The Zinc-Finger Domain Was Essential for Porcine Reproductive and Respiratory Syndrome Virus Nonstructural Protein-1 α to Inhibit the Production of Interferon- β

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Porcine reproductive and respiratory syndrome virus (PRRSV) has caused one of the most economically devastating and pandemic diseases of swine. Previous studies have documented that PRRSV nonstructural protein-1 α (nsp1 α) was an interferon antagonist, but the mechanism by which nsp1 α inhibited the interferon (IFN)- β production was unclear. Here, by site-directed mutagenesis of the predicted zinc-coordinating residues of the zinc-finger (ZF) domain of nsp1 α or by deletion of the ZF domain of nsp1 α , we explored whether the ZF domain was required for nsp1 α to disrupt the IFN- β production. The results showed that both mutagenesis of the predicted zinc-coordinating residues of the ZF domain and deletion of the ZF domain made nsp1 α lose its interferon antagonism activity. In conclusion, our present work indicated that the ZF domain of nsp1 α was necessary for nsp1 α to inhibit the IFN- β induction.

Introduction

PORCINE REPRODUCTIVE AND RESPIRATORY syndrome virus (PRRSV), a small, enveloped virus, including a single positive-strand RNA genome, is a member of family *Arteriviridae*, which, along with the *Coronaviridae*, is classified in the order *Nidovirales* (Cavanagh 1997; Meulenberg 2000). Since it was first identified in the United States in 1987 and in Europe in 1990, PRRSV has caused one of the most economically important diseases of swine that is characterized by severe reproductive failure in sows and respiratory distress in piglets and growing pigs (Rossow 1998). Infection with PRRSV also predisposes pigs to a secondary infection by bacterial and viral pathogens, which may be due to the immunosuppression induced by the virus (Feng and others 2001; Mateu and Diaz 2008).

Type I interferon (IFN- α and IFN- β) is the first responder against animal virus infections (Muller and others 1994; Weber and others 2004). When a virus infects, the virus could be recognized by the pattern-recognition receptors (PRRs) such as membrane-bound Toll-like receptors (TLRs) (including TLR3, TLR7, TLR8, and TLR9), retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) [including the retinoic

acid-inducible gene I (RIG-I), melanoma differentiation-associated 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2)]. Among these, TLR3 and MDA5 detect the viral dsRNA, and then, TLR3 recruits the adaptor molecule TRIF (Toll/IL-1 receptor domain-containing adaptor-inducing IFN), while MDA5 recruits VISA (virus-induced signaling adaptor; also known as CARDIF, IPS-1, or MAVS) to induce activation (phosphorylation) of IFN regulatory factor 3 (IRF-3) and finally induces IFN- β transcription. IRF-3 is a constitutively expressed, and latent transcription factor for the transcription of IFN- β and the activation of IRF-3 needs its phosphorylation by 2 noncanonical I κ B kinases, TANK-binding kinase 1 (TBK1) and the noncanonical I κ B kinases (IKK- ϵ) (Bowie and Unterholzner 2008). Then, IFN- β binds to the IFN- α/β receptor and induces the IFN-regulated genes responsible for the antiviral response (Sadler and Williams 2008). However, during the coevolution with the host cells, many viruses have developed defensive mechanisms to inhibit IFN- β production, making it difficult for host cells to defeat viral infection (Weber and others 2004; Bowie and Unterholzner 2008).

The PRRSV genome has 9 open reading frames (ORFs) composed of ORF1a, ORF1b, ORF2a, ORF2b, and ORF3–7.

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ORF1a and ORF1b could produce 14 nonstructural proteins (nsp1 α , nsp1 β , nsp2, nsp3, nsp4, nsp5, nsp6, nsp7 α , nsp7 β , nsp8, nsp9, nsp10, nsp11, and nsp12) (den Boon and others 1991, 1995; Chen and others 2010; Shi and others 2011a). Moreover, these nsps had been screened for type I IFN suppression, and 4 nsps were identified to contain the suppressive activities: nsp1 α , nsp2, nsp4, and nsp11 (Kim and others 2010). nsp2 is a membrane-anchored protein that contains a 3C-like cysteine protease activity that inhibited IRF3 phosphorylation and nuclear translocation (Li and others 2010). nsp11 contains a domain designated the NendoU domain and functions as an endoribonuclease, which was essential for nsp11 as the IFN antagonist (Shi and others 2011a), while nsp1 α possessed 3 parts: an amino-terminal zinc-finger (ZF) domain [1–65 amino acids (aa)], a papain-like cysteine protease (PCP)- α domain (66–166 aa), and a C-terminal extension (CTE) (167–180 aa) (Sun and others 2009), and previous studies showed that the nsp1 α PCP- α domain and CTE were essential for nsp1 α to antagonize the IFN- β induction (Song and others 2010; Shi and others 2011b). However, whether the ZF domain was essential for nsp1 α as the antagonist of IFN- β production was unclear. Therefore, the aim of our present work was to explore whether the ZF domain was essential for nsp1 α to disrupt the IFN- β production.

Materials and Methods

Cell and virus

MARC-145 cell, a fetal green monkey fibroblast cell line derived from MA-104 (Kim and others 1993), was maintained in the Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone). PRRSV strain BJ-4 was a kind gift from Dr. Hanchun Yang (China Agricultural University).

Plasmids

The plasmids pcDNA3.1-VISA, pcDNA3.1-TRIF, p-284 Luc (positions –284 and +19 in the IFN- β gene before the luciferase report gene), pcDNA3.1-FLAG, pcDNA3.1-FLAG-nsp1 α , and phRL-TK have already been described (Shi and others 2010, 2011b). Full-length GFP cDNA was amplified by using pEGFP-C1 (Clontech) as a template and was digested with *NheI* and *HindIII*, and then cloned into pcDNA3.1. The nsp1 α ZF domain mutants, nsp1 α Cys-8 $\rightarrow$ Ala (nsp1 α C8A)

and nsp1 α C10A, were constructed by a standard PCR method using the primers that the forward primers included the mutation bases. While nsp1 α C25A and nsp1 α C28A mutants were constructed by using the overlap PCR, and the mutation bases were in the primers nsp1 α C25A forward, nsp1 α C25A reverse, nsp1 α C28A forward, and nsp1 α C28A reverse. To generate a GFP-tagged nsp1 α ZF domain, the ZF domain cDNA was digested with *HindIII* and *EcoRI* and then cloned into pcDNA3.1-GFP. All primers used in our present work are listed in the Table 1. p55C1B Luc (Yoneyama and others 1996, 2004; Devaraj and others 2007), a firefly luciferase reporter gene plasmid containing repetitive pIRF-3-binding sites, was kindly provided by Dr. Takashi Fujita. pcDNA3-FLAG-IKK- ϵ was kindly provided by Dr. Katherine A. Fitzgerald (Fitzgerald and others 2003). All constructs were fully sequenced.

Luciferase assay

All newly prepared plasmids were verified by sequencing. Lipofectamine2000 (Invitrogen) was used for transient transfection. Cells grown in 24-well plates were transfected in triplicate with the p-284 Luc plasmid (200 ng) or p55C1B Luc plasmid (500 ng), phRL-TK (80 ng), and expression vector (600 ng) or supplied with an equivalent control vector. At the appointed time, cells were harvested, and the luciferase activity was measured using the dual luciferase reporter assay system (Promega) in the MicroBeta[®] TriLux liquid scintillation and luminescence counters (Microbeta-1450; Wallac).

Cell extracts and immunoblotting

MARC-145 cells grown in 24-well plates were transfected in triplicate with the various expression plasmids, and 48h later, transfected cells were lysed in a lysing buffer [1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM Na₃VO₄, 2 mM NaF, and a protease inhibitor cocktail]. The detailed procedure for immunoblots has been described (Shi and others 2008). Briefly, the total protein concentration was quantified with the Bradford protein assay (Biocolor Bioscience & Technology Company). Equal proteins were separated on SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore Company), and then probed with anti-FLAG (Sigma F1804) or anti-GFP (Clontech).

TABLE 1. THE PRIMERS USED IN PLASMID CONSTRUCTION

Primers	5' $\rightarrow$ 3'
nsp1 α	Forward: CCAAGCTTCGCCACCATGGGCATGTCTGG
Nsp1 α C8A	Forward: AAGCTTCATGGGCATGTCTGGGATACTTGATCGGGCCACGTGTACC
nsp1 α C10A	Forward: CCAAGCTTCATGGGCATGTCTGGGATACTTGATCGGTGCACGGCTACCCCAATGCCAGGGT
nsp1 α C25A	Forward: CAAGTCTACGCCACACGATGCCTCAGTGCACG
nsp1 α C25A	Reverse: GCATCGTGTGGCGTAGACTTGGCCCTCCGCGC
nsp1 α C28A	Forward: TGCACACGAGCCCTCAGTGCACGGTCTCTC
nsp1 α C28A	Reverse: GTGCACTGAGGGCTCGTGTGCAGTAGACTTGG
nsp1 α DZF	Forward: CAAGCTTCCCCACTGTTGAGTGCTCCC
nsp1 α ZF	Reverse: CCGGAATTCTTAGAATGCACGTGGCAACGT
nsp1 α	Reverse: GCGGAATTCTCACATAGCACACTCAAAGGGGCG
GFP	Forward: CTAGCTAGCGCCACCATGGTGAGCAAGGGCGAG
GFP	Reverse: GCAAGCTTGGCTTGTACAGCTCGTCCATGCCGAG

Proteins were detected by using an ECL detection system (Cell Signaling Technology).

Statistical analysis

Student's *t*-test was used for statistical analyses, and the comparisons were considered as statistically significant when $P < 0.05$.

Results

The mutant that deleted the ZF domain in *nsp1α* failed to inhibit the induction of *IFN-β*

nsp1α contained 3 parts: the N-terminal ZF domain (Met1-Glu65), the PCP domain (PCPα domain, Pro66 to Gln166), and the C-terminal extension (CTE; Arg167 to Met180) (Sun and others 2009). Previous studies have demonstrated that *nsp1α* inhibited the production of *IFN-β* (Chen and others 2010; Shi and others 2011b). To explore whether the ZF domain was essential for *nsp1α* as the antagonist to the *IFN-β* production, we deleted the ZF domain in *nsp1α* and constructed the expression plasmid—pcDNA3.1-FLAG *nsp1α* 66–180 (*nsp1α* DZF)—the expressions of which were confirmed by western blot (Fig. 1A), and found that the mutant that deleted the ZF domain in *nsp1α* failed to block Poly (I:C) (a synthetic dsRNA analog)-induced activation of the *IFN-β* promoter (Fig. 1B).

Because the pIRF-3 was a necessary component to the activation of the *IFN-β* promoter (Peters and others 2002), p55C1B-Luc (Yoneyama and others 1998, 2004; Devaraj and others 2007), the pIRF-3-dependent synthetic promoter, was

detected after the Poly (I:C) treatment or the mock treatment. As shown in Fig. 1C, *nsp1α* 66–180 (*nsp1α* DZF) could not inhibit the activation of p55C1B-Luc; that is, the results in Fig. 1C confirmed that in Fig. 1B.

Poly (I:C), a double-stranded RNA, could be recognized by TLR3 (Yamamoto and others 2003) and MDA5 (Gitlin and others 2006; Kato and others 2006; Onoguchi and others 2011). Then, through TBK1 and IKK-ε, TLR3 recruited TRIF, and MDA5 recruited VISA, to phosphorylate IRF-3, and finally activate the *IFN-β* promoter (Bowie and Unterholzner 2008). Overexpression of VISA, TRIF, or IKK-ε could induce the activation of IRF-3 and activate the *IFN-β* promoter (Yoneyama and others 2004; Devaraj and others 2007; Zhong and others 2008). Our previous study has shown that *nsp1α* inhibited the *IFN-β* production induced by overexpression of VISA, TRIF, or IKK-ε (Shi and others 2010, 2011b), so we investigated whether deleting the ZF domain could also influence the *nsp1α* to inhibit the *IFN-β* production induced by overexpression of VISA, TRIF, or IKK-ε. The results showed that the mutation that deleted the ZF domain in *nsp1α* could not suppress *IFN-β* promoter activation induced by ectopic expression of VISA, TRIF, or IKK-ε (Fig. 1D). Similar results were also obtained when p55C1B Luc was in place of p-284 Luc (Fig. 1E).

Mutations that mutagenesis of the predicted zinc-coordinating residues of the ZF domain of *nsp1α* could not inhibit the induction of *IFN-β*

The crystal structure of PRRSV *nsp1α* documented that the ZF domain of PRRSV *nsp1α* belonged to the 4-Cys ZF

FIG. 1. The *nsp1α* mutant that deleted the zinc-finger (ZF) domain failed to inhibit the activities of the interferon (*IFN-β*) promoter (p-284 Luc) and the pIRF-3-dependent promoter (p55C1B Luc). (A) Western blots analyzed the expression of *nsp1α* and *nsp1α* 66–180 (*nsp1α* DZF) by anti-FLAG antibody in MARC-145 cells transfected with pcDNA3.1-FLAG (Vector), pcDNA3.1-FLAG-*nsp1α* (*nsp1α*), or pcDNA3.1-FLAG-*nsp1α* 66–180 (*nsp1α* DZF). MARC-145 cells were cotransfected with p-284 Luc (B) or p55C1B Luc (C), pRL-TK, and different expression plasmids. Twenty hours later, cells were either mock-treated (Con) or transfected with poly (I:C) for 6 h, and then the cells were harvested for the dual luciferase reporter assay. MARC-145 cells were cotransfected with p-284 Luc (D) or p55C1B Luc (E), pRL-TK, and different expression plasmids. Twenty-four hours later, the cells were harvested for dual luciferase reporter assay. Con: cells transfected with pcDNA3.1. *nsp1α* DZF: deletion of the ZF domain in *nsp1α*. Data represented means of 3 replicates, and experiments were repeated 3 times. Error bars represented the standard deviations. * $P < 0.05$ compared with the activity of the cells with the vector.

superfamily (Tijms and others 2001; Sun and others 2009) and the Cys8, Cys10, Cys25, and Cys28 participated in binding with the zinc ion (Sun and others 2009) (Fig. 2A).

The sequence blast showed that the ZF domain in the *Arteriviridae* viruses, which included PRRSV, mouse lactate dehydrogenase-elevating virus, equine arteritis virus (EAV), and simian hemorrhagic fever virus, was conserved (Tijms and others 2007; Sun and others 2009). Moreover, the ZF domain in nsp1 of EAV, another arteritis virus, played important role for the transcription of the EAV; that is, mutation in the zinc-binding residues (Cys25, His27, Cys41, and Cys44 in Fig. 2A) of the ZF domain influenced the transcription of the EAV. Therefore, next, we made single-amino-acid substitutions into the zinc-coordinating residues of the ZF domain and investigated whether the mutants could inhibit the induction of IFN- β or not. Fig. 2C shows that any of the mutant nsp1 α Cys-8 $\rightarrow$ Ala (nsp1 α C8A), nsp1 α C10A, nsp1 α C25A, and nsp1 α C28A failed to inhibit IFN- β promoter activation induced by Poly (I:C) or the ectopic expression of VISA, TRIF, or IKK- ϵ (Fig. 2E), and the results in Fig. 2D and F also confirmed that in Fig. 2C and E.

The ZF domain of nsp1 α was not sufficient to inhibit the induction of IFN- β

Since the ZF domain only contained 65 amino acids, and it was difficult to detect by western blot, we fused the ZF domain to a new tag protein GFP, the molecular weight of which was about 25 kiloDalton (kDa). We constructed the

expression plasmid pcDNA3.1-GFP-ZF, and the protein expression was confirmed by the western blot (Fig. 3A), and also the GFP-nsp1 α and the GFP-ZF had the same location in the cells (data not shown): both nsp1 α and nsp1 α ZF mainly localized in the nucleus, but also some of them retained in the cytoplasm; a scattered punctate dot-like fluorescent-staining pattern was consistently observed, and these results were similar to the previous report about the location of PRRSV nsp1 α (Chen and others 2010). Next, we tested whether only the ZF domain itself could inhibit the induction of IFN- β . The results in Fig. 3 show that the ZF domain itself could not inhibit the Poly (I:C) or the ectopic expression of VISA-, TRIF-, or IKK- ϵ -induced activation of the IFN- β promoter (Fig. 3B, D) and the Poly (I:C) or the ectopic expression of VISA-, TRIF-, or IKK- ϵ -induced activation of the pIRF-3-dependent synthetic promoter (Fig. 3C, E).

Discussion

PRRS is a disease of pigs that is characterized by negligible induction of type I IFNs and viral persistence for an extended period (Sun and others 2012). Previous studies have shown that nsp1 α inhibited the production of IFN- β by suppression of NF- κ B and inhibition of the phosphorylation of IRF-3 in the absence of other genes (Chen and others 2010; Shi and others 2010, 2011b; Song and others 2010). However, it remains to be determined exactly how nsp1 α antagonized the production of IFN- β . Since the recombinant IFN- β not only protected swine alveolar macrophages and MARC-145 cells

FIG. 2. The nsp1 α mutants that mutagenesis of the predicted zinc-coordinating residues of the ZF domain failed to inhibit the activities of the IFN- β promoter (p-284 Luc) and the pIRF-3-dependent promoter (p55C1B Luc). (A) Bold italic word showed 4 residues that were proposed to coordinate zinc in EAV nsp1 and PRRSV nsp1 α . (B) Western blots analyzed the expression of nsp1 α , nsp1 α C8A, nsp1 α C10A, nsp1 α C25A, and nsp1 α C28A by anti-FLAG antibody in MARC-145 cells transfected with pcDNA3.1-FLAG (Vector), pcDNA3.1-FLAG-nsp1 α C8A (nsp1 α C8A), pcDNA3.1-FLAG-nsp1 α C10A (nsp1 α C10A), pcDNA3.1-FLAG-nsp1 α C25A (nsp1 α C25A), or pcDNA3.1-FLAG-nsp1 α C28A (nsp1 α C28A). MARC-145 cells were cotransfected with p-284 Luc (C) or p55C1B Luc (D), phRL-TK, and different expression plasmids. Twenty hours later, cells were either mock-treated (Con) or transfected with poly (I:C) for 6 h, and then the cells were harvested for the dual luciferase reporter assay. MARC-145 cells were cotransfected with p-284 Luc (E) or p55C1B Luc (F), phRL-TK, and different expression plasmids. Twenty hours later, the cells were harvested for the dual luciferase reporter assay. Con: cells transfected with pcDNA3.1. Data represented means of 3 replicates, and experiments were repeated 3 times. Error bars represented the standard deviations. * $P < 0.05$ compared with the activity of the cells with the vector.

FIG. 3. The ZF domain of nsp1 α was not sufficient to inhibit the activations of the IFN- β promoter (p-284 Luc) and the pIRF-3-dependent promoter (p55C1B Luc). **(A)** Western blots analyzed the expression of nsp1 α and nsp1 α 1–65 (nsp1 α ZF) by an anti-GFP antibody in MARC-145 cells transfected with pcDNA3.1-GFP (Vector), pcDNA3.1-GFP-nsp1 α (GFP-nsp1 α), or pcDNA3.1-GFP-nsp1 α 1–65 (GFP-ZF). MARC-145 cells were cotransfected with p-284 Luc **(B)** or p55C1B Luc **(C)**, pRL-TK, and different expression plasmids. Twenty hours later, cells were either mock-treated (Con) or transfected with poly (I:C) for 6h, and then the cells were harvested for the dual luciferase reporter assay. MARC-145 cells were cotransfected with p-284 Luc **(D)** or p55C1B Luc **(E)**, pRL-TK, and different expression plasmids. Twenty hours later, the cells were harvested for dual luciferase reporter assay. Con: cells transfected with pcDNA3.1. GFP-nsp1 α : the plasmid pcDNA3.1-GFP-nsp1 α ZF that expressed nsp1 α . GFP -ZF: the plasmid pcDNA3.1-GFP-nsp1 α ZF that expressed ZF domain. Data represented means of 3 replicates, and experiments were repeated 3 times. Error bars represented the standard deviations. * P < 0.05 compared with the activity of the cells with the vector.

from infection with PRRSV (Overend and others 2007), but also could reduce the yield of PRRSV *in vivo* (Buddaert and others 1998), identifying the viral components that inhibited the production of IFN- β , and their mechanism of this action was important. PRRSV nsp1 α had 2 ZF motifs: one motif (ZF1) of C8–C10–C25–C28 and another motif (ZF2) of C70–C76–H146–M180. Sun and others (2012) considered that the PCP α activity was self-inactivated by conformational changes, and thus was less likely responsible for suppression of the IFN response and suggested that 2 ZF motifs may be associated with nsp1 α -mediated inhibition of IFN- β , although our previous work shows that the PCP α activity was essential for nsp1 as the IFN- β antagonist (Shi and others 2011b). Our recent work shows that deletion of the M180 did not influence nsp1 α to inhibit the induction of IFN- β (Shi and others 2012), which indicated that ZF2 may not take part in the inhibition of IFN- β , while our present results, especially which were obtained from the mutants of the putative zinc-binding amino acid of the ZF domain, provided the first evidence that the ZF domain was necessary for nsp1 α to inhibit the production of IFN- β (Fig. 1 and Fig. 2). Moreover, we also found that only the ZF domain itself was not sufficient to inhibit the production of IFN- β (Fig. 3), which suggested that the ZF domain in nsp1 α was not the only region participated in the inhibition of IFN- β production.

nsp1 α contained 3 parts: the N-terminal ZF domain (Met1–Glu65), the PCP domain (PCP α domain, Pro66 to Gln166), and the C-terminal extension (CTE; Arg167 to Met180) (Sun and others 2009). Our and other previous studies have shown that both the activity of the PCP α and the region of the CTE were required for nsp1 α to inhibit the production of IFN- β (Song and others 2010; Shi and others 2011b, 2012),

while our present work gave the new information that the N-terminal ZF domain was also essential for nsp1 α to inhibit the production of IFN- β . The above results indicated that all the 3 parts participated in the function of nsp1 α to inhibit the production of IFN- β ; that is, if there is no help of any part, the nsp1 α will not inhibit the production of IFN- β , which indicated that it was possible that all the 3 parts determined the structure of nsp1 α or influenced the interaction of nsp1 α with the cellular proteins or the cellular components.

The ZF domain, which was one of most prevalent protein domains, was always short and was independently folded by some amino acids that needed the coordination with the zinc atom to stabilize its structure (Laity and others 2001). Moreover, the ZF domain was the common motif that participated in the interaction between protein and DNA (Klug 2010), protein and RNA (Lu and others 2003), and protein and protein (Liew and others 2005), so it was not surprising that many viruses encoded ZF proteins that help virus antagonize the cell antiviral response. For example, the ZF domain in the infected cell protein 0 (bICP0) encoded by *Bovine herpesvirus 1* (BHV-1) was essential for inhibiting the IFN-dependent transcription (Lin and others 2004; Henderson and others 2005; Saira and others 2007), and the ZF domain in EAV nsp1 was essential for the transcription of EAV (Tijms and others 2007). Therefore, our present work provided another evidence that the nsp1 α ZF domain encoded by PRRSV was essential for nsp1 α to inhibit the cellular antiviral response. However, the precise mechanism that the ZF domain was essential for nsp1 α to inhibit the production of IFN- β warranted further investigation.

In conclusion, our present work demonstrated that the ZF domain was essential for PRRSV nsp1 α to inhibit the IFN- β

induction. Mutation, that is, mutagenesis of the predicted zinc-coordinating residues of the ZF domain made nsp1 α lose its interferon antagonism activity. Along with the previous studies that the recombinant IFN- β not only protected swine alveolar macrophages and MARC-145 cells from infection with PRRSV (Overend and others 2007) but also could reduce the yield of PRRSV *in vivo* (Buddaert and others 1998), the ZF domain of nsp1 α may be an exciting target for exploiting new drugs for PRRSV treatment or for choosing the modified live-virus PRRSV vaccine development.

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Author Disclosure Statement

No competing financial interests exist.

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