Localization of human (pro)renin receptor lacking the transmembrane domain on budded baculovirus of *Autographa californica* multiple nucleopolyhedrovirus

Tatsuya Kato・Dongning Du・Fumiaki Suzuki・Enoch Y. Park

Tatsuya Kato

Laboratory of Biotechnology, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

Dongning Du・Enoch Y. Park(✉)

Integrated Bioscience Section, Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan

e-mail: acypark@ipc.shizuoka.ac.jp

Fumiaki Suzuki

Laboratory of Animal Biochemistry, Faculty of Applied Biological Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

Abstract Human (pro)renin receptor (hPRR), a construct with native transmembrane and cytoplasmic domains (hPRR-wTM), and hPRR lacking both (hPRR-w/oTM) were expressed using insect cells. The hPRR-wTM was expressed in the peripheral domains of the nucleus in infected Sf-9 cells and its localization was observed in endoplasmic reticulum (ER). However, it could not be extracted from recombinant *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) by Triton X-100 treatment at 4°C. In contrast, hPRR-w/oTM was observed in punctate domains in the cytoplasm of infected Sf-9 cells, but intracellular hPRR-w/oTM did not co-localize in the Golgi apparatus and lysosomes. This indicates that hPRR-wTM and hPRR-w/oTM is localized in the ER and cytoplasmic organelles of Sf-9 cell respectively. Moreover, the localization of hPRR-w/oTM in budded baculovirus of recombinant AcMNPV was confirmed by Western blotting. This is the first finding of the association of a foreign protein lacking a transmembrane domain with a baculovirus. If this finding is available for double displaying system, being capable of expression on the envelope and the capsid of baculovirus, it will lead to new methodology of baculovirus display system for tissue- and cell-specific targeting, and intracellular targeting.

Keywords Human (pro)renin receptor · AcMNPV · Transmembrane domain · Budded baculovirus · Localization

Introduction

The baculovirus has been used as an expression vector for the production of eukaryotic proteins in insect cell culture, since the insect cells are capable of performing various co- and post-translational modifications including glycosylation (James et al. 1995), phosphorylation (Smith et al. 2007), fatty acid acylation (Zhang et al. 2003) and processing (Alam et al. 2002). In addition to recombinant protein production, surface display of recombinant proteins on the surface of baculoviruses has been developed lately (Grabherr et al. 2001; Oker-Blom et al. 2003). Such viruses displaying foreign proteins are used for monoclonal antibody production (Lindley et al. 2000; Saitoh et al. 2007), subunit vaccine production (Peralta et al. 2007) and construction and screening of eukaryotic epitope libraries (Ernst et al. 1998). In most cases, the display of foreign proteins on the surface of budded baculoviruses is achieved by the fusion of a foreign protein with a gp64 protein, which is a major baculovirus envelope protein and required for virion entry and exit. Occasionally, biologically active β2-adrenergic receptors are expressed on baculovirus particles without fusion with a gp64 protein (Loisel et al. 1997). Moreover, the reconstitution of leukotriene B4 receptors and trimeric G-proteins or γ-secretase complexes on the baculovirus envelope can be achieved (Masuda et al. 2003; Hayashi et al. 2004). Sterol regulatory element-binding protein-2 (SREBP-2) and SREBP cleavage-activating protein (SCAP) in the endoplasmic reticulum (ER) and/or Golgi apparatus have also been found to be expressed on the baculovirus surface without gp64 (Urano et al. 2003).

In a previous study, human (pro)renin receptor (hPRR) was reported to be expressed in insect cells with the baculovirus expression system. The (pro)renin receptor,

hPRR, complete with native transmembrane and cytoplasmic domains (hPRR-wTM) was purified from its microsomal fraction (Kato et al. 2008). hPRR lacking the native transmembrane and cytoplasmic domains (hPRR-w/oTM) can also be expressed, but was found to be secreted only slightly into the culture medium and could not be adequately purified. hPRR binds to either prorenin or renin and this binding activates the former without cleavage of the pro-segment. It has been reported that a decoy peptide which interferes with the binding of hPRR to prorenin can inhibit diabetic nephropathy and the development of glomerulosclerosis in diabetic angiotensin II type 1a receptor deficient mice (Ichihara et al. 2004; 2006).

In this study, hPRR-wTM or hPRR-w/oTM was expressed in insect cells and its expression on the surface of budded baculoviruses was investigated. Surprisingly, hPRR-w/oTM, which does not have a native transmembrane domain, was detected in the budded baculovirus (BV) fraction of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), but hPRR-wTM was not. This is the first report of the association of a foreign protein lacking a transmembrane domain with a baculovirus and this system will propose a new baculovirus display system.

Materials and methods

Insect cells, baculoviruses, cell cultures and infection

Sf-9 cells (Invitrogen, Carlsbad, CA, USA) were maintained in SF-900 II Serum Free Medium (Invitrogen) supplemented with 1% antibiotic-antimycotic (Invitrogen). Recombinant baculoviruses containing the hPRR-wTM or hPRR-w/oTM gene

(AcMNPV/hPRR-wTM or AcMNPV/hPRR-w/oTM) were constructed prior to the study (Kato et al., 2004). Sf-9 cells were infected with 0.5 ml transfection solution containing the recombinant baculovirus AcMNPV/hPRR-wTM or AcMNPV/hPRR-w/oTM and cultivated for 1-2 d. Culture supernatant was separated from infected cells by centrifugation at 8000×g for 10 min, and used for further recovery of recombinant viruses.

Baculovirus isolation and hPRR-w/oTM extraction from recombinant baculoviruses using detergent

The culture broth was centrifuged at 8000×g for 10 min and culture supernatant was recovered. It was overlaid upon 25% sucrose solution (25% sucrose in 5 mM NaCl and 10 mM EDTA) and centrifuged again at 114000×g for 1 h. The supernatant was removed and the pellets were washed with phosphate-buffered saline (PBS, pH 6.2) and suspended with a small volume of PBS for further experiments.

To extract hPRR-w/oTM from recombinant baculoviruses using detergent, the Sf9 cells grown for 2 d were suspended with PBS containing 1% Triton X-100 and incubated for 30 min at 4°C. This suspension was centrifuged at 114000×g for 1 h, the pellet was washed with the same buffer, and both supernatant and pellet were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.

Western blot

Proteins were separated by SDS-PAGE and subjected to Western blotting. After SDS-PAGE, proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking in 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), the membrane was incubated in a 1:10000 diluted mouse anti-FLAG M2 antibody solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. hPRR could be detected by anti-FLAG antibody due to the insertion of FLAG peptide sequence between the signal peptide and extracellular domain of hPRR (Kato et al. 2008). The membrane was then washed with TBST and incubated in 1:20,000 diluted anti-mouse IgG antibody labeled with horseradish peroxidase for 1 h. Detection was performed using ECL Plus Western blotting reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) and specific bands were detected using a Fluor-S/MAX multi-imager (Bio-Rad).

Immunofluorescence microscopy

Sf-9 cells were infected with transfection solution and, after cultivation for 2 d, the culture was sampled and infected cells were adhered to polylysine-coated slide glass. The cells were fixed in 8% formalin solution (Wako Pure Chem. Ind. Ltd., Osaka, Japan) for 20 min and washed with PBS (pH 7.0), whilst the remaining aldehyde was quenched by 50 mM NH_4Cl in PBS. The fixed cells were washed again with PBS, blocked overnight by 8% (w/v) bovine serum albumin (BSA) dissolved in PBS and incubated with a 1:1000 dilution of mouse anti-FLAG M2 antibody and 2% BSA for 2-3 h. Following this process, the cells were washed with PBS and incubated with 1:100 dilution of FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch

1 Laboratories Inc., West Grove, PA, USA) or 1:100 Cy3-conjugated goat anti-mouse
2 antibody (Jackson ImmunoResearch Laboratories, Inc.) and 2% BSA for 1 h. The ER,
3 Golgi apparatus, and lysosomes were stained with 0.5 μM ER-Tracker Red (Molecular
4 Probes, Carlsbad, CA, USA), 5 μM NBD C_6-ceramide complexed to BSA (Molecular
5 Probes), and 50 nM Lysotracker (Molecular Probes), respectively. After staining, the
6 cells were observed by Confocal laser-scanning microscopy (TCS-LS, Leica
7 Microsystem, Heidelberg, Germany). .
8
9 Surface plasmon resonance (SPR) experiment
10
11 A surface plasmon resonance experiment was performed with an SPR optical biosensor
12 (IAsys plus, IASYS Affinity Sensors Ltd. Saxon Hill, Cambridge, UK) using
13 carboxymethyl (CM) sensor cuvette. On the surface of the cuvettes, 2.5 mg of renin
14 (Cayman, Ann Arbor, MI, USA) was immobilized and those CM groups on the sensor
15 that had not reacted with the immobilized protein were blocked with ethanolamine as a
16 control for non-specific binding. PBS (pH6.2) was used as running buffer.
17
18 **Results**
19
20 Expression of hPRR-w/oTM associated with BV
21
22 The receptor consists of 350 amino acids with a signal peptide at the N-terminus and a
23 single transmembrane domain and a cytoplasmic domain at the C-terminus (Fig.1A).
24 hPRR-w/oTM, which lacks the transmembrane and cytoplasmic domains, could not be

adequately purified from a culture supernatant of insect cells. The inability to purify hPRR-w/oTM might reflect its low expression level in the insect cells. However, a significant level of hPRR-w/oTM could be detected in the culture supernatant fraction at 2 d P.I.T. (Fig. 1B), but hPRR-wTM was not.

To investigate whether the hPRR-w/oTM was displayed on BV, its expression in the BV fraction was assessed by Western blotting. Budded baculoviruses were isolated from the supernatant of Sf-9 cell cultures 2 d P.I.T. Whilst hPRR-w/oTM was detected in the BV fraction of both cell cultures; hPRR-wTM was not (Fig. 1C). Two bands were present above hPRR-w/oTM which, since they were detected in both BV fractions, may be derived from viral proteins by non-specific binding with the antibody. Degraded fraction of hPRR-w/oTM or non-specific binding to anti-FLAG antibody was also detected in the BV fraction. Cell viability at the recovery point was 99%, and therefore, it could be understood that cell debris has not contaminated the BV fraction.

When the BV fraction in the Sf-9 cell culture was stained by CBB after SDS-PAGE, gp64 was mainly detected (Fig. 1D). If the cell debris has contaminated the BV fraction, hPRR-wTM might also have been detected in its fraction, but, since it was absent, the possibility of contamination by cell debris was eliminated. On the other hand, AcMNPV-gp-GFP_{uv}-β3GnT2 (Kato et al., 2004), which has the GFP_{uv}- β3GnT2 gene containing a melittin signal sequence without transmembrane domain was isolated from cell culture supernatant. The GFP_{uv}- β3GnT2 fusion protein was secreted into the culture supernatant but was not detected in the BV fraction (data not shown).

Localization of hPRR in Sf-9 cells

1 Immunofluorescent analysis was performed to determine the localization of hPRR in
2 Sf-9 cells. The cells were infected with recombinant baculovirus and sampled at 2 d
3 P.I.T. with 98% viability. hPRR-wTM was detected predominantly in the peripheral
4 regions of the nucleus (Fig. 2A), and its expression was merged into ER image stained
5 with ER-Tracker Red. This indicates that it localized in the ER. Above result agrees
6 with that of a previous study which reports that full-length hPRR is localized in the ER
7 in HeLa-S3 cells (Schefe et al. 2006). However, in contrast, hPRR-w/oTM was detected
8 predominantly in the cell organelles, with punctate patterns in infected cells. Slight
9 peripheral fluorescence was detected when cells were infected with
10 AcMNPV-hPRR-w/oTM.

11 For further analysis, cells infected with AcMNPV-hPRR-w/oTM were stained
12 with fluorescent structural markers for the Golgi complex (NBD C_6-ceramide
13 complexed to BSA) and lysosomes (Lysotracker) (Fig. 2B, C). hPRR-w/oTM was
14 detected by immunostaining with mouse anti-FLAG M2 antibody, followed by Cy3 or
15 FITC-conjugated goat anti-mouse IgG antibody. Sf-9 cells expressing hPRR-w/oTM
16 exhibited punctate red or green fluorescence in the cytoplasm. However, with the
17 exception of a small amount of overlap between the expression pattern of hPRR-w/oTM
18 and the fluorescence pattern of the Golgi complex, the majority of hPRR-w/oTM was
19 not found to be overlapping with either the Golgi complex or lysosomes. Fluorescence
20 was not detected in the peripheral regions of infected cells, indicating that
21 hPRR-w/oTM resided in the cytoplasm, but not in the Golgi complex or lysosomes.
22

23 Triton X-100 treatment of infected cells and recombinant baculoviruses
24

1 To determine whether hPRR-wTM also exists in the plasma membrane, Sf-9 cells
2 infected with each recombinant baculovirus were suspended with 1% Triton X-100 in
3 PBS at 4 ^{o}C (Fig. 3A). Unlike hPRR-wTM, hPRR-w/oTM was detected in the soluble
4 fraction whilst remaining in the insoluble fraction of Sf-9 cells. The solubilization
5 pattern of hPRR-wTM was different from that of hPRR-w/oTM which was solubilized
6 by Triton X-100 in a similar manner to that of gp64 protein (Zhang et al. 2003). The
7 latter was solubilized from AcMNPV-infected cells by Triton X-100 at 4°C and found
8 to be localized in the plasma membrane. This indicates that hPRR-wTM did not exist in
9 the plasma membrane and that its localization in the ER might result due to its inability
10 to associate with the baculovirus. It is also possible that hPRR-w/oTM localized in the
11 plasma membrane or that the extraction of hPRR-w/oTM by Triton X-100 was caused
12 by the leakage from cellular organelles by the Triton X-100 treatment itself.
13 To determine the localization of hPRR-w/oTM in AcMNPV-hPRR-w/oTM, the
14 latter was treated by Triton X-100 and analyzed by Western blotting and CBB staining
15 of gp64 (Fig. 3B). The gp64 protein, which localizes in the viral envelope, can be
16 extracted by Triton X-100 from the viral fraction, but vp39 protein, which localizes in
17 the nucleocapsid, cannot. hPRR was not detected in the solubilized fraction of
18 AcMNPV-hPRR-w/oTM by Triton X-100 whilst gp64 protein was found in the
19 solubilized fraction, suggesting that hPRR-w/oTM might be associated with the
20 nucleocapsid rather than the viral envelope.
21
22 SPR experiment of recombinant baculoviruses
23
24 To confirm the localization of hPRR expressed in recombinant baculoviruses, SPR

1 experiment was performed. In a previous report (Kato et al. 2008), the result of SPR
2 experiment was obtained from purified hPRR-wTM bound to human renin immobilized
3 to a sensor chip. AcMNPV-hPRR-wTM, AcMNPV-hPRR-w/oTM and
4 AcMNPV-gp-GFP_{uv}-β3GnT2 were isolated and 125 μg of each baculovirus was
5 subjected to SPR experiment. AcMNPV-gp-GFP_{uv}-β3GnT2 was used as a control.
6 AcMNPV-hPRR-w/oTM, which contained hPRR-w/oTM, did not show a greater
7 specificity of binding to human renin than AcMNPV-hPRR-wTM and
8 AcMNPV-gp-GFP_{uv}-β3GnT2 (Fig. 4), indicating that specific binding of hPRR to renin
9 was not observed and hPRR-w/oTM was localized in virus nucleocapsid rather than
10 virus envelope.
11
12 **Discussion**
13
14 A partial PRR peptide from bovine chromaffin granules was firstly isolated as a protein
15 associated with V_0 ATPase (Ludwig et al. 1998). hPRR (full-length hPRR) has been
16 reported to localize in the ER in HeLa-S3 cells (Schefe et al. 2006) and in the
17 intracellular vesicular complexes and plasma membrane in cardiomyocytes (Saris et al.
18 2006). hPRR has two theoretical targeting signals, a tyrosine-based motif (Y^{335}DSI) and
19 a conventional C-terminal dibasic motif (K^{346}IRMD) (Burckle et al. 2006). The
20 tyrosin-based motif is defined as YxxØ (x is any amino acid, Ø is a large hydrophobic
21 amino acid), is in the cytosolic domain and mediates the sorting of transmembrane
22 proteins towards endosomes and lysosomes (Bonifacino et al. 2003). The conventional
23 C-terminal dibasic motif in the cytosolic domain is K(x)Kxx or R(x)Rxx (x is any
24 amino acid) and functions as an ER retention/retrieval signal (Trombetta et al. 2003).

1 These two motifs may be involved in the localization of hPRR-wTM but are deleted in
2 hPRR-w/oTM form.
3 In this report, hPRR was expressed in Sf-9 cells in two forms; a transmembrane
4 form (hPRR-wTM) and a transmembrane domain-deleted form (hPRR-w/oTM).
5 Surprisingly, only hPRR-w/oTM was expressed in the BV fraction (Fig. 1C), but not in
6 the plasma membrane. When a baculovirus buds, the host's plasma membrane is used as
7 a viral envelope. However, hPRR-w/oTM was not detected in the viral envelope
8 fraction isolated from AcMNPV-hPRR-w/oTM by Triton X-100 treatment (Fig. 3B).
9 Moreover, specific binding of AcMNPV-hPRR-w/oTM to renin immobilized to a
10 sensor cuvette was not shown (Fig. 4). The lack of expression of hPRR-w/oTM in the
11 plasma membrane indicates that the hPRR-w/oTM may not be present in the viral
12 envelope and may be associated with another specific area on the BV. These results
13 support that hPRR-w/oTM may be associated with another locality, maybe capsid, in
14 the BV rather than in the viral envelope.
15 In several reports, the display of foreign protein on the surface has been achieved
16 by fusion with baculovirus gp64 protein which resides in a viral envelope. However,
17 transmembrane proteins are sometimes displayed on the surface of BV with its native
18 transmembrane domains without fusion to gp64 (Loisel et al. 1997; Masuda et al. 2003;
19 Urano et al. 2003; Hayashi et al. 2004). The mechanism of this surface display system
20 has not been clearly understood yet, but the transmembrane proteins, which can be
21 displayed on the BV surface, do not localize in the capsid of BV. Capsid display of
22 foreign proteins is usually achieved by the fusion with VP39 nucleocapsid protein of
23 AcMNPV (Kukkonen et al. 2003). However, we did not fuse hPRR-w/oTM with VP39
24 nucleocapsid protein, but the hPRR-w/oTM might be localized in the capsid of BV.

1 It was found that budded baculoviruses could enter into mammalian cell lines *in*
2 *vitro* and *in vivo* efficiently (Pieroni et al. 2001; Chuang et al. 2007). It shows that
3 budded baculoviruses can be used as a gene delivery particle. Capsid display system is
4 also applicable for the intracellular site-specific targeting. If the envelope and the capsid
5 of baculoviruses are modified for tissue- and cell-specific targeting and intracellular
6 targeting, efficient intracellular site-specific transduction system may be established. To
7 establish this double targeting system, capsid display system is essential, and further
8 analysis of the display of hPRR-w/oTM on the capsid will lead to new methodology of
9 baculovirus capsid display system.
10

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15

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1 **Figure legends**

2

3 **Fig. 1** Illustration of hPRR which was expressed in this experiment (A). Extracellular
4 expression of hPRR-wTM and hPRR-w/oTM in Sf-9 cell cultures supernatants by
5 Western blot (B). Analysis of expression of each hPRR in the BV fraction isolated from
6 Sf-9 culture (C) by Western blot and Coomassie Brilliant Blue (CBB) staining of
7 samples in (C) (D).
8 Lane 1 and 2 denote the expression of hPRR-wTM and hPRR-w/oTM, respectively.
9 Arrows indicate hPRR-w/oTM.

10

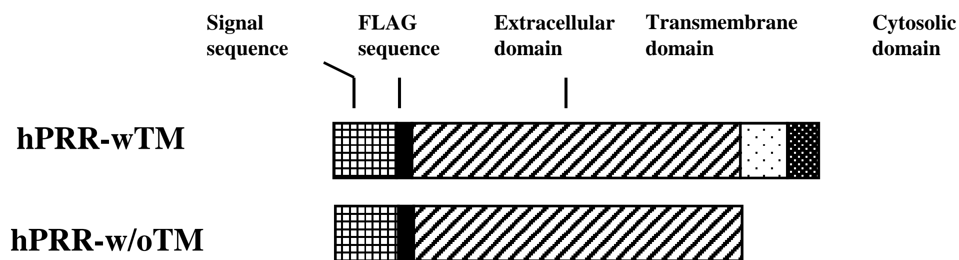
11 **Fig.2** Immunofluorescence microscopic image of expression of hPRR-wTM and
12 hPRR-w/oTM in baculovirus-infected Sf-9 cells (A). Intracellular localization of
13 hPRR-w/oTM in baculovirus-infected Sf-9 cells (B, C). The cells were infected with
14 AcMNPV-hPRR-wTM or AcMNPV-hPRR-w/oTM and cultivated for 2 d before being
15 stained with mouse anti-FLAG M2 antibody followed by Cy3-conjugated goat
16 anti-mouse IgG antibody. ER, Golgi apparatus, and lysosomes were stained with
17 ER-Tracker Red, NBD C_6-ceramide complexes to BSA, and Lysotracker, respectively.
18 Cells were observed by Confocal laser-scanning microscopy.

19

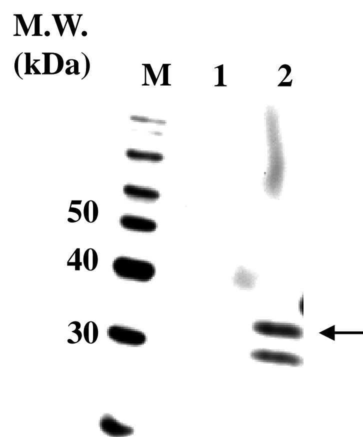
20 **Fig. 3** Analysis of cold Triton X-100 extraction of hPRR-wTM and hPRR-w/oTM in
21 infected Sf-9 (A) or AcMNPV-hPRR-w/oTM (B) by Western blot and CBB staining.
22 Baculovirus-infected cells or AcMNPV-hPRR-w/oTM were harvested, washed,
23 suspended with PBS containing 1% Triton X-100 and incubated for 30 min at 4°C. The
24 suspension was centrifuged and the pellet washed with the same buffer. The supernatant

1 (1) and pellet (2) were analyzed by Western blot. N indicates untreated
2 AcMNPV-hPRR-w/oTM.
3
4 **Fig.4** Analysis of binding of AcMNPV-gp-GFP$_{uv}$-β3GnT2 (A), AcMNPV-hPRR-wTM
5 (B) and AcMNPV-hPRR-w/oTM (C) to human renin. AcMNPV-gp-GFP$_{uv}$-β3GnT2,
6 AcMNPV-hPRR-wTM, and AcMNPV-hPRR-w/oTM were isolated and 125 μg of each
7 baculovirus was subjected to SPR experiment. On the surface of the CM sensor cuvette,
8 2.5 μg of human renin was immobilized and any unreacted CM groups on the sensor
9 cuvette lacking immobilized protein were blocked with ethanolamine as a control for
10 non-specific binding. PBS (pH6.2) was used as a running buffer. The arrows indicate
11 the point at which samples were added to the sensor cuvettes.

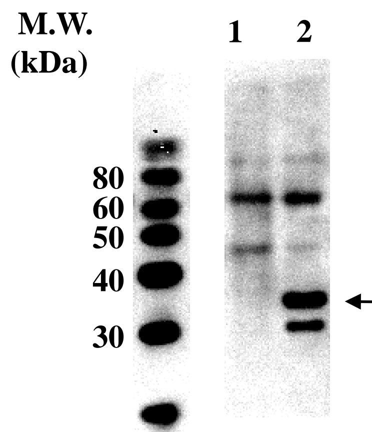
(A)



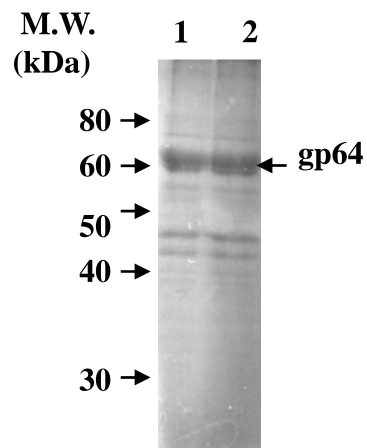
(B)



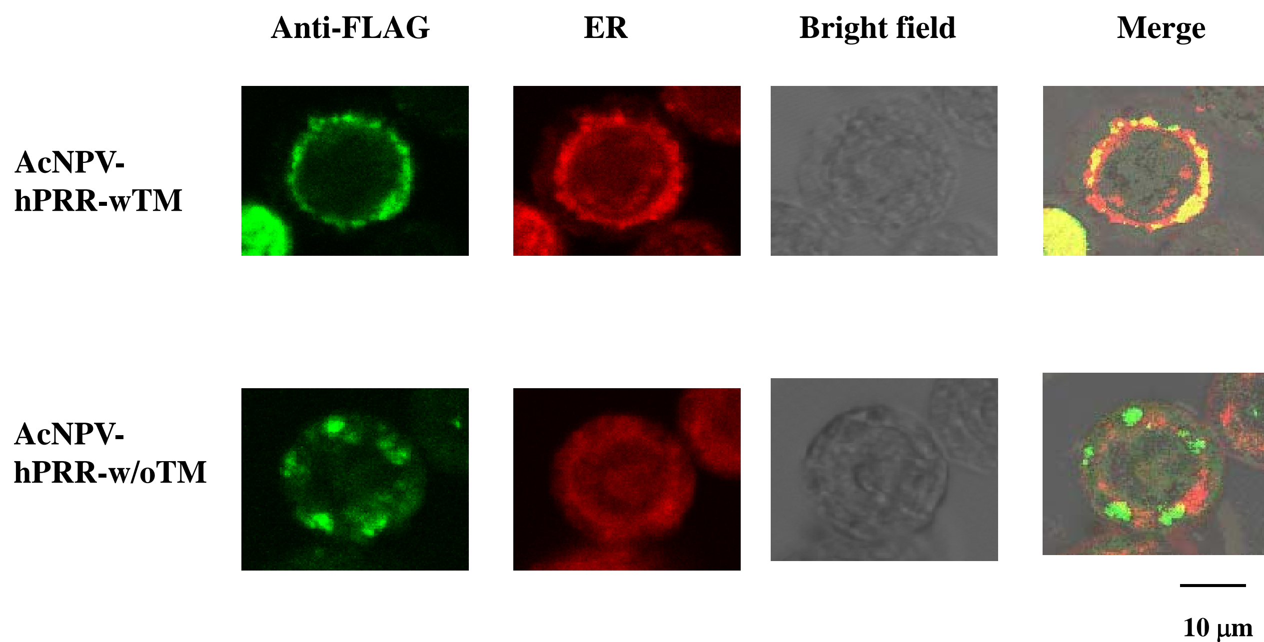
(C)



(D)



(A)



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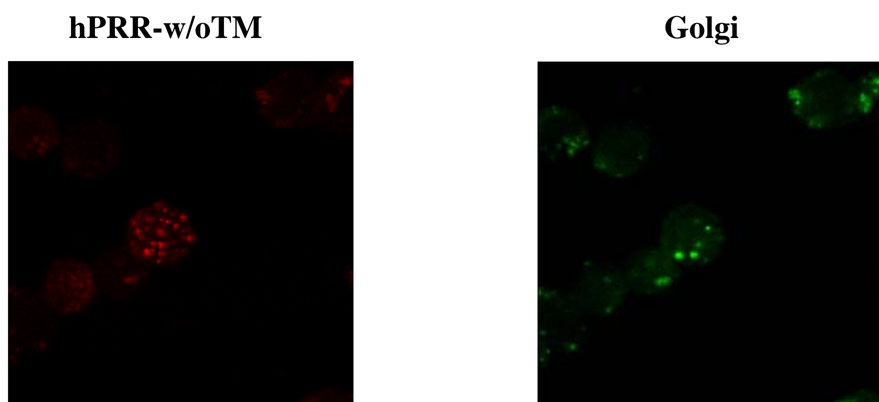
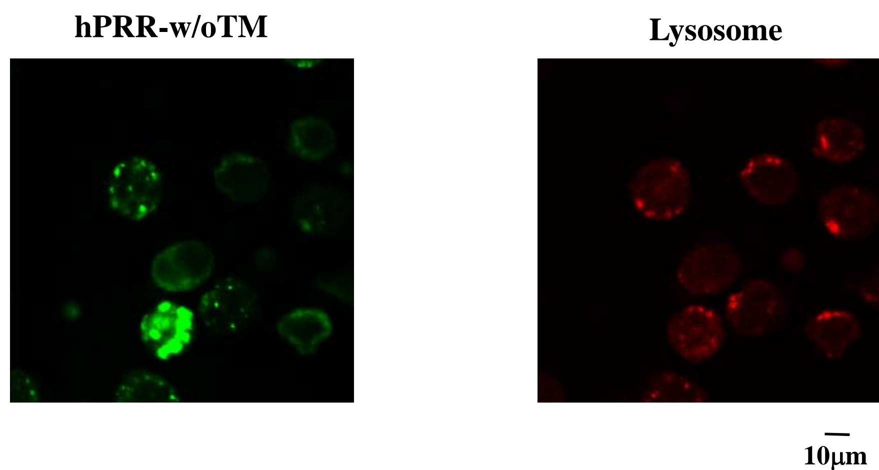
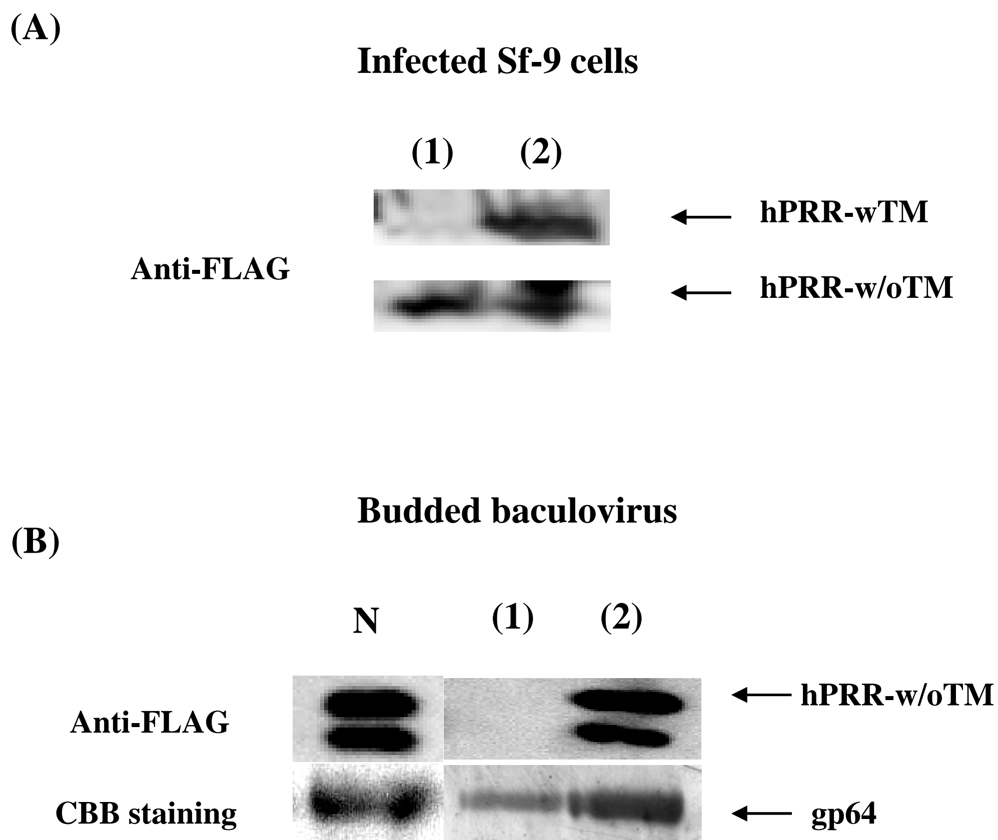
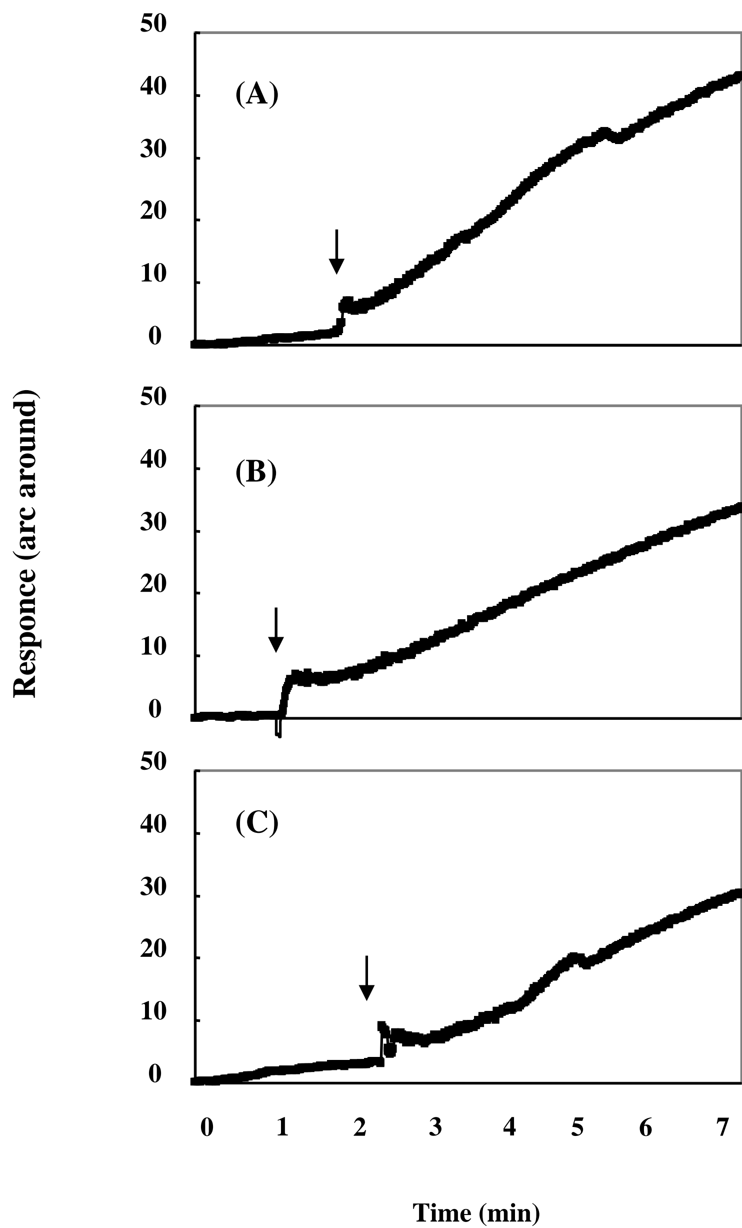


(C)




(A)
Infected Sf-9 cells
(1)
(2)
Anti-FLAG
hPRR-wTM
hPRR-w/oTM
Budded baculovirus
(B)
N
(1)
(2)
Anti-FLAG
CBB staining
hPRR-w/oTM
gp64


(A)
(B)
(C)
Responce (arc around)
Time (min)
0
10
20
30
40
50
0
1
2
3
4
5
6
7