Regulated IRE1-dependent decay pathway is activated during Japanese encephalitis virus-induced unfolded protein response and benefits viral replication

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Japanese encephalitis virus (JEV) infection-induced encephalitis causes extensive death or long-term neurological damage, especially among children, in south and south-east Asia. Infection of mammalian cells has shown induction of an unfolded protein response (UPR), presumably leading to programmed cell death or apoptosis of the host cells. UPR, a cellular response to accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) lumen, is initiated by three ER-lumen-resident sensors (PERK, IRE1 and ATF6), and involves transcriptional and translational regulation of the expression of several genes. The sensor IRE1 possesses an intrinsic RNase activity, activated through homo-dimerization and autophosphorylation during UPR. Activated IRE1 performs cytoplasmic cleavage of Xbp1u transcripts, thus facilitating synthesis of XBP1S transcription factor, in addition to cleavage of a cohort of cellular transcripts, the latter initiating the regulated IRE1-dependent decay (RIDD) pathway. In this study, we report the initiation of the RIDD pathway in JEV-infected mouse neuroblastoma cells (Neuro2a) and its effect on viral infection. Activation of the RIDD pathway led to degradation of known mouse cell target transcripts without showing any effect on JEV RNA despite the fact that both when biochemically purified showed significant enrichment in ER membrane-enriched fractions. Additionally, inhibition of the IRE1 RNase activity by STF083010, a specific drug, diminished viral protein levels and reduced the titre of the virus produced from infected Neuro2a cells. The results present evidence for the first report of a beneficial effect of RIDD activation on the viral life cycle.

INTRODUCTION

Japanese encephalitis virus (JEV) infects a wide range of animals which serve either as a reservoir (pigs and horses) or disseminator (mosquito) of the virus. Transmission of JEV to human beings is solely through the saliva of an infected mosquito, injected during a blood meal. The encephalitic disease symptoms are visible after an incubation of 4–15 days of infection in 1:250 virus-infected individuals. JEV-induced encephalitis is responsible for ~10,000 deaths every year in addition to producing long-term neurological damage in one-third of the survivors. JEV neuropathogenesis is thought to be largely an outcome of the unregulated inflammatory response of the host neuronal immune system. In particular, an increased expression of IL-10 and reduced synthesis of IFN-γ, STAT1 and STAT2 correlate with better survival in mice (Biswas et al., 2010). However, damage to neuronal tissue is an outcome of host cell death by direct virus infection and that induced by the proinflammatory factors (Ghoshal et al., 2007; Swarup et al., 2007). JEV-infected cultured mammalian cells undergo apoptotic cell death, indicating this to be one of the potential mechanisms of neuronal tissue damage (Su et al., 2002).

JEV, belonging to the group of flaviviruses, is a close relative of many clinically important human pathogenic viruses, such as West Nile virus, Yellow fever virus, Dengue virus, St Louis encephalitis virus, Murray Valley encephalitis virus and tick-borne encephalitis virus. The enveloped virion particle contains a single-stranded positive-sense RNA ~11,000 nt long as the genome. The 5′-capped genomic RNA is without a poly(A) tail at the 3′ end and contains a single ORF flanked by UTRs at both termini. Translation of the viral RNA produces a single polypeptide that is cleaved by host-encoded and virus-encoded proteases into three structural (core, envelope (E) and prM) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Fernandez-Garcia et al., 2009; Rice, 1996). As in other flaviviruses, the JEV life cycle revolves around a close association with the endoplasmic reticulum (ER). The structural proteins E and prM along with the non-structural protein NS1 are modified by glycosylation and/or disulphide bond formation in the ER.
Further, a non-covalent complex of NS2B and NS3, with a critical role in viral replication, has been reported to associate closely with the rough ER membrane. Additionally, the viral genome is replicated by the NS5 RNA-dependent RNA polymerase in ER-associated vesicles (Fernandez-Garcia et al., 2009). Therefore, the host ER plays a very important role in replication of the viral genome and the maturation of virion particles.

In response to JEV infection an ER stress in the form of an unfolded protein response (UPR) is induced in host cells and is suggested to lead eventually to apoptotic death (Su et al., 2002). Induction of UPR after infection has also been observed with other flaviviruses, such as West Nile virus (Medigeshi et al., 2007), hepatitis C virus (Merquiol et al., 2011; Saeed et al., 2011) and Dengue virus (Peña & Harris, 2011; Umareddy et al., 2007). The UPR, which is sensed by three ER membrane-resident sensors, PERK, IRE1 and ATF6, is induced by accumulation of unfolded/misfolded protein in the ER lumen (Hetz, 2012; Schröder & Kaufman, 2005). Stress sensing by the IRE1 sensor leads to homomultimerization followed by autophosphorylation and activation of an RNase function, which performs cytoplasmic splicing of Xbp1u transcripts producing Xbp1s (Yoshida et al., 2001). Translation of Xbp1s leads to synthesis of XBP1S, a transcription factor responsible for activating the expression of many genes that are involved in folding, maturation and degradation of proteins in the ER (Lee et al., 2003). In addition to splicing Xbp1u mRNA, activated IRE1 cleaves a cohort of mRNAs, encoding polypeptides destined for cotranslational migration into the ER lumen. This selective degradation pathway has been termed the regulated IRE1-dependent decay (RIDD) pathway (Hollien & Weissman, 2006). The internally cleaved mRNAs are rapidly degraded through activity of XRN1 and Skil-3-8 complex which are responsible for degradation of cytoplasmic RNA through cleavage in a 5′→3′- and 3′→5′-dependent manner, respectively (Hollien & Weissman, 2006). This cleavage reduces the flux of polypeptides that are not necessary for ER functions and mostly destined for the plasma membrane or extracellular secretion (Hollien & Weissman, 2006). JEV infection has been shown to activate IRE1-mediated splicing of Xbp1u transcripts which influences survival of the host cells without showing any effect on viral life cycle (Yu et al., 2006).

In this report, we show activation of the RIDD cleavage pathway followed by degradation of known host gene transcripts in JEV-infected Neuro2a cells upon UPR induction. The endogenous RIDD target transcripts co-purified with the ER-enriched fractions in JEV-infected Neuro2a cells fractionated by biochemical methods. JEV RNA which is also associated with the ER membrane for a major part of the infectious cycle was, however, found to be unaffected by RNA cleavage activity of activated IRE1. Furthermore, an inhibition of this pathway diminished the steady-state level of multiple viral proteins and significantly reduced the JEV titre in the infected cell supernatant, suggesting a beneficial role for the RIDD pathway in viral RNA translation and thereby in replication of the virus.

**RESULTS**

**RIDD pathway is activated during JEV infection-induced UPR**

The RNase activity of IRE1 performing cytoplasmic splicing of Xbp1u mRNA has been previously characterized extensively in the context of JEV infection of mammalian cells (Yu et al., 2006). However, any role in JEV-infected cells of the more recently discovered activity of IRE1 in initiating the RIDD pathway is not yet known yet. Hollien et al. (2009) characterized a number of gene transcripts as valid targets of IRE1 cleavage in response to drug-induced UPR, which can serve potentially as markers of activation for this pathway. In order to investigate a potential activation of the RIDD pathway in JEV-infected cells, the relative transcript level of a few known targets were compared between mock- and JEV-infected Neuro2a cells by quantitative PCR (qPCR) using total RNA isolated 24 h post-infection (p.i.) (Fig. 1a). The results showed a significant downregulation of all the marker transcripts to a similar degree as observed by Hollien et al. (2009). In order to confirm that the downregulation observed is due to the UPR-inducing effect of JEV infection, a similar comparison for these transcripts was performed between Neuro2a cells treated with either DMSO (vehicle) or thapsigargin (Tg), a pharmacological inducer of UPR. The results showed that indeed in Neuro2a cells the transcripts from these genes are downregulated by induction of UPR even in the absence of a virus infection (Fig. 1b).

**Targets of RIDD activity and JEV genomic RNA are bound to the ER membrane**

The RIDD pathway reduces the flux of newly synthesized polypeptides destined for the ER lumen, in order to relieve the protein folding burden and thereby facilitate homeostasis (Hollien & Weissman, 2006). It is known that polypeptides which are co-translationally transferred into the ER lumen are translated on ER-bound ribosomes (Blobel & Dobberstein, 1975a, b). Since multiple JEV proteins mature in the ER lumen, it is possible that the infecting JEV RNA would associate with the ER membrane for translation and thereby be susceptible to IRE1 cleavage upon UPR induction (Rice, 1996). In this context it is important to note that Xbp1u transcripts can be biochemically purified along with the ER membrane (Yanagitani et al., 2009). In order to investigate a potential enrichment of the RIDD target transcripts mentioned above and the JEV genomic RNA on the ER membrane, a biochemical fractionation of JEV-infected Neuro2a cells was performed, 1 h after addition of the inoculum, using lysis buffer supplemented with digitonin. Digitonin disrupts membranes that are rich in cholesterol and other β-hydroxysteroids by inducing the formation of pores in them. Therefore, the cholesterol-rich plasma membrane of the cell can be disrupted leaving membranes like that of the ER intact (Holden & Horton, 2009; Lerner et al., 2003). The
fractionation was performed 1 h after addition of inoculum because at this time point the predominant signal would be expected to be from the infecting positive-strand JEV RNA instead of the same formed by replication in the infected host cells. Using total RNA isolated from the input (20 % of lysate), ER-enriched and cytoplasm-enriched fractions, reverse transcription followed by comparative qPCR was performed for the RIDD target transcripts and JEV RNA along with transcripts that are known to associate or not associate with the ER membrane (Chen et al., 2011). As shown in Fig. 2, similar to earlier reports, the transcripts corresponding to Hspa5 and Lamp1 showed predominant enrichment into in ER fractions compared with Actb, Lap3, Actb and Ap3ml which exhibited either an equal or a preferential distribution into the cytoplasmic fraction (Fig. 2). In this context, it was interesting to note that all the RIDD targets (with the exception of Bloc1s1) and JEV RNA exhibited a significant enrichment into the ER fractions (Fig. 2). This suggested that the JEV RNA, which purifies in the same biochemical subcellular fraction as the RIDD targets, is a potential target for cleavage by activated IRE1, thereby limiting viral growth in infected cells. Interestingly, the level of those transcripts that showed limited or no enrichment on the ER membrane (i.e. Actb, Lap3 and Ap3ml) was not negatively regulated by JEV infection or Tg treatment (data not shown).

**Fig. 1.** Targets of the RIDD pathway are downregulated during UPR in Neuro2a cells. Total RNA isolated from Neuro2a cells (a) mock-infected (white bars) or JEV-infected (grey bars) at an m.o.i. of 5 or (b) treated with either 0.1 % DMSO (white bars) or 1 μM Tg (grey bars) for 6 h, was reverse transcribed and analysed by qPCR for the transcript levels of indicated genes normalized to that of Rpl19. The relative level of transcripts in control samples was taken arbitrarily as 1 and that in the others represented as a fraction of that. The level of transcripts in the experimental and the control samples was compared using Student’s t-test; *P<0.05, **P<0.01 and ***P<0.001.

**Inhibition of IRE1 RNase activity rescues endogenous targets but does not affect JEV RNA level**

The RNase activity of IRE1 can be specifically inhibited by a small-molecule inhibitor STF083010 (STF) in a dose-dependent manner (Papandreou et al., 2011). As observed by Papandreou et al. (2011), simultaneous addition of STF and Tg was able to block the Tg-induced splicing of Xbp1u transcripts through IRE1 activation in a dose-dependent manner (Fig. 3a). To prove conclusively downregulation of the potential RIDD target transcripts in Neuro2a cells to be solely through IRE1 cleavage, the relative level of these transcripts was compared in total RNA isolated from Neuro2a cells that had been treated with either DMSO alone, Tg alone or Tg with STF. The results showed a distinct and significant rescue of the Tg-induced downregulation observed among the potential RIDD target transcripts by co-administration of STF (Fig. 3b). Subsequently, a similar analysis was performed for these transcripts using total RNA from cells that were mock-infected or JEV-infected and incubated in growth media supplemented with either DMSO or STF. As observed earlier, the analysis showed a similar rescue of the relative level of the transcripts (with the exception of St3gal5) in JEV-infected cells when compared with that in mock-infected cells by the addition of STF (Fig. 3c). The cytoplasmic splicing of Xbp1u to Xbp1s by IRE1 is followed by translation of the latter transcripts to synthesize XBP1S, a transcription factor that trans-activates the expression of a cohort of genes including Dnaic3. In fact, Dnaic3 transcription has been shown to be exclusively activated by XBP1S, without mediation by any other transcription factors, such as ATF4, which are also upregulated during the UPR (Lee et al., 2003). Therefore, to further confirm inhibition of the IRE1 cleavage activity, relative transcript levels of Dnaic3 and Atf3 (a target of ATF4 transcription factor) were estimated by qPCR 24 h p.i. in Neuro2a cells which had been either mock-infected or JEV-infected and maintained in the presence of DMSO or STF. As expected,
the result showed an upregulation of both Dnajc3 and Atf3 transcripts upon JEV infection, with STF treatment reversing the level of only the former to that in mock-infected cells (Fig. 3d). If the JEV RNA is also targeted by IRE1 in a manner similar to the endogenous RIDD target transcripts, an inhibition by STF would be expected to increase the relative level of this RNA. To investigate such a potential regulation, the JEV RNA level in infected cells maintained in DMSO or STF for up to 24 h p.i. was visualized by Northern blotting. The genomic RNA of many flaviviruses has been shown to produce a small (~0.5 kb) RNA corresponding to the 3′UTR, derived through partial digestion by the host exonuclease XRN1 (Pijlman et al., 2008). However, the result of the Northern blotting showed no change in the level of JEV positive-strand RNA (genomic RNA) or subgenomic flavivirus RNA (sfRNA), suggesting the RNA to be refractory to IRE1 cleavage (Fig. 3e). Additionally, a qPCR-based estimation of the JEV RNA also showed similar results (data not shown). Since the activation of IRE1, as evidenced by Xbp1u splicing, could be detected only ~12 h p.i. it is possible that by this time point the JEV RNA is protected within replication complexes which form in ER vesicles (Yu et al., 2006). Therefore, in order to activate IRE1 immediately after infection, Neuro2a cells were allowed to be infected by JEV and treated immediately with either DMSO or Tg and the total RNA isolated at 1, 2 or 3 h after drug addition. The relative level of the JEV RNA or Dnajc3 or Pbxip1 transcripts was compared between DMSO- and Tg-treated cells at each time point by qPCR, and the results plotted. Comparison of the fold change in transcript level showed evidence of IRE1 activation at 2 and 3 h after Tg addition through a significant downregulation of the Pbxip1 transcripts and upregulation of the Dnajc3 transcripts, without any change in the level of JEV RNA at these time points (Fig. 3f).

**Inhibition of IRE1 RNase activity in infected cells reduces JEV titre in culture supernatant**

RNA interference-mediated silencing of Xbp1u in JEV infection has been shown to negatively affect host cell survival without any effect on viral replication (Yu et al., 2006). In order to investigate whether inhibition of the IRE1 RNase activity would show a similar trend, viral titres in culture supernatants from Neuro2a cells infected with JEV and subsequently incubated with either DMSO or STF were determined by p.f.u. assay. The results showed a distinct and significant reduction in the titre of JEV produced from cells infected at all the tested m.o.i. upon addition of STF (Fig. 4a). To ascertain that this inhibitory effect on virus titre was not due to cytotoxic effects of the drug, a cytotoxicity assay was performed for different concentrations of the drug on infected cells for 24 h p.i. The result did not indicate a toxic effect of STF as compared with DMSO at all the concentrations tested (Fig. 4b). Similarly, no toxic effect of these drug concentrations compared with DMSO was observed in uninfected Neuro2a and mouse embryonic fibroblasts (data not shown). Since STF did not show any negative effect on the accumulation of JEV positive-strand genomic RNA we assumed that the observed reduction in viral titre is due to an effect on viral protein synthesis. Indeed, a comparison between infected Neuro2a cells treated with either DMSO...
or STF showed a distinct reduction in the level of non-structural protein NS1, NS3 and NS5 (Fig. 4c).

DISCUSSION

As part of the UPR homeostatic response, synthesis of polypeptides that are destined for translocation into the ER lumen is attenuated by phosphorylation of eIF2α by PERK and cleavage of a cohort of coding RNA transcripts by IRE1. The positive-sense genomic RNA of JEV initiates translation by a cap-dependent method, encoding many proteins destined for the ER lumen, and therefore could be a potential target of cleavage by IRE1. Our results show activation of the RIDD pathway and cleavage of known host gene target transcripts without any effect on the JEV RNA, which interestingly, however, shows a similar subcellular localization as observed through biochemical fractionation. Further, replication of JEV is significantly attenuated by drug-mediated inhibition of this RNase activity. Analysis of viral proteins showed a drastic reduction in the steady-state level of multiple non-structural proteins without any effect on accumulation of the positive-sense genomic RNA, indicating a negative effect on viral RNA translation. The results of this report indicate a beneficial effect of the RIDD mRNA degradation pathway executed by the UPR sensor IRE1 on the JEV life cycle through an influence on viral protein synthesis.

Cellular transcripts encoding polypeptides that co-translationally migrate into the ER lumen for their maturation are translated by ribosomes on the cytoplasmic face of the ER membrane. Among these, those coding proteins which are not required for functioning of the ER are particularly susceptible to cleavage by IRE1 (Hollien & Weissman, 2006). Interestingly, the coding region of the mRNA has been shown to have a role in marking transcripts for cleavage (Hollien & Weissman, 2006). Since the nature of such signals is not yet clear, it is difficult to predict the susceptibility of JEV RNA to IRE1 cleavage. However, we did not detect any cleavage of JEV RNA in our experiments, which would suggest either the absence of such ‘targeting’ signals or the protection of viral RNA by an as-yet undefined mechanism. This suggestion is strengthened by observation of the effect of drug-mediated UPR on JEV RNA at early time points post-infection. Whilst degradation of Pbxip1 through direct cleavage by IRE1 and upregulation of Dnajc3 transcripts through activity of XBPS were observed, no effect was observed on the viral genomic RNA. It is, however, possible that the difference in susceptibility to IRE1 cleavage between the cellular transcripts and viral genomic RNA rests on the fact that whilst there is stringent regulation of transcription of the former, the latter is generated constantly or replenished by virus-encoded polymerase. Interestingly, through an unknown mechanism, the downregulation of Stxgal5 transcript could be rescued in the case of Tg-induced but not in infection-induced UPR.

The mechanism for the reduction in viral titre upon inhibition of IRE1 cleavage activity would potentially be mediated through abrogation of either Xbp1u splicing or the cleavage of one or more RIDD targets. Yu et al. (2006) demonstrated convincingly the absence of any potential benefit of XBP1 for the JEV life cycle (Yu et al., 2006), suggesting the attenuating effect of IRE1 inhibition is not due to an abrogation of Xbp1u splicing. As part of the cross-talk between UPR signalling pathways, the sensor ATF6 has been shown to trans-activate expression of the Xbp1 gene, generating more Xbp1u mRNAs for cleavage by IRE1 (Yoshida et al., 2001). This indicates that drug-mediated inhibition of IRE1 RNase activity would lead to an accumulation of Xbp1u transcripts and a potential overexpression of XBP1U. Interestingly, XBP1U is known to interact with XBPS and inhibit its trans-activation of transcription from the Nos2 gene (inducible nitric oxide synthase) (Guo et al., 2010). Overexpression of Nos2 in host cells is observed after infection by a wide range of viruses, although we did not observe any deregulation of the Nos2 transcript in Neuro2a cells after JEV infection (data not shown) (Akaike & Maeda, 2000). Therefore, it is not yet clear whether the impaired overexpression of Nos2 is responsible for the observed effect of STF on the JEV titre. It is also possible that in the cohort of transcripts that are cleaved by the RIDD pathway there exists one or multiple species which encode potential antiviral proteins and stabilization of these mRNAs through inhibition of IRE1 RNase activity attenuates the virus titre produced from infected cells. An ~0.5 kb fragment of the viral genomic RNA, derived from the 3′ UTR of the positive strand and termed sfRNA, has been shown to accumulate in cells infected by different members of the family Flaviviridae, including JEV (Lin et al., 2004; Scherbik et al., 2006; Urosevic et al., 1997). The generation of this sfRNA has been shown to have a strong correlation with the cytotoxicity of the viral infection in addition to having an effect on virus growth (Pijlman et al., 2008). However, in this report we did not observe any impedance to sfRNA generation upon STF treatment. Interestingly, sfRNA has been shown to be generated through cleavage of the positive-sense RNA by the cellular 5′→3′ exonuclease XRN1 (Pijlman et al., 2008). Since the JEV fragment RNA has been observed to be enriched in P-bodies, a subcellular site for RNA degradation, it has been speculated that the 5′-capped viral RNA is de-capped by the cellular de-capping enzyme DCP1/2 and digested subsequently by XRN1 (Pijlman et al., 2008; Sheth & Parker, 2003). However, it is still not clear if DCP1/2 is the only host factor which initiates the XRN1-mediated degradation of JEV genomic RNA. Nonetheless, speculation about an endonucleolytic cleavage of the viral RNA by activated IRE1 followed by XRN1 digestion to generate the JEV sfRNA is nullified by the lack of any effect on the sfRNA level upon STF treatment of infected cells.

The 5′-capped genomic RNA of flaviviruses is known to initiate protein synthesis through a canonical cap-dependent mechanism. As part of the host antiviral defence mechanism the dsRNA-activated PKR (EIF2AK2) is activated upon interaction with viral double-stranded replication intermediates and phosphorylates EIF2S1,
Fig. 3. Effect of IRE1 inhibitor upon degradation of RIDD targets and JEV genomic RNA. (a) Total RNA isolated from cells either untreated (lanes 1 and 2), treated with Tg only (lanes 3 and 4), treated with Tg and 50 µM STF (lanes 5 and 6) or treated with Tg and 100 µM STF (lanes 7 and 8) was reverse transcribed and used for PCR amplification of Xbp1 transcripts. The undigested (U) PCR products (lanes 1, 3, 5 and 7) or those digested (D) with Pst I (lanes 2, 4, 6 and 8) were resolved on an agarose gel. The numbers on the right indicate the size of the undigested and Pst I-digested products and those on the left indicate relative migration of DNA size markers (M). (b) Total RNA isolated from Neuro2a cells, 6 h after addition of complete medium supplemented with DMSO alone (white bars), Tg alone (grey bars) or Tg supplemented with STF (black bars), were reverse transcribed and analysed by qPCR for relative transcript levels of the indicated genes normalized to that of Rpl19. The relative level of transcripts in DMSO-treated cells was taken arbitrarily as 1 and that in others represented as (continued)
which reduces drastically the rate of protein synthesis on almost all mRNAs. However, host cells also encode DNAJC3 or p58IPK, which is transcriptionally activated by XBP1 and inhibits this phosphorylation activity of EIF2AK2. In fact, the class of molecules represented by DNAJC3 have been termed by Goodman et al. (2009) as the ‘cellular inhibitors of the host defence system’. In our study we observed a reversal of the transcriptional upregulation of DNAJC3 during JEV-induced UPR, by STF, indicating a requirement for this host factor for optimal synthesis of viral protein. This report adds another example of how viruses have evolved to exploit the host cellular processes while simultaneously evading the antiviral defence arsenals (Ambrose & Mackenzie, 2011; Burnett et al., 2012).

**METHODS**

**Cell lines, virus culture and titration.** Mouse neuroblastoma (Neuro2a) and porcine stable kidney (PS) cells were maintained in Dulbecco’s modified Eagle’s medium and minimum essential medium, respectively, supplemented with 10% FBS (Invitrogen), 2 mM glutamine, Dulbecco’s modified Eagle’s medium and minimum essential medium, (Neuro2a) cells and porcine stable kidney (PS) cells were maintained in respect to that of Rp19. The relative level of transcripts in mock-infected sample was taken arbitrarily as 1 and that in JEV-infected samples plotted as fraction of that. The transcript levels under different treatment conditions were compared using Student’s t-test; **P**<0.05 and **P**<0.01.

**Reverse transcription and quantitative PCR.** Reverse transcription was performed using random hexamers in the Superscript III RNase inhibitor containing reaction mix (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR using SYBR Green for detection was performed using 2 × Fast SYBR mix (ABI) and that using TaqMan probes with 2× Fast TaqMan mix (ABI). The sequences of qPCR primers for RDR target genes were: Hgsnat1- F (5’-CTGTGACGTCAACAGGGCTG-3’), Col6a1- F (5’-CCCATAGGTTGGTGTTAGCTC-3’), Sst3gal5- F (5’-ATGCCAAATGTCTGCCTCTC-3’), Sst3gal5- R (5’-ACTCAGAAAAATGGACCCACGATG-3’); Pbxip1- F (5’-AGATCCTGAAATTAGCTGGTGGTAC-3’), Pbxip1- R (5’-TGTCAGAACAGCATTTCTC-3’); Rp19- F (5’-ATGGAATGCTCGTACAGAG-3’), Rp19- R (5’-GCATTGGC-GATTTCGCGT-3’). The sequence of the TaqMan probe and oligonucleotide primers used for detection of JEV RNA was adapted from earlier reports (Huang et al., 2004). All qPCR experiments were performed on an ABI 7500 Fast qPCR instrument according to the manufacturer’s recommendations.

**Fractionation of Neuro2a into ER and cytosolic fractions.** Neuro2a cells (1 × 10⁶) were split into each 6 cm dish, and incubated overnight at 37 °C and 5% CO₂. The day after, cells were treated using PBS and infected with JEV at an m.o.i. of 1. At h p.i., the cells were sequentially washed using ice-cold PBS, ice-cold acetate buffer (pH 5.5) and ice-cold PBS. The cells were then lysed on ice by addition of 0.5 ml ice-cold lysis buffer (150 mM potassium acetate, 20 mM K-HEPES (pH 7.5), 2.5 mM magnesium acetate, 2 mM DTT, 200 U RNase inhibitor ml⁻¹ (Promega) and EDTA-free protease inhibitor cocktail (Roche)) supplemented with 40 μg digitonin ml⁻¹ (Sigma) followed by scraping with a cell scraper. The extract was transferred to a microcentrifuge tube and incubated on ice for 15 min for complete lysis; 20% of the lysate was saved as input and the rest centrifuged at 500 g for 5 min at 4 °C. The supernatant (S1) was transferred to a new tube and the pellet (P1) washed three times using ice-cold lysis buffer without digitonin to obtain pellet P2, which was saved as the ER-enriched fraction. The supernatant was centrifuged at 7500 g for 10 min at 4 °C and the supernatant saved as S3 or the cytoplasmic fraction. Total RNA from the input or P2 or S3 fraction was isolated using QIAzol reagent (Qiagen) and RNeasy Mini Kit (Qiagen) with in-column DNase digestion, according to the manufacturer’s instructions.

**Assay for cytoplasmic splicing of Xbp1u transcripts.** The Xbp1u splicing assay was performed as described previously (Calfon et al., 2002). Total RNA was reverse transcribed using random hexamers, and the cDNA used for nested PCR amplification of Xbp1 transcripts (both unspliced and spliced) using the forward primer Xbp1- F (5’-AAAACAGGATAGCACGCGCAACTG-3’) and Xbp1- R (5’-TCCTCTGCGTGATACCTTGGAAG-3’). The PCR product was purified, digested with the restriction enzyme PstI (NEB) and the digested product along with the corresponding undigested ampiclon resolved in 1.8% agarose gel.
Northern blotting. Total RNA (1 μg) was denatured and resolved in formamide/1 % agarose gel, and blotted onto nylon membranes (Millipore). The RNA was cross-linked on the membrane by UV light and probed with in vitro-transcribed DIG-labelled riboprobe corresponding to the anti-sense strand of JEV 3’ UTR RNA using the DIG Northern kit (Roche). The actin probe supplied as part of the kit was used for the purpose of normalization.

Cytotoxicity assay. The cell survival assay was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based In Vitro Toxicology Assay Kit according to the manufacturer’s instructions (Sigma). Cells incubated with increasing concentration of DMSO or STF were treated with MTT and absorbance at 570 nm measured.

Immunoblotting assay. Equal quantities of total protein extracted from mock-infected or JEV-infected cells were resolved by SDS-PAGE, blotted onto nitrocellulose membranes and probed with primary antibodies against the JEV proteins NS1 or NS3 or NS5 or the loading control glyceraldehyde 3-phosphate dehydrogenase, and subsequently by HRP-conjugated secondary antibodies. The bands were visualized by chemiluminescence using Western Blotting Luminol Reagent (Santa Cruz).

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr Manjula Kalia and Dr Arup Banerjee for critical reading of the manuscript. The antibodies against JEV proteins NS1 and NS3 were developed jointly by Vishal Gupta and Dr Manpreet Kaur in our group. The anti-NS5 antibody was a generous gift from Dr R.-Y. Chang, Taiwan. The research work was supported by the Department of Biotechnology, Government of India (grant BT/MB/01/VIDRC/08) to S.V.

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