

Article

ZIKA Virus Envelope Protein Forms Disulfide Bond-Dependent Polymers: Evidence for A Redox Imbalance during Infection

Grégoire Lebeau¹, Jonathan Turpin¹, Etienne Frumence^{1,a}, Daed El Safadi¹, Wissal Harrabi¹, Philippe Desprès¹, Pascale Krejbich-Trotot^{1,*} and Wildriss Viranaicken^{1*}

¹PIMIT, Processus Infectieux en Milieu Insulaire Tropical, Université de La Réunion, INSERM UMR 1187, CNRS 9192, IRD 249, Plateforme CYROI, 97490 Sainte-Clotilde, Ile de La Réunion, France

^a Present address : Laboratoire de Microbiologie - CNR Arbovirus CHU de la Réunion - site Félix Guyon

* Correspondence: wildriss.viranaicken@univ-reunion.fr; pascale.krebich@univ-reunion.fr

Abstract: Flaviviruses replicate in membrane factories associated with the endoplasmic reticulum (ER). Significant levels of flavivirus viral protein accumulation contribute to ER stress. As a consequence, the host cell exhibits an Unfolded Protein Response (UPR), subsequently stimulating appropriate cellular responses such as adaptation, autophagy or apoptosis. The correct redox conditions of this compartment are essential to form native disulfide bonds in proteins. ZIKA virus (ZIKV) has ability to induce persistent ER stress leading to activation of UPR pathways. In this study, we wondered whether ZIKV affects the redox balance and consequently the oxidative protein folding in the ER. We found that ZIKV replication influences redox state leading to aggregation of viral envelope protein as amyloid-like structures in the infected cells.

Keywords: Zika virus; unfolded protein response; ER stress; oligomer; disulfide bond, amyloid aggregates

1. Introduction

Zika virus (ZIKV) is a pathogenic single-stranded RNA virus belonging to the *Flaviviridae* family together with Yellow Fever virus (YFV), Dengue virus (DENV), West Nile virus (WNV) and Japanese Encephalitis virus (JEV). Among the pathogenic *flaviviruses*, ZIKV has gained notoriety in the last ten years, due to several outbreaks and serious clinical concerns. Notably, neurological complications have been described, including Guillain-Barré syndrome (GBS) and congenital malformations (Congenital Zika Syndrome – CZS), prompting specific vigilance for pregnant women in case of ZIKV epidemic [1,2]. Otherwise, the ability of ZIKV to be transmitted sexually alongside to vector-borne transmission, and the evidence of its persistence in some tissues in contrary of other *flaviviruses*, raised the interest on specific ZIKV-host interactions. This has led to a growing number of studies to decipher these interactions at the cellular and molecular levels. Similar to other *flaviviruses*, ZIKV replication occurs in Endoplasmic Reticulum (ER) invaginations of infected cells and induces an accumulation of viral proteins [3–5]. Genomic RNA translation leads to the viral polyprotein incorporation in the ER membrane, followed by its proteolytic cleavage. Translation of the polyprotein, initially enclosed through the ER membrane, results in three structural (C, prM, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS) proteins. Six of these proteins remain membrane-bound, as do the prM and E proteins that constitute the future viral envelope.

In addition to its key roles in cell physiology and homeostasis, the ER is an essential cellular component in the execution of the viral cycle. The main function of the ER is the regulation of folding and post-translational modifications of neo-synthesised proteins. Quality control of folding by the ER determines the outcome of proteins destined for centrifugal transport and the secretory pathway. Indeed, protein folding involves N-glycosylation, an oxidative environment favorable to disulfide bridge formation in the ER lumen, and the presence of several Ca²⁺-dependent molecular chaperones (*e.g.*, calreticulin,

GRP78/BiP and GRP94) [6,7]. The latter are in particular involved in the stabilization of protein folding intermediates. ER homeostasis can be disrupted by many factors like glucose deprivation, hypoxia, calcium imbalance or protein accumulation [8]. The resulting ER stress causes cellular adaptive responses, such as the Unfolded Protein Response (UPR) which is activated in an attempt to restore ER homeostasis.

Upon virus infection, the sudden and massive influx of ER-processed viral proteins typically results in ER stress and UPR in infected cells. Such cell responses contribute to several modes of host defense, *i.e.*, antiviral programs, immune responses [9,10] and commitment in autophagy or cell suicide [11,12]. The crosstalk between ER stress, UPR, autophagy and apoptosis are particularly important during infection. Each of these responses can affect cellular homeostasis and consequently the efficiency of viral replication and spread.

The ability of ZIKV to induce UPR in response to ER stress in several cell types has been previously studied [13–19]. The infection was accompanied by morphological changes in cell organelles, including remodeling of the ER into a swollen shape [20,21]. As aforementioned, during viral infection, ER stress is usually the result of an increasing amount of viral proteins to be processed by the ER. This influx of proteins disrupting ER homeostasis is likely to trigger UPR and a translational shutdown which will have negative effects on the replicating virus. We have previously shown that ZIKV is able to hijack the ATF6 and CHOP pathways during UPR [18,19]. This control by ZIKV results in persistent unresolved ER stress and delayed induction of cell death, which benefit the virus and may even promote its long-term persistence in the body [22].

Data from the literature indicate that persistent ER stress is associated with a loss of glutathione activity which in turns provokes an imbalance in redox homeostasis [23]. Indeed, the ER provides a relatively oxidizing thiol-disulfide environment suitable for the oxidative folding of co-translationally translocated proteins. The stability of the thiol-disulfide system in the ER relies on the glutathione redox pair, which acts as a powerful guardian of the redox balance. Thus, the ratio of the glutathione reduced form (GSH) to the oxidized glutathione disulfide form (GSSG) in the ER, is an indicator of the redox homeostasis [24]. It should be noted that the specific redox conditions in the ER allow the formation of disulfide bonds within the neo-synthesized polypeptides. These covalent bonds are necessary for the functional three-dimensional configurations of proteins in progress in the ER. Due to the importance of an oxidative environment for the correct folding of proteins [25], a redox imbalance could be a trigger for protein misfolding and formation of non-native disulfide bonds during ER stress [26].

We therefore wondered whether, during ZIKV infection, persistent ER stress might be related to an imbalance of the redox status. We questioned to what extent this would contribute to the accumulation of misfolded proteins. Under infection conditions, we examined the GSH/GSSG balance and showed that it significantly decreased, subsequently impairing the redox balance. We showed that in ZIKV-infected cells, the envelope protein (ZIKV-E) can oligomerize in large molecular weight complexes with insoluble characteristics. The presence and accumulation of such oligomers seem to correlate with the decrease in the redox potential of glutathione in infected cells. The redox imbalance generated during infection could be responsible for the generation of amyloid-like protein structures, linked by inappropriate disulfide bonds. This hypothesis would be consistent with fluorescence microscopy observations of thioflavin T labeling which reveal the presence of protein aggregates that colocalize with the ZIKV-E protein. All of these data suggest a change in oxidative protein folding in the ER due to persistent ER stress and ER redox imbalance during ZIKV infection. Misfolded proteins would cause persistent stress, promoting a spiral in which more inappropriate disulfide bonds would form, promoting protein aggregation.

2. Materials and Methods

2.1. Cells, virus and reagents

The clinical isolate PF-25013-18 (PF13) of ZIKV [27] was used for all infections. A549 cells (ATCC, CCL-185) were cultured at 37 °C under a 5% CO₂ atmosphere in Dubecco's modified eagle medium (P04-3500, PAN-Biotech™) supplemented with 10% heat-inactivated fetal bovine serum (FBS, P40-37500, PAN-Biotech™). Briefly, A549 cells were infected for 48h with MOI ranging from 0.5 to 5.

Immunodetection of the viral proteins was performed using the mouse anti-pan flavivirus E protein 4G2 (RD Biotech®, Besançon, France), or with the rabbit anti-EDIII ZIKV which was described previously [28]. Donkey anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 IgG antibodies were purchased from Invitrogen (AB_141607 and AB_2556547, Thermofisher®, Les Ulis, France). Horseradish peroxidase-conjugated anti-rabbit (ab97051) and anti-mouse (ab6789) antibodies were from Abcam® (Cambridge, UK). Thioflavin T, a benzothiazole dye that increases in fluorescence upon binding to amyloid fibrils and protein aggregates was purchased from Sigma-Aldrich® (243.003516.10, Humeau, La Chapelle-Sur-Erdre, France). GSH/GSSG-Glo™ assay was purchased from Promega (V6611, Charbonnières-les-Bains, France). Tris(2-carboxyethyl)phosphine (TCEP) is a reducing reagent used at 5 µM and purchased from Sigma-Aldrich® (243.075259.02, Humeau, La Chapelle-Sur-Erdre, France).

2.2. Cell fractionation and western blot

For fractionation, cells were washed with PBS and lysed at the concentration of 1x10⁴ cells.µl⁻¹ in buffer A (0.2% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2.5 mM EDTA) as before [29]. Following lysis, the insoluble fraction was separated by centrifugation at 3400g for 10 min. Pellets were enriched in non-folded proteins and the supernatant enriched in soluble proteins. All fractions were used in corresponding western blots.

For other western blot assays, samples were either lysed with TEN buffer (0.1M Tris-Cl pH 8.0, 0.01M EDTA pH 9.0 and 1M NaCl) or RIPA buffer. Cell lysates were then sonicated, treated in Laemmli buffer with or without DTT/TCEP and heat-treated at 95°C for 5 minutes. Finally, samples were processed by SDS-PAGE, transferred onto nitrocellulose membrane as previously reported [30], and subsequently incubated with aforementioned antibodies at following dilutions: 1:1000 for 4G2, rabbit anti-EDIII and β-actin, 1:2000 for HRP-conjugated anti-rabbit and anti-mouse.

2.3. Immunofluorescence and thioflavin T staining

A549 cells were grown, infected or treated on glass coverslips. They were further fixed with 3.7% formaldehyde at room temperature for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. Coverslips were incubated with primary antibodies (1:1000 dilution) in 1x PBS 1% BSA for two hours and with Alexa Fluor-conjugated secondary antibodies (1:1000, Invitrogen) for 1h. Nucleus morphology was revealed by DAPI staining. According to Beriault and Werstuck [31], after immunodetection of ZIKV EDIII and DAPI staining, amyloid indicator thioflavin-T was added to the coverslips at 5 µM for 10 min before proceeding to mounting. The coverslips were mounted with VECTASHIELD® (Clinisciences, Nanterre, France) and fluorescence was observed using a Nikon Eclipse E2000-U microscope. Images were captured and processed using a Hamamatsu ORCA2 ER camera and the imaging software NIS-Element AR (Nikon, Tokyo, Japan).

2.4. Glutathione abundance measurement

A549 cells were either infected or not with PF13 at MOI 5. After 48h of infection, the antioxidant capacity of glutathione was assessed in infected cells versus mock infected cells, using the luminescence-based GSH/GSSG-Glo™ assay kit, following manufacturer recommendations. Briefly, after treatment, cell culture supernatant was removed and cells were lysed either with Total Glutathione Lysis Reagent or Oxidized Glutathione Lysis Reagent. After 5 minutes of agitation, Luciferase Generation reagent was added. 30 minutes after incubation at RT, Luciferin Detection Reagent was added. Luminescence

emitted was measured after brief agitation and 15 minutes of incubation, using a micro-plate reader (Tecan FLUOstar® Omega). The GSH/GSSG ratio for a sample was calculated using the following formula:

$$\frac{\text{Net total glutathione luminescence} - \text{Net oxidized glutathione luminescence}}{\text{Net oxidized glutathione luminescence} / 2}$$

2.5. Protein sequence alignment

Flavivirus prM/E proteins were aligned using Clustal Omega. Following sequences were used: POLG_YEFVA – Yellow Fever virus, POLG_DEN2N – Dengue virus, ARB08102.1 – Zika virus PF13.

2.6. Statistical analyses

All values are expressed as mean \pm SD of at least three independent experiments. After normality tests, comparisons between infected and mock infected cells were analyzed using a t-test. Values of $p < 0.05$ were considered statistically significant. All statistical tests were done using Graph-Pad Prism version 9 software.

3. Results

3.1. Glutathione abundance in the course of ZIKV infection

We and others have reported that ZIKV infection, like many other enveloped viruses, can trigger ER stress and UPR in the host cell [13–19]. It is still unknown whether a viral factor is more specifically involved in ZIKV-mediated ER stress. Disruption of ER homeostasis may be followed by altered redox state and may be associated with impaired glutathione availability and sulfur tripeptide metabolism [23,32]. We wondered whether ZIKV-mediated ER stress could be related to a similar phenomenon. Therefore, we infected A549 cells with ZIKV at MOI 5 for 48h. As shown in **Figure 1A**, western blot analysis (in reducing conditions) of cell extracts harvested 48 hours post-infection (h.p.i.) shows ZIKV envelope protein (~55 kDa) indicating infection and viral replication in A549 cells. At the same time, infected and mock infected A549 cells were lysed to compare the relative level of oxidized and reduced glutathione under these two conditions. Interestingly, following infection, approximately 3-fold decrease of the GSH/GSSG ratio is observed in infected cells (**Figure 1B**). The decrease of glutathione reduced form observed presumably relates to a loss of glutathione activity during ZIKV infection.

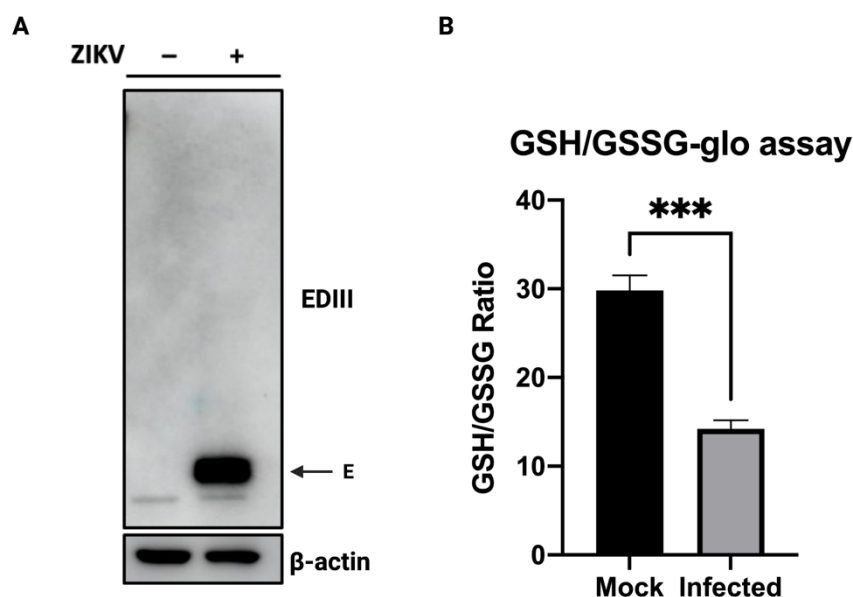


Figure 1. ZIKV infection is associated with redox imbalance. (A) ZIKV infection of A549 cells. A549 cells were either mock infected or infected with ZIKV at MOI 5 for 48h. Cell extracts were reduced or not using DTT and heat-treated at 95°C for 5 minutes. Western blot analysis of cell extracts following infection leads to detection of ZIKV envelope protein (~55 kDa). ZIKV-E protein is detected using rabbit anti-EDIII antibody. This is representative of three independent experiments. (B) ZIKV leads to a decrease of the GSH/GSSG ratio, indicating a loss of glutathione activity in infected A549 cells. 48 h.p.i. glutathione activity was assessed in mock infected vs. infected cells. ***: $p < 0.01$. h.p.i: hour post-infection.

3.2. Detection of amyloid-like structures in ZIKV-infected cells

The imbalance in the GSH/GSSG ratio suggests an alteration of redox state during ZIKV infection. Redox imbalance is known to participate in ER stress [33], which has to be resolved by UPR. However, it has been shown that during ZIKV infection, incomplete UPR resulted in persistent and poorly resolved ER stress [18,19]. Under these conditions of persistent stress and redox imbalance, the accumulation of oxidized protein could lead to the formation and the aggregation of amyloid-like structures [33].

To assess a such hypothesis, we infected A549 cells with ZIKV at MOI 1 for 48h. Cells were fixed and examined by immunofluorescence. Intracellular virus replication was examined using a monospecific anti-E polyclonal antibody and fluorescent dye Thioflavin-T (THT) was used to detect protein aggregates and any form of amyloid-like structures [31]. At 48 h.p.i., only A549 cells positively stained for the E protein showed a positive signal for THT as compared to mock-infected cells, suggesting that amyloid-like protein aggregates can occur in ZIKV-infected cells (**Figure 2**). Interestingly, the staining of amyloid-like structures and the staining of the ZIKV-E protein overlap (**Figure 2**). We have previously demonstrated that ZIKV-E localizes to the ER by co-immunodetection of calnexin [18] and it is validated that E protein is processed and folded in the ER, before the assembly of viral particles in the secretory pathway [34]. Thus, colocalization of ZIKV-E and THT labeling suggest an accumulation of amyloid-like structures in the stressed ER of infected cells. Furthermore, these amyloid-like structures may be related to misfolding of the viral envelope protein.

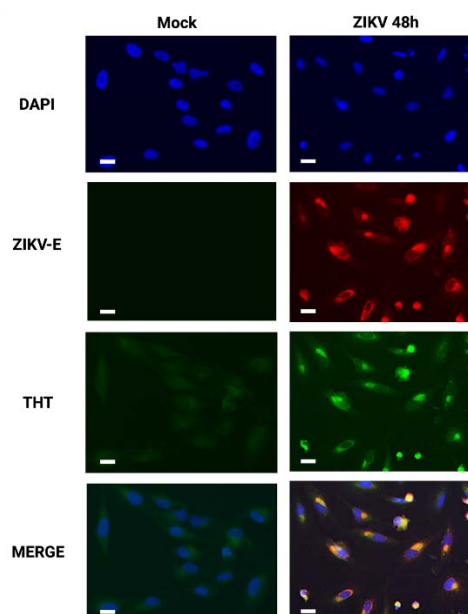


Figure 2. Amyloid-like protein accumulation during ZIKV infection. A549 cells, infected with ZIKV at MOI 1 for 48h, were incubated with Thioflavin-T (THT), a green fluorescent ER stress indicator that binds aggregated proteins. Immunodetection of ZIKV envelope protein confirmed the infection of the cells. Scale bar: 5 μ m. All images are representative of three independent experiments.

3.3. Detection of disulfide-bonded E oligomers in ZIKV-infected cells

To determine whether a relation exists between expression of E protein and formation of amyloid-like structures in infected cells, A549 cells were infected with increasing inputs of ZIKV (Figure 3). Protein extracts were collected 48 h.p.i. and either reduced or not using Tris(2-carboxyethyl) phosphine (TCEP) as a reducing agent. Protein samples were then analyzed by immunoblot assay (Figure 3A). Under non-reducing conditions, we can observe a single band when we use the 4G2, an anti-pan-flavivirus envelope protein antibody. The signal corresponds to the monomeric form of ZIKV-E protein (M, ~55 kDa) and it increases in a MOI dependent manner (Figure 3A). Interestingly, this band is no longer observed when using TCEP (Figure 3A). The lack of recognition of ZIKV-E protein under reducing conditions is consistent with literature data indicating that the 4G2 antibody binds a conserved conformational epitope overlapping the E fusion loop [35]. This suggests that oxidized disulfide bonds may stabilize the tertiary structure of ZIKV-E protein and be necessary for conformational epitope recognition.

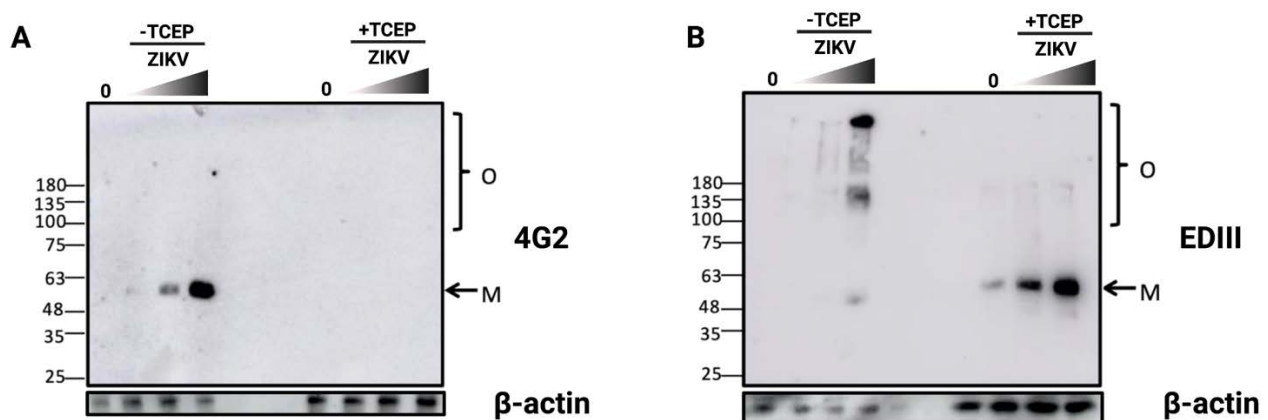


Figure 3. ZIKV infection results in the formation of disulfide-crosslinked E oligomers. (A) ZIKV-E in native conformation contains intermolecular disulfide bridges. After infection of A549 cells with ZIKV in a dose-dependent manner (MOI 0.5, 1 and 5), protein extracts were either treated or not with TCEP before SDS-PAGE. Western blot analysis was performed with mouse 4G2 antibody, which recognizes a conformational epitope of the E protein. (B) ZIKV-E protein forms disulfide-cross linked oligomers. A549 cells were infected with increased quantities of ZIKV (MOI 0, 0.5, 1 and 5) and the total fraction were treated or not with TCEP before western blotting. Western blot assay was achieved with a rabbit anti-EDIII antibody. M indicates the 55 kDa monomer, O indicates high molecular weight oligomers of ZIKV-E. These images are representative of three independent experiments.

Immunoblotting was repeated in the same conditions as described above, but this time using anti-EDIII antibody. Under non-reducing conditions, the monomeric form of ZIKV-E protein was not predominantly detected. In contrast, high molecular weight signals of ZIKV-E protein are detected. These forms of over 100 kDa in size could correspond to oligomers of E protein (O, Figure 3B). Conversely, after TCEP treatment and immunodetection with anti-EDIII antibody, the monomeric form of ZIKV-E (M, Figure 3B) prevails and the oligomeric forms are no longer detected. These observations suggest that during ZIKV infection, E proteins are produced and accumulated in different forms, including high molecular weight forms that are not detectable when reduced. These oligomeric forms would therefore be stabilized by disulfide bonds.

During infection, the envelope protein of flavivirus is mainly folded in the ER and its monomeric tertiary structure depends on the generation of intramolecular disulfide bonds that occur in this cellular compartment [36]. Oligomeric forms resulting from weak interactions between the individual monomers have also been described [37]. To the best of

our knowledge, we are the first to demonstrate the possibility of E-oligomers formation in the context of ZIKV infection due to disulfide covalent bonds formation.

Thus, we wondered whether this ability was a specificity of the ZIKV-E protein among the different flaviviral envelope proteins. We therefore aligned the prM/E amino acid sequences of YFV, DENV, WNV and ZIKV, as shown in **Figure 4A**. All *flaviviruses* assessed have cysteine positions highly conserved with 6 residues belonging to the prM protein and 12 to the E protein. Interestingly, ZIKV-E contains a 13th cysteine residue. As illustrated, in **Figure 4B** for DENV2 or in the literature for WNV, the 12 well conserved cysteine residues within E protein are all adjacent and involved in disulfide proximity bonds [36,38]. This feature is also found in ZIKV prM/E (**Figure 4C**), where 12 of the 13 cysteine residues in E are highlighted and form disulfide bonds through their adjacent positions, the thirteenth residue not being accessible for an oxidized bond formation in the native ZIKV-E form. The monomer of ZIKV-E would then be stabilized for its proper folding by 6 disulfide bonds, as are the E proteins of other *flaviviruses*. In contrast, the presence of an additional cysteine specific to ZIKV-E could account for intermolecular disulfide bond formation when the protein incorporated into the stressed ER is misfolded. We propose that oligomerization of E proteins is then due to non-native disulfide bonds cross-linking between polypeptides and that the additional cysteine could play a role in increasing the combinatorial possibilities for the formation of these non-native bonds.

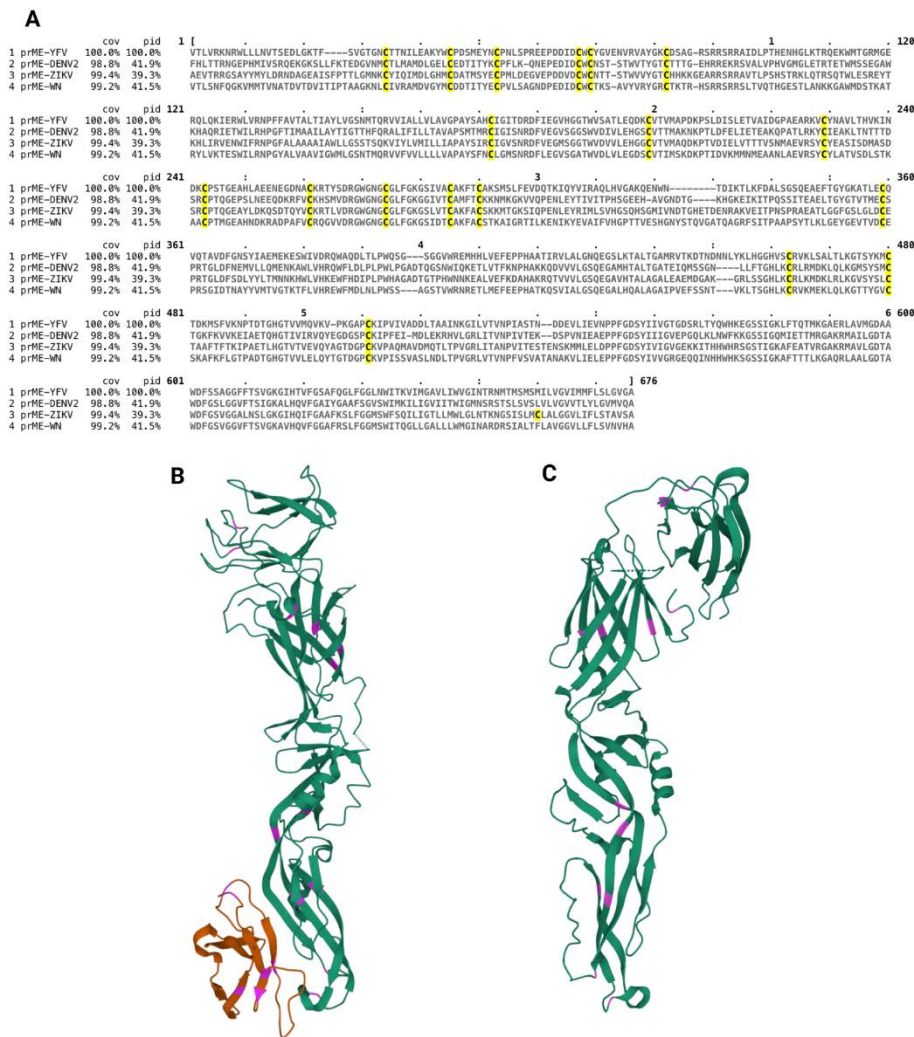


Figure 4. Disulfide bonds in *flavivirus* prM/E protein. (A) Alignment of prM/E protein sequence from YFV (POLG_YEFVA), DENV2 (POLG_DEN2N), WNV (POLG_WNV9) and ZIKV (ARB08102.1). The cysteine positions are highlighted in yellow. (B) Crystallography structure of

DENV2 prM/E (3C6E), with cysteine positions involved in disulfide bonds formation highlighted in pink. (C) Crystallography structure of ZIKV-E (5JHM), with cysteine positions involved in disulfide bonds formation highlighted in pink.

3.4. ZIKV E protein forms insoluble oligomers during infection

Oligomerization of proteins due to cross-linking of non-native disulfide bonds may lead to the formation of protein aggregates, one hallmark of which will be a loss of solubility [39]. As previously stated, we have been able to show that ZIKV induces unresolved ER stress in infected cells that have a prolonged lifespan [18,19]. These conditions, which are combined with the redox imbalance (**Figure 1**), are likely to influence ER function and protein folding. Indeed, during infection, a large amount of misfolded ZIKV-E protein accumulates in the form of oligomers. In agreement with the results obtained with the THT-amyloid-like imaging (**Figure 2**), the detection of E-protein oligomers (**Figure 3**) could be consistent with the formation of insoluble aggregates. To test this hypothesis, we prepared protein extracts from ZIKV infected cells, using a buffer that contains a detergent able to solubilize integral membrane proteins. Protein extracts were then fractionated according to their solubility by low-speed centrifugation (3400g) to prevent microsomes sedimentation. Total protein extract, soluble fraction and insoluble fraction were then used for western blot analysis. Immunoblot using 4G2 antibody shows a unique band at ~55 kDa in both total protein extract and soluble fraction, corresponding to the monomeric form of E protein (**Figure 5**). The anti-EDIII antibody detected the E oligomers in insoluble fraction (**Figure 5**) suggesting that ZIKV envelope protein can be engaged in formation of insoluble aggregates. These latest results confirm that ZIKV infection leads to an accumulation of high molecular weight oligomers of the E protein, in which misfolded polypeptides are associated with each other by disulfide bonds, thus resulting in protein aggregation with insoluble and amyloid features.

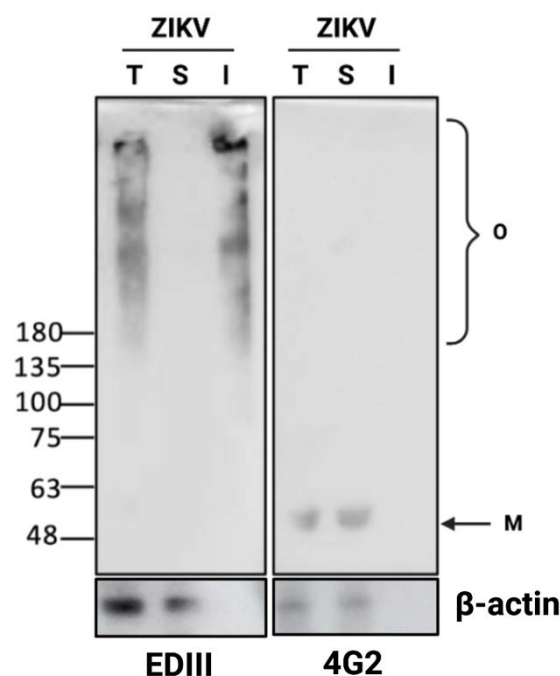


Figure 5. ZIKV E disulfide-crosslinked oligomers are insoluble. A549 cells were infected with ZIKV at MOI 5. 48 h.p.i. cells were lysed in TX100 buffer A and fractionated by centrifugation at 3400g. An equal volume of total (T), soluble (S) and insoluble (I) fractions were separated under non-reducing conditions and immuno-blotted for ZIKV-E with anti-EDIII or 4G2 antibodies. M indicates the 55 kDa monomer, O indicates high molecular weight oligomers of ZIKV-E. This is representative of three independent experiments.

4. Discussion

The shaping of proteins that transit in the ER is a crucial step in their maturation, necessary for them to adopt a native conformation that will condition their future biological functions. This 3D configuration relies on the correct folding of polypeptide chains and involves both weak interactions and the formation of specific covalent bonds between cysteine residues. Disulfide bond formation is a reversible process that is achieved by a thiol-disulfide exchange reaction [40]. This exchange is mediated by a catalytic redox cycle which involves ERO-1 (ER oxidoreductin 1), PDI (Protein Disulfide Isomerase) and GSH (reduced glutathione) to ensure formation and isomerization of disulfide bonds in proteins [41] (**Figure 6**). This oxidative protein folding occurs in an oxidized environment to maintain native protein conformation. However, a significant decrease in GSH/GSSG ratio is known to lead to protein misfolding and inappropriate formation of disulfide bonds. In eukaryotic cells, GSH is the most abundant non-protein thiol [42], which is oxidized to glutathione disulfide (GSSG) when it assists in disulfide-bond reduction (**Figure 6**). Upon ER stress and UPR, ER environment becomes increasingly reduced due to consumption of GSH [43]. Thus, the ratio change observed during ZIKV infection (**Figure 1**) can be related to prolonged UPR and an unresolved ER stress. During ER stress, ERO-1 and PDI are induced through UPR to favor protein folding as an adaptive response. The induction of ERO-1 during UPR involves the transcription factor CHOP [44]. However, CHOP expression and activity are downregulated during ZIKV infection [19], thus probably affecting ERO-1 expression (**Figure 6**). In addition, homeostasis impairment imposed by the UPR, like during ZIKV infection [18,19], is known to be associated with a compromised ER protein oxidation and a PDI chaperone activity rather than a disulfide isomerase activity for PDI [45]. All of these points (ERO-1 downregulation, PDI activity switch and decreased GSH) illustrate that during ZIKV infection the redox state is affected in relation to a decreased thiol-disulfide exchange reaction (**Figure 6**). Accordingly, protein misfolding and formation of non-native disulfide bonds during ZIKV infection can occur. Such changes in ER homeostasis and activities are likely to lead to formation of aggregates and amyloid-like structures, as shown with THT staining in ER upon ZIKV infection (**Figure 2**).

During the infection process, viral proteins incorporated into the ER are subject to the same folding and control rules and the organized formation of disulfide bridges contributes to their functional shaping. ZIKV-E contains cysteine residues which allow the formation of disulfide bonds important for the protein's native conformation. Using the monoclonal pan-flavivirus 4G2 antibody, we were then able to detect a conformational epitope in the monomeric form of E protein (**Figure 3A**). Conversely, after treatment with a reducing agent the epitope became undetectable with the 4G2 antibody. This confirmed that intramolecular disulfide bridges shape the native E protein. These observations are in agreement with data on other *flavivirus* (e.g., WNV, DENV...) indicating that all cysteine in E protein are engaged in intramolecular disulfide bonds [36,38]. Unexpectedly, the use of a monospecific antibody raised against the antigenic domain III of the E protein (EDIII), allowed us to reveal that the E protein exists in several forms during ZIKV-infection. One is the native monomeric form with intrachain disulfide bonds and the others are high molecular weight oligomers with unfitted intermolecular disulfide bonds (**Figure 3B**). The formation of such E-related structures can be related to a change in redox state associated to a thiol-disulfide exchange reaction reduction in link with a persistent ER stress, as described earlier. Sequence analysis of prM/E for cysteine content highlights the presence of an additional cysteine in ZIKV-E compared to other *flavivirus* (**Figure 4**). This free cysteine could be related to a greater susceptibility of ZIKV-E protein to form non-native disulfide-bonded oligomers compared to the other *flavivirus* in case of redox imbalance and misfolding. Finally, the non-native disulfide-bonded oligomers of E remain in an insoluble fraction (**Figure 5**) which is consistent with protein aggregation. As THT staining co-localized with ZIKV-E protein (**Figure 2**), we can therefore argue that these aggregates of ZIKV-E form amyloid-like structures.

CHOP	C/EBP Homologous Protein
DENV	Dengue Virus
EDIII	Domain III of E protein
ER	Endoplasmic Reticulum
ERO-1	ER oxidoreductin 1
GSH	Reduced glutathione
GSSG	Oxidized glutathione
PDI	Protein Disulfide Isomerase
TCEP	Tris(2-carboxyethyl)phosphine
THT	Thioflavin T
UPR	Unfolded Protein Response
WNV	West Nile Virus
YFV	Yellow
ZIKV	Zika Virus
ZIKV-E	Zika Virus envelope protein

and P.K.-T.; funding acquisition, P.D. All authors have read and agreed to the published version of the manuscript.

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