine if this viral protein was involved in preventing the translation of the UPR genes, the expression of NSP3 was silenced by RNA interference (RNAi) in rotavirus-infected cells. For this, MA104 cells were cotransfected with an siRNA directed to NSP3 together with each of the luciferase reporters previously described; 24 h later the cells were infected with RRV, and 9 hpi the luciferase activity was determined. As a control, MA104 cells were transfected with an irrelevant siRNA. The siRNA directed to NSP3 efficiently knocked down the expression of this protein in infected cells, as shown by a representative Western blot assay (Fig. 4A, inset). Silencing NSP3 resulted in increased luciferase activities from the 5'×ATF6-luc and GRP78-luc reporters that were similar to that found in cells treated with thapsigargin and 3- to 4-fold higher than the luciferase activity detected in infected cells transfected with the control siRNA (Fig. 4A), suggesting that in the absence of NSP3, the activation and subsequent binding of the endogenous ATF6 to its target sequence present in the 5'×ATF6-luc construct and the upregulation of the GRP78 promoter took place in these cells. We also found that under these same conditions the expression of the translational reporter mUTR-ATF4-luc did not change, whereas there was a 2-fold increase in the luciferase activity
from the CHOP-luc construct, suggesting an ATF4-independent activation of the CHOP promoter, which correlated with the increased level of CHOP mRNA previously detected by quantitative RT-PCR (Fig. 3B). To discard the possibility that NSP3 could be interfering with the activity of luciferase, rather than on its translation, we performed a Western blot assay using an antiluciferase antibody in cells transfected with the GRP78-luc reporter that were infected or not with rotavirus, and we found that the absence of luciferase activity in rotavirus-infected cells correlated with the absence of the protein, based on Western blotting (results not shown).

Since the splicing of XBP1 mRNA, but not the transcriptional activity of this factor, was detected in rotavirus-infected cells (Fig. 1A), we suspected that the inhibition of XBP1 activity could also be at the translational level and that NSP3 could be involved. Thus, we quantified by qRT-PCR the mRNA level of EDEM and P58 (whose synthesis is under the control of XBP1) in NSP3-silenced RRV-infected cells. Since the XBP1 gene also has an ERSE sequence that can be recognized by both ATF6 and XBP1 itself (66), the level of XBP1 mRNA was also determined. As a control, IRE1 mRNA levels were also quantitated. We found that in cells where the expression of NSP3 was silenced, the level of all XBP1 target genes tested increased to levels similar to those observed in thapsigargin-treated cells: XBP1 mRNA increased more than 3-fold, whereas EDEM and P58 mRNAs increased 2- and 4-fold, respectively (Fig. 4B). As expected, the level of IRE1 mRNA did not change significantly under these conditions. These findings indirectly suggested that even though the primary transcript of XBP1 is spliced, it is not translated in rotavirus-infected cells, most likely due to the presence of NSP3, and thus the transcriptional activity of this factor is absent in infected cells. Overall, our data indicate that the infection of MA104 cells with rotavirus causes an ER stress that leads to the activation of the UPR. However, this response seems to be modulated at the translational level by the viral nonstructural protein NSP3.

Transfection of double-stranded RNA into cells does not induce UPR. PKR is considered a component of the ER stress-mediated pathway, since treatment of cells with ER stress inducers renders PKR active, suggesting a role for this kinase in the ER stress-mediated signaling (39). As previously mentioned, we recently reported that PKR is the kinase responsible for the phosphorylation of eIF2α in rotavirus-infected cells and that viral dsRNA is its most likely activator (47). To determine if the activation of PKR contributed to the ER stress response induced by rotavirus infection, we transfected cells either with purified viral dsRNA or with a synthetic dsRNA [poly(I·C)], a known activator of PKR, and analyzed which of the UPR pathways became activated under these conditions. Again, thapsigargin was used as a positive UPR inducer.

Since the phosphorylation of eIF2 by PKR results in a severe shut off of cellular protein synthesis, the activation of PKR in poly(I·C)-transfected cells was indirectly monitored following the metabolic labeling of proteins. As expected, in cells transfected with poly(I·C), there was a strong shut off of cellular protein synthesis, as judged by the poor incorporation of 35S in proteins (Fig. 5A). The splicing of XBP1 mRNA mediated by IRE1 was then determined by RT-PCR in cells transfected with either viral dsRNA or poly(I·C) or in cells treated with thapsigargin. We found that there was a weak but consistent signal of the spliced form of XBP1 in the poly(I·C)- and dsRNA-transfected cells at all times tested; however, this slight processing was more likely due to the stress caused by the transfection reagent, since the same amount of XBP1 mRNA was found in cells treated with the transfection reagent alone (Fig. 5B, lane T). In contrast, in thapsigargin-treated cells there was a gradual increase in the production of the XBP1s form and a corresponding decreased amount of the unspliced form.

![Figure 4](http://jvi.asm.org/Downloadedfrom)
Next, the expression levels of the ER stress reporters in cells transfected with poly(I·C) were evaluated. The luciferase activity from the four reporters tested was not affected by transfection of the synthetic dsRNA at any time analyzed (only shown for 9 hpt), while thapsigargin increased significantly the luciferase activity from all constructs (Fig. 5C); similar results were obtained using purified viral dsRNA (data not shown), suggesting that neither the dsRNA nor the poly(I·C) per se, were responsible for the activation of the UPR. Interestingly, similar to our observations in rotavirus-infected cells, the transfection of dsRNA did not result in the upregulation of eIF2α in transfected cells (data not shown) (47), suggesting that the phosphorylation of eIF2α might not be the only requirement to activate the translation of ATF4 in MA104 cells.

Several viral proteins are involved in the induction of ER stress in rotavirus-infected cells. After discarding dsRNA as the main elicitor of UPR in rotavirus-infected cells and to identify the viral protein(s) responsible for inducing ER stress, we silenced the expression of each viral protein by RNA interference and quantitated by qRT-PCR the level of GRP78 mRNA as an indicator of ER stress. For this, MA104 cells were transfected with siRNAs directed to each viral protein for 24 h and then infected with rotavirus. Nine hours postinfection cells were harvested and total RNA was obtained for qRT-PCR, or the cells were metabolically labeled with 35S for 30 min prior to lysis. The knockdown of rotavirus proteins was analyzed by SDS-PAGE and autoradiography (Fig. 6A and B), by qRT-PCR analysis (for NSP1 [data not shown]), or by Western blotting for NSP5 (Fig. 6C). As shown, the expression of the rotavirus proteins was significantly reduced (Fig. 6A, B, and C). When the level of GRP78 mRNA was quantitated under these conditions and compared to its level in mock-infected cells, it was found that there was an increase of about 10-fold in the amount of GRP78 mRNA in infected cells transfected with an irrelevant siRNA (as previously observed [Fig. 2B]), while in cells where the expression of VP1, VP2, VP6, and NSP5 was silenced, the level of GRP78 mRNA was not induced, suggesting that these viral proteins could participate in the induction of ER stress (Fig. 6B). The knockdown of VP3, VP4, NSP1, and NSP2 resulted in different degrees of ER stress, as indicated by a slight increase in the level of GRP78 mRNA, but was clearly lower than that observed in a control infection. In the absence of NSP3, the level of GRP78 mRNA increased to a level similar to the level detected during the infection. Interestingly, neither VP7 nor NSP4, the two viral glycoproteins, were apparently involved in the induction of ER stress, since when knocked down the level of GRP78 mRNA increased 5 to 7 times above that in the control mock-infected cells.
DISCUSSION

Increasing evidence supports the notion that UPR signaling pathways influence the pathogenesis associated with viral infections. Interestingly, several viruses have developed specific strategies to prevent, modify, or benefit from each of the pathways induced during the UPR (1, 3, 7, 23, 36, 43, 53, 55). To better understand the molecular pathogenesis induced by rotavirus, we examined the activation of each one of the three UPR arms following infection. For this, we used luciferase reporters driven by promoters of ER stress-responsive genes and qRT-PCR to determine the levels of the stress-induced mRNAs. Taken together, our results suggest that rotavirus infection induces a UPR that is suppressed by the viral nonstructural protein NSP3 at the translational level. This response seems to be triggered by a viral product synthesized during the replication cycle of the virus, more likely by one or more viral proteins, but not by the viral dsRNA, since cells transfected with psoralen-inactivated virions, or with naked viral dsRNA, did not start a UPR.

The IRE1-XBP1 pathway is involved in several functions related to ER stress, specifically, the activity of XBP1 coordinately augments the synthesis of the ER-resident chaperones,
degradation factors, and lipid components of the membrane. The control of this pathway at different levels has been described in cells infected with various viruses; for example, in enterovirus 71-infected cells, even though the level of XBP1 mRNA increases during infection, it is not spliced, suggesting an inhibition at the level of IRE1 activity (24). Similar to our findings, cell infection with hepatitis C virus (55), human cytomegalovirus (23), or severe acute respiratory syndrome (SARS) coronavirus (3) induces a progressive increase in the amount of spliced XBP1 mRNA; however, the upregulation of XBP1 target genes is not detected, indicating that either the translation or the transcriptional activity of XBP1 is blocked. In cells carrying hepatitis C virus repli- cos it has been observed that XBP1 is targeted for proteasomal degradation, thus blocking the induction of the ERAD pathway (26). In contrast, in cells infected with West Nile virus (36) or Japanese encephalitis virus or dengue virus (68), the IRE1-XBP1 pathway is activated, remains functional, and is associated with the survival of stressed cells, since the knockdown of XBP1 correlates with an enhanced cytopathic effect (36, 54). On the other hand, the efficient replication of coxsackievirus B3 has been associated with the activity of XBP1, since silencing the expression of XBP1 inhibits the synthesis of the viral protein VP1 and enhances the virus-induced cell death (69).

In this work we found that rotavirus infection of MA104 cells activates the first step of the IRE1-XBP1 pathway, since XBP1 mRNA became spliced. However, the translation of the XBP1s mRNA was prevented as a result of the general translational inhibition mediated by rotavirus NSP3 and, as a consequence, its downstream products were not activated (Fig. 1). The knockdown of NSP3 resulted in translation of the XBP1s mRNA and induction of the transcription of EDEM and P58. We previously observed that silencing the expression of NSP3 in RRV-infected MA104 cells resulted in an increased yield of viral progeny, which was associated with a delayed cytopathic effect (37). This extended viability of rotavirus-infected cells could be due to activation of XBP1, which might increase the folding capacity of the ER, promoting cell survival.

Recent studies have shown that the production of XBP1 protein is required for the enhanced cell survival induced by HSP70 and phosphatidylinositol 3-kinase (PI3K) under ER stress conditions (14, 22). PI3K-mediated signaling has been reported to occur early after rotavirus infection, and it has been proposed as a viral protection mechanism from cell apoptosis (2, 16). It will be interesting to characterize the expression of HSP70 and the activation of PI3K signaling in rotavirus-infected cells in which the expression of NSP3 is silenced.

Our results also indicated that in RRV-infected cells the ATF6 pathway is activated upon infection, since the transcription of GRP78 and CHOP, two of its gene targets, was induced. However, these results were not obvious when the expression of the luciferase reporters was initially used to monitor UPR in infected cells; it was necessary to look for the levels of transcription of the ATF6 target genes to find that there was indeed an induction of the ATF6 arm. The lack of translation of the luciferase reporters was due to translational inhibition mediated by NSP3. Thus, it is important to characterize both RNA and protein expression when studying UPR induction in viral infections, especially in the case of those viruses known to decrease or inhibit host protein synthesis. A similar observation was recently made by Bechill et al. (3) when characterizing the UPR induced by coronavirus, which also induces a shut-off of cell protein synthesis.

In general, the activation of the UPR also leads to activation of PERK, which phosphorylates eIF2α, with the consequent attenuation of cell translation that in turn reduces the protein load in the ER. Under these restrictive conditions the translation of ATF4, CHOP, and GADD34 is stimulated. The mRNA of ATF4 is translated using a translation shunting mechanism which functions preferentially under conditions of limiting amounts of eIF2α; ATF4 then activates the transcription of the CHOP and GADD34 genes. In cells infected with different viruses, the phosphorylation of eIF2α plays a pivotal role in the translational control of viral and cellular mRNAs (62). However, different viruses, including rotavirus, have evolved mechanisms to either dephosphorylate eIF2α (7) or to translate their mRNAs efficiently in the presence of phosphorylated eIF2α (38, 46, 60). We have previously found that in rotavirus-infected cells eIF2α becomes phosphorylated early during infection and remains in this state throughout the replication cycle of the virus. In that same study we found that when NSP3 was silenced the phosphorylation of eIF2α was not affected (38). We also showed that the kinase responsible for the phosphorylation of eIF2α is PKR, not PERK (47). In this work we found that the mUTR-ATF4-luc reporter protein was not synthesized in rotavirus-infected cells, even when NSP3 was knocked down, suggesting that the phosphorylation of eIF2α is not the only requisite for ATF4 translation in MA104 cells, or that its translation is selectively prevented in RRV-infected cells.

In contrast with the previous findings, while the CHOP-luc reporter was not translated, its mRNA increased during infection, suggesting that the limited synthesis of CHOP-luc was due to the NSP3 inhibition of translation. Indeed, when the expression of NSP3 was silenced we found that the activity of CHOP-luc increased in rotavirus-infected cells. Since it has been observed that both ATF4 and ATF6 can activate CHOP expression (9, 67), it is possible that in rotavirus-infected cells the upregulation of CHOP and hence GADD34 might be induced mainly by ATF6.

Despite the fact that we found an increased transcription of the GADD34 mRNA during infection, we were not able to detect an increased amount of GADD34 by Western blotting in rotavirus-infected cells (results not shown). Furthermore, we previously showed that treatment of RRV-infected MA104 cells with salubrin, a selective inhibitor of the dephosphorylation of eIF2α by the GADD34/PP1 phosphatase complex (4), did not change the phosphorylation state of eIF2α, suggesting that GADD34 was not active under those conditions (47). Taken together, these results suggest that either the synthesis or the function of GADD34 is blocked in rotavirus-infected cells. The mechanism of this modulation is unclear, but it appears not to depend on the known role of NSP3 in translation.

Another negative regulator of the phosphorylation of eIF2α is P58, which is an inhibitor of protein kinases PERK and PKR (29, 65). We believe that the low expression of P58 (Fig. 1C), together with the lack of GADD34 function observed during rotavirus infection, might contribute to a persistent PKR signaling and a maintained phosphorylation state of eIF2α. In
coxsackievirus B3-infected cells, overexpression of P58 increased VP1 protein production and decreased cell death, suggesting that P58 serves to promote overall host cell health and thereby viral protein production (69).

It has been shown that the spike protein of SARS coronavirus (6), the NS2B-3 of dengue 2 virus (68), the pUL38 protein of human cytomegalovirus (64), and the hydroporphic nonstructural proteins NS4A and NS4B of West Nile virus (1) are the viral components responsible for the induction of UPR during infection, since heterologous expression of the mentioned proteins was sufficient to induce ER stress in transfected cells. In an attempt to identify the viral protein responsible for ER stress induction in rotavirus-infected cells, we silenced the expression of each viral gene by RNAi and analyzed the level of GRP78 mRNA as an indicator of ER stress. We found that silencing the expression of the two viral glycoproteins NSP4 and VP7 (which were the natural UPR inducer candidates, since they accumulate in the ER) did not result in a severe reduction in the level of GRP78 mRNA; similarly, NSP3 knockdown had little or no effect on UPR induction. In contrast, silencing the rest of the viral proteins caused different degrees of reduction in the activation of GRP78 transcription, where VP1, VP2, VP6, and NSP5 knockdown had the most severe effects. We propose that in RRV-infected cells, the activation of the UPR is not triggered by a single viral protein, but rather it could be a multifactorial event in which either the budding of the DLPs into the ER, the formation of viroporins, or the activation of genome replication could be responsible for triggering the UPR. It remains to be determined which viral process during the replication cycle of rotaviruses is the main inducer of ER stress.

Although the UPR is primarily a survival response, the continuous activation of this response also results in cell death. The surprising finding that rotavirus replicates most efficiently under stressful conditions that shut off host protein synthesis and cellular responses, such as the UPR, raises interesting questions about the mechanisms involved in viral translation and the relationship between cell death and viral growth. It is interesting that viruses causing chronic infections have developed strategies to modulate ER stress signaling, while for viruses that cause acute infections the activation of these pathways represents a mechanism to induce cell death. The work of our group and that of other groups suggest that the activation of the cellular UPR and its modulation is an important determinant for viral replication/pathogenesis. Therefore, it is relevant to characterize the cellular responses triggered during infection in order to better understand the pathogenesis of viruses.

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