

Full Length Research Paper

Hsp70 protein can enhance the immunogenicity of FMDV VP1 epitopes fused with PTD-J domain

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An artificial FMDVepi polypeptide assembled by T_H- and B-cell epitopes of FMDV VP1 capsid protein was prepared to study its immunogenicity. FMDVepi, PTD-J-FMDVepi and PTD-J-FMDVepi/mHsp70 complex were separately used to immunize mice to determine their antigenicities to elicit antibodies against FMDVepi and VP1. The immunogenicity of PTD-J-FMDVepi was stronger than that of FMDVepi. Moreover, addition of mHsp70 to PTD-J-FMDVepi significantly enhanced its antigenicity to elicit antibodies against both FMDVepi and VP1. The VP1 protein expressed in HeLa cells was detected locating in autophagosome by using the prepared antibodies.

Key words: Hsp70, FMDV VP1, antigenicity, autophagosome.

INTRODUCTION

Foot-and-mouth disease virus and autophagy

Foot-and-mouth disease virus (FMDV) is the etiological agent of FMD, which is a highly contagious and economically devastating disease affecting cloven-hoofed animals such as cattle, pig, deer, goat and sheep.

FMDV is a positive single-stranded RNA virus with a genome of about 8500 nucleotides encoding a polyprotein which can be self-processed into structural capsid proteins VP1-VP4 and non-structural proteins such as L proteinase, 3C protease and 3D RNA-dependent RNA polymerase.

FMDV belongs to the genus *Aphthovirus* of the family Picornaviridae (Grubman and Baxt, 2004; Grubman et al., 2008). FMDV binds to its target cells through interactions between RGD tripeptide motif on the GH-loop of VP1 and integrin receptors on the target cells (Jackson et al., 2002). VP1 protein, but not FMDV particle, could induce the treated BHK-21 cells apoptosis through the Akt signaling pathway (Peng et al., 2004).

Like many positive single-stranded RNA viruses that can subvert the cellular defense autophagy machinery to

favor their replication and release (Klein and Jackson, 2011; Shi and Luo, 2012), the non-structural 2B and C and 3A as well as, the structural VP1 proteins of FMDV are localized in autophagosomes and autolysosomes of FMDV infected cells (O'Donnell et al., 2011).

The PTD-J-X system

We took advantages of the specific association ability of the J domain of Hsp40 with the nucleotide binding domain of Hsp70 and the cell membrane penetrating activity of the protein transduction domain (PTD) of HIV-1 Tat protein to construct an expression vector pET22b-PTD₁J₁ which was able to express higher level and more soluble chicken IGF-I than pET32b vector did.

An HpNC peptide containing two fragments of human heptoprotein was expressed by this vector. The PTD-J-HpNC recombinant polypeptide product could effectively elicit rat antisera specific to subtype Hp1 and Hp2 heptoproteins in human serum samples; however, the counterpart, TrxA-HpNC, could not (Chuang et al., 2009).

Mouse inducible form of Hsp70 protein and innate immunity

There are two major types of heat shock protein 70 in

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mouse somatic cells: constitutive Hsc70 which is involved in the folding of nascent proteins and stress-inducible Hsp70 (also signed as Hsp72, Hsp70.1 or Hspa1b). Hsc70 and Hsp70 can associate with lipid bilayer (Arispe et al., 2002). Extracellular Hsp70 can be in a soluble form released from injured cells.

Moreover, Hsp70 can translocate into plasma membrane and release into the extracellular environment in a membrane-bound form (De Maio, 2011; Vega et al., 2008). Extracellular Hsp70 acts as a functionally significant danger signal for the immune system (Campisi et al., 2003) to induce a pro-inflammatory response through CD91 receptor (Basu et al., 2001) and/or TLR-4 receptor (Chase et al., 2007) followed by NF- κ B-dependent pathway (Anand et al., 2010; Pawaria and Binder, 2011).

In this study, we took an artificial polypeptide composed of FMDV VP1 T_H and B epitopes (FMDVepi) as antigen, either conjugated with the PTD-J carrier or alone to test the ability of mouse Hsp70 recombinant protein (mHsp70) as an enhancer of immunity in a mouse animal model.

MATERIALS AND METHODS

T_H- and B-cell epitopes analysis

BALB/c mouse was chosen as animal model; therefore, the items of H-2^d alleles were picked up in the T_H epitopes prediction program. ABCpred (Saha and Raghava, 2006) and BepiPred (Larsen et al., 2006) were used to predict B epitopes and consensus method (Yang and Yu, 2009) was used to predict MHC-II-binding peptides. Published B- (Cedillo-Barron et al., 2003; Pfaff et al., 1988; Taboga et al., 1997; Wang et al., 2002) and TH-epitopes (Collen et al., 1991; Taboga et al., 1997) of cattle were selected as references. The predicted and selected data are summarized in supplementary Figure S₁.

Vectors construction and recombinant proteins expression

Three peptides of FMDV VP1 (O-TW-253-99) with amino acid 4-27 predicted B- and TH- epitopes and with amino acid 143 to 160 (NVRGDLQVLAQKAERTLP), containing known swine and cattle B-epitopes (Cedillo-Barron et al., 2003; Pfaff et al., 1988; Taboga et al., 1997; Wang et al., 2002) were assembled to be an FMD Vepi peptide (Figure 1) having the amino acid sequence "AGESADPVTATVENYGGGETQVQRRVKHEGDLTWVPN GAPETALNVRGDLQVLAQKAERTLP". FMDVepi cDNA with optimized *Escherichia coli* codons flanked by EcoRI and NdeI restriction enzyme sites and XhoI site at 5' and 3' end, respectively, and a VP1 cDNA encoding full-length FMDV VP1 protein were synthesized. The sequences of

these cDNAs are shown in supplementary Figure S₂. The FMDVepi cDNA was either digested by EcoRI and XhoI or NdeI and XhoI before insertion into the same restriction enzyme sites of pET22b-PTD1J1 (Chuang et al., 2009) to obtain pET22b-PTD1J1-FMDVepi and pTE22b-FMDVepi, respectively. The FMDVepi cDNA digested by EcoRI and XhoI were also inserted into the same restriction sites of pET22b-PTD1 and pET22b-J1 vectors to obtain pET22b-PTD-FMDVepi and pET22b-J-FMDVepi, respectively.

The VP1 cDNA was digested with EcoRI and XhoI and inserted into the same sites of pET32b and pCX-MCS1-Flag2 vectors (supplementary Figure S₃) to obtain pET32b-VP1 and pCX-VP1-Flag2, respectively. pET22b-FMDVepi, pET22b-PTD1J1-FMDVepi, pET32b-VP1 and pET22b-mHspa1 were transformed into *E. coli* Rosetta-gami B(DE3)pLysS to express FMDVepi, PTD-J-FMDVepi, TrxA-VP1 and recombinant mHsp70 proteins.

The FMDVepi, PTD-J-FMDVepi, and recombinant mHsp70 proteins were expressed in a soluble form, whereas, the TrxA-VP1 was expressed in an inclusion body form. HeLa cells were grown on cover slips and transfected with pCX-VP1-Flag2 plasmid by using Turbofect (Fermentas, #R0531) to express VP1-Flag2 recombinant protein. Forty-eight hours after transfection, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline at 4°C for 10 min.

Immunization and immunological analysis

Three BALB/c mice in a group were immunized equimolarly with 20 μ g of FMDVepi (mice # 801-803), 35 μ g of PTD-J-FMDVepi (mice # 80-806) or 35 μ g of PTD-J-FMDVepi plus 135 μ g of mHsp70 recombinant protein (mice # 807-809) with complete Freund's adjuvant, followed by three boosts with incomplete Freund's adjuvant every third week. Sera were collected 6 days after each boost.

For dot-blot analysis, serially diluted FMDVepi recombinant protein was spotted on PVDF membrane. After blotting in 5% skimmed milk/TBST (25 mM Tris-HCl, pH 7.5/150 mM NaCl/0.05% Tween 20), the membranes were incubated with immunized sera (1:1000 dilution) in a blotting solution for 1 h at room temperature. Next, the membranes were washed 4 times with TBST and incubated with HRP-conjugated goat anti-mouse IgG antibody (1:2000 dilution, Millipore, Cat. No. AP132P) for 1 h at room temperature.

Signals were performed using a Western lightning Plus-ECL kit (Perkin Elmer, NEL105-001 KT). For immunofluorescence assay, the pCX-VP1-Flag₂ transfected cells fixed on cover slips were incubated with the prepared sera (1: 100 dilutions), M2 mAb (Sigma, Cat. No. F-1804) or rabbit anti-LC3-II antibody (1: 100, Cell Signaling, #3868). Then, the cover slips were incubated with either DyLight488-conjugated goat anti-mouse IgG antibody (1:200 dilutions, GeneTex,

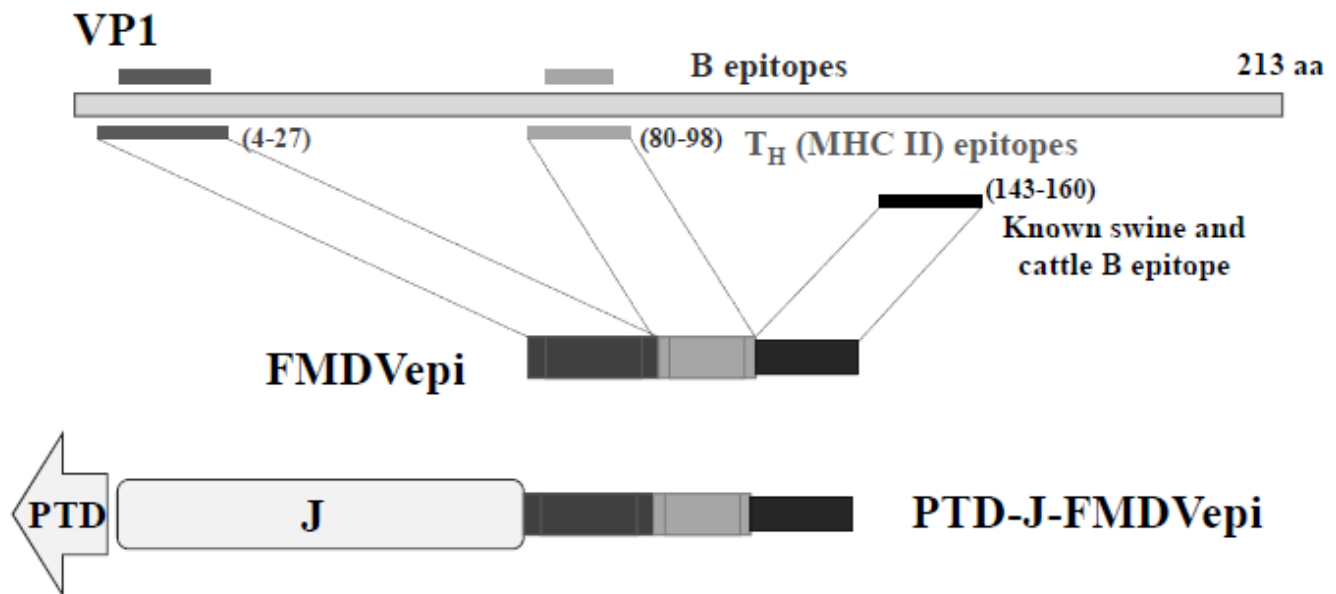


Figure 1. FMDVepi, VP1, and PTD-J-FMDVepi; FMDVepi is an artificial polypeptide assembled by the linking amino acid 4 to 27, 80 to 89 and 143 to 160 of the VP1 protein. The first two protein fragments contain one expected B- and one T_H -cell (MHC II) epitope for BALB/c mouse, and the third fragment contains a known B-cell epitope for cattle and swine.

GTX76717) or DyLight594-conjugated goat anti-rabbit IgG antibody (1:200 dilution, GeneTex, GTX76759) for 1 h. Nuclei were counterstained by DAPI in a mounting reagent (Invitrogen, Cat. No. P36935). The immunofluorescence images were taken by a fluorescence microscope (Personal DV Applied Precision, Issaquah, WA) and processed by the software softWORX for deconvolution.

***In vitro* TLR2 and TLR4 stimulation assays**

HEK-Blue-hTLR2 cells which is stably transfected with TLR2 receptor gene, CD14 co-receptor gene and SEAP (secreted embryonic alkaline phosphatase) reporter gene (InvivoGen, Cat. No. hkb-hltr2). The SEAP reporter gene was placed under the control of the IFN- β minimal promoter fused to five NF- κ B and AP-1-binding sites.

The stimulation of TLR2 can activate NF κ which turns on the expression of SEAP. Like the HEK-Blue-hTLR2 cells, HEK-Blue-hTLR4 cells are stably transfected with hTLR4 gene instead of hTLR2 gene (InvivoGen, Cat. No. hkb-hltr4). The assays were followed by the manufacturer's instruction manual. FSL-1 (Invivo Gen, Cat. No. tlr1-fsl) was used as positive stimulator for TLR2, while recombinant LH (attenuated heat-labile enterotoxin, a kind gift from Dr. Jiunn-Horng Lin at Division of Animal Medicine, Animal Technology Institute, Taiwan) and recombinant DsRed proteins isolated by the same procedure of mHsp70 isolation were used for comparison

in the assays.

The HEK-Blue-hTLR2 cells were treated with seriously diluted recombinant proteins for 24 h. The activities of secreted alkaline phosphatase in media were measured by using QUANTI-Blue as chromogenic substrate with specific product absorption at 630 nm. The stimulating activities of the recombinant mHsp70, LH and DsRed on HEK-Blue-hTLR4 cells were tested with the same procedures.

RESULTS

Expression of the PTD-J-FMDVepi, J-FMDVepi, PTD-FMDVepi and FMDVepi recombinant proteins

Because expression of recombinant proteins by the pET22b-PTD₁J₁ vector was higher than that by other pET vectors (Chuang et al., 2009), we suggested that the PTD-J domain stabilized the fused recombinant proteins to increase the final yield. However, such effect is whether dependent on the PTD, the J domain or the combination of them remains undetermined. To answer this question, four plasmids, pET22b-PTD₁J₁-FMDVepi, pET22b-PTD-FMDVepi, pET22b-J-FMDVepi and pET22b-FMDVepi, were constructed and transformed into *E. coli* Rosetta-gami B(DE3)pLysS cells.

Three colonies from each transformant were picked. These clones were grown in 2 × YT supplemented with Ap until OD₆₀₀ reached 0.6. Then, production of

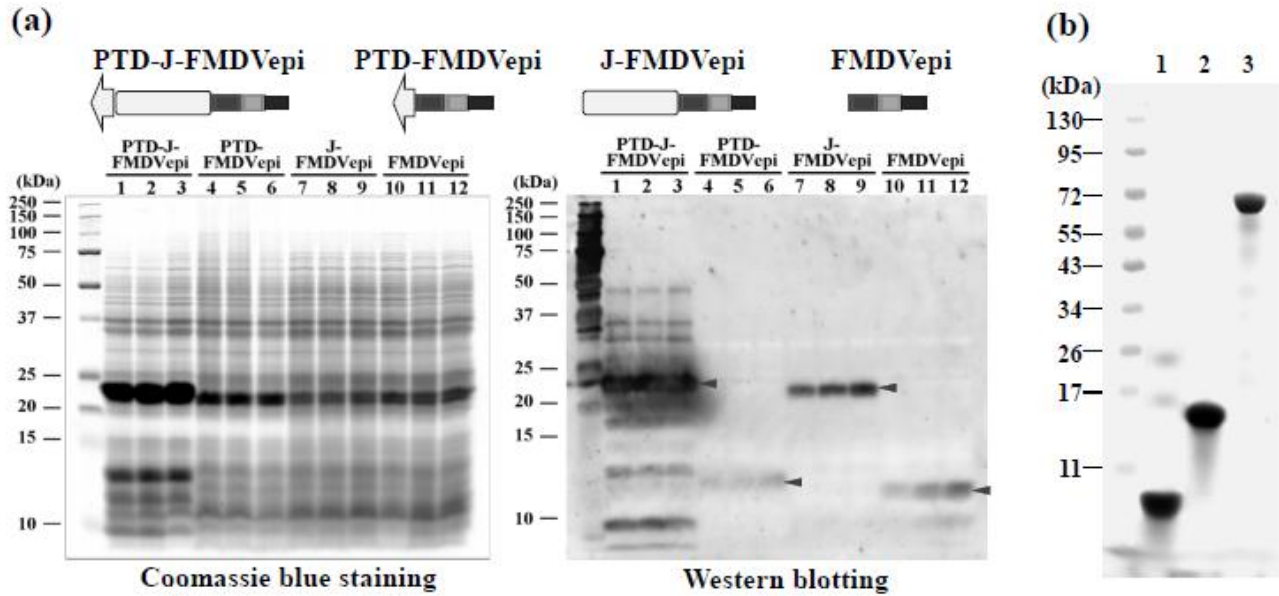


Figure 2. Expression of PTD-J-FMDVepi, PTD-FMDVepi, J-FMDVepi, and FMDVepi in *E. coli* host cells; (a) All of the four recombinant proteins contain a (His)₆-tag at the C-terminal end; three colonies from each transformant were picked. After IPTG induction, total lysate from each 0.1 OD₆₀₀ units of cells was loaded in different wells of a polyacrylamide gel. After electrophoresis, the gel was stained directly by Coomassie blue dye (left panel) or was blotted by semi-dry electro-transfer to detect signals by using an anti-His-tag antibody (right panel). (b) Purified FMDVepi (lane 1), PTD-J-FMDVepi (lane 2) and mHsp70 (lane 3) recombinant proteins were analyzed by SDS PAGE and stained by Coomassie blue.

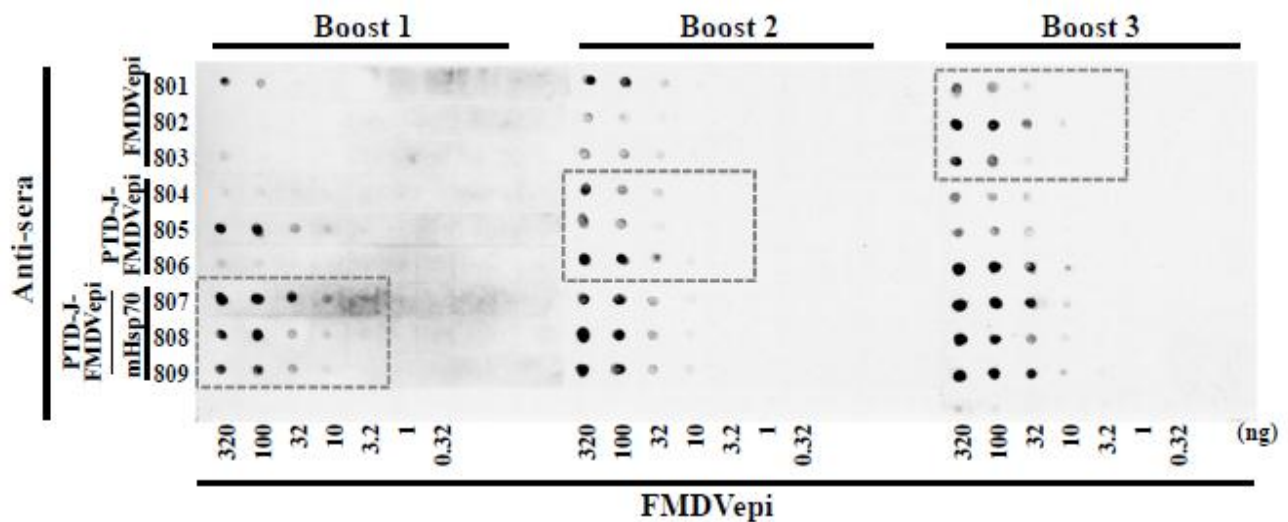


Figure 3. Dot blot assay of sera against FMDVepi; membrane dots with different amount of FMDVepi protein as indicated were used to analyze the strength of the sera from mice immunized with FMDVepi, PTD-J-FMDVepi, and PTD-J-FMDVepi/mHsp70. Sera after first, second and third boosts were collected.

recombinant proteins was induced by addition of 1 mM IPTG and culture for 3 h. Total lysates from 0.1 OD unit of cells were loaded in each lane of a 15% polyacrylamide gel.

The amount of expressed recombinant proteins was

analyzed using Coomassie blue staining or western blotting with an antibody against the His-tag on the C-terminus of all the 4 recombinant proteins. The expression level of PTD-J-FMDVepi recombinant protein was significantly higher than that of PTD-FMDVepi, J-

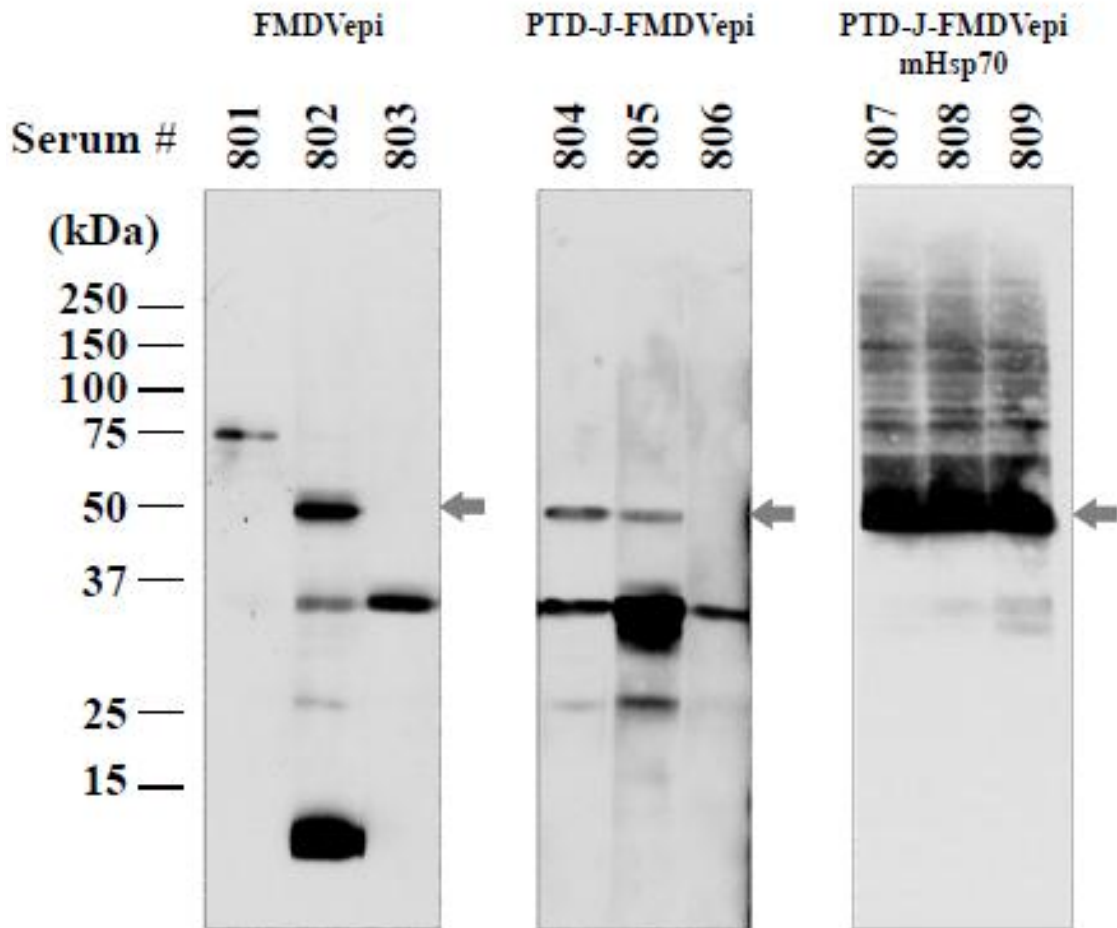


Figure 4. Western blot assay of sera against TrxA-VP1; total lysate of 0.01 OD₆₀₀ unit of *E. coli* transformed with pET32b-VP1 was separated in different lanes of a polyacrylamide gel. Once the proteins were transferred to a PVDF membrane, each slot of membrane was incubated with boost 3 sera (1:1000 dilution) for 1 h, followed by incubation with HRP-conjugated secondary antibody (1:2000 dilution) for 1 h. Signals were detected by chemo-luminescence image performance.

FMDVepi and FMDVepi both in mass (detected using Coomassie blue staining; Figure 2a, left panel) and in molar ratio (detected using western blotting; Figure 2a, right panel).

This result supports our hypothesis that the combination of PTD and J domain could enhance the expression of fused recombinant proteins in *E. coli* cells. The purities of the FMDVepi, PTD-J-FMDVepi and mHsp70 recombinant proteins were higher than 95% as analyzed by scanning after SDS PAGE and Coomassie blue staining as shown in Figure 2b.

Immunogenicity

Previously, we reported that PTD-J-HpNC, but not TrxA-HpNC, could elicit the production of rat antibodies against heptoprotein (Chuang et al., 2009). Whether PTD-J domain could enhance immunogenicity of its fused

polypeptide was a question to be resolved. FMDVepi (Figure 1) was designed according to the genetic background of BALB/c mouse, which was chosen as the animal model in this study.

Mice were immunized with equimolecular amounts of FMDVepi, PTD-J-FMDVepi and PTD-J-FMDVepi/mHsp70 as described in materials and methods. As shown in Figure 3, high titers of antibodies elicited by FMDVepi against itself were only found in the boost 3 sera; however, similar effects were illustrated in boost 2 sera as PTD-J-FMDVepi was used. It is interesting to note that only one boost is necessary in the PTD-J-FMDVepi/mHsp70 cases to elicit antibodies of the same, even stronger activities.

Western blotting assay

FMDVepi is composed of three VP1 fragments and as

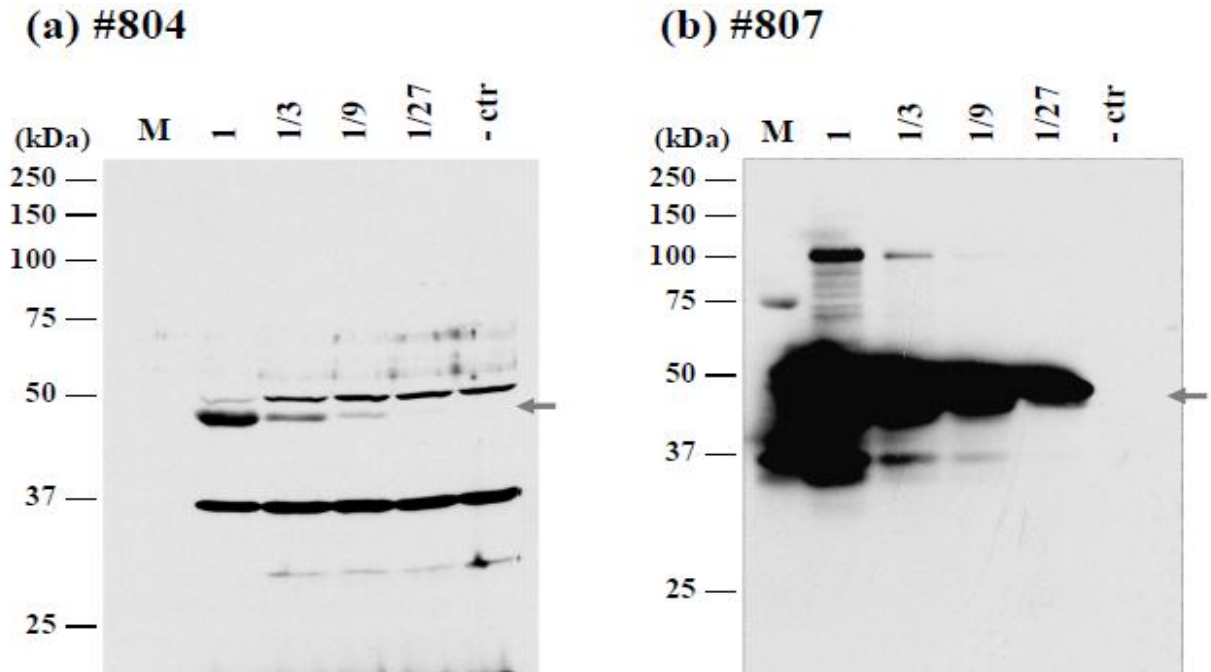


Figure 5. Effect of mHsp70 on the immunogenicity of PTD-J-FMDVepi; one-fold TrxA-VP1 lysate (total lysate of 0.01 OD₆₀₀ unit of *E. coli* cells transformed by pET32b-VP1) was serially diluted to 3 folds by using normal *E. coli* host cell lysate (also used as negative control, “-ctr”) and separated by SDS-PAGE. Western blot assay was performed using boost 3 #804 and #807 serum as described earlier.

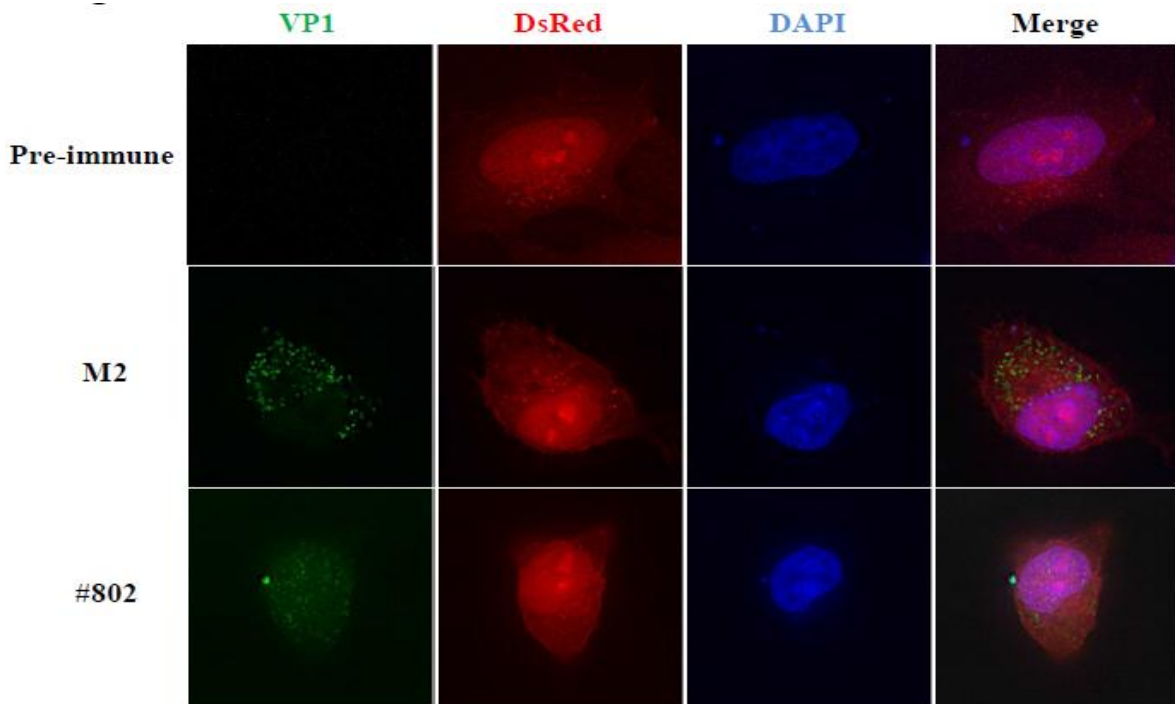


Figure 6. Localization pattern of overexpressed VP1 protein in cells; plasmid pDsRed-monomer N1 was cotransfected with pCX-VP1-Flag₂ into HeLa cells to indicate the transfected cells. Forty-eight hours after transfection, the cells were fixed and treated with pre-immune serum (row 1), M2 mAb (row 2) or boost 3 #802 serum (row 3). Next, DyLight488-conjugated goat anti-mouse IgG antibody were used to produce the fluorescence signals. Cellular nuclei were counterstained with DAPI.

such the antisera elicited by FMDVepi or PTD-J-FMDVepi are expected to recognize the full length VP1 protein. Initially, TrxA-VP1 expressed by pET32b in total lysate of host cells was used to analyze the boost 3 sera by western blotting. Only one serum in the FMDVepi set (#802) and two sera in the PTD-J-FMDVepi set (#804 and #805) could weakly recognize TrxA-VP1.

In contrast, all of the sera in the PTD-J-FMDVepi/mHsp70 set (#807, #808 and #809) gave very strong signals (Figure 4). To determine the enhancement of immunogenicity by mHsp70, TrxA-VP1 samples serially diluted to 3-fold with normal host cell lysates with normal host cell lysates were detected using 1000-fold diluted serum #804 and #807. Signal from 27-fold diluted TrxA-VP1 sample detected using the #807 serum was comparatively stronger than that from undiluted TrxA-VP1 signal detected using the #804 serum (Figure 5), indicating that mHsp70 is a very good adjuvant for the PTD-J domain-conjugated FMDVepi antigen.

Immuno-fluorescence assay

In addition to western blotting assays, the VP1-recognizing activity of these boost 3 sera were also tested in HeLa cells by using immunofluorescence assay. Plasmid pDsRed-monomer N1 (BD Biosciences, Cat. No. 632465) was cotransfected with pCX-VP1-Flag₂ to indicate transfected cells. The expressed VP1-Flag₂ recombinant protein was detected using M2 mAb against the Flag tag or boosts 3 sera. DyLight488-conjugated goat anti-mouse IgG antibody was used as a secondary antibody to produce fluorescence signals.

It is interesting to note that spotted green fluorescence signals were found in the transfected cells as detected by using M2 mAb as well as #802, #804, #805, #807, #808 and #809 sera. Images of fluorescence signals detected by M2 mAb and #802 serums are shown in Figure 6.

VP1 associated with autophagosome

Positive single-stranded RNA virus such as Picornavirus can subvert cellular autophagy pathway and use autophagosome as platform for viral replication (Klein and Jackson, 2011; Shi and Luo, 2012). FMDV, which belongs to the family Picornaviridae was also found utilizing autophagy pathway for its replication (O'Donnell et al., 2011).

The spotted pattern of FMDV VP1 capsid protein expressed in HeLa cells may be because of the association of VP1 with autophagosome. LC3-II, a protein marker of autophagosome, was immuno-stained to differentiate between the spacial distribution patterns of VP1 and LC3-II containing vesicles. High percentage of the VP1 signals obtained using serum #802, #804 and #807 overlapped the LC3-II signals (Figure 7). Moreover, signals obtained by serum #807 were much stronger than those obtained using the other two sera.

DISCUSSION

mHsp70 can enhance the immunogenicity of PTD-J-FMDVepi

The extremely high expression of PTD-J-FMDVepi relative to PTD-FMDVepi, J-FMDVepi and FMDVepi indicates that the combination of PTD and J domain produces a special conformation that stabilizes the PTD-J-FMDVepi recombinant protein, which accumulates abundantly in *E. coli* cells. Such conformation is an interesting topic for further study.

As the model we had introduced (Chuang et al., 2009), specific interactions between the J domain and the nucleotide-binding domain of Hsp70, polypeptide conjugated to the PTD-J domain could have a higher chance to be trapped by the substrate domain of Hsp70. Because Hsp70 isolated from tumor lysate can trap a tumor-specific peptide and this Hsp70/peptide complex along with dendritic cells can induce cellular immune response (Flechner et al., 2006; Pandya et al., 2009).

mHsp70 was mixed with PTD-J-FMDVepi to check whether mHsp70 could alter the immunogenicity of PTD-J-FMDVepi during a humoral immune response in mice. Figure 3 shows that the three boosts for FMDVepi and two boosts for PTD-J-FMDVepi were necessary to elicit antibodies that were strong enough to recognize the FMDVepi protein itself. However, only one boost was needed to elicit the same, even higher, antibody titers. This result indicates that mHsp70 could evidently enhance the immunogenicity of PTD-J-FMDVepi.

We immunized three mice with a set with FMDVepi and FMDVepi/mHsp70 and did not observe significant differences between the two sets of antisera (data not shown). Taken together, our results suggest that the enhancement of the immunogenicity of PTD-J-FMDVepi on FMDVepi by mHsp70 is PTD-J domain dependent.

Although, it had been suggested that Hsp70 could stimulate innate immunity through TLR2 and/or TLR4 (Tsan and Gao, 2009), direct evidences are necessary to further confirm it. We used HEK-Blue-hTLR2 cells which express TLR2 and CD14 uniquely to analyze the stimulation effect of mHsp70. As shown in Figure 8A, like FSL-1, a positive control, which could activate the SEAP expression with a half-stimulation concentration of 9 nM, mHsp70 could stimulate TLR2 to elevate SEAP activity with a half-stimulation concentration of 20 nM.

The recombinant LH and DsRed proteins could not stimulate TLR2. On the other hand, both mHsp70 and LH could stimulate TLR4 with a half-stimulation concentration of 6 and 4 nM, respectively. It is suggested that TLR2 and TLR4 can recognize the cell wall components of Gram positive and Gram negative bacteria, respectively. The recombinant proteins, mHsp70, LH and DsRed, prepared from *E. coli* may be contaminated with trace amount of the cell wall components (O'Neill et al., 2013), such as LPS, therefore, high concentration of DsRed could

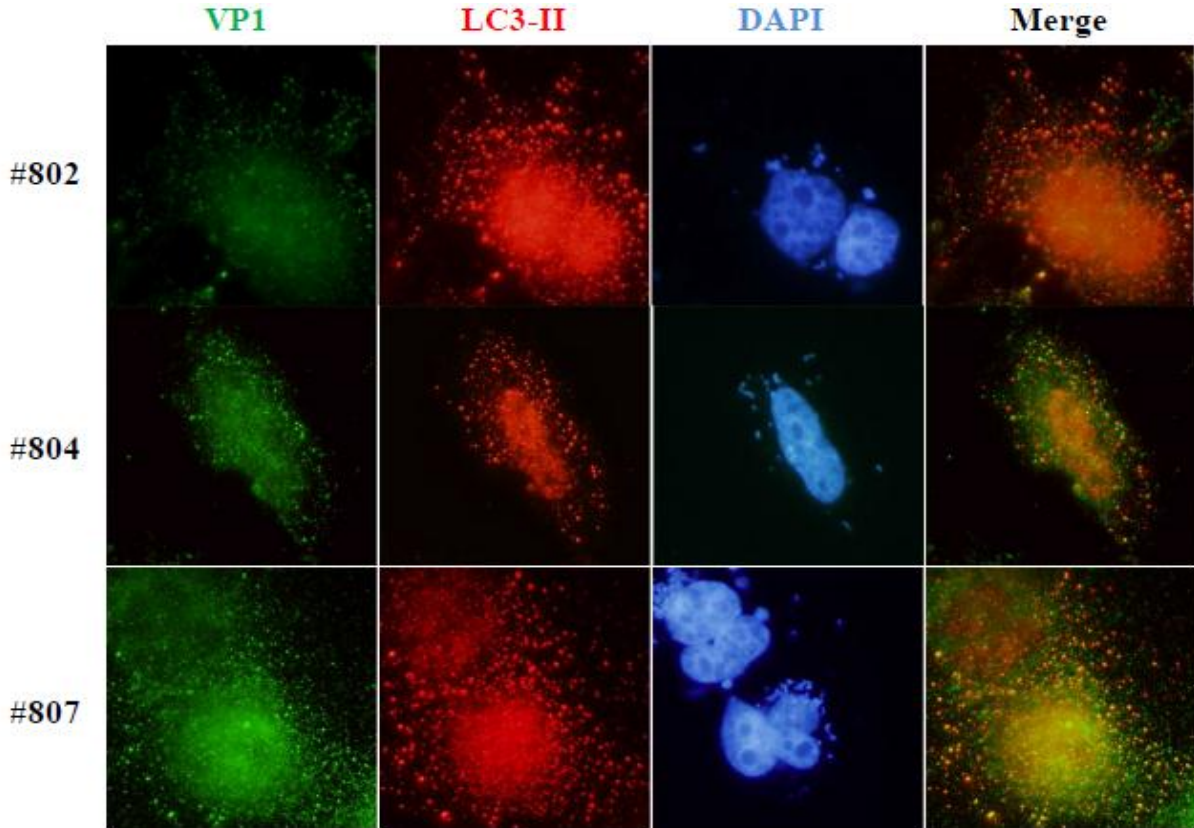


Figure 7. VP1 was colocalized with autophagosome-specific LC3-II; HeLa cells transfected with pCX-VP1-Flag2 were double labeled with the boost 3 sera (#802, #804 and #807) and rabbit anti-LC3-II antibody. Next, DyLight488-conjugated goat anti-mouse IgG antibody and DyLight594-conjugated goat anti-rabbit IgG antibody were added to produce the VP1 and LC3-II signals, respectively. Cellular nuclei were counterstained with DAPI.

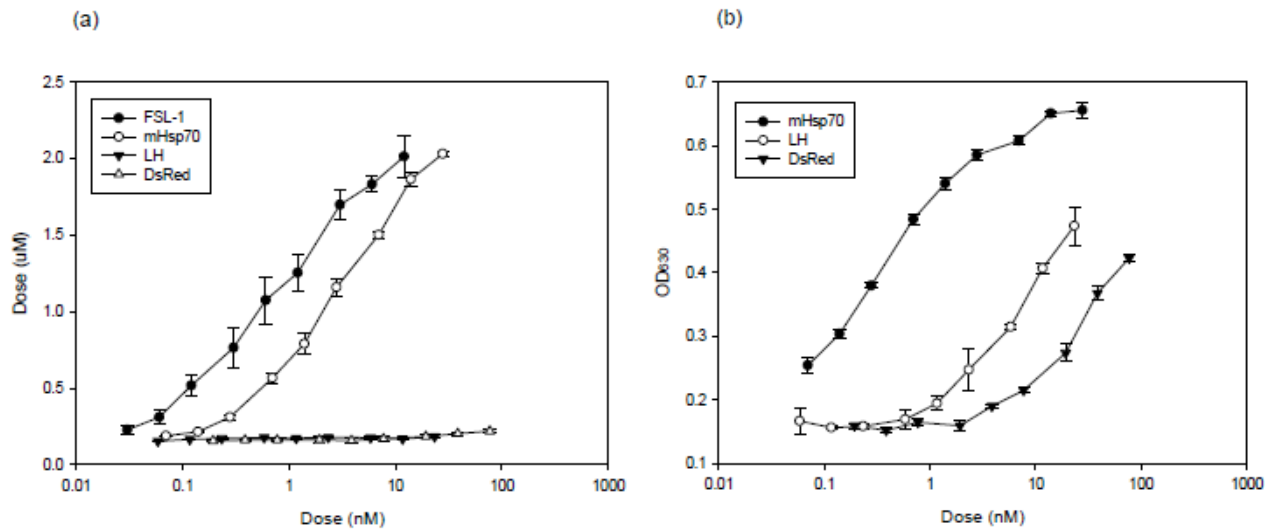


Figure 8. Stimulation of TLR2 and TLR4 by mHsp70. (A) HEK-Blue-hTLR2 cells were treated with 0.00005, 0.0001, 0.0002, 0.0005, 0.001, 0.002, 0.005, 0.01 and 0.02 $\mu\text{g/ml}$ of FSL-1 (M.W. 1666.2) or 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 $\mu\text{g/ml}$ of mHsp (72 kDa), LH (86 kDa) and DsRed (26 kDa) for 24 h. Then, alkaline phosphatase activities in the media were analyzed by using QUANTI-Blue as a chromogenic substrate. (B) Like procedures described earlier, HEK-Blue-hTLR4 cells were seriously treated with diluted mHsp, LH and DsRed.

partially stimulate TLR4 (Figure 8B).

The endotoxin concentrations of mHsp70, LH and DsRed recombinant proteins were measured as 960 ± 30 , 1280 ± 20 and 810 ± 20 units per mg of recombinant protein, respectively. In comparison with the data shown in Figure 8, the effect of endotoxin was not obvious and the stimulations of TLR2 and TLR4 should be activated by mHsp70 and by LH and mHsp70, respectively.

Antibodies elicited by PTD-J-FMDVepi/mHsp70 can recognize the full-length VP1 protein more specifically and strongly than those elicited by FMDVepi and PTD-J-FMDVepi

Because FMDVepi was created by linkage of three epitope-containing VP1 fragments, the amino acid sequences around the junctions of the linked peptides were not present in native VP1 protein, indicating that antibodies elicited by FMDVepi may not recognize the full-length VP1 at all. Only one (#802) antiserum elicited by FMDVepi and two (#804 and #805) antisera elicited by PTD-J-FMDVepi could recognize TrxA-VP1 by Western blotting.

However, all of the three antisera elicited by PTD-J-FMDVepi/mHsp70 could bind to TrxA-VP1 more strongly and specifically (Figure 4). This result suggested that mHsp70 not only augmented the immunogenicity of PTD-J-FMDVepi but also improved the ability of the antisera to recognize the epitopes on full-length VP1 protein. The other antisera which could recognize FMDVepi only may contain immune-globulins elicited by the peptide motifs at the junctions between the fused epitopes.

FMDV VP1 might associate with autophagosome

Many positive single-stranded RNA viruses replicate on the autophagosome membrane (Klein and Jackson, 2011; Shi and Luo, 2012). IRGM is a common target of RNA viruses that subvert the autophagy network (Grégoire et al., 2012, 2011). Replication of pig positive-stranded RNA viruses, such as FMDV and porcine reproductive and respiratory syndrome virus (PRRSV), was also demonstrated to be autophagy dependent (Chen et al., 2012; O'Donnell et al., 2011).

The capsid protein VP1 of FMDV alone in HeLa cells displayed a punctate pattern, as analyzed by the immunofluorescence assay (Figure 6); moreover, these spots highly overlapped the autophagosome spots marked by LC3-II (Figure 7).

The VP1 protein alone could induce apoptosis of treated BHK-21 cells through the Akt signaling pathway (Peng et al., 2004). Some scraps of nuclei could also be detected (Figure 7). Correlation between the association of VP1 with autophagosome and the ability of VP1 to induce apoptosis is still unclear and is a topic for future studies.

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