Research article

Role of Nucleocapsid Protein of Hantaviruses in Intracellular Traffic of Viral Glycoproteins

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Summary

To understand the role of nucleocapsid protein (NP) of hantaviruses in viral assembly, the effect of NP on intracellular traffic of viral glycoproteins Gn and Gc was investigated. Double staining of viral and host proteins in Hantaan virus (HTNV)-infected Vero E6 cells showed that Gn and Gc were localized to cis-Golgi, in which virus particles are thought to be formed. When HTNV Gn and Gc were expressed by a plasmid encoding glycoprotein precursor (GPC), which is posttranslationaly cleaved into Gn and Gc, Gn was localized to cis-Golgi, whereas Gc showed diffuse distribution in the cytoplasm in 32.9% of Gc-positive cells. The ratio of the diffused Gc-positive cells was significantly decreased to 15.0% by co-expression of HTNV NP. Co-expression of HTNV GPC with NPs of other hantaviruses, such as Seoul virus, Puumala virus and Sin Nombre virus, also reduced the ratios of diffused Gc-positive cells to 13.5%, 25.2%, and 11.6%, respectively. Among amino- and carboxyl-terminally truncated HTNV NPs, NP75-429, NP116-429, NP1-333, NP1-233, and NP1-155 possessed activity to reduce the ratio of diffused Gc-positive cells, while NP155-429 and NP1-116 did not. NP30-429 has partial activity. These results indicate that amino acid region 116-155 of NP is important for the activity, although amino acid region 1-30 is partially related. Truncation of the HTNV Gc cytoplasmic tail caused an increase in diffused Gc-positive cells. In addition, the effect of coexpression of HTNV NP was weakened. These results suggest that HTNV NP has a role to promote Golgi localization of Gc through a mechanism possibly mediated by the Gc cytoplasmic tail.

Keywords

HFRS; HPS; Bunyaviridae; Assembly; Transport; Golgi

1. Introduction

Hantaviruses are classified into the family *Bunyaviridae*, genus *Hantavirus*. Hantaviruses have been found in animals belonging to the Orders Rodentia, Soricomorpha and Chiroptera. To date, only rodent-borne hantaviruses have been thought to be pathogenic to humans. Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) are severe diseases caused by infection of hantaviruses. HFRS is characterized by renal dysfunction and hemorrhage and is caused by Old World hantaviruses such as Hantaan virus (HTNV), Seoul virus (SEOV), Dobrava virus (DOBV) and Puumala virus (PUUV). HPS is characterized by acute respiratory distress and is caused by New World hantaviruses such as Sin Nombre virus (SNV) and Andes virus (ANDV). The number of HFRS cases is estimated to be more than 100,000 per year with a case-fatality rate of less than 10%. In contrast, although the number of HPS cases is estimated to be only a few thousand per year, the case-fatality rate of HPS has reached as high as 40%. Specific treatments and vaccines against HFRS and HPS remain to be developed (Jonsson et al., 2010).

Hantaviruses are enveloped spherical or polymorphic viruses with a diameter of 80 to 120 nm. The genomes of hantaviruses are composed of tri-segmented single-stranded, negative sense RNA, designated as small (S), medium (M) and large (L) segments. Each segment encodes nucleocapsid protein (NP), glycoprotein precursor (GPC) and RNA-dependent RNA polymerase (RdRp), respectively. GPC is posttranslationally cleaved into Gn and Gc (Lober et al., 2001). NP is associated with the genome and is involved in transcription, translation and replication together with RdRp (Mir et al., 2008a, 2008b, 2010). Glycoproteins Gn and Gc are transmembrane proteins and constitute an envelope with a lipid membrane derived from host cells. Gn and Gc are

involved in receptor binding, membrane fusion and induction of protective immunity (Arikawa et al., 1992; Ogino et al., 2004; Ray et al., 2010). In some hantaviruses such as Tula virus, PUUV, and ANDV, nonstructural protein (NSs) is also encoded in S segment (Jaaskelainen et al., 2007; Vera-Otarola et al., 2011).

Formation of progeny particles of hantaviruses is thought to take place in the Golgi complex as is the case in representative viruses of the Bunyaviridae family (Schmaljohn and Nichol, 2007). However, the mechanism leading to assembly of viral components remains unclear. In many other viruses, matrix protein has a key role in the process of assembly and particle formation (Chen et al., 2008; Craven et al., 1999; Harty et al., 2000). However, matrix protein is not encoded in the hantaviral genome, raising the possibility that other viral protein has a role alternatively. Formation of virus-like particles of HTNV was shown in both GPC and NP expressing cells (Li et al., 2010), but not in GPC expressing cells, implying that NP has a role in particle formation. Indeed, interaction between NP and cytoplasmic tails of Gn and Gc has been reported (Hepojoki et al., 2010b). In addition, it was reported that NP is localized at endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC) prior to its movement to the Golgi compartment and that inhibition of transport of NP to ERGIC resulted in reduction of virus replication (Ramanathan et al., 2007). Taking into account these findings, we hypothesized that NP has alternative role of matrix protein in the process of assembly and particle formation. In this study, to elucidate the role of NP in viral assembly, the effect of GPC and NP expression on their intracellular traffic was investigated.

2. Materials and Methods

2.1. Cells and viruses

Vero E6 cells (CRL-1586; American Type Culture Collection) were maintained in Eagle's minimum essential medium (Life Technologies) supplemented with 5% fetal calf serum, 1% insulin-transferrin-selenium (Life Technologies) and 1% MEM non-essential amino acids (Life Technologies). HTNV strain 76-118-derived clone-1 (Tamura et al., 1989), which was provided by Dr. K. Yamanishi (Osaka University Medical School, Osaka, Japan), was propagated in Vero E6 cells.

2.2. Preparation of virus-infected cells

2×10^5 cells/ml of Vero E6 cells were mixed with a equal volume of medium containing 2×10^3 focus-forming units (FFU)/ml of HTNV (multiplicity of infection = 0.01). 20 μl of the mixture was seeded on 24-well 4 mm HTC slides (Thermo Scientific) (2×10^3 cells/well). After incubation for 3 days, cells were fixed with 3% paraformaldehyde in PBS for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 4 min at room temperature.

2.3. Construction of plasmids

pCAGGS/MCS (Niwa et al., 1991) was used as a vector for expression of GPC and NP in Vero E6 cells. pCHTNM and pCHTNS encoding GPC and NP of HTNV strain 76-118, and pCSEOS encoding NP of SEOV strain SR-11 were constructed previously (Ogino et al., 2003; Yoshimatsu et al., 2003). pCPUUS encoding NP of PUUV strain Kazan were constructed by digesting the pGEM-T-based plasmid including cDNA of the NP gene of PUUV (Lundkvist et al., 1997) with *Sph*I and *Spe*I and cloning the

cDNA fragment into *Sph*I and *Nhe*I sites of pCAGGS/MCS. pCSNS encoding NP of SNV strain SN77734 was constructed by digesting the pFastBac-based plasmid including cDNA of the NP gene of SNV (Koma et al., 2012) with *Eco*RI and *Xho*I and cloning the cDNA fragment into pCAGGS/MCS. A series of amino and carboxyl terminally-truncated HTNV NP expressing vectors, pCAGGS-HTNV-NP30-429, pCAGGS-HTNV-NP75-429, pCAGGS-HTNV-NP116-429, pCAGGS-HTNV-NP155-429, pCAGGS-HTNV-NP1-333, pCAGGS-HTNV-NP1-233, pCAGGS-HTNV-NP1-155 and pCAGGS-HTNV-NP1-116, were constructed by amplifying cDNA fragments with primers flanked by *Eco*RI and *Xho*I sites and cloning into pCAGGS/MCS. For expression of NPΔ35-74 lacking amino acid region 35-74 of HTNV NP, pCAGGS-HTNV-NPΔ35-74 was constructed by utilizing overlap extention PCR (Higuchi et al., 1988). pCAGGS-HTNV-GPC-ZF1 and pCAGGS-HTNV-GPC-ZF2 encoding HTNV GPC with mutations in CCHC-type zinc finger motif 1 (H to Q and C to S at amino acid positions 561 and 565) and 2 (H to Q and C to S at amino acid positions 587 and 591) in the Gn cytoplasmic tail, respectively, was constructed by utilizing overlap extension PCR (Higuchi et al., 1988). Primers flanked by *Eco*RI and *Xho*I sites and primers for introducing the mutations were used. pCAGGS-HTNV-GPC-ΔGc-3 and pCAGGS-HTNV-GPC-ΔGc-6 encoding HTNV GPC with 3 or 6 amino acid deletions in the carboxyl terminal region of the Gc cytoplasmic tail, respectively, were constructed by amplifying cDNA fragments with primer containing stop codon and *Xho*I site and then cloning into *Hind*III and *Xho*I sites of pCHTNM. Primer sequences are available upon request.

2.4. Expression of recombinant proteins

Vero E6 cells on 24-well 4 mm HTC slides (Thermo Fisher Scientific Inc.) were transfected with various combinations of pCAGGS-based plasmids using TransIT LT1 (Takara Bio Inc.) according to the manufacturer's instructions. After incubation for 24 hours, the cells were fixed and permeabilized as described in section 2.2.

2.5. Immunofluorescence assay

To stain Gn, Gc and NP, mouse monoclonal antibodies 3D5, 5B7 and E5G6 (Arikawa et al., 1989; Yoshimatsu et al., 1996) were used as primary antibodies, respectively, and Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) was used as a secondary antibody. To stain ER and cis-Golgi, rabbit anti-Protein disulfide isomerase (PDI) polyclonal antibodies (Sigma-Aldrich) and rabbit anti-Mannosidase II (Man II) polyclonal antbodies (Merck KGaA) were used as primary antibodies, respectively, and Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies) was used as a secondary antibody. Cells were incubated with primary antibodies at room temperature for 1 hour. After washing with PBS three times, cells were incubated with secondary antibodies at room temperature for 1 hour. After washing, slides were mounted with glycerol for fluorescence microscopy (Merck KGaA) diluted with PBS (1:1), covered, and sealed with clear nail polish. Fluorescence was observed using ECLIPSE E600 (Nikon) and confocal laser microscopy system A1 (Nikon).

2.6. Evaluation of the effect of NP on diffuse localization of Gc

Ratio of diffused Gc-positive cells was determined by counting Gc-positive cells and diffused Gc-positive cells in each well. The number of Gc-positive cells in each well was more than 300.

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161 2.7. Statistical analysis

162 Student's *t* test was used to determine statistical significance. *P* values of <0.05 were

163 considered statistically significant.

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3. Results

3.1. Localization of Gn, Gc and NP of HTNV in infected cells

To determine the localization of Gn, Gc and NP of HTNV in infected cells, the viral proteins and the cis-Golgi or ER markers were doubly stained with Alexa Fluor 488- and Alexa Fluor 594-conjugated antibodies, respectively. Gn was stained with a granular pattern and colocalized with the cis-Golgi marker but not with the ER marker (Fig. 1). Similarly, Gc was colocalized with the cis-Golgi marker. On the other hand, NP was also stained with a granular pattern but only partially colocalized with the cis-Golgi marker. These results indicated that Gn and Gc were localized to cis-Golgi, while NP was localized to cis-Golgi as well as other compartments in infected cells.

3.2. Localization of Gn, Gc and NP of HTNV in GPC or NP-expressing cells

To determine whether the localization of Gn, Gc and NP in GPC or NP-expressing cells was the same as that in infected cells, Vero E6 cells were transfected with a HTNV GPC or NP-expressing vector and their localization was analyzed in the same way. Expressed GPC is posttranslationally cleaved into Gn and Gc. Most of Gn was localized to cis-Golgi in GPC-expressing cells as in infected cells (Fig. 2 and Fig. 3A). In contrast, some Gc showed diffuse distribution in the cytoplasm in GPC-expressing cells (Fig. 2 and Fig. 3A). The ratio of diffused Gc-positive cells among total Gc-positive cells was 32.9%. Diffused Gc was not observed in infected cells. NP was localized to the cytoplasm diffusely in NP-expressing cells (Fig. 2B). Thus, the localization of Gc and NP in GPC or NP-expressing cells was different from that in infected cells.

3.3. Effect of coexpression of GPC and NP of HTNV on their localization

To determine whether Gn, Gc and NP each affect their localization, Vero E6 cells were co-transfected with HTNV GPC and NP-expressing vectors. Co-expression of NP caused reduction of the ratio of diffused Gc-positive cells in a dose-dependent manner (Fig. 3A and 3B). To show time course of the effect of NP, cells were examined at 15~48 hours post transfection. The ratio of diffused Gc-positive cells reached a plateau level (12.9~14.9%) around 24 hours post-transfection, which was a significantly lower level than that in cells without NP (Fig. 3C). These results indicate that NP has activity to reduce the diffused localization of Gc. On the other hand, the localization of NP was not affected by co-transfection of a GPC-expressing vector (data not shown). In later experiments, cells were examined at 24 hours post-transfection.

3.4. Activity of NPs of SEOV, PUUV, and SNV to reduce diffused localization of HTNV Gc

To determine whether the activity of NP to reduce diffused localization of Gc was conserved among other representative hantaviruses, NPs of SEOV, PUUV and SNV were co-expressed with HTNV GPC. NPs of SEOV and SNV caused reduction of the ratio of diffused Gc-positive cells to 13.5% and 11.7%, respectively, which were comparable with that in the case of HTNV NP (Fig. 4). PUUV NP also caused reduction of the ratio of diffused Gc-positive cells to 25.2%, though the degree of reduction was lower than others (Fig. 4). To confirm the expression level of NPs, NPs were stained by immunefluorescence assay (Supplementary Fig. 1). Expression level of NP of PUUV was relatively low compared to those of NPs of HTNV, SEOV and SNV. To examine whether the low expression level of PUUV NP was related to the low activity to reduce diffuse distribution of Gc, increasing amount of pCPUUS was cotransfected with

213 pCHTNM. As a result, the ratio of diffused Gc-positive cells was decreased in a
214 dose-dependent manner (Supplementary Fig. 2B), indicating that PUUV NP also has the
215 activity. Thus, despite the heterologous relationship between NP and GPC, each NP
216 reduced the ratio of diffused Gc-positive cells.
217
218 3.5. Amino acid region of HTNV NP that is important for activity to reduce diffused
219 localization of Gc
220 To determine the amino acid region of NP that is important for activity to reduce
221 diffused localization of Gc, a series of truncated NP-expressing vectors were transfected
222 with a GPC-expressing vector. In the case of amino-terminally truncated HTNV NPs,
223 NP155-429 did not reduce the ratio of diffused Gc-positive cells, while NP75-429 and
224 NP116-429 reduced the ratio of those cells as did whole NP (Fig. 5). NP30-429 showed
225 only a slight reduction of the ratio of diffused Gc-positive cells. In the case of
226 carboxyl-terminally truncated HTNV NPs, NP1-333, NP1-233 and NP1-155 reduced
227 the ratio of diffused Gc-positive cells, while NP1-116 did not (Fig. 5). When expression
228 vector of NPΔ35-74 lacking amino acid region 35-74 was transfected, the ratio of
229 diffused Gc positive cells was reduced. To confirm the expression level of NPs, NPs
230 were stained by immunefluorescence assay (Supplementary Fig. 3). Expression level of
231 NP30-429, NP155-429, and NP1-233 were relatively low. To examine whether the low
232 expression level of NP30-429 and NP155-429 were related to the low or no activity to
233 reduce diffuse distribution of Gc, increasing amount of the expression plasmids of
234 NP30-429 and NP155-429 were transfected. As a result, slight reduction of the ratio of
235 diffused Gc-positive cells was observed in NP30-429 expressing cells (Supplementary
236 Fig. 2C). In contrast, the ratio of those cells was not changed in NP155-429 expressing

237 cells (Supplementary Fig. 2D). These results indicate that amino acid region 116-155 of
238 HTNV NP is important for activity to reduce diffused localization of Gc, although
239 amino acid region 1-30 is partially related.
240
241 3.6. Effect of mutations in HTNV Gn and Gc cytoplasmic tails on their localization
242 Glycoproteins Gn and Gc of hantaviruses are transmembrane proteins consisting of
243 an ectodomain, transmembrane domain and cytoplasmic tail and they form a hetero
244 complex. Considering the topology of NP and glycoproteins, NP may affect the
245 localization of Gc by binding to the cytoplasmic tails of Gn and Gc. To examine this
246 possibility, the effect of mutations in two CCHC-type zinc finger motifs in the HTNV
247 Gn cytoplasmic tail and the effect of deletions of short HTNV Gc cytoplasmic tail were
248 investigated. Both of the regions are thought to be important for the binding of NP to
249 cytoplasmic tails (Hepojoki et al., 2010b; Wang et al., 2010). ZF1 and ZF2 were
250 mutants with amino acid mutations in zinc finger motif 1 (H561Q and C565S) or 2
251 (H587Q and C591S), respectively. ΔGc-3 and ΔGc-6 were mutants with deletion of 3 or
252 6 amino acids in the carboxyl terminal of Gc cytoplasmic tail, respectively. Expression
253 levels of wt GPC, ZF1, ZF2, ΔGc-3, and ΔGc-6 were similar (Supplementary Fig. 4).
254 Ratio of diffused Gc-positive cells in ZF1 or ZF2 expressing cells were comparable
255 with that in wild type GPC expressing cells (Fig. 6). In contrast, deletion of 6 amino
256 acids in the carboxyl terminal of Gc cytoplasmic tail caused an increase in the ratio of
257 diffused Gc-positive cells in the absence of NP. In addition, the effect of coexpression of
258 NP was weakened in ΔGc-6 expressing cells (Fig. 6). These results indicate that the
259 HTNV Gc cytoplasmic tail is important for efficient cis-Golgi localization of Gc.
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4. Discussion

Particles of hantaviruses are thought to be formed in the Golgi complex (Schmaljohn and Nichol, 2007). Therefore, Golgi localization of viral glycoproteins Gn and Gc should be an important step for their life cycle. We showed that Gn and Gc were mainly detected in cis-Golgi in HTNV-infected cells (Fig. 1). We speculate that the quantity of Gn and Gc in ER is very small, since Gn and Gc are transported to Golgi complex immediately after synthesis in ER. In contrast, when HTNV GPC was expressed by transfection, Gc showed diffuse distribution in some cells (Fig. 2 and Fig. 3A), indicating that factors other than GPC are involved in efficient Golgi localization of Gc. In this study, we found that HTNV NP has a role in promoting the Golgi localization of Gc.

Some studies have shown that Gn and Gc form a hetero complex (Antic et al., 1992; Hepojoki et al., 2010a). It has been also reported that the presence of both Gn and Gc is essential for their Golgi localization (Pensiero and Hay, 1992; Ruusala et al., 1992). Based on the results of those studies, we speculate that formation of a hetero complex is a prerequisite for their transport to the Golgi complex. Interestingly, Gn was localized to cis-Golgi in GPC-expressing cells regardless of the presence of NP, suggesting that formation of a hetero complex before transport to the Golgi complex is accomplished without NP and that Gc may be dissociated from the hetero complex after transport to the Golgi complex. Therefore, NP may play a role in increasing stability of the hetero complex after transport to the Golgi complex.

The instability of Gc may be due to its short cytoplasmic domain. The cytoplasmic domain of Gc consists of only 6 amino acids. Deletion of the cytoplasmic domain resulted in an increase of diffused distribution of Gc. In addition, the deletion decreased

285 the effect of NP. It has been reported that NP interacts with the short cytoplasmic
286 domain of Gc (Hepojoki et al., 2010b). Therefore, NP may stabilize the hetero complex
287 through interaction with the Gc cytoplasmic domain.
288 Analysis of deletion mutants of HTNV NP showed that amino acid regions 1-30 and
289 116-155 in NP were important to promote Golgi localization of Gc. The amino acid
290 region 116-155 seemed to be more important for the activity, since NP30-429 caused
291 slight reduction of diffused localization of Gc. In addition, NP75-429 and NP116-429,
292 which lacked amino acid region 1-30, were still functional. In the amino-terminal region,
293 there was an intramolecular coiled-coil structure consisting of two alpha-helixes (Wang
294 et al., 2008). Alpha-helixes 1 and 2 were located in amino acid regions 1-34 and 39-74,
295 respectively. Deletion of amino acid region 1-30 may disrupt folding of the
296 amino-terminal region and interfere with the activity of NP to promote Golgi
297 localization of Gc. The amino acid region 116-155, especially 135-148, in NP was
298 relatively conserved among hantaviruses including Thottapalayam virus that is one of
299 the shrew-borne hantaviruses and genetically distant from rodent-borne hantaviruses
300 (Fig. 7). These conserved amino acids may have an important role in the mechanism.
301 Old and New World hantaviruses have evolved differences in their interaction with host
302 cell machinery as well as in pathogenesis. HTNV enters cells via clathrin-mediated
303 endocytosis, while ANDV entry is clathrin-independent (Ramanathan et al., 2008).
304 HTNV requires an intact microtubule network for replication, while ANDV requires
305 actin. However, in this study, we found that NPs of SEOV, PUUV and SNV exerted a
306 similar effect over HTNV glycoproteins, suggesting that the activity of NP may be a
307 primitive feature common to hantaviruses. In other members of the *Bunyaviridae* family,
308 importance of cytoplasmic tails of the glycoproteins in viral assembly has been reported.

Shi et al. showed that the cytoplasmic tails of both Gn and Gc of Bunyamwera virus, member of the *Orthobunyavirus* genus, play crucial role in virus assembly and morphogenesis. The cytoplasmic tail of glycoprotein G_N of Uukuniemi virus, member of the *Pholebovirus* genus, is important for Golgi retention, binding of nucleoprotein, and genome packaging (Andersson et al., 1997; Overby et al., 2007). Overby et al. showed the importance of a lysine at position -3 from the C terminus of the short cytoplasmic tail of Uukuniemi virus G_C in Golgi localization and particle formation. Interestingly, the lysine is highly conserved among members of the *Phlebovirus*, *Hantavirus*, and *Orthobunyavirus* genera. In this study, we showed that the short cytoplasmic tail of Gc is important for the correct localization of Gc and for receiving support from NP. Considering these reports and findings, interaction between the cytoplasmic tails of glycoproteins and nucleoprotein may be conserved feature for the *Bunyaviridae* family that lacks matrix protein.

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448 **Legends to figures**

449 **Fig. 1.** Viral glycoproteins Gn and Gc were localized to cis-Golgi in HTNV-infected
450 Vero E6 cells. (A) Localization of viral proteins (left column) and ER marker (center
451 column). (B) Localization of viral proteins (left column) and cis-Golgi marker (center
452 column). Right columns show merged images. Mouse monoclonal antibodies 3D5, 5B7,
453 and E5G6 were used as primary antibodies for staining of Gn, Gc, and NP, respectively.
454 Rabbit anti-PDI polyclonal antibodies and rabbit anti-Man II polyclonal antibodies were
455 used as primary antibodies for staining of ER and cis-Golgi markers, respectively. Alexa
456 Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG were used as
457 secondary antibodies.

458

459 **Fig. 2.** Appearance of diffused Gc in HTNV GPC-expressing cells. (A) Localization of
460 Gn, Gc (left column) and cis-Golgi marker (center column) in HTNV GPC-expressing
461 Vero E6 cells. (B) Localization of NP (left) and cis-Golgi marker (center) in HTNV
462 NP-expressing Vero E6 cells. Right columns show merged images. Vero E6 cells were
463 transfected with 30 ng/well of pCAGGS/MCS and 30ng/well of pCHTNM or pCHTNS.
464 After 24 hours, cells were fixed, stained and examined under microscope.

465

466 **Fig. 3.** Co-expression of HTNV GPC and NP promoted cis-Golgi localization of Gc.
467 (A) Localization of Gn and Gc in cells transfected with 30 ng/well of pCHTNM and 30
468 ng/well of pCAGGS/MCS (left column) or pCHTNS (right column) at 24 hours post
469 transfection. (B) Dose-dependent effect of NP on localization of Gc. Vero E6 cells were
470 co-transfected with pCHTNM (40 ng/well) and increasing amounts of pCHTNS. After
471 incubation for 24 hours, ratios of diffused Gc-positive cells were determined by

counting Gc- and diffused Gc-positive cells. (C) Time course of the ratio of diffused Gc-positive cells. Vero E6 cells were co-transfected with 30 ng/well of pCHTNM and 30 ng/well of pCAGGS/MCS (open bar) or pCHTNS (filled bar) and incubated for indicated periods. Error bars represent standard deviation of values determined by three independent experiments.

Fig. 4. Co-expression of GPC of HTNV and NP of HTNV, SEOV, PUUV or SNV promoted cis-Golgi localization of Gc. Vero E6 cells were co-transfected with 30 ng/well of pCHTNM and 30 ng/well of pCAGGS/MCS, pCHTNS, pCSEOS, pCPUUS or pCSNS and incubated for 24 hours. Error bars represent standard deviation of values determined by three independent experiments. Asterisks indicate significant differences between empty and each NP expressing plasmid. *p<0.05. **p<0.01.

Fig. 5. Identification of the amino acid region in NP that is important for activity to reduce diffuse localization of Gc. Vero E6 cells were co-transfected with 30 ng/well of pCHTNM and 30 ng/well of pCAGGS/MCS, pCHTNS or pCAGGS-based truncated HTNV NP-expressing plasmids and incubated for 24 hours. The bars on the left of the graph are pictorial representations of the portions of truncated NPs. Error bars represent standard deviation of values determined by three independent experiments. Asterisks indicate significant differences between empty and each NP expressing plasmid. *p<0.05. **p<0.01.

Fig. 6. Effect of Gn and Gc cytoplasmic tail mutations on localization of Gc. Vero E6 cells were co-transfected with 30 ng/well of pCAGGS-based wild type (wt) or mutant

HTNV GPC-expressing plasmids and 30 ng/well of pCAGGS/MCS (open bar) or pCHTNS (filled bar) and incubated for 24 hours. Error bars represent standard deviation of values determined by three independent experiments. Asterisks indicate significant differences. *$p<0.05$. **$p<0.01$.

Fig. 7. Alignment of partial amino acid sequences of NPs of representative hantaviruses. Asterisks indicate conserved amino acid residues.

Legends to Supplementary figures

Supplementary Fig. 1. Expression level of NP in Vero E6 cells transfected with 30 ng/well of pCHTNM and 30 ng/well of pCHTNS, pCSEOS, pCPUUS or pCSNS at 24 hours post transfection. Mouse monoclonal antibody E5G6 and Alexa Fluor 488 goat anti mouse IgG were used for staining of NP.

Supplementary Fig. 2. Dose-dependent effect of HTNV NP (A), PUUV NP (B), HTNV NP30-429 (C) and HTNV NP155-429 (D) on localization of Gc. Vero E6 cells were transfected with 20 ng/well of pCHTNM and increasing amount of pCHTNS, pCPUUS, pCAGGS-HTNV-NP30-429 or pCAGGS-HTNV-NP155-429. After incubation for 24 hours, ratios of diffused Gc-positive cells were determined by counting Gc- and diffused Gc-positive cells. Error bars represent standard deviation of values determined by three independent experiments. Asterisks indicate significant differences between empty and each quantity of NP expressing plasmid. *$p<0.05$. **$p<0.01$.

Supplementary Fig. 3. Expression level of NP in Vero E6 cells transfected with 30 ng/well of pCHTNM and 30 ng/well of pCAGGS-based truncated HTNV NP-expressing plasmids at 24 hours post transfection. Mouse monoclonal antibody E5G6 was used for staining of NPs except for NP1-155 and NP1-116. Mouse monoclonal antibody ECO2 was used for staining of NP1-155 and NP1-116. Alexa Fluor 488 goat anti mouse IgG was used as secondary antibody.

Supplementary Fig. 4. Expression level of Gc in Vero E6 cells transfected with 30

528 ng/well of pCAGGS-based wild type (wt) or mutant HTNV GPC-expressing plasmids
529 and 30 ng/well of pCAGGS/MCS at 24 hours post transfection. Mouse monoclonal
530 antibody 5B7 and Alexa Fluor 488 goat anti mouse IgG were used for staining of Gc.
531

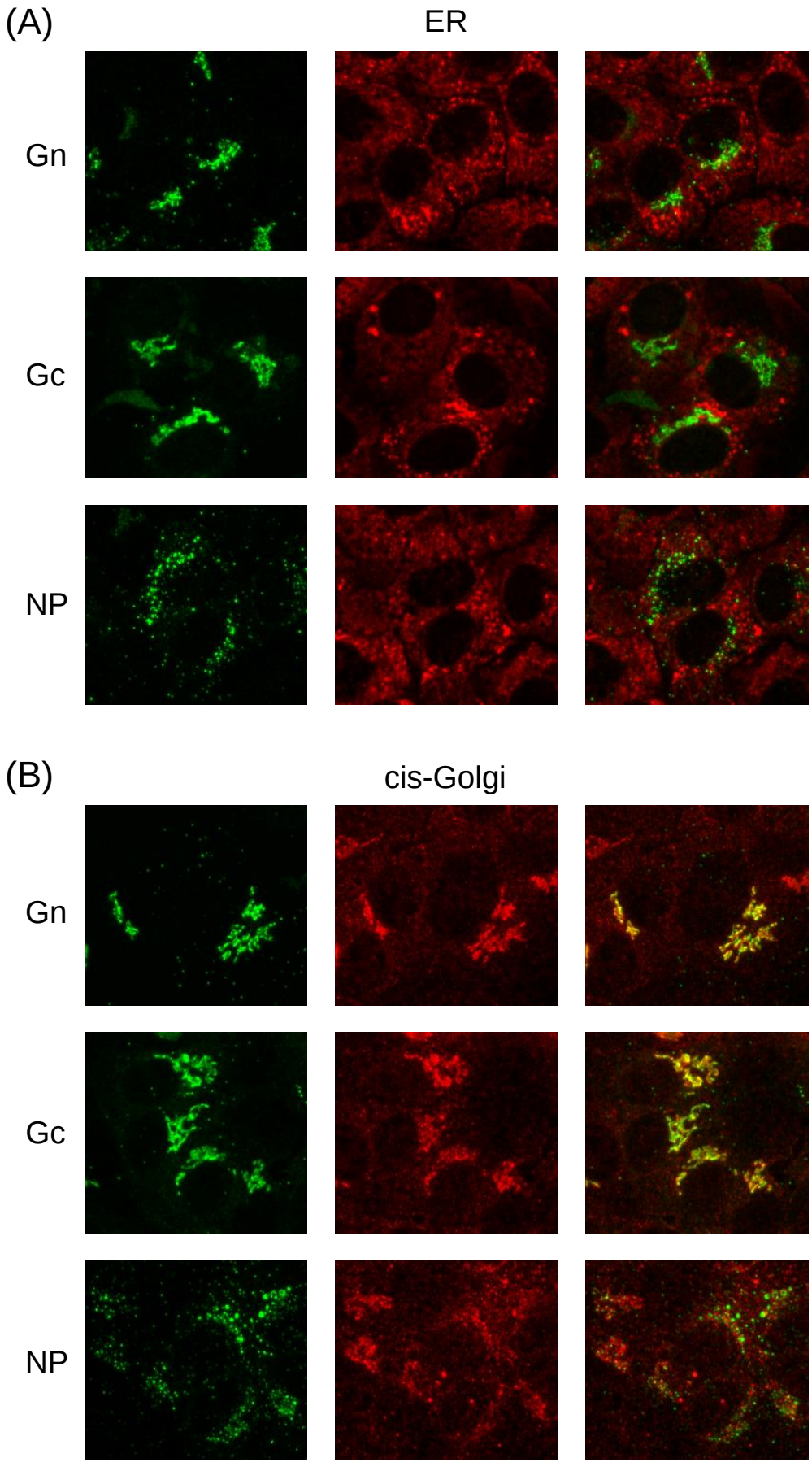

Fig. 1

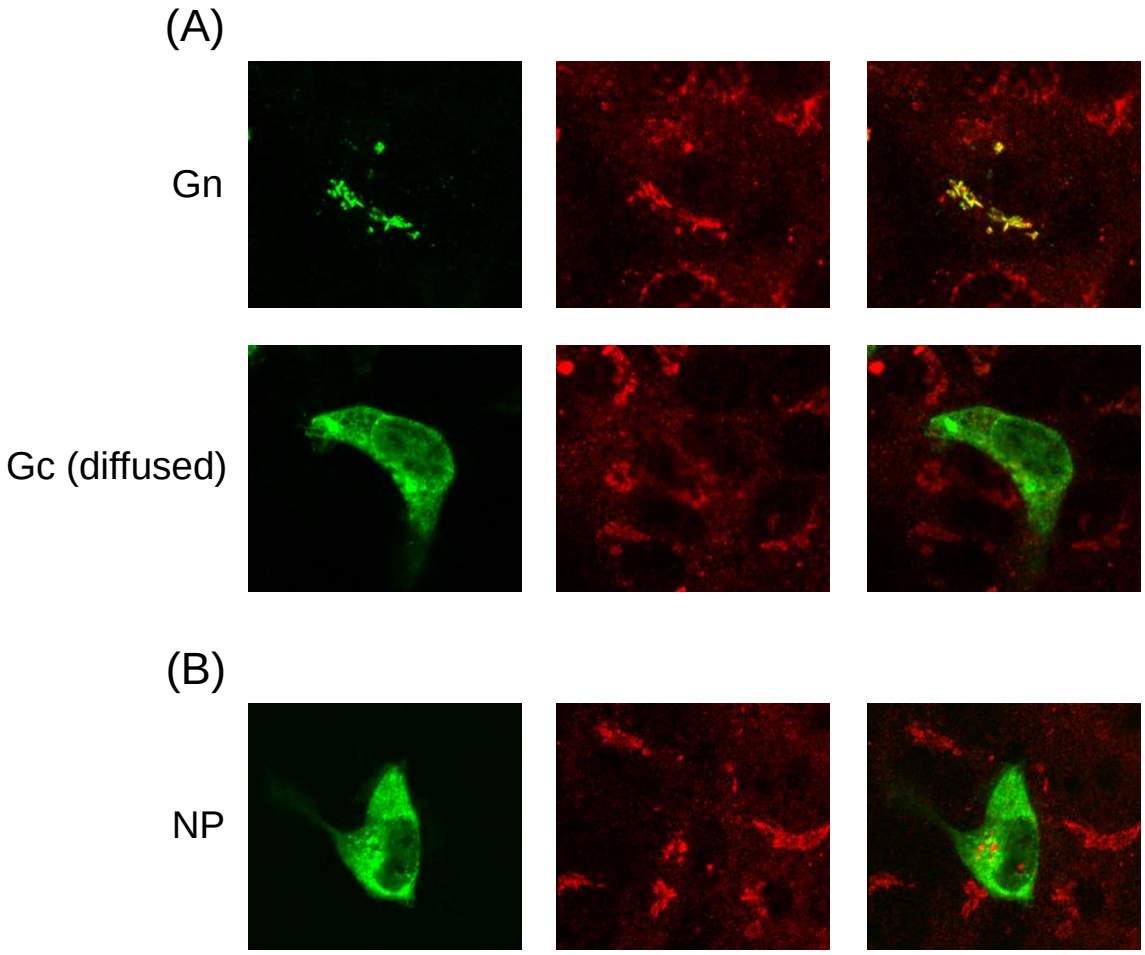

Fig. 2

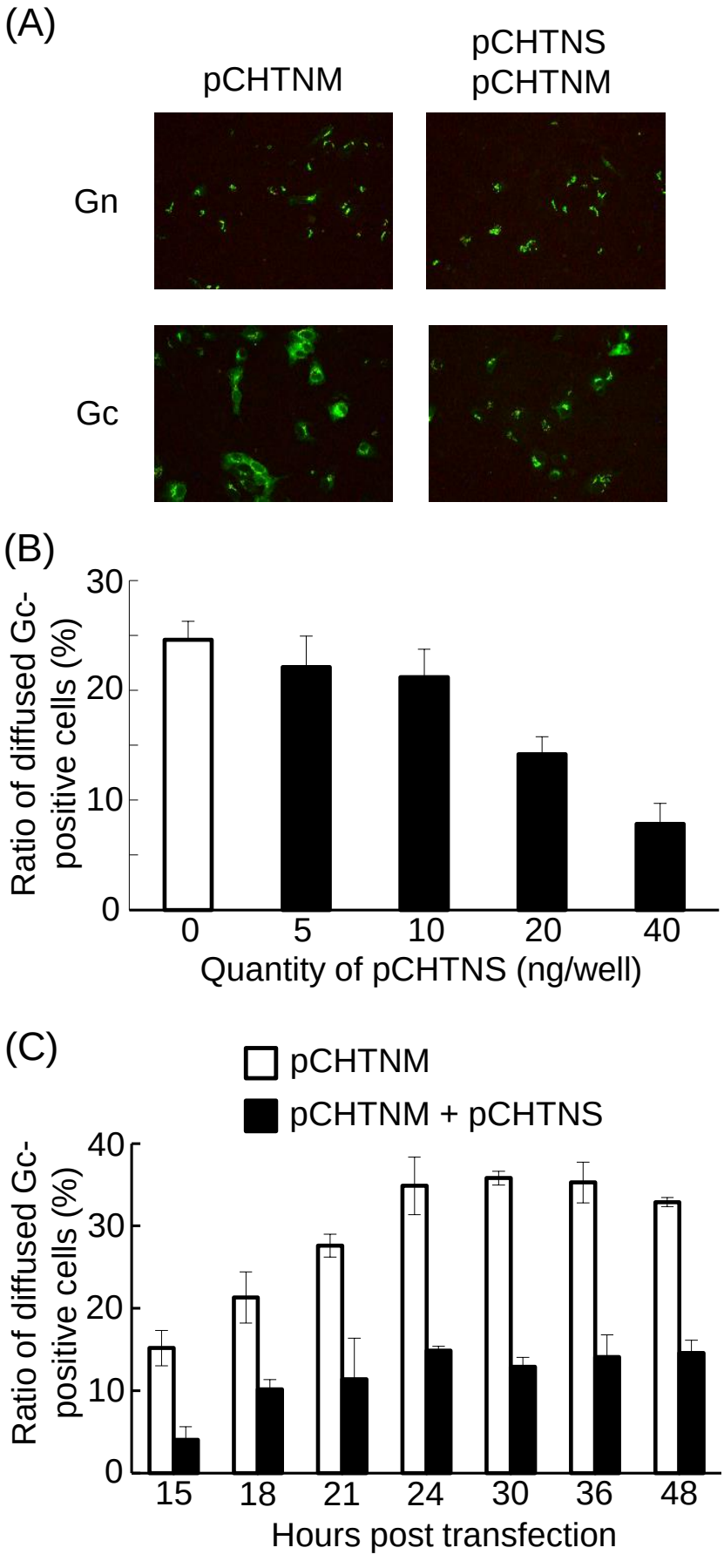
(A)
pCHTNM
pCHTNS
pCHTNM
Gn
Gc
(B)
Ratio of diffused Gc-positive cells (%)
30
20
10
0
0
5
10
20
40
Quantity of pCHTNS (ng/well)
(C)
pCHTNM
pCHTNM + pCHTNS
Ratio of diffused Gc-positive cells (%)
40
30
20
10
0
15
18
21
24
30
36
48
Hours post transfection

Fig. 3

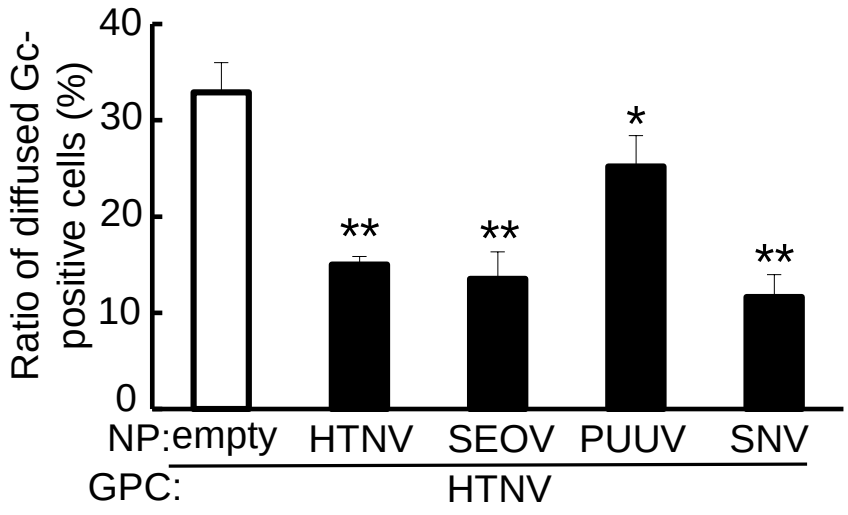
Ratio of diffused Gc-positive cells (%)
40
30
20
10
0
**
**
*
**
NP:
empty
HTNV
SEOV
PUUV
SNV
GPC:
HTNV

Fig. 4

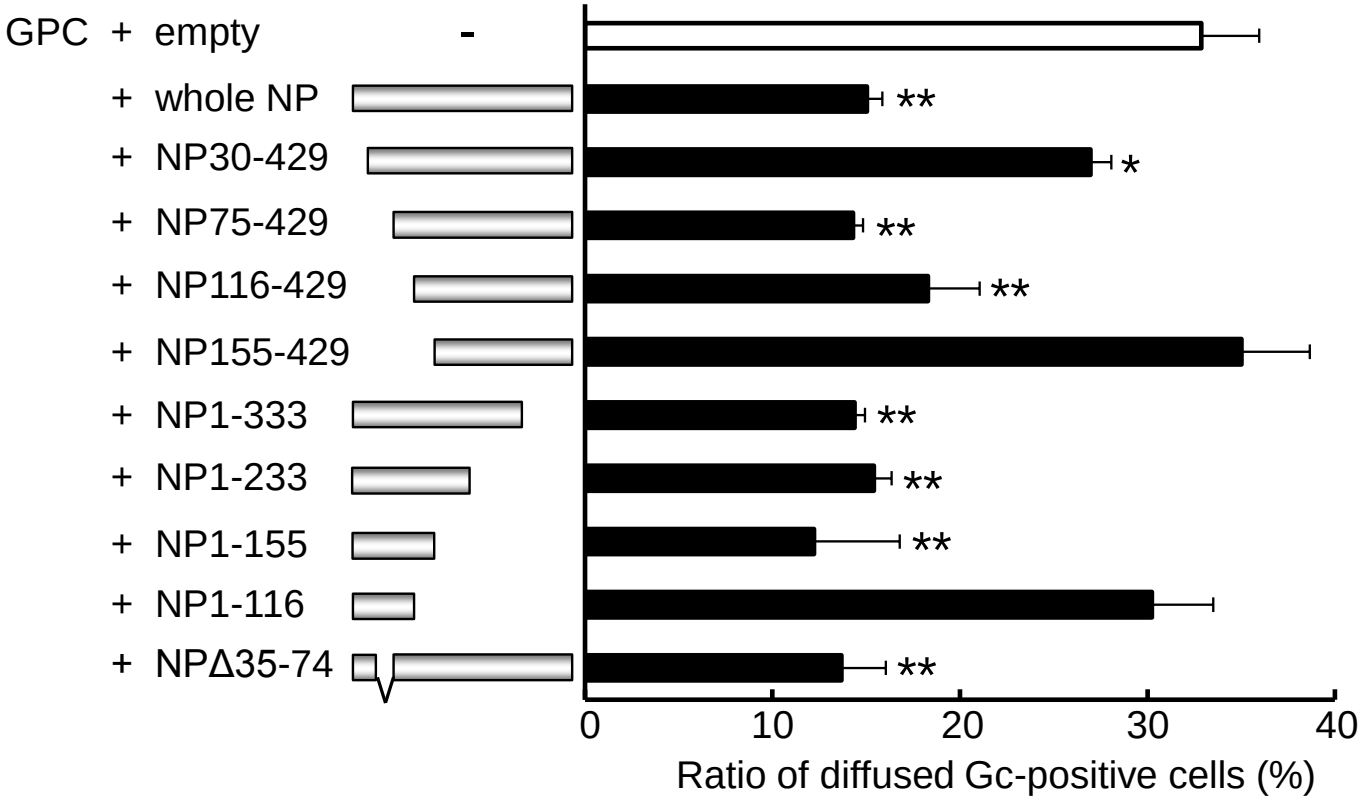
GPC
+ empty
-
+ whole NP
+ NP30-429
+ NP75-429
+ NP116-429
+ NP155-429
+ NP1-333
+ NP1-233
+ NP1-155
+ NP1-116
+ NPΔ35-74
**
*
**
**
**
**
**
0
10
20
30
40
Ratio of diffused Gc-positive cells (%)

Fig. 5

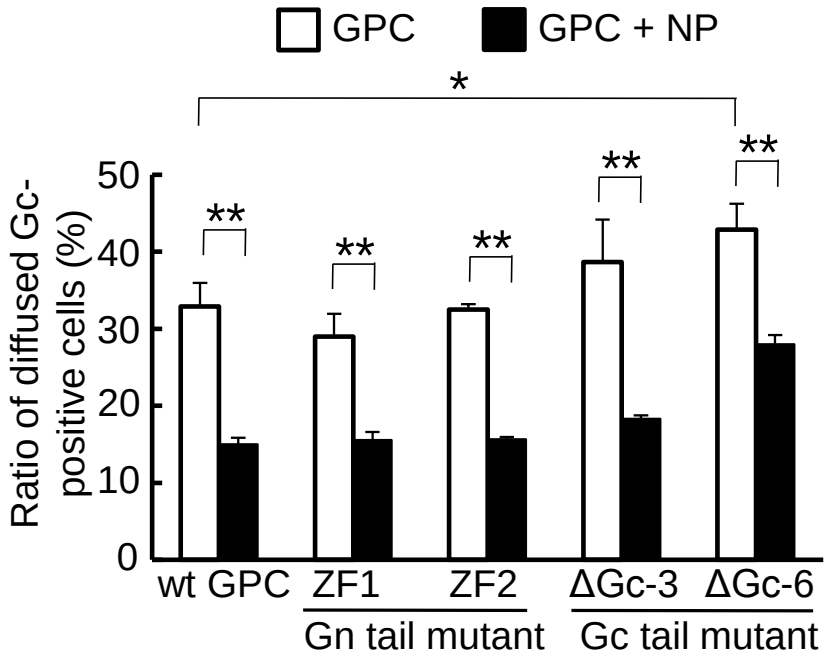
GPC
GPC + NP
*
**
**
**
**
Ratio of diffused Gc-positive cells (%)
50
40
30
20
10
0
wt GPC
ZF1
ZF2
ΔGc-3
ΔGc-6
Gn tail mutant
Gc tail mutant

Fig. 6

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                          116                                       155
Hantaan virus             TADWLSIIVYLTSFVVPILLKALYMLTTRGRQTTKDNKGT
Seoul virus               .....T............I..............S.....M
Thailand virus            .....T.........I..I....................M
Dobrava virus             .......V..............................M
Saaremaa virus            .......V..............................M
Puumala virus             ....YT.G..VIG.TI..I.......S......V.E....
Topografov virus          ....FT.G..IV..TL..I.......S......V.E....
Khabarovsk virus          ....FT.G..II..TL..I.......S......V.E....
Tula virus                ....F..GQ.I.G.ALA.I.......S......I.E....
Prospect Hill virus       .....K.GS.IIG.AL..I.......S......V.E....
Isla Vista virus          .....Q.GS.II..AL..I.......S......V.E....
Sin Nombre virus          ....K..GL.IL..AL..I.......S......I.E....
New York virus            ....KA.GM.IL..AI..I.......S......V.E....
Rio Segundo virus         ....R..GM.IL..TL..V.......S......V.E....
El Moro Canyon virus      ....K..GL.IL..TL..V.......S......VQE....
Cano Delgadito virus      ....KT.G..VLG.AI..I.......S......V.E....
Andes virus               ....KA.GA.ILG.AI..I.......S......V......
Laguna Negra virus        ....KA.GA.ILG..I..I.......S......V.E....
Rio Mamore virus          ....KA.GA.ILG..I..V.......S......V.E....
Black Creek Canal virus   ....KA.GT.IL...L..V.......S......V.E....
Bayou virus               ....KA.G..IL...L..V.......S......V.E....
Muleshoe virus            ....KA.G..VL...L..V.......S......V.E....
Thottapalayam virus       ..N.GK.FE.ILTLTQVL...G..I........S......
                          ** *  *  *         ** ** * ******   ***
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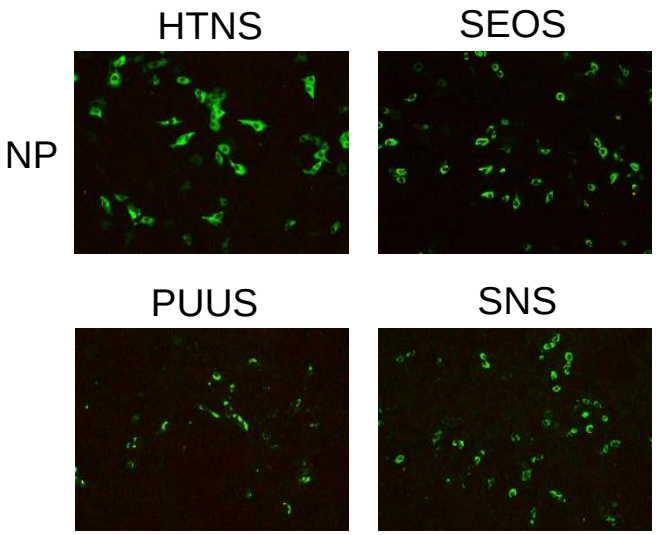
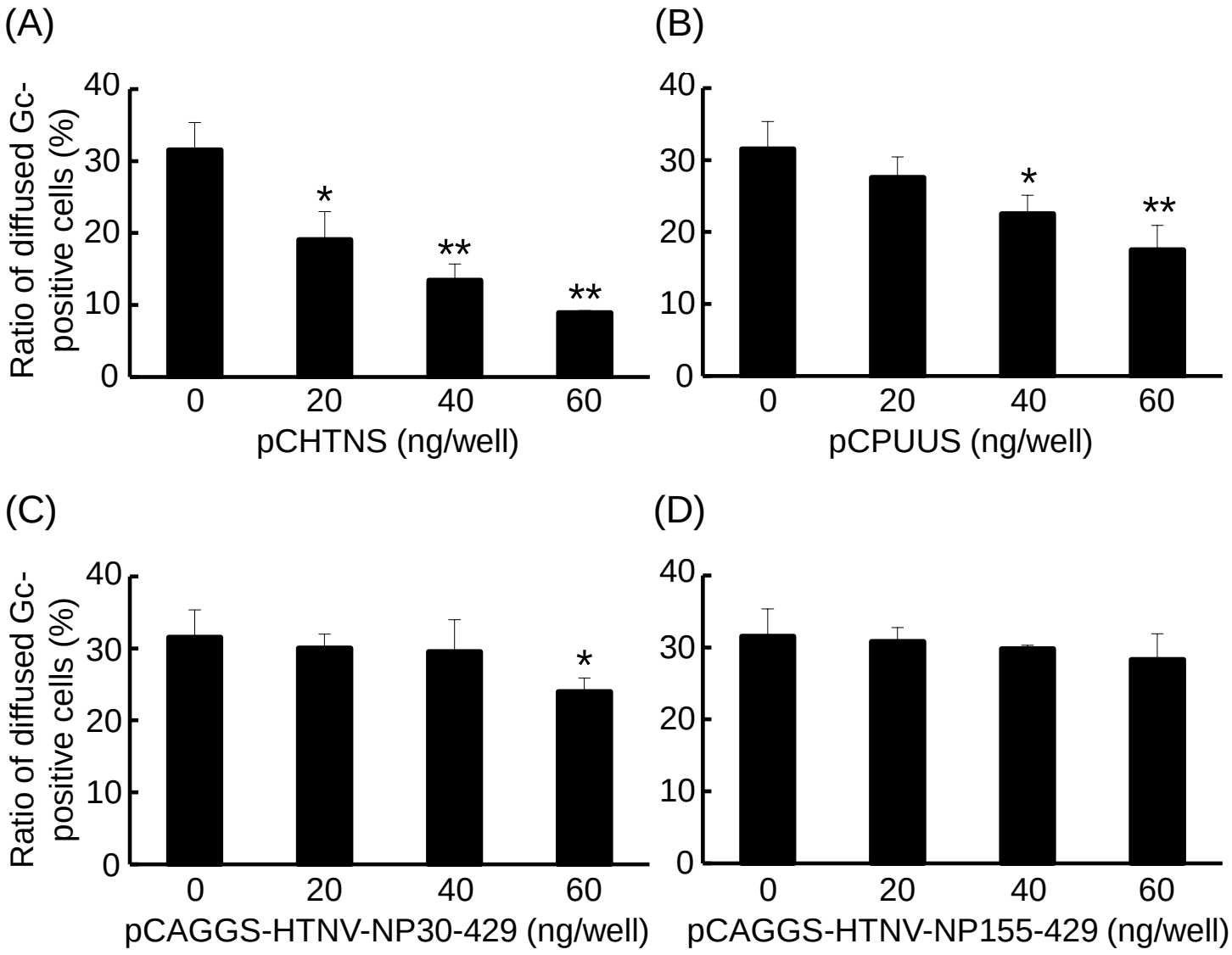
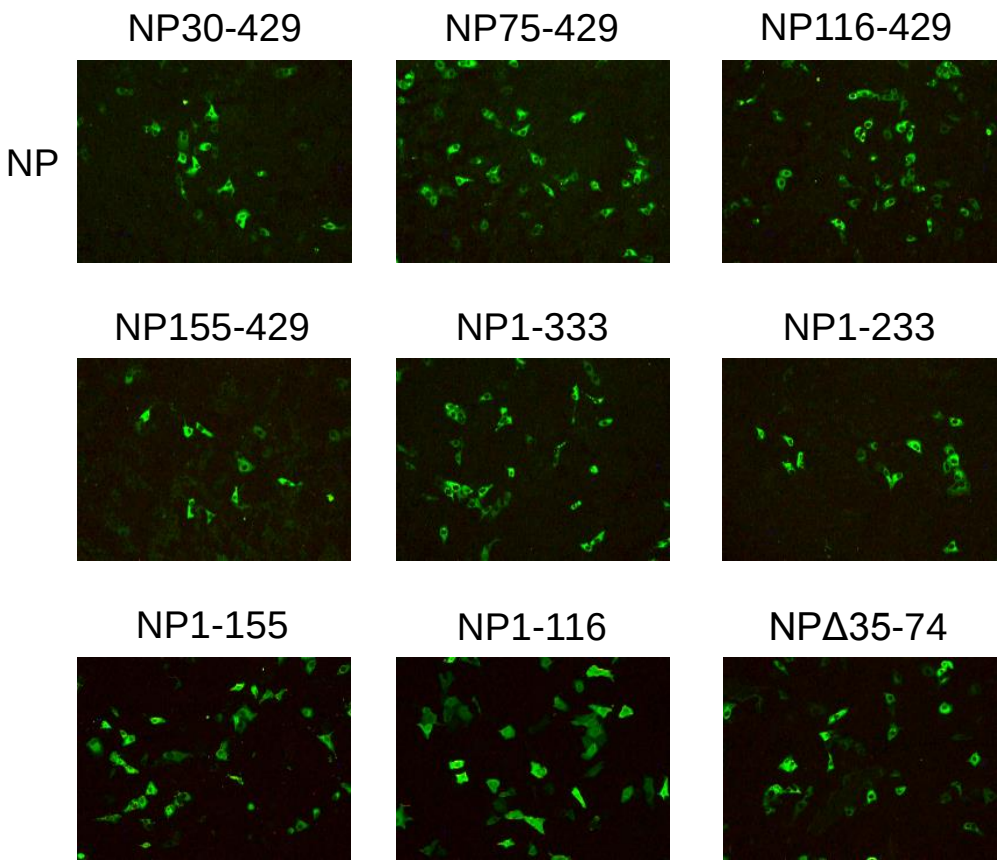
Fig. 7



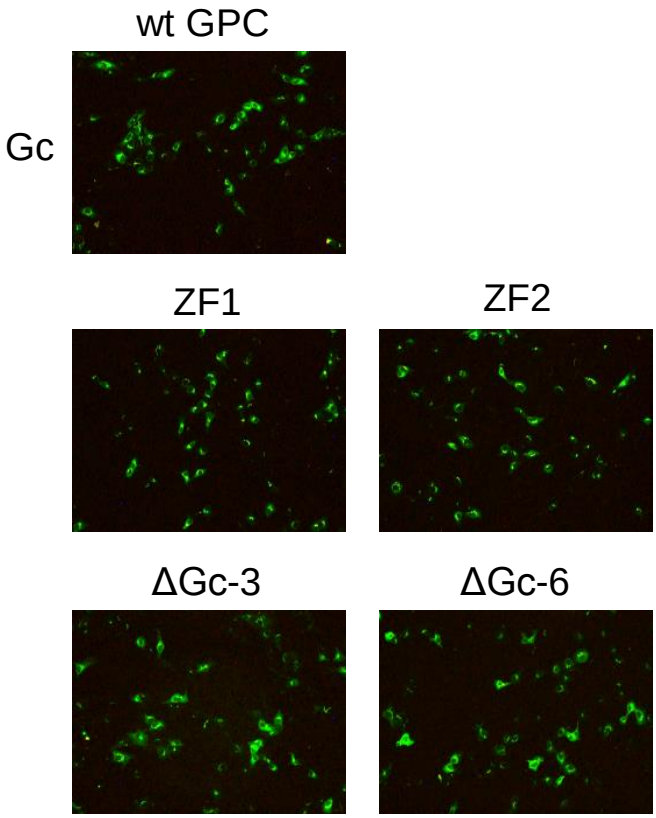
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3



Supplementary figure 4