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Display of the human (pro)renin receptor on *Bombyx mori* nucleopolyhedrovirus (BmNPV) particles using Bm cells

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Abstract

The human prorenin receptor (hPRR) was displayed on the surface of *Bombyx mori* nucleopolyhedrovirus (BmNPV) with and without fusion to glycoprotein 64 (GP64) of the BmNPV. hPRR1 is a native hPRR with an additional FLAG peptide sequence inserted between the signal peptide and prorenin-binding domain. hPRR2 has the prorenin-binding domain inserted between amino acid residues ^{81}Asp and ^{82}Pro of GP64. hPRR4 has the prorenin-binding domain inserted in ^{81}Asp and ^{320}Met of partially deleted GP64. Incorporation of hPRR was confirmed in recombinant BmNPV (rBmNPV) but not in cysteine protease-deleted rBmNPV. hPRR1 was observed in ER, but hPRR2 and hPRR4 were observed around the endoplasmic reticulum (ER) and in its periphery. rBmNPV-hPRR1 and -hPRR2, carrying hPRR1 and hPRR2 respectively, showed binding affinity to human renin, but rBmNPV-hPRR4 did not. The presence of hPRR4 of rBmNPV-hPRR4 was confirmed in western blotting under nonreducing conditions, suggesting that although hPRR4 was incorporated in rBmNPV-hPRR4, it behaved as a nonfunctional aggregate. This rBmNPV display system can also be used for analyzing a ligand-receptor interaction.

Keywords: BmNPV; surface display; prorenin receptor; GP64; fusion protein

Baculoviruses are widely used as expression vectors of recombinant proteins in insect cells and larvae. They permit high-level expression of recombinant proteins that are co- and post-translationally modified. Baculoviruses can also be used to display recombinant proteins on their surfaces (1, 2). A common method for displaying proteins on the surface of baculoviruses involves the use of the transmembrane domain of GP64 present in the baculovirus. The GP64 transmembrane domain can be substituted with that of hemagglutinin (HA) or vesicular stomatitis virus G glycoprotein (VSVG) (3, 4). The N-terminal domain of neuraminidase from influenza virus has also been used as the transmembrane domain (5). To date, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) that can infect *Spodoptera frugiperda, Trichoplusia ni*, and cabbage loopers, has been the most widely used baculovirus for baculovirus surface display. The display system using *Bombyx mori* nucleopolyhedrovirus (BmNPV) has been employed, but it is in a few (6, 7). BmNPV is a pathogen of the silkworm *B. mori*, and both AcMNPV and BmNPV belong to the family Baculoviridae. BmNPV is also used for recombinant protein production, especially when insect larvae, silkworms, are used as hosts. Silkworms are bigger than and superior to cabbage loopers for recombinant protein production. The baculovirus titer in the hemolymph of BmNPV-infected silkworm larvae is higher than that in the BmNPV-infected Bm5 cell culture supernatant. When insect larvae are used, sterile conditions and culture medium, including serum and growth factors, are not required during the protein production process. Therefore, the insect larvae are advantageous to mass production of virus titer and recombinant protein.

In this paper, the human (pro)renin receptor (hPRR) was employed to display on the surface of BmNPV in the culture of *B. mori*-derived Bm5 cells. Three types of constructs

were used for display of hPRR. One was the native form of hPRR. Others were fused to GP64 of BmNPV; the prorenin-binding domain of hPRR was either inserted between ^{81}Asp and ^{82}Pro of GP64 or between ^{81}Asp and ^{320}Met of partially deleted GP64. These three constructs were investigated for insect cell localization and binding for prorenin. The BmNPV display system can be used for the large-scale production of recombinant proteins displayed on the surface of the baculovirus. Infection of silkworm larvae with such baculoviruses that display various proteins may then be carried out and used for the functional analysis of receptors, drug delivery system, and vaccines against infectious viruses and protozoa.

MATERIALS AND METHODS

Cell line, cell culture, and baculovirus infection Bm5 cells derived from *Bombyx mori* were cultured in NIM-Ex medium (Nihon Nosan Co. Ltd., Yokohama, Japan) supplemented with 1% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA, USA). Bm5 cells were infected with 10 μl hemolymph from recombinant bacmid-injected silkworm larvae. The multiplicity of infection (M.O.I.) was 0.1–1.

Construction of hPRRs and bacmids and preparation of hemolymph from silkworm larvae Three hPRRs were constructed as shown in Fig. 1. hPRR1 is the native form and has an additional FLAG peptide sequence behind the signal peptide sequence, as reported previously (8). To fuse hPRR with *gp64* from BmNPV, the gp64 gene was amplified by PCR with the Bmgp64-F and Bmgp64-R primers (Table 1). The amplified gp64 gene product was

88 inserted in the *Kpn* I–*Hin*d III site in pUC18, and the resulting plasmid was named pBmgp64.
89 hPRR2 gene, in which the prorenin-binding domain of hPRR is inserted between the N-
90 terminal domain between ^{81}Asp and ^{82}Pro of the complete gp64 gene was constructed. The
91 hPRR gene was amplified with the bgl-hPRR-F and bgl-hPRR-R primers (Table 1), and the
92 amplified hPRR gene was inserted into the *Bam*H I site in pBmgp64. hPRR4 gene, in which
93 the prorenin-binding domain of hPRR is inserted between ^{81}Asp and ^{320}Met of partially
94 deleted gp64, was constructed. In hPRR4, the prorenin-binding domain of hPRR was put
95 between N-terminal (amino acid 1-81) and C-terminal (amino acid 320-511) domains of
96 GP64. This is for investigation of the deletion effect of internal domain of GP64 (amino acid
97 82-319) in hPRR4. The hPRR PCR product was inserted into the *Bam*H I–*Sph* I site in
98 pBmgp64. The hPRR1, hPRR2, and hPRR4 genes were amplified with the Bmgp64-F and
99 Bmgp64-R primers (Table 1), and the three amplified hPRR genes were inserted into
100 pENTR/D/TOPO by the TOPO cloning method. Recombinant bacmids were constructed
101 using *Escherichia coli* BmDH10Bac (9) and BmDH10Bac (BmNPV-CP$^-$) that lacks its own
102 cysteine protease (10). Hemolymph containing recombinant BmNPV (rBmNPV) was
103 prepared using constructed recombinant bacmids according to a previous study (9, 10).
104 **Baculovirus isolation** Bm5 cells were infected with each hemolymph containing
105 rBmNPV and cultivated for 1–2 days. The culture media were centrifuged at 8000 × *g* for 10
106 min, and the culture supernatants were recovered and overlaid on a 25% sucrose solution
107 (25% sucrose in 5 mM NaCl and 10 mM EDTA). These were further centrifuged at 114000 ×
108 *g* for 1 h. The supernatants were removed, and the pellets were washed with phosphate-
109 buffered saline (PBS, pH 6.2). The pellets were resuspended in small volumes of PBS and
110 used for further experiments.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting Proteins were separated by SDS-PAGE using 12% polyacrylamide or 5–15% gradient polyacrylamide gels that were subsequently subjected to western blotting. For electrophoresis under nonreducing conditions, the samples were mixed with sample buffer without β-mercaptoethanol and boiled. After SDS-PAGE, the proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), the membrane was incubated for 1 h in either 1: 10000 diluted mouse anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO, USA) or 1: 4000 diluted rabbit anti-Bmgp64 polyclonal antibody (BioGate Co., Ltd., Gifu, Japan). The membrane was washed with TBST and then incubated for 1 h in 1: 20000 diluted anti-mouse or anti-rabbit IgG antibody labeled with horseradish peroxidase (GE Healthcare UK Ltd., Buckinghamshire, England). Detection was carried out using the ECL Plus Western blotting reagent (GE Healthcare UK Ltd.). Specific bands were detected on a Fluor-S MAX MultiImager (Bio-Rad).

Immunofluorescence microscopy Bm5 cells were infected with hemolymph and cultivated for 2 days. The culture medium was sampled, and infected cells were attached to polylysine-coated slide glass. These cells were fixed with 8% formalin solution (Wako Pure Chem. Ind. Ltd., Osaka, Japan) for 20 min and washed with PBS (pH 7.0). Any remaining aldehyde was quenched with 50 mM NH_4Cl in PBS. The fixed cells were washed with PBS and blocked overnight with 8% (w/v) bovine serum albumin (BSA) in PBS. After blocking with BSA, the cells were incubated for 2–3 h with 1: 1000 dilution of mouse anti-FLAG M2 antibody in PBS and 2% BSA. The cells were washed with PBS and incubated for 1 h with 1:

100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in PBS and 2% BSA. The endoplasmic reticulum (ER) was stained with 1 μM ER-Tracker^{TM} Red (Molecular Probes, Carlsbad, CA, USA) at 4°C for 30 min. After staining, the cells were examined by confocal laser scanning microscopy (TCS-LS, Leica Microsystems, Heidelberg, Germany).

Surface plasmon resonance (SPR) experiments The surface plasmon resonance experiments were performed in an SPR optical biosensor (IAsys plus; IASYS Affinity Sensors Ltd., Saxon Hill, Cambridge, UK) using a carboxymethyl (CM) sensor cuvette. Renin (2.5 μg; Cayman, Ann Arbor, MI, USA) was immobilized on the surface of the CM sensor cuvette, and unreacted CM groups on the sensor cuvette, which did not have any immobilized protein, were blocked with ethanolamine to control nonspecific binding. PBS (pH 6.2) was used as the running buffer.

Results

Expression of hPRRs in Bm5 cells using rBmNPV and rBmNPV-CP$^-$ Production of the GFP_{uv}-β1,3-*N*-acetylglucosaminyltransferase2 fusion protein (GGT2) was significantly improved when the rBmNPV-CP$^-$ bacmid was used because GGT2 degradation by the cysteine protease was reduced (10). Therefore, to express hPRRs on rBmNPV surfaces, rBmNPV-CP$^-$ was used. However, there are no reports on display on the surface of BmNPV-CP$^-$.Three bacmids rBmNPV-CP$^-$-hPRR1, -hPRR2, and -hPRR4 were constructed, and hemolymph from each bacmid-injected silkworm larvae was prepared. Bm5 cells were infected with each rBmNPV-CP$^-$, and the cell cultures were sampled at a post-infection time

157 (P.I.T.) of 2 days. During this period, the viability of Bm5 cells was 95%–97%. The Bm5
158 cells were separated from the cultures, and the intracellular expression of each hPRR in Bm5
159 cells was confirmed by western blotting (Data not shown). The estimated molecular weights
160 of the hPRRs in the Bm5 cells are as follows: hPRR1, 38.4 kDa; hPRR2, 95.8 kDa; and
161 hPRR4, 64.7 kDa. The culture supernatant was overlaid on a 25% sucrose solution, and each
162 rBmNPV-CP$^-$ was recovered by centrifugation. The expression of each hPRR in each
163 rBmNPV-CP$^-$ was confirmed by western blotting. GP64 was observed in all samples, but the
164 hPRR was not detected (Fig. 2A).
165 Three types of rBmNPVs (-hPRR1, -hPRR2, and -hPRR4) were prepared by silkworm
166 expression system, and used for infection to Bm5 cells. Each hPRR was expressed
167 intracellularly (Data not shown). The presence of each hPRR in each rBmNPV was examined
168 (Fig. 2B) and compared against that in rBmNPV-CP$^-$. In particular, hPRR1 seemed to be
169 incorporated in rBmNPV-hPRR1 with its own native single transmembrane domain (Fig. 2B,
170 left, lane 1). Recently, it has been reported that several transmembrane proteins could be
171 displayed on the surface of baculoviruses through their native transmembrane domains (11–
172 13). hPRR2 and 4 were incorporated in rBmNPV using C-terminal domain (amino acid 320-
173 511) of GP64 (Fig. 2B, left, lanes 2 and 3). This domain, including transmembrane domain, is
174 enough for incorporation of hPRR into rBmNPV. GP64s in rBmNPV-hPRR1, -hPRR2, and -
175 hPRR4 were detected by the polyclonal anti-gp64 antibody, which is against the 400–416
176 amino acid sequence of GP64 of rBmNPV (Fig. 2B, right). hPRR2, in which the prorenin-
177 binding domain is inserted into complete GP64, was examined together with GP64 (Fig. 2B,
178 right lane 2). However, the hPRR4 couldn't be detected with the same anti-GP64 antibody

because its molecular weight is similar to that of GP64 (Fig. 2B, right, lane 3). These results show that hPRR, hPRR2 and hPRR4 was incorporated in rBmNPV.

Analysis of hPRRs under nonreducing conditions GP64 is present intracellularly in the form of two types of homotrimers (trimers I and II) under nonreducing conditions (14). Trimers I and II of GP64 with molecular weights above and below 220 kDa, respectively, were observed in each rBmNPV. This was confirmed by western blot using the anti-Bmgp64 polyclonal antibody under nonreducing conditions (Fig. 3A). The hPRR1 (38.4 kDa) was present as a monomer under both reducing and nonreducing conditions (Fig. 3B). The hPRR2 (288 kDa) was also observed above GP64 trimers I and II (around 220 kDa) when the anti-Bmgp64 (Fig. 3A) and anti-FLAG M2 antibodies were used under nonreducing conditions (Fig. 3B). Under reducing conditions it was observed between 80 and 100 kDa (95.8 kDa) (Fig. 3A). This suggests that hPRR2 is also a trimer. The hPRR4 was not clearly observed by the anti-Bmgp64 antibody under either reducing or nonreducing conditions because its molecular weight is similar to that of GP64 (Fig. 3A). The hPRR4 (64.7 kDa) was detected between 60 and 80 kDa under reducing conditions by using the anti-FLAG M2 antibody (Fig. 3B). Under nonreducing conditions, the hPRR4 was stacked in a well. This suggests that hPRR4 formed aggregates. A protein band at 220 kDa was detected under nonreducing conditions, which were also observed in rBmNPV-CP$^-$-GGT2. This means that this band was nonspecific (data not shown).

Intracellular distribution of hPRRs Expressed GP64 enters the cellular secretory pathway where it is glycosylated, oligomerized, and transported to the plasma membrane (14).

During the budding and assembly of budded baculoviruses, GP64 is localized in discrete areas on the plasma membrane, and these sites appear as budding sites (15, 16). Therefore, whether each hPRR is able to localize in the plasma membrane in a manner similar to that of GP64 or not was investigated. The hPRR (hPRR1 in this report) has been reported to localize in the ER in HeLa-S3 cells (17) and in the intracellular vesicular complexes and plasma membrane in cardiomyocytes (18). In this study, hPRR1 was observed in ER, at the same site stained by ER tracker. However, hPRR2 and hPRR4 were observed in the periphery of ER (Fig. 4). It indicates that expressed hPRR1, hPRR2 and hPRR4 entered into the secretory pathway in Bm5 cells and the transmembrane domain in each variant is essencial for its distribution in Bm5 cells.

Renin–binding by hPRR-displaying rBmNPVs To confirm the expression of each hPRR on the surface of each rBmNPV, SPR experiments were performed. Human renin was immobilized in the wells of the CM sensor cuvette, and 5 μg of each rBmNPV was added to the wells. Control rBmNPV (rBmNPV-CP$^-$-GGT2) was found to bind to immobilized renin to a slight extent (Fig. 5). In the cases of hPRR1 and hPRR2, the response increased with the incubation time, indicating that rBmNPV-hPRR1 and -hPRR2 bound to the immobilized renin, indicating that hPRR1 and hPRR2 were localized on the surfaces of the rBmNPVs respectively. The response of rBmNPV-hPRR4 was at the same level as that of the control, indicating that the binding of rBmNPV-hPRR4 to renin was nonspecific. This suggests that although hPRR4 might be incorporated in rBmNPV-hPRR4 through the transmembrane domain of GP64, it may not be functional.

Discussion

Surface display of recombinant proteins and functional peptides in baculovirus systems has been reported earlier. The AcMNPV baculovirus has been mainly used in these systems, but BmNPV has been used in very few cases (6, 7). In general, display of recombinant proteins has been achieved by fusion with GP64 present on the baculovirus surface. However, recombinant proteins with their own native transmembrane domains without fusion to GP64 was also expressed and displayed on the surface of baculoviruses (11–13, 19, 20). In this study, we tried to express hPRR on the surface of BmNPV by fusing it with GP64 of BmNPV or in its native form.

hPRR1 and 2 were expressed on the surface of rBmNPV. The hPRR1 without fusing to GP64 was displayed on the surface of rBmNPV but not on that of AcMNPV (21). This suggests that the mechanism of protein display on the surface of BmNPV was differed from AcMNPV. All three hPRRs were not incorporated in rBmNPV-CP$^-$, which lacks the cysteine protease v-cath. This protease has a cathepsin L-like sequence, and its substrate specificity is similar to that of cathepsin B (22). When insect cells are infected with a baculovirus, this protease accumulates as a propeptide, and the death of host cells activates this propeptide (23). Moreover, in baculovirus-infected larvae, this cysteine protease plays an important role in the liquefaction of infected larvae. The relationship between this cysteine protease and the incorporation of recombinant proteins has not yet been investigated.

The hPRR2, which was constructed by inserting the prorenin-binding domain of hPRR into the site between ^{81}Asp and ^{82}Pro in GP64, could be displayed on the surface of rBmNPV in an active form. Deletion of the N-terminal half of AcMNPV GP64 did not prevent its

248 oligomerization. However, deletion of the predicted alpha-helical region (amino acids 298 to
249 346) and hydrophobic region II (amino acids 330 to 338) prevented the oligomerization of
250 *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (OpMNPV) GP64 (14). hPRR2,
251 composing of the prorenin-binding domain of hPRR, inserted into full-length GP64, was
252 displayed on the surface of BmNPV, and its rBmNPV-hPRR2 was able to bind to human
253 renin. However, hPRR4, which was constructed by inserting the prorenin-binding domain of
254 hPRR into the site between ^{81}Asp and ^{320}Met of GP64, was expressed, but rBmNPV-hPRR4
255 did not bind to renin (Fig. 5). In previous report, purified hPRR4 was not able to bind to
256 human renin (24). The hPRR4 was detected under nonreducing conditions in the upper part
257 of the SDS-PAGE gel in this study (Fig. 3), suggesting that it is present in rBmNPV particles
258 as non-functional aggregates. GP64 from AcMNPV or BmNPV has 15 cysteine residues and
259 is expressed as a trimer. Moreover, the oligomerization domain, which is in the form of a
260 leucine zipper motif, is located between amino acids 298 and 339 in GP64 of AcMNPV (25).
261 This oligomerization domain is also present in GP64 of BmNPV. Twenty-five amino acids
262 from this domain in GP64 were deleted in hPRR4, which may result in the aggregation of
263 hPRR4 (25).

264 Incorporation of hPRRs in rBmNPVs and immunofluorescent microscopic observation
265 suggest that hPRR1 was expressed in ER, but hPRR2 and hPRR4 were in the periphery of ER
266 (Fig. 4). This indicates that the C-terminal domains of hPRR and BmNPV GP64, which
267 include the transmembrane and cytoplasmic domains, direct proteins to go through the
268 secretory pathway in Bm5 cells and be expressed in the membrane fraction in the secretory
269 pathway. Previously, it has been reported that hPRR1 localizes in the ER in HeLa-S3 and Sf-
270 9 cells (17) and in the intracellular vesicular complexes and plasma membrane in

271 cardiomyocytes (18). In this study, most hPRR1 was observed mainly at ER. During the
272 budding and assembly of budding baculoviruses, nucleocapsids are thought to migrate
273 through the cytoplasm and bud through the plasma membrane at sites where GP64 is
274 concentrated (16). It suggested hPRR1 might be also expressed in the plasma membrane and
275 then hPRR1 may be displayed on the surface of rBmNPV. However, proteins expressed in
276 ER were also displayed on the surface of baculoviruses (20). It is possible that hPRR1
277 expressed in ER was displayed on the surface of rBmNPV, not expressed in the plasma
278 membrane.
279 hPRR1, with its native single transmembrane domain, and hPRR2 with the
280 transmembrane domain of GP64, were successfully displayed on the surface of rBmNPV and
281 showed biological function. The BmNPV-based surface display system may be useful for
282 analyzing receptors and the baculovirus drug delivery system, which require very high titers
283 of baculoviruses.
284
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376

377 **Figure legends**

378

379 FIG. 1. Constructions of hPRRs. hPRR1 is native hPRR with an additional FLAG peptide
380 sequence inserted between the signal peptide and prorenin-binding domain. hPRR2 was
381 constructed by inserting the prorenin-binding domain of hPRR between ^{81}Asp and ^{82}Pro of
382 GP64 from BmNPV. hPRR4 was constructed by inserting the prorenin-binding domain of
383 hPRR into the site between ^{81}Asp and ^{320}Met of partially deleted GP64.

384

385 FIG. 2. Expression of individual hPRRs in rBmNPV-CP$^-$ (A) and rBmNPV (B). Infected
386 Bm5 cells and culture supernatant were separated from cell cultures. Each rBmNPV was
387 isolated from its culture supernatant by ultracentrifugation. An aliquot of each sample was
388 analyzed by CBB staining and western blotting with the mouse anti-FLAG M2 antibody or
389 rabbit anti-BmGP64 antibody. Lanes 1, 2, and 3 in (A) represent rBmNPV-CP$^-$-hPRR1, -
390 hPRR2, and -hPRR4, respectively, while lanes 1, 2, and 3 in (B), rBmNPV-hPRR1, -hPRR2,
391 and -hPRR4, respectively.

392

393 FIG. 3. Analysis of oligomerization under both reducing and nonreducing conditions. Each
394 sample was electrophoresed on SDS-PAGE gels under both reducing and nonreducing
395 conditions and then analyzed by western blotting using the rabbit anti-Bmgp64 antibody (A)
396 and the mouse anti-FLAG M2 antibody (B). Lanes 1, 2, and 3 represent rBmNPV-hPRR1, -
397 hPRR2, and -hPRR4, respectively.

398

399 FIG. 4. Immunofluorescence localization of each hPRR in Bm5 cells infected with rBmNPVs.

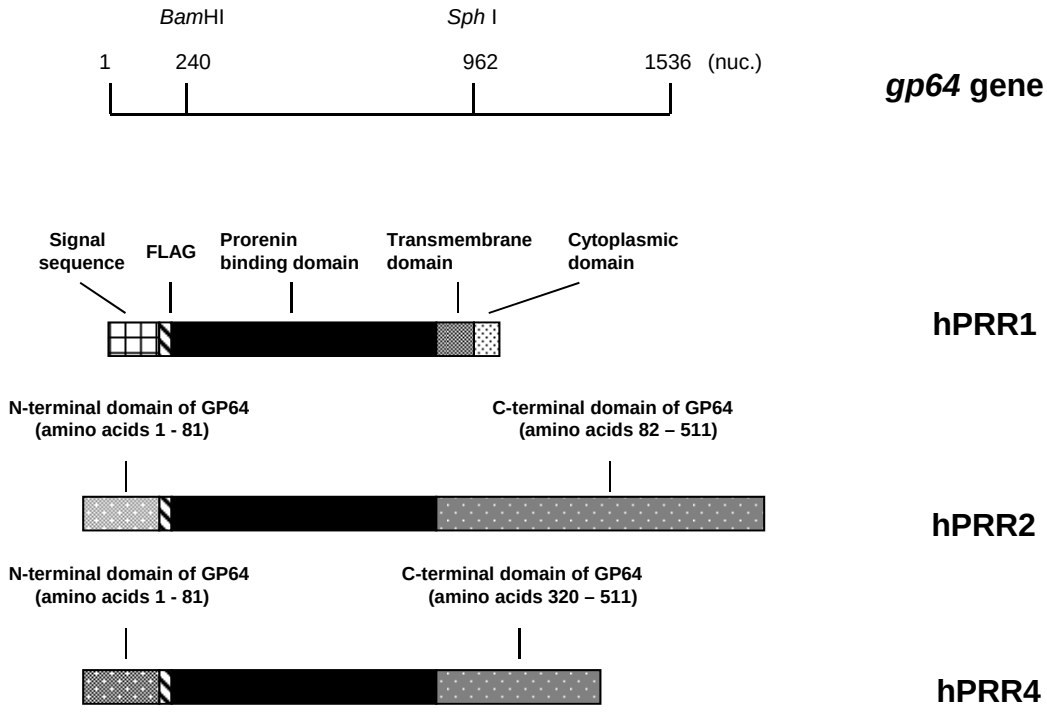
400 Bm5 cells were infected with each rBmNPV (rBmNPV-hPRR1, -hPRR2, and -hPRR4) and
401 cultivated for 2 days prior to staining with the mouse anti-FLAG M2 antibody, and followed
402 by staining with the FITC-conjugated goat anti-mouse IgG antibody and ER-Tracker^{TM} Red.
403 Bar indicates 10 μm. Each hPRR was shown to be green fluorescence by staining with the
404 mouse anti-FLAG M2 antibody. ER was stained as red fluorescence.
405
406 FIG. 5. SPR analysis of the binding of rBmNPV-hPRR1, -hPRR2, and -hPRR4 to human
407 renin. Each rBmNPV was isolated by ultracentrifugation, and 5 μg was subjected to SPR
408 analysis. Human renin (2.5 μg) was immobilized on the surface of the CM sensor cuvette,
409 and unreacted CM groups were blocked with ethanolamine. The sensor cuvette that had no
410 immobilized human renin was also blocked with ethanolamine and used as a control for
411 nonspecific binding. PBS (pH 6.2) was used as the running buffer. The arrows indicate the
412 point at which the samples were added to the sensor cuvettes. rBmNPV-CP^{-}-GGT2 was used
413 as the negative control.

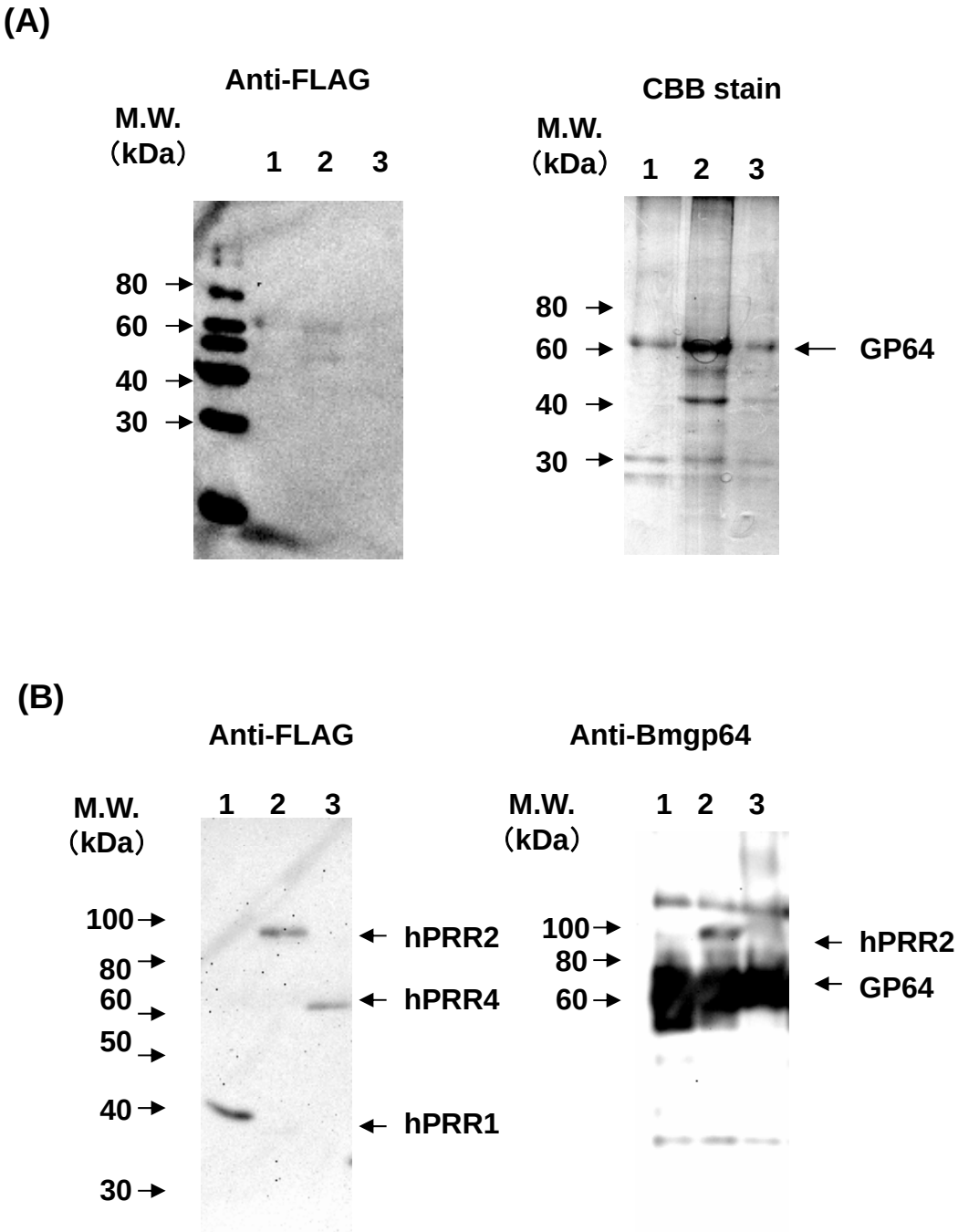
414 Table 1. Primers

Name	5′ → 3′
Bmgp64-F	caccggtaccatggtaggcgctattgttttatacg
Bmgp64-R	cccaagcttttaatattgtctactattacggtttc
bgl-hPRR-F	gaagatctccgactacaaggacgacgacgacaag
bgl-hPRR-R	gaagatctagatattcaaaattatacttatatgc
sph-hPRR-R	acatgcatgcatatattcaaaattatacttatatgc

415

Fig. 1, Kato et al.




(A)
Anti-FLAG
M.W.
(kDa)
1 2 3
80
60
40
30
CBB stain
M.W.
(kDa)
1 2 3
80
60
40
30
GP64
(B)
Anti-FLAG
M.W.
(kDa)
1 2 3
100
80
60
50
40
30
hPRR2
hPRR4
hPRR1
Anti-Bmgp64
M.W.
(kDa)
1 2 3
100
80
60
hPRR2
GP64

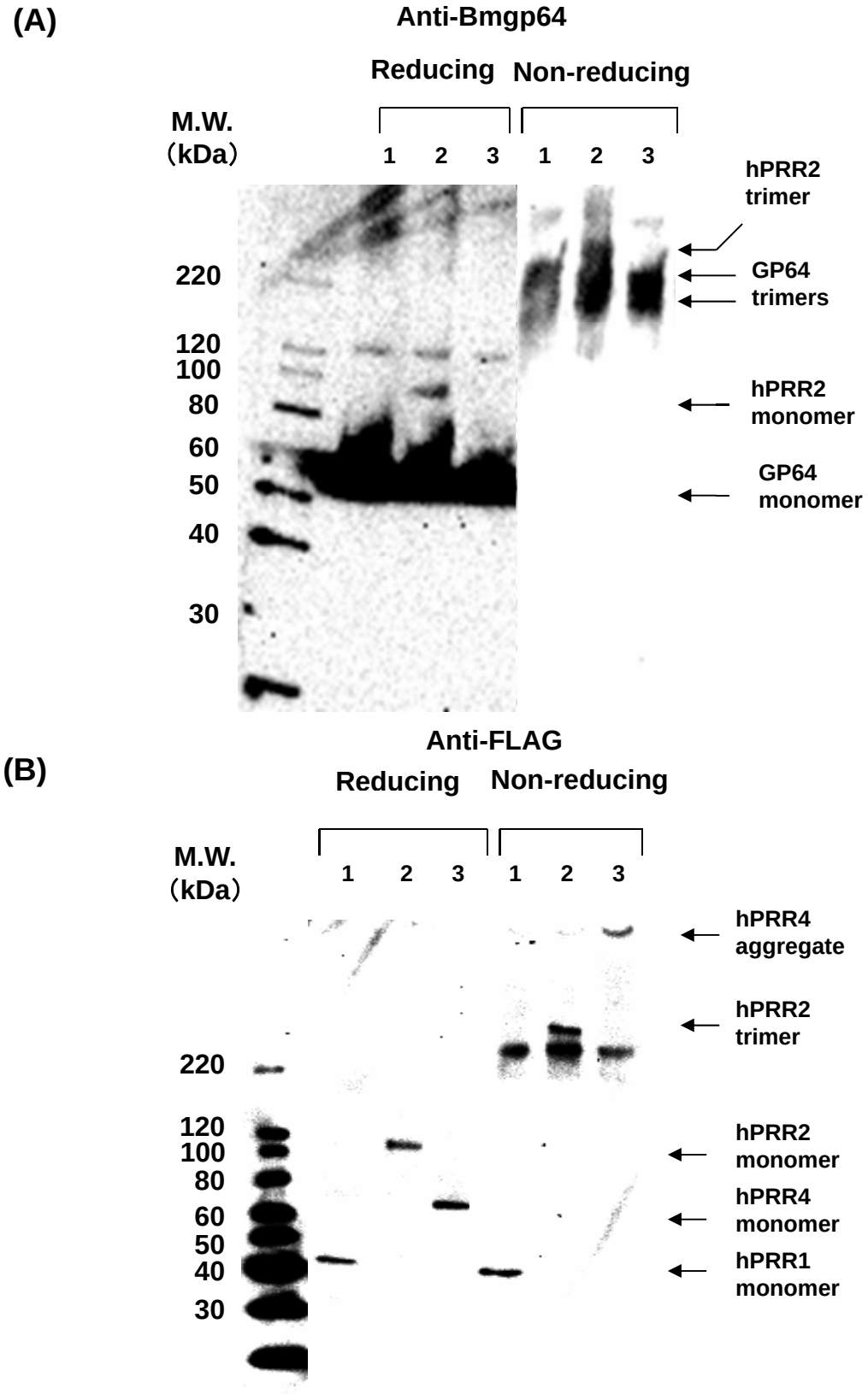

(A)
Anti-Bmgp64
Reducing
Non-reducing
M.W.
(kDa)
1 2 3 1 2 3
220
120
100
80
60
50
40
30
hPRR2
trimer
GP64
trimers
hPRR2
monomer
GP64
monomer
(B)
Anti-FLAG
Reducing
Non-reducing
M.W.
(kDa)
1 2 3 1 2 3
220
120
100
80
60
50
40
30
hPRR4
aggregate
hPRR2
trimer
hPRR2
monomer
hPRR4
monomer
hPRR1
monomer

Fig. 4, Kato et al.

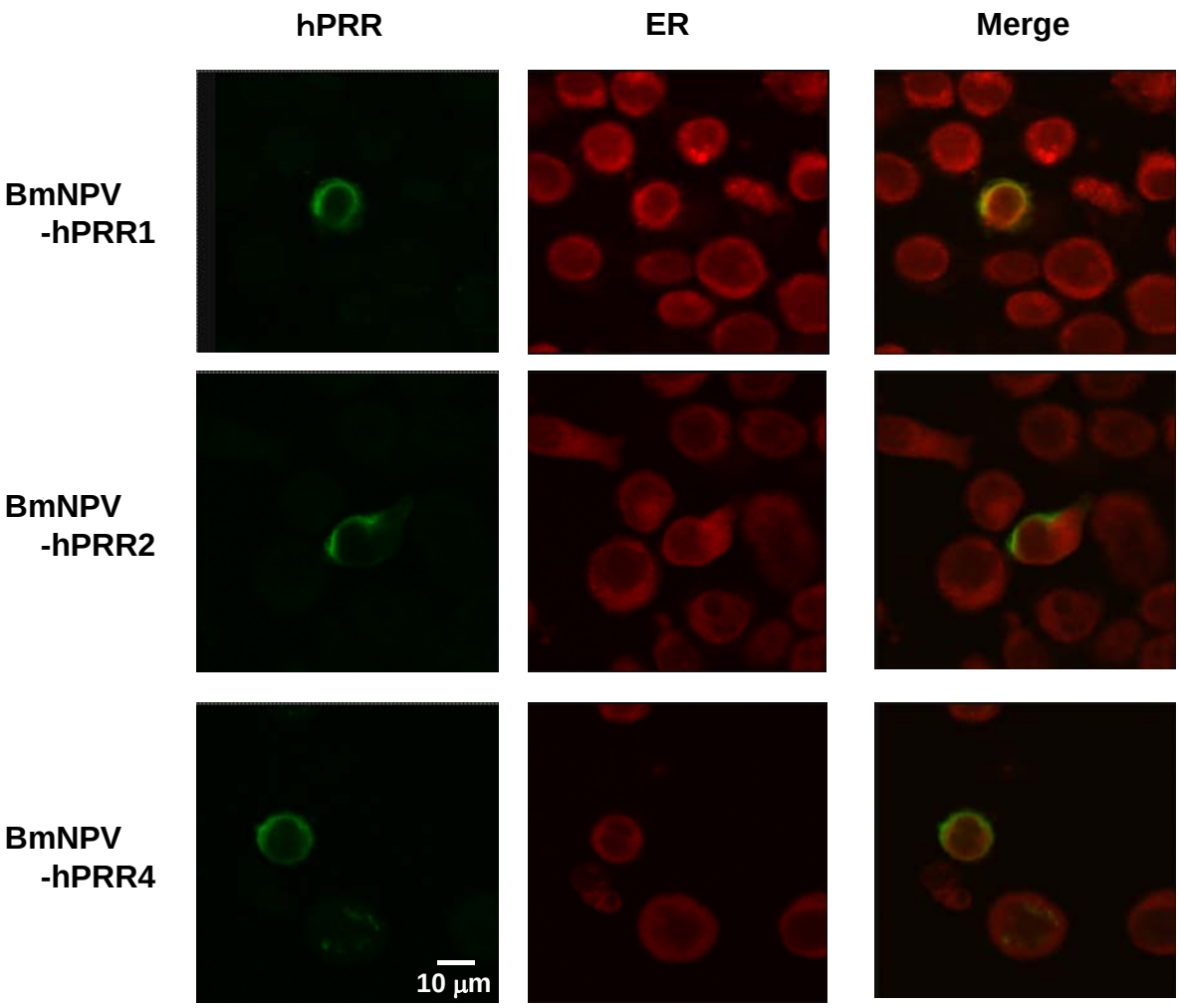


Fig. 5, Kato et al.