590 **Figure legends**

591 **Fig. 1.** Design of HBVpp packaging system. A. Construct of the retroviral genome.

592 A MLV-based retroviral vector was constructed. As commonly used, this vector was

593 two LTRs at the 5' and the 3' end. A Packaging signal (Ψ), a selection marker (Hyg^R),

594 a CMV immediate early enhancer and promoter followed by a *GFP* gene are

595 represented. B. An established packaging cell line is shown. This cells was

596 generated in MLV gag-pol expressing GP2 (Clontech) cells, where the retroviral vector

597 (see Fig. 1A) was integrated. As a result, the packaging cells express the Hyg^R and the

598 *GFP* in addition to MLV gag-pol. C. A strategy of the generation of HBVpp. The

599 established packaging cells could produce HBV membrane protein enveloped retroviral

600 capsids, when HBV membrane proteins were successfully expressed.

601

602 **Fig. 2.** HBV membrane proteins and their expressing plasmid, pCEP4 LS-S. A.

603 Three HBV membrane proteins are shown. The S region is shared by all HBV

604 membrane proteins. A hexagon and a diamond represent an O-glycosylation and an

605 N-glycosylation site, respectively. B. An expression map of HBV membrane

606 proteins. Arrows represent putative transcription start sites for each HBV membrane

607 gene. C. HBV membrane protein expression was analyzed by immunoprecipitation

608 with rabbit polyclonal anti-HBs antibodies followed by Western blot with a mouse

609 monoclonal anti-HBs antibody. Input: lysate from the transfected cells. UB:

610 unbound fractions with goat polyclonal anti-HBs antibodies (Austral Biologicals). B:

611 bound fractions with the same antibodies. Arrowheads show authentic HBV

612 membrane proteins.

613

614 **Fig. 3.** HBV membrane bound particles contains retroviral genomes inside. A.

615 Culture medium of either HBV LS-S or VSV-G transfected packaging cells was

616 immunoprecipitated with anti-S antibodies or anti-VSV-G antibodies. Putative RNA

617 genomes were extracted from the immunoprecipitates and subjected to RT-PCR for the

618 *EGFP* gene. Ab: antibody, IP: immunoprecipitation, RT: reverse transcription, +ve:

619 positive. B. CsCl density gradient ultracentrifugaion profile, ELISA and RT-PCR of

620 the each fraction. (Upper) Profiles of the density, ELISA for HBV membrane proteins

621 (HBs and preS1). The left longitudinal axis shows the density of each fraction. ρ:

622 density, mg/ml. The right longitudinal axis shows OD_{450} values for HBs and preS1

623 measured with ELISA kits. (Lower) An agarose-gel electrophoresis of RT-PCR

624 products of the *EGFP* gene as a target (about 320bp).

625 **Fig. 4.** Electronmicroscopy of intracellular sub-viral particles and secreted virus-like

626 particles. a. Sub-viral particles accumulation were seen in the ER of LS-S

627 expressing packaging cells. b. Secreted virus-like particles are shown.

628

Fig. 1

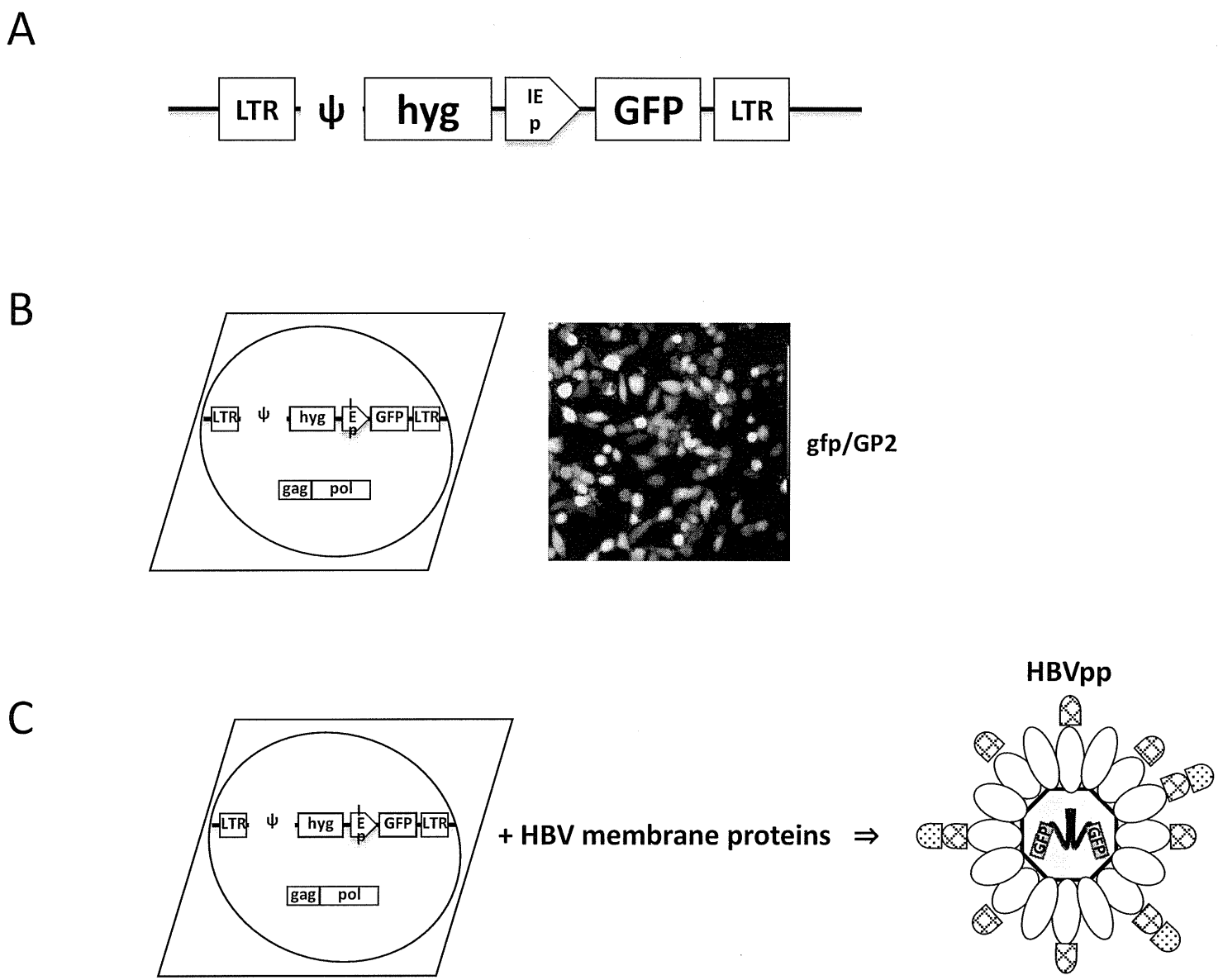
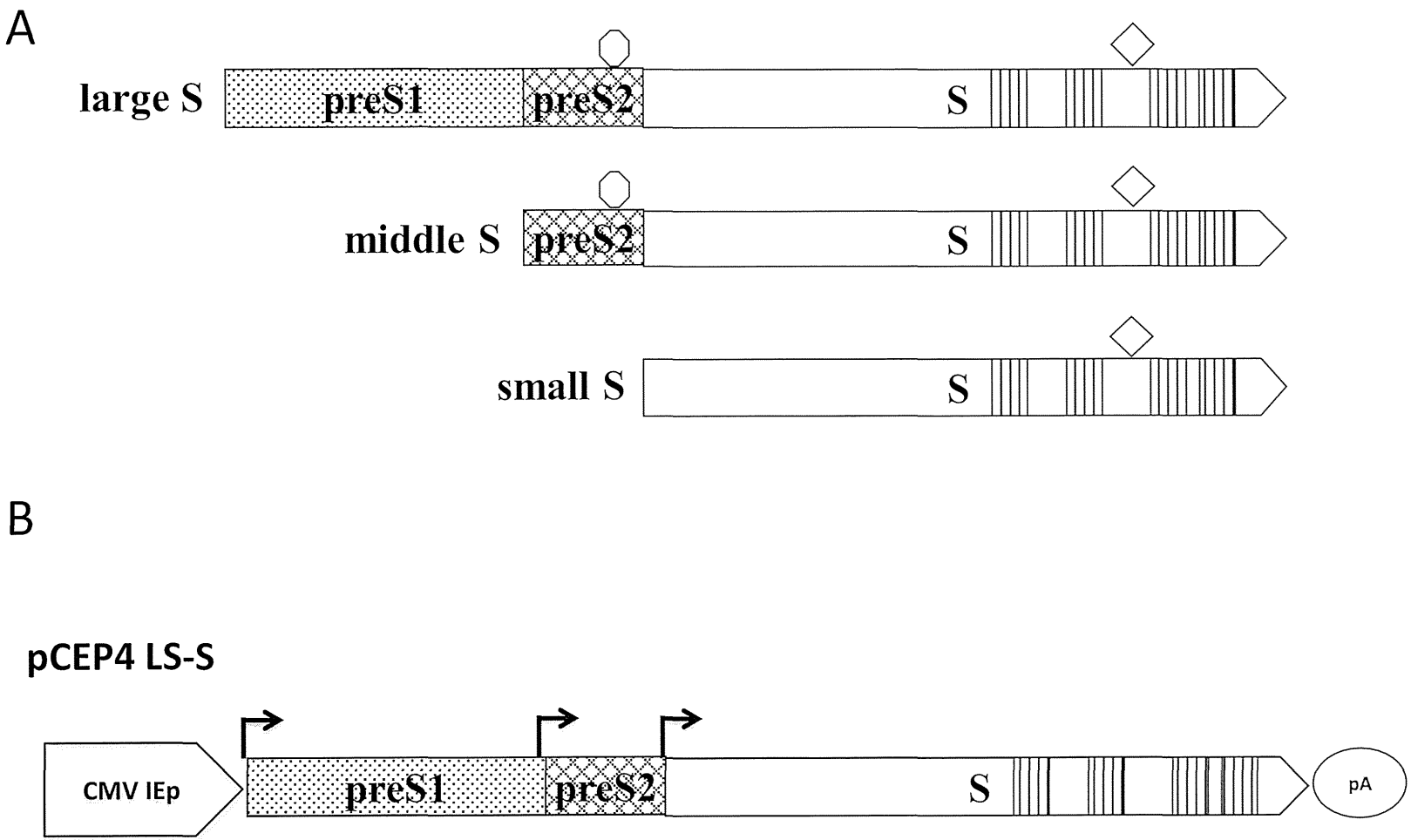


Fig. 2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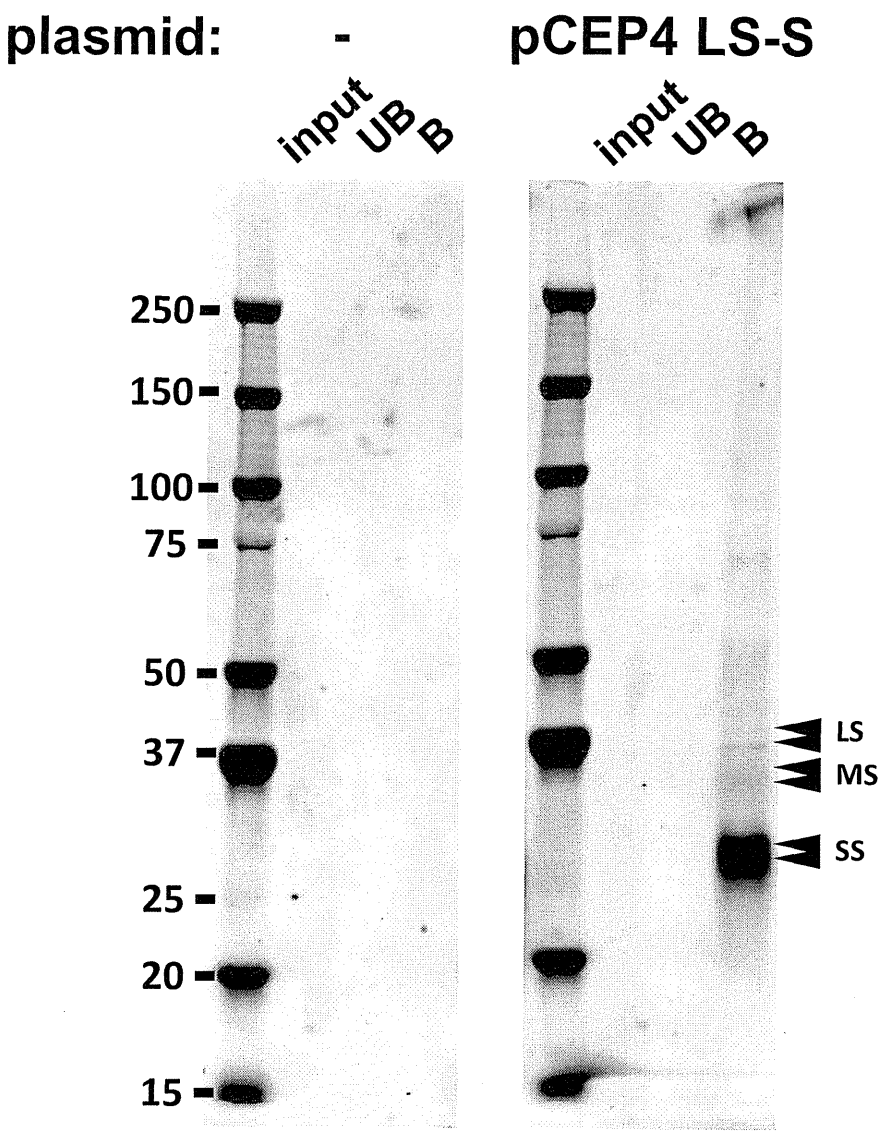
large S
preS1
preS2
S
middle S
preS2
S
small S
S
B
pCEP4 LS-S
CMV IEp
preS1
preS2
S
pA

Fig. 2

C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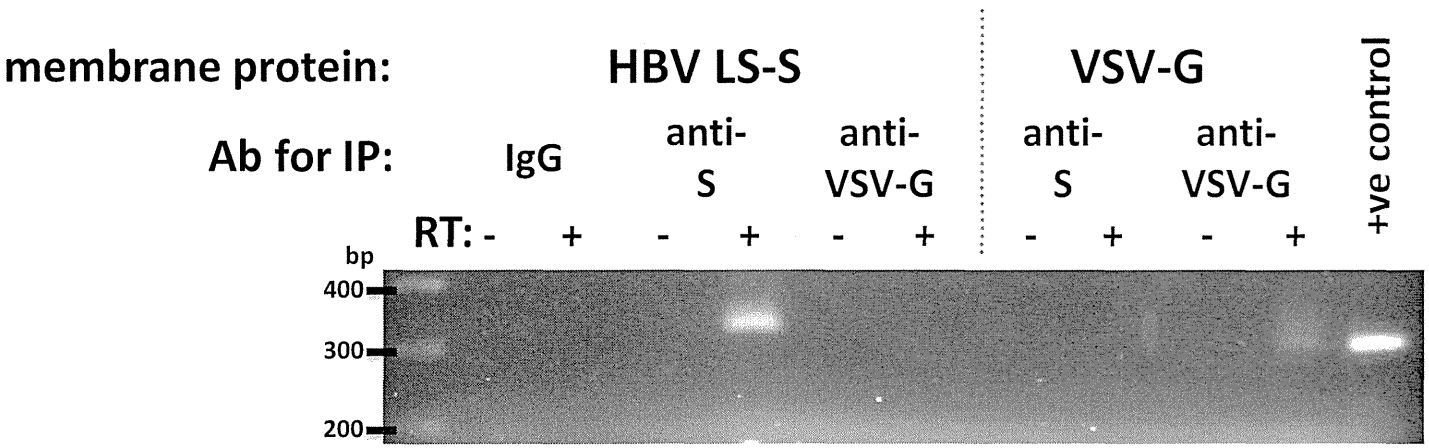
-
pCEP4 LS-S
input
UB
B
input
UB
B
250
150
100
75
50
37
25
20
15
LS
MS
SS

Fig. 3

A.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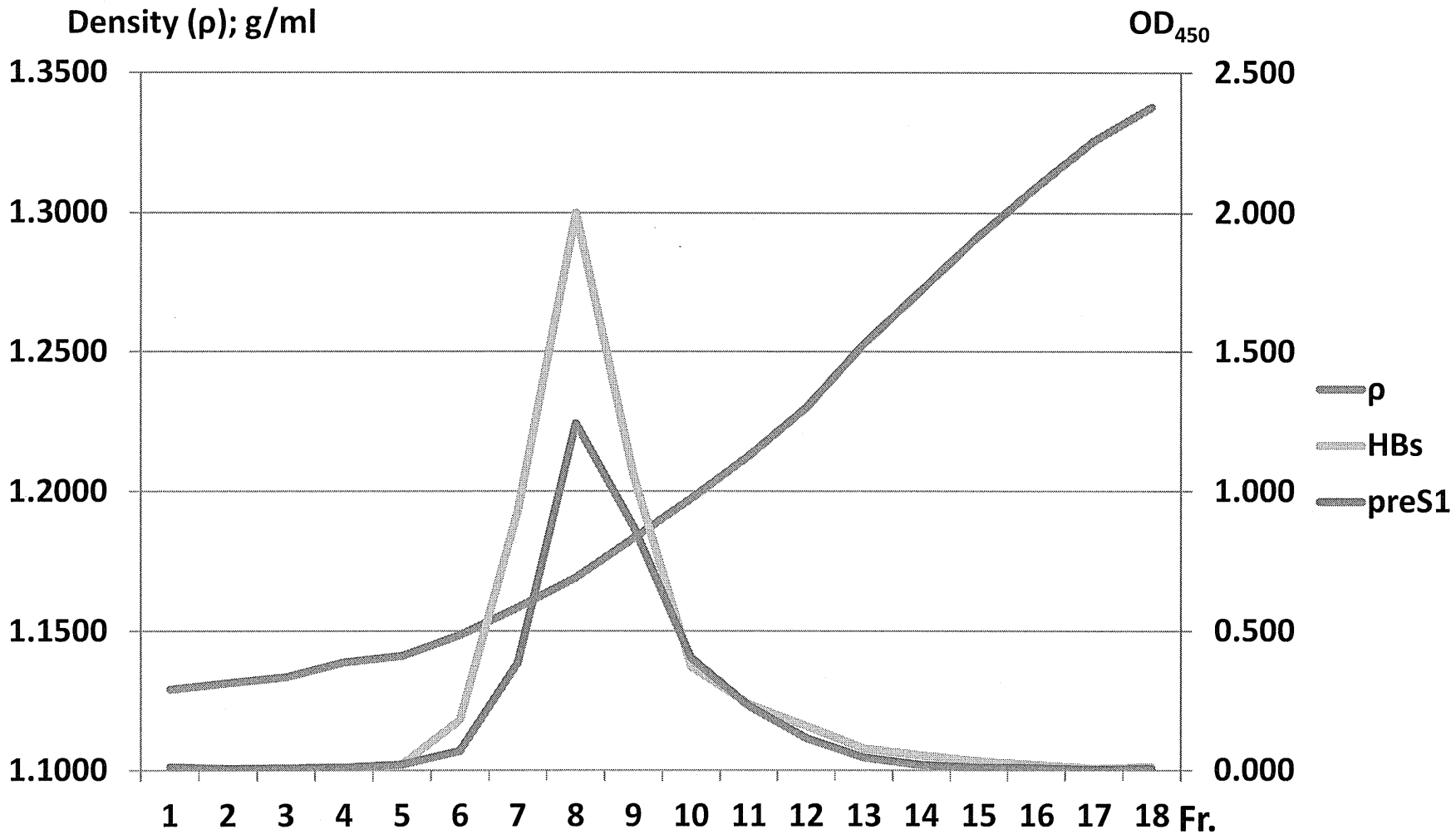
HBV LS-S
VSV-G
Ab for IP:
IgG
anti-S
anti-VSV-G
anti-S
anti-VSV-G
+ve control
RT: - + - + - + - + - + - +
bp
400
300
200

Fig. 3

B.


Density (ρ); g/ml
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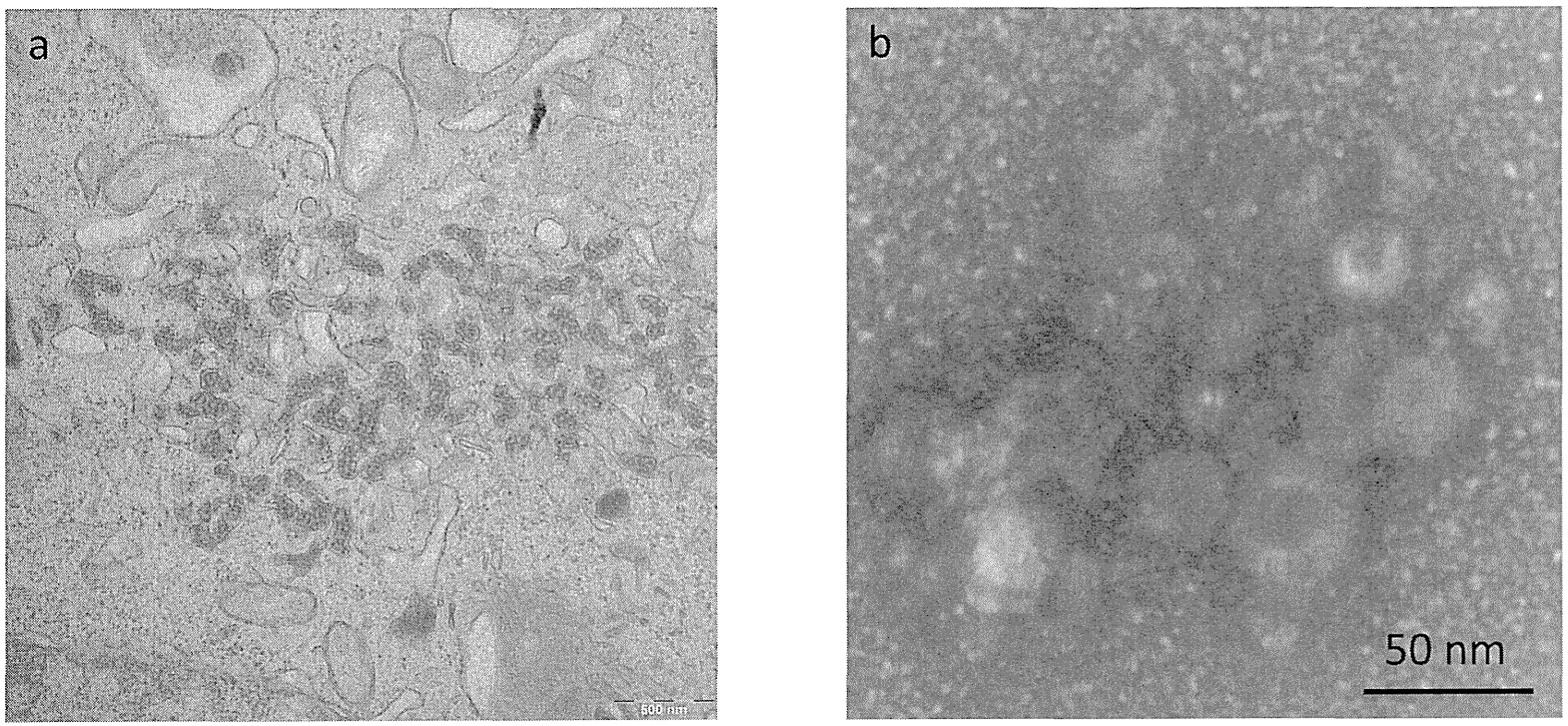
1.3000
1.2500
1.2000
1.1500
1.1000
2.500
2.000
1.500
1.000
0.500
0.000
ρ
HBs
preS1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Fr.
RT-PCR
bp
400
300

Fig. 4



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The Mechanism of Angiopoietin-1 Up-regulation in KSHV-infected PEL Cell Lines

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Running title: Up-regulation of *ANGPT-1* in KSHV-infected PEL cells

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ABSTRACT

Angiopoietin-1 (ANGPT-1) is a secreted glycoprotein that was first characterized as a ligand of the Tie-2 receptor. In a previous study using microarray analysis, we reported that the expression of ANGPT-1 was up-regulated in Kaposi's sarcoma-associated herpesvirus (KSHV)-infected primary effusion lymphoma (PEL) cell lines compared with uninfected Burkitt and other leukemic cell lines. Other authors have also reported focal expression of ANGPT-1 mRNA in biopsies of Kaposi's sarcoma (KS) from patients with acquired immune deficiency syndrome (AIDS). Here, to confirm these findings, we examined the expression and secretion levels of ANGPT-1 in KSHV-infected PEL cell lines and address the transcriptional regulation mechanisms of *ANGPT-1*. We also showed that ANGPT-1 was expressed and localized in the cytoplasm and was secreted into the supernatant in KSHV-infected PEL cells. Deletion studies of the regulatory region revealed that a -143 to -125 nt region of the *ANGPT-1*-regulating sequence was responsible for the up-regulation. Moreover, an electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) followed by qPCR suggested that some KSHV-infected PEL cell line-specific DNA-binding factors, such as OCT-1, should be involved in the up-regulation of *ANGPT-1* in a sequence-dependent manner.

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47 **Importance**

48 We confirmed that ANGPT-1 was expressed and secreted in and from KSHV-infected
49 PEL cells, respectively, and the transcriptional activity of *ANGPT-1* was up-regulated.
50 A 19 bp fragment was identified as a responsible region for *ANGPT-1* up-regulation,
51 through binding with OCT-1 as a core factor in the PEL cells. This study suggests that
52 ANGPT-1 is overproduced in KSHV-infected PEL cells, which could affect the
53 pathophysiology of patients harboring PEL under AIDS setting.

INTRODUCTION

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), belongs to the gamma-2 herpesvirus family, which was first identified in Kaposi's sarcoma lesions (1). Epstein-Barr virus (EBV), which belongs to the gamma-2 herpesvirus family, is frequently associated with malignancies such as Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC) (2). KSHV is also associated with several malignancies, i.e., two lymphoproliferative disorders, primary effusion lymphoma (3) and multicentric Castleman's disease (MCD), as well as KS (4, 5).

It has been reported that KSHV infects various kinds of cell types, such as B cells, blood vessel endothelial cells (BECs), lymphatic endothelial cells (LECs), Vero cells and HEK293 cells (6-9). After infection, KSHV utilizes latency as a default pathway of replication (1, 7). Though viral gene expression profiles might differ between BECs and LECs (10), KSHV infection is predominantly in latency with the genome binding to chromosome (10, 11), and governs the host gene expression profiles (12) as other viruses do (13, 14). Most KSHV-infected cells are latently infected, and only a limited number of viral genes are expressed in latency: latency-associated nuclear antigen (LANA), viral cyclin (v-CYC), viral FLICE inhibitory protein (v-FLIP), kaposin (10, 11, 15-18) and viral interferon regulatory factor-3 (v-IRF-3) (12).

Several viral products of KSHV have been reported to exert pivotal effects that contribute to the proliferation of endothelial cells, the viral life cycle and the secretion of cytokines associated with angiogenic and inflammatory properties; these products includeLANA, vIL6, vGPCR, K15, and vIRF3 (12, 19-24). These latency-related viral

77 products may also be involved in enhancing the expression of various cytokines and
78 growth factors, such as angiopoietin-1 (ANGPT-1), angiopoietin-2 (ANGPT-2), vascular
79 endothelial growth factor (VEGF), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor
80 necrosis factor-alpha (TNF-α) (6, 25-29). The angiogenic and inflammatory cytokines
81 regulated by viral proteins or KSHV infection could lead to the induction of
82 lymphangiogenesis, angiogenesis and anti-apoptosis and likely play an important role in
83 KSHV pathogenesis (12, 26, 30-33).
84 In a previous study, we compared the gene expression profiles of the KSHV-infected
85 cell lines BC1, BCBL1 and BC3 with those of the uninfected cell lines Daudi, AKATA,
86 Raji, Ramos, and Namalwa, and the leukemic cell lines MT4, SupT1 Jurkat, Molt3. We
87 found that ANGPT-1, a pro-angiogenic and pro-inflammatory cytokine, was expressed at
88 significantly higher levels only in the KSHV-infected primary effusion lymphoma (PEL)
89 cell lines (6). ANGPT-1, isolated as a ligand for Tie-2, is a glycoprotein secreted from
90 subendothelial stromal cells and hepatic stellate cells (34, 35), and is involved in vascular
91 remodeling, lymphangiogenesis, angiogenesis, and extravasation through the
92 ANGPT-1/Tie2 signaling (35, 36). These functions are convincing associations with
93 various oncologic diseases.
94 Here, we found that ANGPT-1 was expressed in the cytoplasm of KSHV-infected
95 PEL cell lines and actually secreted into the culture medium. Further, we identified a
96 regulatory region affecting *ANGPT-1* transcription activity and found that OCT-1 could
97 bind to this region *in vitro* as well as *in vivo*. These findings suggest that the cellular
98 environment established by KSHV infection and/or the PEL cell environment should be
99 involved in the facilitation of *ANGPT-1* expression and should affect the pathophysiology

100 of PEL-harboring individuals with AIDS.

101

MATERIALS AND METHODS

Cells. The cell lines BCBL1, TY1, BC3, BC1, Raji, Namalwa and BJAB were maintained in RPMI 1640 medium (Nakalai Tesque, Kyoto, Japan) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 10 i.u. per ml penicillin G and 10 μg per ml streptomycin under a 5% CO2 atmosphere. HEK293 (or just 293) and GP2 cells (Takara-Clontech®, Tokyo), which express a murine leukemia virus gag-pol protein, were maintained in DMEM medium-high glucose (Nakalai Tesque) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 i.u. per ml penicillin G and 10 μg per ml streptomycin (Nakalai Tesque). The cell lines LacZ-VH/BJAB and ANGPT-1-VH/BJAB were maintained in RPMI 1640 medium (Nakalai Tesque) supplemented with 20% heat-inactivated FBS, 10 i.u. per ml penicillin G, 10 μg per ml streptomycin and 500 μg/ml hygromycin B under a 5% CO_2 atmosphere. The cell lines LacZ-VH/293 and ANGPT-1-VH/293 were maintained in DMEM medium (Nakalai Tesque) supplemented with 10% heat-inactivated FBS, 10 i.u. per ml penicillin G, 10 μg per ml streptomycin and 500 μg/ml hygromycin B under a 5% CO_2 atmosphere.

Plasmids and retrovirus production. The *ANGPT-1* transcription regulatory region was amplified the gene information (e! Ensemble Human GeneSeqView; ENSG00000154188). We initially cloned the -2000 to +490 nt region (where +1 is the transcription start site) by PCR with the synthetic primers (Greiner, Tokyo) ANGPT1-ups Fw1; 5'-ggaagcttcaaatcaagcattattggaaag-3' and ANGPT1-ups RV1; 5'-aaaagctt cacactccttccgtgcctctcg-3'. We cloned the -898 to +490 nt region with the primers

ANGPT1-ups Fw4; 5'-ggaagctttatacgctgcctgtgggaaatc-3' and ANGPT1-ups RV1, using 100 ng of genomic DNA from BCBL1 as a template. The regulatory sequence and the deleted versions of the regulatory sequence (described below) were cloned into the pGL-3b vector (Promega, Madison, WI). Then, we verified that the -2000 nt upstream and -898 nt upstream regions did not show clear differences in transcription activity in BCBL1 cells. Therefore, in this experiment, the -898 nt upstream region was used as a starting reporter construct to determine the element responsible for the *ANGPT-1* up-regulation in KSHV-infected PEL cell lines.

Each deletion mutant was constructed by PCR amplification, with the -898 nt reporter plasmid used as a template. For the -579, -178, -109, -84 and -58 nt reporter constructs, ANGPT1-ups Fw5.5; 5'-ggaagcttcagaacatgaagggttgcattc-3', ANGPT1-ups Fw8; 5'-ggaagctttgctatattttagtaggtcagc-3', ANGPT1-ups Fw8.55; 5'-aaaagctttgccatgaatctgctaaaggc-3', ANGPT1-ups Fw9; ggaagcttaggcaattgtctgtggaaag-3', ANGPT1-ups Fw9.5; 5'-ggaagcttagggccatacatgatcgaggtc-3' were used, respectively, and ANGPT1-ups RV1 was used as a downstream primer. The D1 fragment (-143 to -110 nt) was generated by annealing two synthetic oligonucleotides, ANGPT1-ups FW8.5 SmaI; 5'-aacccgggagtgtattaaggtggactgctc-3' and ANGPT1-ups RV8.5 SmaI; 5'-aacccgggatcaataaatagagcagtccac-3', followed by repair reaction with a Klenow fragment (Takara-Clontech), digestion with SmaI and insertion of a SmaI site just upstream of the -58 nt reporter construct. The D2 fragment (-130 to -102 nt) was generated in the same way except using two synthetic primers, ANGPT1-ups FW8.5 SmaI-2; 5'-aacccgggtggactgctctatttattgatt-3' and ANGPT1-ups RV8.5 SmaI-2; 5'-aacccgggttcatggcaaatcaataaatag-3'. The D1-A (-143 to -125 nt) and D1-B (-128 to

-110 nt) fragments were generated by annealing phosphorylated synthetic DNA, ANGPT1-ups D1-A S; 5'-gggaagtgtattaaggtggactccc-3' and ANGPT1-ups D1-A AS; 5'-gggagtccaccttaatacacttccc-3' for D1-A, ANGPT1-ups D1-B S; 5'-ggggactgctctatttattgatccc-3' and ANGPT1-ups D1-B AS; 5'-gggatcaataaatagagcagtcccc-3' for D1-B, respectively, and insertion of a SmaI site from the -58 reporter construct.

D1-A mutant plasmids (Mut1 to 6) were constructed by inserting each of the annealed phosphorylated synthetic oligonucleotides (Mut1: 5'-gggaagtgtattaaggtggaaaccc-3'/ 5'-gggtttccaccttaatacacttccc-3', Mut2: 5'-gggccccgtattaaggtggactccc-3'/ 5'-gggagtccaccttaatacggggccc-3', Mut3: 5'-gggaagtcccctaaggtggactccc-3'/ 5'-gggagtccaccttaggggacttccc-3', Mut4: 5'-gggaagtgtatccccgtggactccc-3'/ 5'-gggagtccacggggatacacttccc-3', Mut5: 5'-gggaagtgtattaagccccactccc-3'/ 5'-gggagtggggcttaatacacttccc-3', Mut6: 5'-gggaagtgtattaaggtggcccccc-3'/ 5'-gggggccaccttaatacacttccc-3') into a SmaI site of the -58 nt reporter plasmid as described above.

The ANGPT-1 ORF clone was purchased from the Kazusa ORFeome Project (Product ID: FHC0948; Promega). The ORF SgfI~PmeI fragment was cloned into the EcoRV site of a pMT V5His B expression vector (Invitrogen®, Carlsbad, CA) with some modifications to generate pMT ANGPT-1-V5His. The ANGPT-1-V5His fragment was re-cloned into a retroviral expression vector, pQC XIH (Clontech, Palo Alto, CA), and pQC XIH ANGPT-1-V5His was generated. For retrovirus production, GP2 cells were co-transfected with pQC XIH ANGPT-1V5His and pVSV-G (Takara-Clontech) using LT1 reagent (Mirus, Madison, WI) for 12 hours. At 72 hours post-transfection, the

171 supernatant was collected to infect the BJAB and 293 cell lines. Hygromycin B (500
172 μg/ml) (Wako Pure Chemicals®, Tokyo) was used to select the virally transduced cells.
173 The OCT-1 expression vector (pCGN OCT-1) and its parental vector (pCGN) were the
174 kind gifts of Dr. Hibi as described .
175 **Transfection and luciferase assay.** For the luciferase assay, 1.5×10^5 PEL cells per 0.5
176 ml of medium were plated overnight, then transfected with 1 μg of each of the reporter
177 plasmids and 0.1μg of the LacZ expression plasmid CMVβ-galactosidase, using
178 TransFectin™ Lipofectin Reagent (BioRad, Hercules, CA). The cells were harvested at
179 48 h post-transfection. After washing with phosphate-buffered saline (PBS) twice, the
180 cells were lysed in 50 μl Glo lysis buffer (Promega). Luciferase activity was measured
181 using a Bright-Glo™ Luciferase Assay System (Promega) according to the
182 manufacturer's instructions and normalized by β-Galactosidase activity (12.5 μg/ml
183 CPRG, Z buffer [0.1 M $NaPO_4$ pH7.5, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-ME]). To
184 test OCT-1 transactivation activity, either pCGN OCT-1 or its parental vector with either
185 the D1A reporter or the -58nt reporter plasmid were transfected into 5 X 10^5 BCBL-1.
186 Effector plasmids (pCGN OCT-1) were increased from 0, 0.3, 0.9 and up to 1.5 μg.
187 **Immunofluorescence assay (IFA).** Cells were fixed for 1 h with 4% paraformaldehyde
188 (Nakalai Tesque)/PBS, and permeabilized in 0.1% Triton X-100 (Nakalai Tesque)/PBS
189 for 30 min. Proteins were detected using the following primary antibodies: a mouse
190 monoclonal antibody against V5 (1:1000 dilution; Nakalai Tesque) in the case of
191 LacZ-VH/BJAB and ANGPT-1-VH/BJAB, and a mouse monoclonal antibody against
192 ANGPT-1 (1:500 dilution; R&D Systems, Minneapolis, MN) for LacZ-VH-/BJAB,
193 ANGPT-1-VH/BJAB, BJAB, BC1, BC3, TY1 and BCBL1. Images were taken with an

Olympus FV1000D confocal microscope (Olympus).

Enzyme-linked immunosorbent assay (ELISA), immunoprecipitation and Western blotting. The culture supernatant of each PEL cell line (10^6/ml cells) was tested using a human angiopoietin-1 ELISA kit (MyBioSource, San Diego, CA) according to the manufacturer's instructions. Angiopoietin-2 (ANGPT-2) was also measured likewise using Quantikine® ELISA Human Angiopoietin-2 Immunoassay (R&D Systems) according to manufacturer's direction. For immunoprecipitation, 30 μl of Protein G Sepharose® 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) were washed with IP reaction solution (25 mM Hepes-KOH, PH 7.9, 200 mM NaCl, 5 mM $MgCl_2$, 0.2% NP-40, 10% glycerol, 1 mM DTT), and mixed with culture supernatants from the LacZ-VH/BJAB, ANGPT-1-VH/BJAB, BC1 and TY1 cell lines. After 1 h of incubation with Protein G Sepharose® to remove nonspecific binding proteins, the Sepharose was excluded and then 10 μl of Protein G Sepharose™ and 2 μg of an ANGPT-1 antibody (R&D Systems) were added into the supernatant with rotation overnight. The Sepharose® was harvested, washed 3 times with 1 ml of IP reaction solution and finally suspended in 50 μl SDS-PAGE sampling buffer and boiled. For Western blotting, each sample (20 μl) was separated on SDS-PAGE (8% polyacrylamide) and transferred onto a PVDF membrane (Immune-Blot PVEF Membrane For Protein Blotting; BioRad). The membrane was blocked with 5% dry milk in TBS-T. A specific antibody against ANGPT-1 (1:1000 dilution; R&D Systems) was added, followed by secondary antibodies with conjugated horseradish peroxidase (HRP) for detection. The membrane was washed with TBS-T three times and the chemiluminescence image was obtained with an imaging system (ChemiDoc™ MP;