Endoplasmic Reticulum (ER) Stress Induced by a Neurovirulent Mouse Retrovirus Is Associated with Prolonged BiP Binding and Retention of a Viral Protein in the ER*

Received for publication, March 24, 2004, and in revised form, May 3, 2004
Published, JBC Papers in Press, June 3, 2004, DOI 10.1074/jbc.M403304200

Derek E. Dimcheff, Mark A. Faasse, Frank J. McAtee, and John L. Portis‡

From the Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, National Institutes of Health, Hamilton, Montana 59840

Some murine retroviruses cause a spongiform neurodegenerative disease exhibiting pathology resembling that observed in transmissible spongiform encephalopathies. The neurovirulence of these “spongionic retroviruses” is determined by the sequence of their respective envelope proteins, although the mechanisms of neurotoxicity are not understood. We have studied a highly neurovirulent virus called FrCasE that causes a rapidly progressive form of this disease. Recently, transcriptional markers of endoplasmic reticulum (ER) stress were detected during the early preclinical period in the brains of FrCasE-infected mice. In contrast, ER stress was not observed in mice infected with an avirulent virus, F43, which carries a different envelope gene, suggesting a role for ER stress in disease pathogenesis. Here we have examined in NIH 3T3 cells the cause of this cellular stress response. The envelope protein of F43 bound BiP, a major ER chaperone, transiently and was processed normally through the secretory pathway. In contrast, the envelope protein of FrCasE bound to BiP for a prolonged period, was retained in the ER, and was degraded by the proteasome. Furthermore, engagement of the FrCasE envelope protein by ER quality control pathways resulted in decreased steady-state levels of this protein, relative to that of F43, both in NIH 3T3 cells and in the brains of infected mice. Thus, the ER stress induced by FrCasE appears to be initiated by inefficient folding of its viral envelope protein, suggesting that the neurodegenerative disease caused by this virus represents a protein misfolding disorder.

Several murine retroviruses cause vacuolar brain disease closely resembling the pathology caused by the transmissible spongiform encephalopathy (TSE) agents. The neurovirulence of these “spongionic retroviruses” is determined by the sequence of their respective envelope genes (1–3), although the molecular interactions of the viral envelope protein and the host that lead to neurodegeneration remain to be elucidated. Previous studies by others (3, 4) have suggested that endoplasmic reticulum (ER) retention of the envelope protein may play a role in the pathogenic process. Supporting this hypothesis, we demonstrated that a transcriptional profile consistent with an ER stress response is activated in the brains of mice infected with a neuropathogenic virus FrCasE but not in mice infected with a nonpathogenic retrovirus F43 (5). The FrCasE and F43 viruses were constructed by using the same retrovirus genetic background but differ in their envelope genes (6). FrCasE contains the envelope gene of CasBrE, a neuropathogenic retrovirus originally isolated from wild mice (7), whereas F43 contains the envelope gene of a nonpathogenic virus FMLV 57. Both viruses utilize the same receptor for virus entry, infect the brain at high levels, and target the same cell populations (6). Most interesting, NIH 3T3 cells infected with FrCasE also exhibit a transcriptional profile consistent with ER stress (5), suggesting that these cells could be used to further analyze the interactions between the virus and the host cell that initiates this response.

ER stress is caused by conditions that perturb ER function including nutrient or energy deprivation, calcium fluxes, and the accumulation of misfolded proteins (8, 9). When misfolded proteins accumulate in the ER, a stereotypical cellular program to eliminate this stress, called the unfolded protein response (UPR), is activated (8). Central to the UPR is the binding of BiP (grp78), an ATP-dependent ER chaperone, to misfolded proteins (10–12). BiP binds transiently to newly synthesized proteins but more persistently to misfolded proteins. Under nonstressed conditions, BiP binds to three ER resident transmembrane proteins called “sensors of ER stress,” which negatively regulates the UPR by keeping these proteins in an inactivated state. When misfolded proteins accumulate in the ER, BiP binds instead to the misfolded proteins and allows activation of these UPR sensors, leading to decreased protein synthesis and increased production of ER chaperones (13). This is followed by activation of ER-associated degradation (ERAD), which shunts misfolded proteins to the proteasome (14). In this way BiP monitors the folding status of proteins in the ER and regulates the UPR in response to ER stress.

Several human neurodegenerative diseases are associated with the accumulation of misfolded host proteins, although the specific role played by misfolded proteins in disease pathogenesis remains elusive. ER stress pathways and the subsequent cellular responses may provide a mechanistic connection between misfolded proteins and disease (11). For instance, ER stress can lead to oxidative stress (15, 16) as well as the initiation of cell death pathways (17). The effects of ER stress may participate in the pathogenesis of a number of degenerative diseases such as Parkinson’s (18), Huntington’s (19), Alzheimer’s (20), and amyotrophic lateral sclerosis (21), although the evidence is still circumstantial.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Rocky Mountain Laboratories, 903 S. 4th St., Hamilton, MT 59840. Tel.: 406-363-9339; Fax: 406-363-9286; E-mail: jportis@nih.gov.

The abbreviations used are: TSE, transmissible spongiform encephalopathy; ER, endoplasmic reticulum; RT-PCR, reverse transcriptase; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; UPR, unfolded protein response; MHC, major histocompatibility complex; ERAD, ER-associated degradation; SU, the surface glycoprotein; dpi, days post-infection; PrP, prion protein.
Given the importance of misfolded proteins in neurodegenerative diseases, and the possible link between ER stress and retrovirus-induced spongiform neurodegeneration (5), we investigated the virus-host interactions in NIH 3T3 cells that initiate this response. FrCasE not only up-regulated BiP RNA and protein expression in NIH 3T3 cells, but its envelope protein bound a greater amount of BiP for a longer time than the F43 envelope protein. The FrCasE envelope protein was found to be retained in the ER and degraded by the proteasome, whereas that of F43 was processed normally through the secretory pathway. Furthermore, the shunting of viral envelope protein to the degradative pathway resulted in its decreased levels both in NIH 3T3 cells and in the brains of FrCasE-infected mice. These results strongly suggest that binding of misfolded envelope protein to BiP is responsible for the transcriptional activation of the UPR in FrCasE-infected cells. Because signs of ER stress are an early event seen during the preclinical period of the FrCasE-induced neurodegenerative disease, the results presented here support the notion that this represents a virus-induced protein misfolding disease.

EXPERIMENTAL PROCEDURES

Cell Culture and Virus Infections—NIH 3T3 cells (ATCC CRL 1658) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum at 37°C in a 5% CO2 humidified incubator. Virus stocks were prepared in Mus dungi cells as described before (22), and titers of infectivity of each virus stock were determined on NIH 3T3 cells. Cells were infected, as described previously (22), in the presence of 8 μg/ml Polybrene at a multiplicity of infection of 2–5. This multiplicity of infection was found empirically to result in the expression of viral envelope protein at the cell surface of >95% of the cells in the culture within 24 h of infection. We found no further increase in viral DNA between 24 and 48 h post-infection suggesting that there was no detectable virus spread after the initial infection. The three viruses used in this study were FrCasE (22), F43 (6), and a new construct FrCasNC described herein. The FrCasNC construct was generated as described previously (6) for the F43 virus, except that NdeI to ClaI sites were used to insert the CasBrE envelope gene into the F43 retrovirus background (Fig. 2).

Real Time Quantitative Reverse Transcription-PCR—Total RNA samples were analyzed using Taqman real time RT-PCR (Applied Biosystems) as described previously (5). The RT-PCR products were performed in triplicate in individual RNA samples, and independent infections were repeated in triplicate. Expression values were normalized using glyceraldehyde-3-phosphate dehydrogenase. Primer and probe sequences used to amplify BiP, GADD153/CHOP, and virus RNA were reported previously (5). Statistical tests were performed on multiple independent infections using analysis of variance with a Tukey’s multiple-comparison post-test and a level of significance of <0.05.

Mice, Inoculations and Immunohistochemistry—Inbred Rocky Mountain White mice were bred and raised at the Rocky Mountain Laboratories Animal Care and Use Committee. Mice were inoculated with virus stocks prepared in M. dungi cells, as described previously (22), or for mock infections with tissue culture medium alone. Mice were inoculated intraperitoneally 24–48 h after birth with 30 μl of virus stocks containing 1.5–3.0 × 10^5 focus forming units of infectivity. Immunohistochemistry using goat antiserum to viral surface glycoprotein (SU) was performed as described previously (6).

Immunoprecipitation and Immunoblot Experiments—NIH 3T3 cells were infected, and 48 h later monolayers were washed twice with Dulbecco’s phosphate-buffered saline and lysed on ice for 10 min using the following lysis buffer: 0.5% Nonidet P-40, 0.5% deoxycholic acid, 50 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, and a protease inhibitor mixture (Sigma). Rabbit anti-BiP was used at a 1:1000 dilution and rabbit anti-p30 (capsid protein) (23) at a 1:1000 dilution. Primary antibodies were detected with species-specific horseradish peroxidase-conjugated antibodies (Bio-Rad) and visualized using the ECL system (Amersham Biosciences) on autoradiography film (LabScientific Inc.).

Metabolic Labeling and Pulse-Chase Experiments—For pulse-labeling experiments, after 48-h infections, cells were washed in warm phosphate-buffered salt, incubated for 30 min in methionine/cysteine-free medium, and then metabolically labeled with 200 μCi/ml [35S]methionine/cysteine for 15 min. Cells were then lysed with buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholic acid, and a protease inhibitor mixture and centrifuged at 6,000 × g for 2 min at 4°C. The supernatant was used for immunoprecipitations. For experiments investigating BiP binding, 10 units/ml aprotinin (Sigma) was added to lysis buffer. For pulse-chase analysis, after the 15-min pulse, the labeling medium was removed, and cells were washed and chased with complete medium supplemented with 2 mM methionine/cysteine at 37°C for various times and then lysed as above. For proteasome inhibitor experiments, cells were incubated in 50 μM MG132 for 2 h prior to methionine/cysteine wash out. During the pulse and chase periods, 50 μM MG132 was included in media. Immunoprecipitations were performed as above except prior to immunoprecipitation cell lysates were normalized to total trichloroacetic acid precipitable counts. Immunocomplexes were solubilized by boiling for 5 min in SDS sample buffer. SDS PAGE sample volume was resolved on 9% SDS-PAGE gels and, after drying, exposed to a phosphor screen (Amersham Biosciences). Bands were quantified using ImageQuant (Amersham Biosciences). For BiP inhibition pulse-chase experiments, the amount of envelope communoprecipitated was normalized to the amount of BiP at each time point prior to determining the half-lives. Data were fit to a nonlinear regression for one-phase exponential decay, and half-lives (t_1/2) were calculated from the equation for the best fit curves.

Immunofluorescence Microscopy—Cells were grown and infected on 12-mm glass coverslips, fixed in 3.7% formaldehyde, and permeabilized with 0.5% Triton X-100, all at room temperature. Cells were stained for 30 min with both primary and secondary antibodies. Viral envelope protein was detected with the same rabbit anti-SU protein used for viral protein studies, and calnexin was detected with goat anti-calnexin (Santa Cruz Biotechnology). The primary rabbit antibody was developed with AlexaFlour 488 chicken anti-rabbit IgG (Molecular Probes), and the primary goat antibody was detected with AlexaFlour 555 donkey anti-goat Ig (Molecular Probes). Both secondary antibodies were highly cross-absorbed to remove interspecies cross-reactive antibodies. Initial studies using conventional fluorescence microscopy (Nikon Microphot SA) were carried out to show that the secondary antibodies were specific for their respective Ig for colocalization studies, coverslips were also stained with the DNA dye DRAQ-5 to stain nuclei and were examined using a PerkinElmer Life Sciences UltraView LCI confocal microscope. Samples were observed with species-specific horseradish peroxidase-conjugated antibodies (Bio-Rad) and visualized using the ECL system (LabScientific Inc.).

Flow Cytometric Analysis—Suspensions of live cells were analyzed for surface expression using a FACStar flow cytometer (BD Biosciences) as described previously (24). For viral envelope protein, cells were stained with polyclonal rabbit anti-SU antiserum diluted 1:1000 and detected with AlexaFlour 488 anti-rabbit IgG (Molecular Probes) diluted 1:2000. For MHC class I and transferrin receptor, cells were stained with mouse monoclonal antibodies 30.5.75 (ATCC) and TIB-219 (ATCC) respectively, and detected with AlexaFlour 488 anti-mouse Ig. For quantification of total protein, cells were fixed with 3.7% formaldehyde and permeabilized with 0.1% saponin in Dulbecco’s phosphate-buffered saline prior to adding primary antibodies. Controls for specificity were performed by staining infected and uninfected cells with
ER Quality Control and Virus-induced Neurodegeneration

RESULTS

ER Stress Is Influenced by the Quality of CasBrE Envelope Protein—FrCasE induces the up-regulation of several markers of ER stress in the brain (5), including BiP, an ER chaperone, and CHOP, a Bzip transcription factor. We quantified these two transcripts in NIH 3T3 cells 48 h after infection, using real time RT-PCR. As reported previously (5), both genes were up-regulated 2–3-fold by FrCasE but not F43 (Fig. 1A). However, the difference in the BiP and CHOP responses paralleled a difference in viral RNA load, which was found to be 2–3-fold higher in FrCasE compared with F43 infection (Fig. 1A). This suggested that the ER stress might simply be a consequence of ER overload. To address this issue directly, we utilized another viral construct, FrCasNC, in which 216 nucleotides in the 3’ regulatory region were replaced with sequences from F43 (Fig. 2A). Both BiP and CHOP were up-regulated by FrCasNC, and both viruses induced comparable spongiform neuropathology (Fig. 2C). However, unlike FrCasE, the viral RNA load in FrCasNC-infected NIH 3T3 cells was comparable to that of F43 (Fig. 1B). Both BiP and CHOP were up-regulated by FrCasNC, and the magnitude of the effect was similar to that induced by FrCasE (Fig. 1B). Nevertheless, we monitored newly synthesized envelope protein to further establish that the ER load in FrCasNC and F43 infected cells was comparable (Fig. 1C). Forty-eight hours after infection, cells were pulsed for 15 min with [35S]methionine/cysteine, and cell lysates were immunoprecipitated with an antisera specific for viral surface glycoprotein (SU). After a 15-min pulse, the only radiolabeled protein detected by this antiserum was the envelope precursor polyprotein pr85env. The CasBrE envelope protein encoded by FrCasE and FrCasNC is slightly smaller than that of F43 because it contains one less glycosylation site and has two deletions of 4 and 7 amino acid residues. As expected, newly synthesized pr85env was 2-fold higher in FrCasE than F43-infected cells, and consistent with the viral RNA levels, the amount of FrCasNC was comparable with that of F43 (Fig. 1C). These results suggested that an intrinsic quality of the CasBrE envelope protein, present in both FrCasE and FrCasNC, was sufficient to induce the ER stress response.

The CasBrE Envelope Protein Is Retained in the ER—The envelope precursor polyprotein of these viruses (pr85env), which is synthesized in the ER, is cleaved by a cellular protease in the Golgi, generating the surface glycoprotein (SU, ~70 kDa) and the transmembrane fusion protein (~15 kDa) (25). We previously showed by immunoblot analysis that the ratio of pr85env to SU protein in cell lysates at steady-state was greater for FrCasE than F43, suggesting a delay in exit of the protein from the ER (5). To further examine this question, we used confocal fluorescence microscopy on fixed and permeabilized cells infected with FrCasE, FrCasNC, and F43 to compare the distribution of envelope protein (Fig. 3). The anti-SU antiserum used to stain viral envelope protein reacts with both the pr85env precursor polyprotein and the SU protein. Although the F43 envelope protein exhibited a largely vesicular staining pattern consistent with staining primarily cleaved, post-ER SU protein, the CasBrE envelope protein of FrCasE and FrCasNC had a reticular pattern that colocalized with the ER marker calnexin. These data suggest that the anti-SU antibody stained predominantly the ER-localized CasBrE pr85env precursor protein. There was very little colocalization of F43 envelope protein and calnexin (Fig. 3). These results support the notion that the CasBrE envelope precursor protein is retained in the ER relative to that of F43.

We next examined the possible role of BiP in the ER retention of this viral protein. BiP binds to exposed hydrophobic regions and retains client protein in the ER, where it assists in protein folding (26). Proteins that do not fold properly remain bound to BiP for prolonged periods. We performed coimmunoprecipitation studies using anti-KDEL (reactive with BiP and grp94) and anti-SU (reactive with pr85env and SU protein). Cell
lysates were split, one-half being subjected to immunoprecipitation (IP) and the other half being boiled directly in sample buffer (Total Extract). Both samples were subjected to immunoblot analysis (see Fig. 4). Immunoprecipitation with anti-KDEL coprecipitated a protein from the virus-infected cells that was reactive with anti-SU and was consistent in size with the respective pr\textsubscript{85}\textsuperscript{env} proteins (Fig. 4\textit{A}, compare IP to Total Extract). In addition, it was clear that the amount of pr\textsubscript{85}\textsuperscript{env} coimmunoprecipitated from lysates of FrCas E and FrCas NC-infected cells was greater than that from F43-infected cells. Immunoprecipitation with anti-SU (Fig. 4\textit{B}) resulted in the coprecipitation of a protein reactive with anti-KDEL that had a molecular mass of 78 kDa, consistent with that of BiP (Fig. 4\textit{B}, compare IP to Total Extract). Most interesting, another KDEL-containing ER chaperone (grp 94) was not coprecipitated with pr\textsubscript{85}\textsuperscript{env}. Finally, it is apparent from the anti-KDEL immunoblot of total extracts (Fig. 4\textit{B}) that BiP protein was up-regulated in FrCas\textsuperscript{E} and FrCas\textsuperscript{NC}-infected cells, consistent with the analyses of m\textit{RNA} levels (Fig. 1) (5).

Kinetic studies of BiP binding further suggested the folding instability of the CasBrE envelope protein in the ER. Pulse-chase analyses were carried out on FrCas\textsuperscript{E} and F43-infected cells, using anti-KDEL antibodies to coimmunoprecipitate BiP and pr\textsubscript{85}\textsuperscript{env} (Fig. 5\textit{A}). The amount of pr\textsubscript{85}\textsuperscript{env} coimmunoprecipitated at each time point was quantified and normalized to BiP. The ratios of pr\textsubscript{85}\textsuperscript{env} to BiP at each chase time were then compared with the ratio at time 0 and converted to a percentage of pr\textsubscript{85}\textsuperscript{env} remaining (Fig. 5\textit{B}). From these measurements, the half-lives of the BiP-Env interaction were estimated. The binding half-life for FrCas\textsuperscript{E} pr\textsubscript{85}\textsuperscript{env} to BiP was 2.5 h., whereas that for F43 was 1.0 h. Results were confirmed in another independent experiment examining several time points in the first h after the pulse. Taken together with results from the steady-state studies, it appears that the ER retention of the CasBrE envelope protein was a function of its prolonged binding to BiP, implying that this protein folds inefficiently in the ER compared with the F43 envelope protein.

FrCas\textsuperscript{E} Envelope Protein Is Subjected to Proteasomal Degradation—Proteins that do not fold properly in the ER are subjected to retrograde transport to the cytosol and degradation by the ubiquitin-proteasome system. Thus, after synthesis pr\textsubscript{85}\textsuperscript{env} exits the ER by vesicular transport to the Golgi for normal proteolytic processing or is shunted to the degradative pathway. It was of interest to determine the relative role of degradation in this process for the FrCas\textsuperscript{E} and F43 envelope proteins. Pulse-chase analysis was used to determine the overall rate of disappearance of the pr\textsubscript{85}\textsuperscript{env} precursor as a measure of the rate of exit of the viral envelope protein from the ER. Cell lysates were immunoprecipitated with anti-SU, and as expected the FrCas\textsuperscript{E} pr\textsubscript{85}\textsuperscript{env} protein decayed at a slower rate than that of F43 (t\textsubscript{1/2} of 2.2 \textit{versus} 1.2 h, respectively) (Fig. 6\textit{A}). In addition, the SU protein of F43 appeared earlier during the
Eukaryotic Protein Quality Control and Virus-induced Neurodegeneration

Differences in the intracellular distribution of the envelope proteins of FrCasE, FrCasNC, and F43. Cells were infected as in Fig. 1 and grown on glass coverslips, fixed with 3% formaldehyde, and permeabilized with 0.5% Triton X-100. Viral envelope protein was detected using rabbit anti-SU developed with AlexaFluor 488-conjugated secondary antibody (green), and calnexin was detected using goat anti-calnexin, developed with a secondary antibody conjugated with AlexaFluor 555 (red). Nuclei were stained with DRAQ-5 (blue). Shown are confocal slices from a Z series. Colocalization of envelope protein and calnexin is seen in the merged images (yellow).

Protein Misfolding Is Restricted to the CasBrE Envelope Protein—We considered the possibility that the ER stress response in FrCasE- and FrCasNC-infected cells was the result of a global perturbation of ER function leading to generalized protein misfolding in the ER. Conceivably, the virus infection could affect calcium stores, amino acid transport, or glycosylation systems in the ER that would secondarily perturb global ER protein folding. To address this issue we quantified the relative cell-surface expression of two other secretory proteins, MHC class I and transferrin receptor, in FrCasE and mock-infected cells (Fig. 8). Cells were either stained live to quantify cell-surface protein or fixed and permeabilized prior to staining to determine total cellular content (Fig. 8A). The latter technique was used to normalize the cell-surface staining (Fig. 8B). The cell-surface expression of neither of these proteins was down-regulated in the infected cells. We observed the same magnitude of up-regulation in F43-infected cells (not shown) suggesting that this likely represented the increased loading of viral peptides onto MHC class I molecules (29). This phenomenon has been observed as well in flaviviruses-infected cells (30). Thus, the ER retention and degradation of the CasBrE envelope protein encoded by FrCasE and FrCasNC was delayed in its exit from the ER and degraded, a portion of the protein was processed normally through the secretory pathway and reached the cell surface.

Evidence That ERAD Down-regulates Envelope Protein in the Brain Stem of FrCasE-infected Mice—To place these results in the context of the neurodegenerative disease caused by FrCasE, we compared the steady-state levels of FrCasE and F43 envelope proteins in the brain stems of infected mice using immunoblot analysis with anti-SU (Fig. 9). Clinical neurologic dis-
ease, initially manifested by tremor and weakness, is observed beginning at 14 days post-infection (dpi) and progresses to paralysis and wasting by 17 dpi (23). As seen in vitro in NIH 3T3 cells (Fig. 4A, Total Extract), the steady-state levels of FrCasE envelope protein in the brain stem (Fig. 9) were on the order of 3-6-fold lower than that of F43 at both time points.
Fig. 7. The FrCasE and FrCasNC envelope proteins reach the cell surface but at lower levels than that of F43. NIH 3T3 cells were infected with FrCasE, FrCasNC, F43, or mock, and 48 h later were stained live with anti-SU antiserum to stain envelope protein exposed at the plasma membrane. Cells were analyzed by flow cytometry. Geometric mean fluorescence intensities are as follows: Mock (52.0), FrCasE (89.3), FrCasNC (99.0), and F43 (293.7).

Fig. 8. Normal export of other secretory proteins to the cell surface in FrCasE-infected cells. NIH 3T3 cells infected for 48 h with FrCasE or mock-infected cells were stained for MHC class I and transferrin receptor (TfR). Cells were fixed and permeabilized prior to staining to determine the total content of the respective protein (A, Total) or were stained live to detect cell-surface protein (A, Surface). Cell-surface staining was then normalized to total staining to generate the bar graphs (B). Data shown are the average of three independent infections, and error bars represent one S.D.

Fig. 9. Level of viral envelope protein in the brainstem is lower in FrCasE than in F43-infected mice. Immunoblot analysis of 10% homogenates of brain stems from mice infected as neonates intraperitoneally with FrCasE or F43 are shown. Mice were sacrificed at 14 and 17 days post-inoculation and brain stems prepared as 10% homogenates. Lysates containing equal amounts of total protein were separated by electrophoresis, and after blotting, the membranes were cut in half horizontally. The upper half was probed with anti-SU and the lower half probed with anti-capsid protein (30-kDa protein). Molecular mass markers are shown on the left.

DISCUSSION

Murine retroviruses containing the envelope gene of CasBrE, a virus originally isolated from wild mice, cause a fatal spongiform neurodegenerative disease. Whereas neurovirulence is determined by the sequence of the envelope protein (1), the nature of its neurotoxicity has yet to be clarified. In wild mice, this is a chronic disease with an incubation period of 6–15 months (7), associated with progressive neuronal dropout and astrogliosis, but not with typical signs of inflammation seen in viral encephalitis. Indeed, these mice were found to be immunologically unresponsive to this virus (31). Thus, in several respects this conventional virus causes a disease that resembles the TSE diseases (32, 33) but without the accumulation of abnormal forms of the prion protein (PrP). We recently reported (5) that mice inoculated with a chimeric retrovirus FrCasE, which contains the CasBrE envelope gene and induces an acute form of this disease, exhibit a transcriptional program in the brain stem indicative of the UPR. Signs of ER stress were detected early during the preclinical period and were disease-specific, because ER stress was not observed in mice infected with the avirulent virus F43, which carries a different envelope gene. Furthermore, activation of the ER stress sensor PERK and its downstream target eIF2-α have recently been demonstrated in the brains and astrocytes of mice infected with another spongiform retrovirus (Moynkery murine leukemia virus ts1) supporting the idea that the UPR is involved in the pathogenesis of these diseases (34, 35).

Because FrCasE and F43 encode different envelope proteins, it was reasonable to assume that the source of UPR induced by
the former virus is related to the nature of the interaction of the CasBrE envelope protein with ER quality control systems. We found a number of signs that cumulatively present compelling evidence that folding of this protein in the ER is inefficient. Most important, BiP, a major ER chaperone that is involved in protein translocation into the ER during synthesis as well as protein folding and ER-associated degradation remained bound to the CasBrE envelope protein for a prolonged period. BiP binds to exposed hydrophobic sequences during the folding process, preventing protein aggregation. As seen for the F43 envelope protein, this binding is normally transient in nature. Prolonged association with BiP, although indirect, is a hallmark of protein misfolding (26). The excessive binding to BiP also probably accounts for the induction of the UPR observed in this system, because BiP is known to be displaced from the sensors of ER stress by the accumulation of misfolded client proteins (13). Next, the CasBrE envelope protein was delayed in its exit from the ER when compared with that of F43. This was manifested by a slower rate of disappearance of the envelope precursor protein pr85env and a slower appearance of the Golgi-dependent SU protein than that of F43. Furthermore, steady-state levels of FrCasE envelope protein were consistently lower than that of F43, both measured by cell-surface staining and by immunoblot analysis of whole cell lysates. This was the case both in cells infected with FrCasE, in which synthesis of the CasBrE envelope protein was comparable with that of F43, and in cells infected with FrCasE, in which it was 2–3-fold greater. Finally, lower steady-state levels of CasBrE envelope protein appear to be explained by higher rates of degradation relative to the envelope protein of F43. The proteasome inhibitor MG132 caused a dramatic slowing in the rate of disappearance of the CasBrE envelope precursor protein pr85env but had a negligible effect on that of F43. Although the F43 envelope protein appeared to fold efficiently and progressed through the secretory system to the cell surface, a larger fraction of the CasBrE envelope protein encountered ER quality control systems and was degraded prior to reaching the cell surface.

Because the murine retroviruses studied here use the cationic amino acid transporter (CAT-1) as their cell-surface receptor (36, 37), it is possible that the transport function of this molecule was compromised by the CasBrE envelope protein, leading to arginine/lysine deprivation. It is clear that nutrient or amino acid deprivation, even of a single amino acid, can activate the expression of some UPR genes such as CHOP (38, 39). Retrovirus-induced nutrient deprivation is not without precedent, because the human retrovirus human T-cell lymphotropic virus, which uses the glucose transporter GLUT-1 from the host as a receptor, has been shown to block glucose transport (40). It is unlikely, however, that amino acid deprivation and general ER dysfunction account for the ER retention of the CasBrE envelope protein, because we found no evidence for down-regulation of two other secretory proteins, MHC class I and transferrin receptor, at the cell surface. Furthermore, it has been shown that amino acid deprivation induces CHOP through a pathway independent of the UPR and BiP (41). Both in vivo and in vitro, infection of cells by FrCasE induces the up-regulation of both CHOP and BiP (5).

The studies discussed above were carried out in vitro in fibroblastic cells, and raise the question whether they are relevant to the virus-host interactions occurring in the brains of FrCasE-infected mice. Although the types of kinetic experiments carried out in NIH 3T3 cells are not feasible in the animal, the steady-state studies reported here strongly suggest that the CasBrE envelope protein encountered similar quality control systems in vivo. Thus, steady-state levels of the FrCasE envelope protein were found to be lower than that of F43 in the brain stems of infected mice. This observation per se appears counterintuitive, because one would expect pathogenicity, which is mechanistically linked to the viral envelope protein, to be directly and not inversely related to its level of expression in the brain. This result can be viewed, however, in the context of the protein misfolding and engagement of ERAD, which was observed in NIH 3T3 cells. As noted previously (23, 42), the level of the CasBrE envelope protein in the brain stem actually decreased between 14 and 17 dpi, the period of clinical progression of this neurodegenerative disease. This appeared to be a property specifically of the envelope protein as the cytosolic viral capsid protein remained at constant levels during the same period. In contrast, in the F43-infected mice, the viral envelope protein increased slightly and capsid proteins remained at constant levels in the brain stem during the same period. Combined with gene expression studies indicating evidence of ER stress in the brain stems of FrCasE-infected mice (5), these results appear to link protein misfolding, protein quality control systems, and ER stress with the neurodegenerative disease caused by FrCasE and support the notion that this represents a virus-induced protein misfolding disease.

A perplexing aspect of neurovirulence of retroviruses, including human immunodeficiency virus (43), is that the cells that degenerate (neurons and astroglia in the case of FrCasE) are not infected, indicating that the neurotoxicity of these viruses is mediated through indirect mechanisms (44). Two main cell types infected by FrCasE in the brain are microglia (brain macrophages) and microvascular endothelial cells (45), and infection of microglia is sufficient to cause neurodegeneration (46, 47).

In view of the apparent linkage of protein misfolding with this disease, one must entertain the possibility that protein aggregates could play a role. Although we have not explored this question in detail, immunofluorescence microscopy did not reveal evidence for aggregosome formation. In addition, preliminary centrifugation studies designed to separate large aggregates (i.e. 18,500 × g for 30 min) (48) have failed to show that such aggregates were present in FrCasE-infected cells within the time frames examined in the current study. Certainly this subject deserves further attention.

It is also of interest to consider possible mechanisms by which ER stress pathways might be indirectly involved in the neurotoxicity caused by this virus. First, although the UPR is an adaptive response, it appears that prolonged activation of the UPR by unrelieved ER stress can initiate cell death pathways. Previous gene expression data from FrCasE-infected mice demonstrated the up-regulation of several molecules involved in cell death pathways, including CHOP and the BH3-only Bel-2 family member DP5 (5), both of which have been shown to play a role specifically in ER stress-induced cell death (49, 50). Thus, it is possible that ER stress causes microglial cell death, and the loss of trophic support provided by these cells (51) could result in neuronal degeneration. Second, ER stress can induce oxidative stress (15, 16). Continued attempts to process misfolded proteins, including re-shuffling of disulfide bonds, may result in the depletion of reducing equivalents in the form of glutathione, leading to the accumulation of reactive oxygen species. In addition, it appears that elevated expression of CHOP, independent of protein misfolding, can lead to glutathione depletion (52). Thus, intracellular accumulation and extracellular release of reactive oxygen species by infected endothelial and microglial cells may damage both the infected cells and neighboring uninfected neurons. Although we have not yet found evidence of oxidative damage in FrCasE-infected mice, excessive protein oxidation has been observed in the brain in a spongiform neurodegenerative disease caused by a...
rat-adapted murine retrovirus (53). In addition, there is indirect evidence suggesting the importance of oxidative stress in two other retrovirus-induced neurodegenerative diseases, one caused by a Moloney murine leukemia virus ts1 (54) and also in human immunodeficiency virus-associated dementia (55). The source of the reactive oxygen species in these diseases, however, has not been clarified.

Our findings may have relevance to recent data regarding the mechanism by which TSE agents cause disease and may link murine retrovirus-induced spongiform lesions to TSE diseases at the molecular level. Hetz et al. (56) demonstrated the up-regulation of UPR markers in TSE infection and implicated ER stress pathways in cell death through activation of caspase-12 during the disease process. Although UPR molecular markers are common to these diseases, the underlying cause of the activation of the UPR does not seem to be the same. In the case of retrovirus-induced spongiosis, it appears that direct binding of BiP to the retrovirus envelope protein is responsible for the initiation of the UPR. The ER stress observed in scrapie infection appears to be caused by changes in ER calcium stores that lead to the activation of the UPR (56). In fact, there is no evidence for interaction between ER chaperones and PrPSc, the form of PrP associated with infectivity, or ER localization of this pathogenic PrP species. The implication of this is that during TSE disease, the ER stress response may be a secondary event. The scenario might be different for the inherited TSE diseases, as it is known that mutant PrP interacts with BiP (57), although it has not been determined whether the PrP-BiP interaction is associated with ER stress or the induction of a stereotypical UPR. It will be interesting to determine whether the activation of the UPR or the consequences of protein misfolding in the form of oxidative stress are responsible for the characteristic lesions seen in these diseases. Ultimately, it may not be the misfolded or aggregated proteins that directly induce neuronal degeneration but instead the deleterious pathways triggered by protein quality control systems.

Acknowledgments—We thank Drs. Bruce Chesebro, Kim Hasenkrag, Rachel LaCasse, and Suzette Priola for critical reading of this manuscript and Gary Hettrick and Anita Mora for assistance with figures. We also thank Ron Messer for technical assistance with flow cytometry and Dr. Olivia Steele-Mortimer for assistance with confocal microscopy.

REFERENCES
1. DesGroseillers, L., Barrette, M., and Jolicoeur, P. (1984) J. Virol. 52, 356–363
2. Masuda, M., Remington, M. P., Hoffman, P. M., and Ruscetti, S. K. (1992) J. Virol. 66, 2788–2806
3. Szurek, P. F., Yuen, P. H., Ball, J. K., and Wong, P. K. Y. (1990) J. Virol. 64, 467–475
4. Shikova, E., Lin, Y. C., Saha, K., Brooks, B. R., and Wong, P. K. Y. (1993) J. Virol. 67, 1137–1147
5. Dundcheff, D. E., Askovic, S., Baker, A. H., Johnson-Fowler, C., and Portis, J. L. (2003) J. Virol. 77, 12617–12629
6. Askovic, S., McAttee, F. J., Favara, C., and Portis, J. L. (2000) J. Virol. 74, 465–473
7. Gardner, M. B., Henderson, B. E., Officer, J. J., Rongey, R. W., Parker, J. C., Officer, J. E., Ihle, J. N., Stanley, A. G., Gardner, M. B., Henderson, B. E., Officer, J. E., Rongey, R. W., Parker, J. C., and Gray, F. (2003) J. Virol. 77, 1408–1414
8. Lynch, W. P., Czub, S., McAtee, F. J., Hayes, S. F., and Portis, J. L. (1991) FEBS Lett. 298, 299–305
