expressing clones. We cultured one clone of the LANA-expressing cells and prepared nuclear extract. This extract was incubated with anti-FLAG affinity gel (M2-agarose), followed by elution with FLAG peptides. Eluate was subjected to SDS-PAGE to detect a prominent 120-kDa band (Fig. 1*A*). Although there was a 75-kDa band, which was a nonspecific binding protein commonly found with the antibody. We determined the sequences of the N-terminal 10 residues of the 120-kDa protein, which revealed the protein to be Daxx. To confirm the identification, the nuclear extract (each 500 μg of protein) was immunoprecipitated with anti-FLAG antibody to apply to immunoblotting with anti-Daxx antibody. As shown in Fig. 1*B*, anti-Daxx antibody recognized a band of 120 kDa. These results indicated that Daxx is a cellular binding protein of exogenously expressed LANA in the HeLa cell. To confirm LANA-Daxx interaction in a physiological context, we immunoprecipitated with anti-Daxx antibody from nuclear extracts of BCBL-1 cells, a PEL cell line infected with KSHV. LANA was co-immunoprecipitated with Daxx as well (Fig. 1*C*). This result suggested that LANA formed a complex with Daxx in KSHV-infected cells.

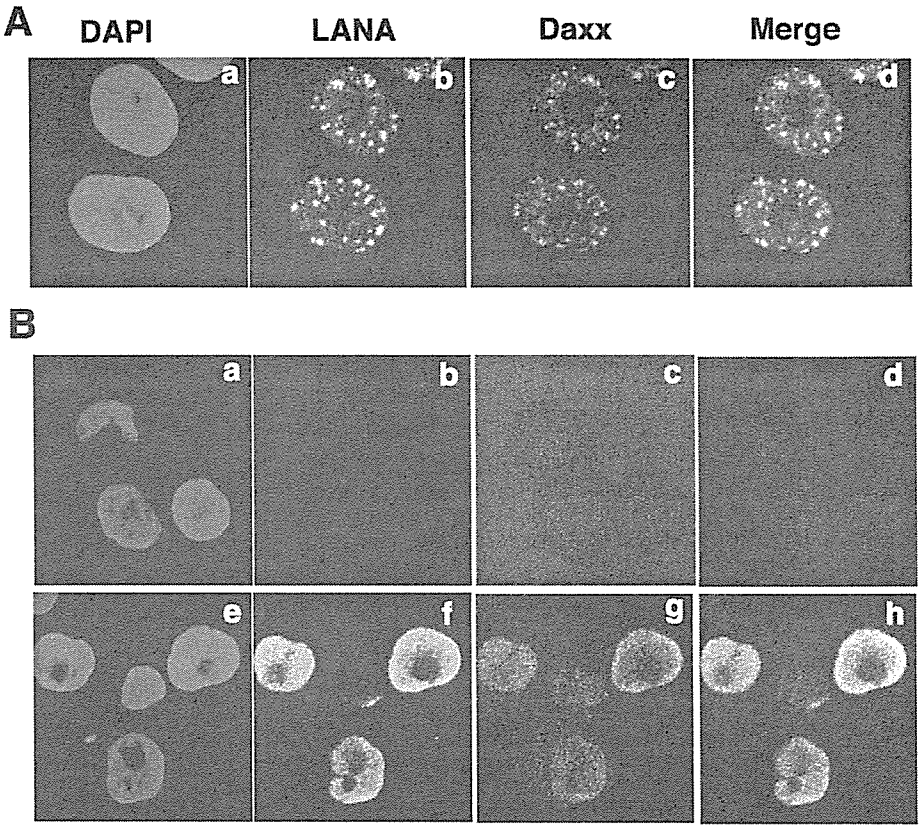

FIGURE 2. **LANA co-localizes with Daxx in BCBL-1 cells and HeLa cells.** Confocal microscopic images of PEL cell line, BCBL-1 cells (*A*), and HeLa cells (*B*, control (*panels a–d*) and LANA-expressing cells (*panels e–h*)). Cells were doubly immunostained with anti-LANA antibody (1:500 for *A*, 1:1000 for *B*) and anti-Daxx antibody (1:100 for *A*, 1:200 for *B*). Images represent cells stained with DAPI (*panels a* and *e*), anti-LANA antibody (*panels b* and *f*), or anti-Daxx antibody (*panels c* and *g*), and merged images of LANA and Daxx staining (*panels d* and *h*).

Colocalization of LANA and Daxx in the Nuclei of KSHV-infected Cell Line BCBL-1—Next we examined the localization of LANA and Daxx in BCBL-1 by immunofluorescence microscopic assay (Fig. 2*A*). LANA gave a characteristic speckled staining pattern in nuclei of the cells (Fig. 2*A*, *panel b*), Daxx also showed some speckles in the nuclei (Fig. 2*A*, *panel c*). The merged image indicated that LANA considerably co-localized with Daxx in the nuclear dots (Fig. 2*A*, *panel d*). We also investigated the localization of Daxx using HeLa cells (Fig. 2*B*). LANA gave fine patchy staining in the nucleus (Fig. 2*B*, *panel f*), which is a typical observation in the absence of KSHV genome (Fig. 2*B*, *panel g*). The parental HeLa cells showed diffused staining of Daxx throughout the cell (Fig. 2*B*, *panel c*). In contrast, Daxx appeared to accumulate in the nuclei of the LANA-expressing cells (used in Fig. 1) (Fig. 2*B*, *panel g*). LANA and Daxx largely localized in the nucleus of the HeLa cells (Fig. 2*B*, *panel h*). We performed biochemical fractionation using three independent clones of LANA-expressing HeLa cells and examined cellular localization of Daxx by Western blotting. The results indicated that the amount of Daxx in the nuclear fraction increased as LANA expression increased, although total amounts of Daxx were comparable in these HeLa clone cells (data not shown).

A Region Containing the Acidic-rich Domain in LANA Is Required for Binding with Daxx—To determine the interacting domain of LANA with Daxx, we constructed a series of LANA deletion mutants (Fig. 3*A*), which were translated *in vitro* and subjected to pull-down assay with GST-Daxx. As shown in Fig. 3*B*, full-length LANA was pulled down with GST-fused full-length Daxx, indicating direct interaction between LANA and Daxx. Three N-terminal mutants of LANA (L1–L3) bound with GST-Daxx, but the shortest N-terminal LANA (aa 1–261) (L4), and C-terminal LANA (aa 496–740) (L5) failed (Fig. 3*B*). We constructed mammalian expression plasmids, LANA-N (aa 1–564), LANA-C (aa 496–1162), LANA-N1 (aa 1–260), LANA-N2 (aa 1–320), LANA-N3 (aa 1–344), and LANA-ΔAD (with aa 322–493 deleted) (Fig. 3*A*). These plasmids were co-transfected with pcDNA-Daxx into 293T cells, and the nuclear extracts were analyzed. Immunoprecipitation with anti-Daxx antibody and Western blotting with anti-FLAG antibody indicated that Daxx formed a complex with full-length LANA and LANA-N, and weakly with LANA-N3, but not with the other LANA fragments (Fig. 3*C*). Taken together, these results suggested that aa 320–344 of LANA, which contains many aspartic acids and glutamic acids, were required for binding with Daxx.

A Central Domain of Daxx Is Required to Interact with LANA—To determine the critical region of Daxx for binding with LANA, a series of GST-fused deletion mutants of Daxx (Fig. 4*A*)

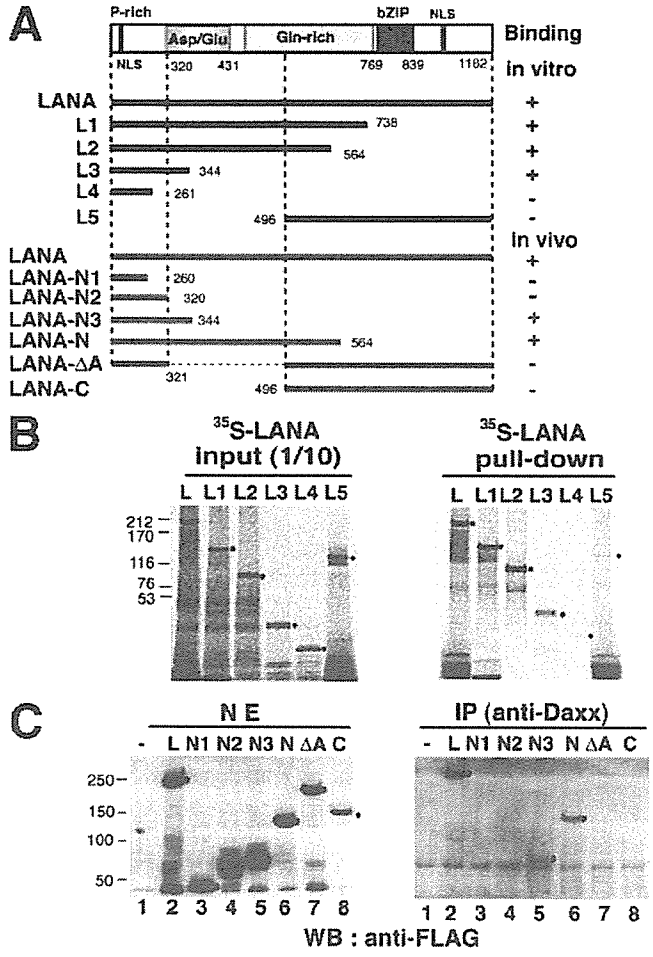

FIGURE 3. **A region containing an acidic-rich domain in LANA is required for binding with Daxx *in vitro* and *in vivo*.** *A*, domain structure of LANA and its deletion mutants. LANA is constituted of domains of proline-rich, acidic-rich, glutamine-rich, and basic leucine-zipper. A series of deletion mutants of LANA and the binding activity *in vitro* and *in vivo* are shown. *B*, result of pull-down assay with GST-fused full-length Daxx of ^{35}S-labeled LANA deletion mutants (*L1–L5*). *C*, co-immunoprecipitation of Daxx and LANA deletion mutants in 293T cells. PFLAG-CMV-2 vector (4.0 μg) (*lane 1*), pFLAG-LANA (4.0 μg) (*lane 2*), pFLAG-LANA-N1 (1.0 μg) (*lane 3*), pFLAG-LANA-N2 (2.0 μg) (*lane 4*), pFLAG-LANA-N3 (2.0 μg) (*lane 5*), pFLAG-LANA-N (2.0 μg) (*lane 6*), pFLAG-LANA-ΔA (4.0 μg) (*lane 7*), or pFLAG-LANA-C (4.0 μg) (*lane 8*) was individually co-transfected with pcDNA-Daxx (1.0 μg) in 60-mm dishes with adjustment of total DNA amount (5.0 μg). The immunoprecipitates (*IP*) with anti-Daxx antibody were followed by immunoblotting (*WB*) with anti-FLAG antibody (M5).

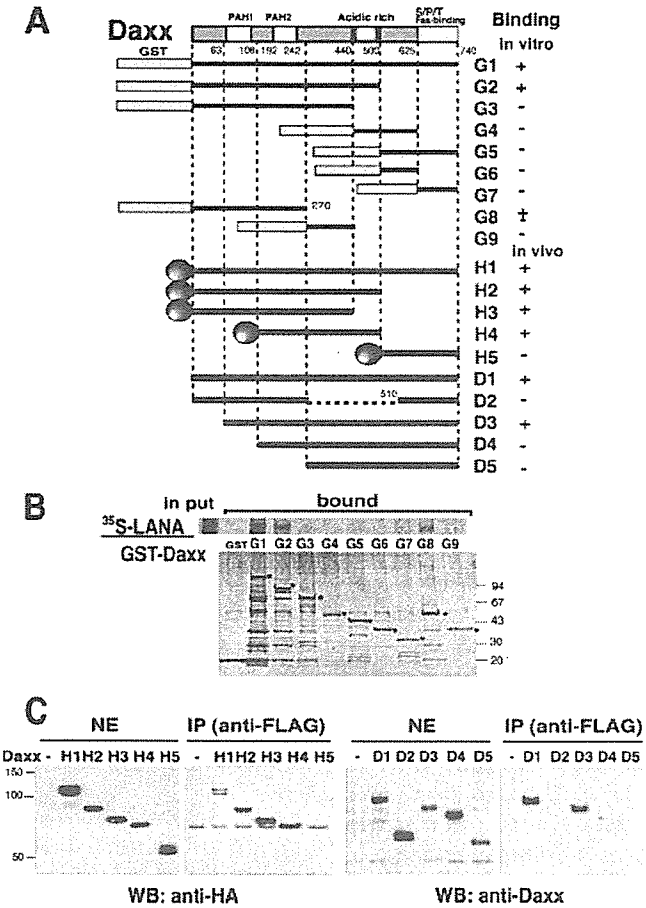

FIGURE 4. *A*, central region containing PAH 2 and acidic-rich domain in Daxx is required to interact with LANA. *A*, domain structure of Daxx and various deletion mutants. Daxx is composed of two PAH and acidic-rich and Ser/Pro/Thr-rich domains. A series of mutants of Daxx and the binding activity *in vitro* and *in vivo* are shown. *B*, purified GST-Daxx variants (*G1–G9*) were applied in *in vitro* pull-down assay with full-length ^{35}S-LANA. *C*, mammalian expression plasmids, pCMV-HA-Daxx-H1 (full-length) (2.0 μg), pCMV-HA-Daxx-H2 (aa 1–500) (2.0 μg), pCMV-HA-Daxx-H3 (aa 1–440) (1.0 μg), pCMV-HA-Daxx-H4 (aa 110–500)(1.0 μg), pCMV-HA-Daxx-H5 (aa 500–740) (1.0 μg) were co-transfected with pFLAG-LANA (1.0 μg) (*left two panels*). PcDNA-Daxx-D1 (full-length) (1.0 μg), pcDNA-Daxx-D2 (deleted aa 271–509) (3.0 μg), pcDNA-Daxx-D3 (aa 63–740)(3.0 μg), pcDNA-Daxx-D4 (aa 111–740)(3.0 μg), and pcDNA-Daxx-D5 (aa 243–740) (2.0 μg) were individually co-transfected with pFLAG-LANA (1.0 μg) (*right two panels*). Immunoprecipitates (*IP*) with anti-FLAG antibody (M2) were followed by Western blotting (*WB*) with anti-HA antibody (*left panels*) or anti-Daxx antibody (*right panels*).

were produced in *E. coli*, and applied to pull-down assay with full-length ^{35}S-labeled LANA. The GST-fused full-length Daxx (G1) and the Daxx-deleted aa 500–740 (G2) bound to LANA, but deleted aa 440–740 (G3) failed (Fig. 4*B*). From the *in vitro* result above, the region of aa 440–500 in Daxx was thought to be critical for the binding. However, GST-fused aa 440–625 of Daxx (G4) did not bind (Fig. 4*B*), nor did any other mutants, although weak binding was observed with GST-fused aa 1–270 of Daxx (G8)(Fig. 4*B*). We constructed a series of deletion mutants of N-terminal HA-tagged Daxx (H2–H5), and co-expressed them with pFLAG-LANA in 293T cells. Immunoprecipitation with anti-FLAG antibody followed by Western blotting with anti-HA antibody showed that all the mutants except H5 bound to LANA (Fig. 4*C*, *left two panels*). The acidic-rich region (aa 440–500) of Daxx was not critical to the binding with LANA in cells, not corresponding with the results *in vitro*. To examine contribution of N terminus of Daxx, a series of mutant Daxx expression vectors with N-terminal deletion (D3–D5) and a deletion mutant without central region aa 271–509 (D2), were constructed and transiently expressed in 293T cells. Experiments of immunoprecipitation with anti-FLAG antibody and Western blotting with anti-Daxx antibody (sc-7152, that recognizes the C terminus of Daxx) showed that D3 bound firmly with LANA, but D4 did very little (Fig. 4*C*, *right two panels*). The first paired amphipathic helix (PAH), aa 63–108 appeared to be of some importance for the binding, although HA-tagged Daxx without PAH1 (H4) bound LANA. These results indicated that a central region aa 63–440 within Daxx,

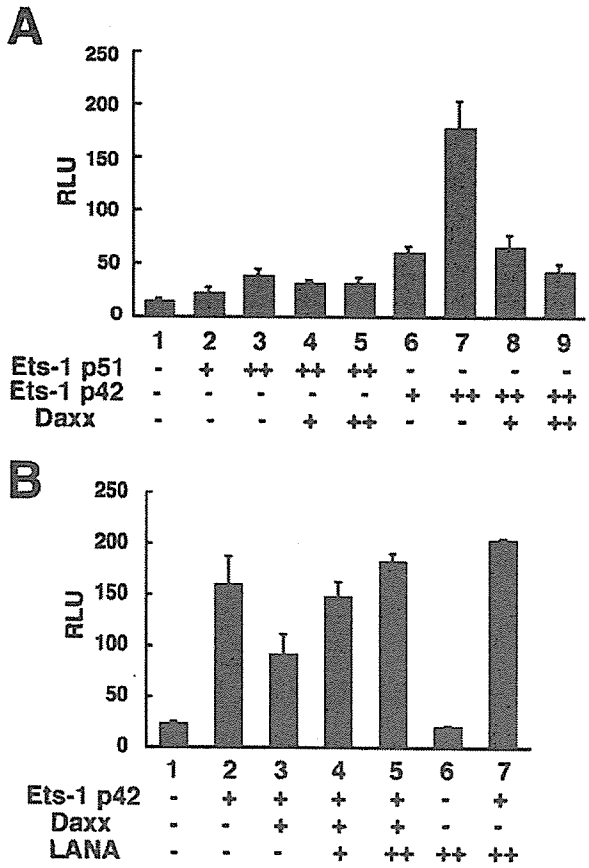

FIGURE 5. **LANA inhibited Daxx-mediated repression on Ets-1-dependent VEGF receptor 1 (Flt-1) gene expression.** *A*, Daxx repressed Ets-1-dependent Flt-1 expression. PcDNA-p51Ets-1 or pcDNA-p42Ets-1 (+; 25 ng, ++; 50 ng) were co-transfected with pcDNA-Daxx (+; 200 ng, ++; 500 ng) and pFlt-luc (100 ng). *B*, LANA counteracts Daxx-mediated repression in Flt-1 expression in the presence or absence of exogenous Daxx. PcDNA-p42Ets-1 (50 ng), pcDNA-Daxx (200 ng), and pFLAG-LANA (0, +; 50, ++; 100 ng, respectively) were co-transfected with pFlt-1-luc (100 ng). The relative luciferase activity (*RLU*) was normalized by β-galactosidase activity. Assays were performed in triplicate, and *error bars* indicate S.D.

containing two paired PAHs and its following 200 aa, was important for the binding with LANA in cells.

LANA Inhibited Daxx-mediated Repression of Ets-1-dependent VEGF Receptor 1 (Flt-1) Gene Expression— To examine the role of Daxx in Kaposi's sarcoma, we focused on Ets-1 transcription factor. It was reported that Daxx interacts with Ets-1 to repress Ets-1-dependent transcriptional activity of MMP-1 and Bcl-2 (12). On the other hand, as a characteristic feature of KS, it is known that VEGF and its receptors, Flt-1 and KDR (VEGF receptor-1 and -2, respectively), are highly expressed in KS (20). There are several Ets-1 motifs in Flt-1 and KDR promoters to regulate the expression (26) (27). We examined the effect of Daxx on Ets-1-dependent Flt-1 expression. We co-transfected a luciferase reporter plasmid pFlt-1-luc driven by Flt-1 promoter, an Ets-1 expression vector, and a Daxx expression vector into 293T cells, to perform luciferase assay. Transcriptional activity on Flt-1 increased depending on the amount of Ets-1 plasmid, although the effect of p51-Ets-1 was quite weak. Daxx evidently repressed Ets-1-dependent activation (Fig. 5*A*). p51 and p42 are two human variants of the Ets-1 molecule. It is reasonable that the activity of p51-Ets-1 is lower than p42-Ets-1 because p42-Ets-1 lacks exon VII, the internal transcriptional regulatory domain (24). This result is similar to the case of MMP-1 and Bcl-2 expression (28). As we observed the repressive activity of Daxx on Ets-1-dependent Flt-1 expression, we examined the effect of LANA on the Daxx-mediated repression with p42Ets-1. Co-transfection with a LANA expression plasmid dose-dependently reactivated the transcriptional activity repressed by exogenous Daxx (Fig. 5*B*, *4* and *5*), although LANA slightly activated it in the absence of exogenous Daxx (Fig. 5*B*, *7*). These results suggested that LANA inhibited the repression via interaction with Daxx.

LANA Activated Expression of VEGF Receptors in Vascular Endothelial Cells—To investigate the possibility that LANA induces Flt-1 in Kaposi's sarcoma lesion, we tried to express LANA in HUVEC, because endothelial cells (ECs) are regarded as the origins of KS lesions. We constructed a plasmid, pIRES2-LANA-GFP, which contains an internal ribosomal entry site (IRES) to express both LANA and GFP from a single mRNA. We transfected pIRES2-LANA-GFP or pIRES2-GFP as control into HUVEC and Flt-1 and KDR expression in GFP-positive cells were analyzed by flow cytometry. Flt-1 of GFP-positive cells in pIRES2-LANA-GFP-transfected cells was significantly increased as compared with that in control cells (Fig. 6*A*, *left*). The number of cells expressing Flt-1 over log intensity 1 (M1) was about 1.9× higher (Fig. 6*A*, *upper, right graph*) than that of control. M1 of KDR also increased 1.4× (Fig. 6*A*, *lower, right graph*). Furthermore, to examine the level of mRNA of the two receptors, we performed real-time PCR with total RNA prepared from the GFP-expressing HUVEC. LANA expression in pIRES2-LANA-GFP-transfected cells was confirmed by using PCR with primers of LANA (data not shown). The relative expressions of Flt-1 and KDR in LANA-expressing cells were 1.4 and 2.0× higher than that of control cells, respectively (Fig. 6*B*). Although there was discrepancy between rise of protein and mRNA, results of both FACS and real-time PCR indicated that LANA induced the two receptors in human endothelial cells. The expression of Ets-1 and Daxx was not altered between LANA-expressing cells and control cells (Fig. 6*B*).

LANA Sequesters Daxx from Ets-1—To resolve the mechanism of the activation of VEGF receptors expression by LANA, we examined the relation of the three molecules, LANA, Daxx, and Ets-1. 293T cells were co-transfected with a constant amount of pcDNA-Daxx and pcDNA-Ets-1, and a variable amount of pFLAG-LANA. Nuclear extracts were prepared and subjected to immunoprecipitation and Western blotting with anti-Ets-1 antibody, anti-Daxx antibody or anti-FLAG antibody. Daxx and Ets-1 were expressed in a fixed amount (Fig. 7*A*, *row a*), *middle and right panel*, respectively) and FLAG-LANA was dose-dependently increased in the nuclear extract (Fig. 7*A*, *row a, left panel*). When we performed immunoprecipitation with anti-FLAG antibody, Daxx was detected in the immune complex in proportion to the amount of LANA (Fig. 7*A*, *row b, middle panel*). On the other hand, we detected no specific interaction between LANA and Ets-1 in the immune complex (Fig. 7*A*, *row b, right panel*). Next, by immunoprecipitation with anti-Daxx antibody, FLAG-LANA was detected in direct proportion to the amount of LANA (Fig. 7*A*, *row c, left panel*). The immune complex also contained Ets-1 in inverse

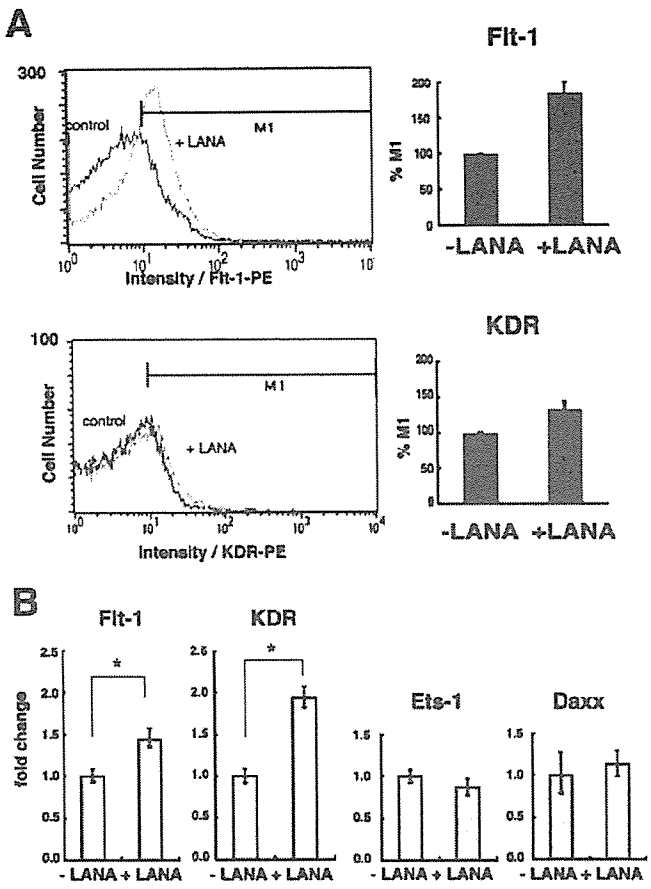

FIGURE 6. **LANA induced VEGF receptors in HUVEC.** *A*, flow cytometric analysis of Flt-1 (*upper graphs*) and KDR (*lower graphs*) expression of control (pIRES2-GFP transfected cells; *black lines*) and LANA-expressing cells (pIRES2-LANA-GFP-transfected cells; *gray lines*). The graphs to the *right* of each indicate percentages of cells that exceed 1 of the relative log intensity (*M1*). Experiments were repeated three times and the M1 values represent means of the three experiments; *error bars* indicate S.D. *B*, real-time PCR analysis of Flt-1, KDR, Ets-1, and Daxx. HUVECs transfected transiently with pIRES2-LANA-GFP or pIRES2-GFP (as a control) were sorted 2 days after transfection. Total RNA extracted from the cells (1 μg) was reverse-transcribed to cDNA (40 μl), and aliquots (0.4 μl) were applied to real-time PCR (20 μl) with each primer (0.4 mM) in triplicate described under "Experimental Procedures." Values represented relative expression of Flt-1, KDR, Ets-1, and Daxx (calculated with threshold cycle number, CT) of LANA-expressing cells compared with that of control cells. Each value was adjusted with CT of internal control (GAPDH). *, p value < 0.02.

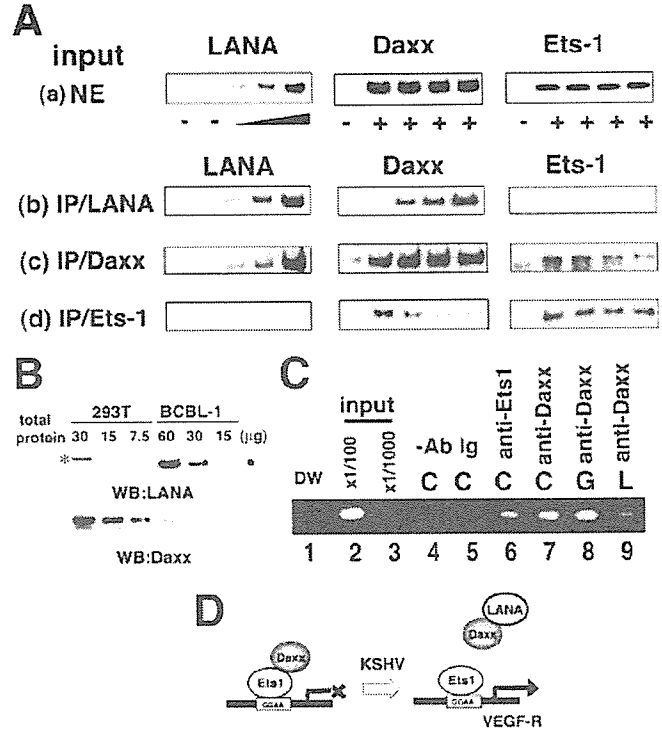

FIGURE 7. **LANA interacted with Daxx to sequester from Ets-1.** *A*, Western blotting analysis of immunoprecipitates with anti-FLAG, anti-Daxx, and anti-Ets-1 antibodies. 293T cells were transfected with a constant amount of pcDNA-Daxx (2 μg) and pcDNA-p42Ets-1 (2 μg), and an increasing amount of pFLAG-LANA (0.25, 0.5, 1.0 μg). Total DNA amounts were adjusted with pFLAG-CMV-2 vector to be 5 μg. Nuclear extracts (*row a*), immune complex using anti-FLAG antibody (*row b*), immune complex using anti-Daxx antibody (*row c*), and immune complex using anti-Ets-1 antibody (*row d*), were subject to Western blotting with anti-FLAG antibody (*left*), anti-Daxx antibody (*middle*) or anti-Ets-1 antibody (*right*). *B*, relative protein amounts of LANA and Daxx in BCBL-1 cells and those of transfected 293T cells. 293T cells were co-transfected with pcDNA-Daxx (2 μg), pcDNA-p42Ets-1 (2 μg), and pFLAG-LANA (1.0 μg). Nuclear extract (30, 15, 7.5 μg of the 293T cells and 60, 30, 15 μg of BCBL-1 cells) were subjected to Western blotting with anti-LANA antibody or anti-Daxx antibody. FLAG-LANA (*) migrated slower than native LANA(●) did. *C*, chromatin immunoprecipitation of Ets-1 and Daxx interaction with Flt-1 promoter in HUVECs. Bands indicate PCR products targeting −90 to +8 of Flt-1 promoter. 2 μl of water (*lane 1*), 1/100 and 1/1000 of input (cross-linked and sonicated pre-immunoprecipitation lysate) (*lanes 2* and *3*), eluate from no antibody (*lane 4*), rabbit IgG (2 μg) (*lane 5*), anti-Ets-1 antibody (2 μg) (*lane 6*), and anti-Daxx antibody (2 μg) (*lane 7*) were applied to the PCR reaction, respectively. Eluate from anti-Daxx antibody of LANA-expressing HUVECs (*L*, *lane 9*) and that from the control GFP-expressing HUVECs (*G*, *lane 8*) were subjected to PCR reaction. *D*, possible mechanism for induction of VEGF receptors by LANA. Daxx interacts with Ets-1, and represses Ets-1-dependent expression in the absence of LANA, while LANA sequesters Daxx from Ets-1 to inhibit the interaction between Daxx and Ets-1, resulting in activation of Ets-1-dependent expression of VEGF receptors.

proportion to LANA expression (Fig. 7*A*, *row c*, *right panel*). Consistently, Daxx was detected in the immune complex with anti-Ets-1 antibody in inverse proportion to LANA expression (Fig. 7*A*, *row d*, *middle panel*). LANA was not detected in the immune complex with the anti-Ets-1 antibody (Fig. 7*A*, *row d*, *left panel*), which implies that increasing LANA caused increase of Daxx-LANA interaction, and reduction of Daxx-Ets-1 interaction. These results suggested that LANA sequesters Daxx from Ets-1, which results in inhibition of the interaction between Daxx and Ets-1.

In the experiments above we used transiently transfected 293T cells (Fig. 7*A*). To address whether the transient expression system for LANA-Daxx interaction is physiologically relevant or not, we analyzed relative expression levels of LANA and Daxx proteins using BCBL-1 and the transfected 293T cells. As shown in Fig. 7*B*, the expression level of exogenous LANA protein in 293T cells in the same condition of Fig. 7*A* was similar to that of endogenous LANA in BCBL-1 cells. In contrast, endogenous Daxx expression level is much lower in BCBL-1 cells than in the 293T cells. These data indicated that relative expression ratio of endogenous LANA to Daxx in BCBL-1 cells was much higher than that of LANA-transfected 293T cells.

Daxx associated with Flt-1 promoter and LANA reduced its association in HUVEC. To investigate the possibility that Daxx affects transcriptional activity of Ets-1 for Flt-1 expression in endothelial cells (ECs), we performed ChIP assay using HUVEC. Cross-linked nuclear extract from HUVECs was immunoprecipitated with anti-Ets-1 antibody or anti-Daxx

antibody, and subjected to PCR to amplify a 98-bp fragment. The PCR product is designed to span the fourth ets motif (−54 to −51) that is thought to be indispensable for Flt-1 promoter activity (26). The anti-Daxx antibody precipitated the Flt-1 promoter as well as anti-Ets-1 antibody (Fig. 7*C*, *lanes* 6 and 7). The result indicated that Daxx as well as Ets-1 associated with Flt-1 promoter in ECs. Furthermore, HUVECs transfected with pIRES2-LANA-GFP or pIRES2-GFP as the control were sorted and subjected to ChIP assay with anti-Daxx antibody. The PCR product from the LANA-expressing cells (Fig. 7*C*, *L*, *lane* 9) was lower than that of control GFP-expressing cells (Fig. 7*C*, *G*, *lane* 8), indicating reduction of Daxx association with the promoter of the *Flt-1* gene in ECs.

DISCUSSION

LANA is reported to have multiple functions in KS lesion. It interacts with many host cellular molecules: p53 (8), pRb (9), ATF4/CREB2 (29), CBP (30), c-Jun (31), RING3 (32), mSin3A (33), HP-1 (33), Dek (34), GSK-3b (10) and so on. In the present study, we identified Daxx as a new member of LANA-binding proteins. Daxx was prominently detected in our immunoaffinity system, but this system also detected previously reported LANA-interacting protein such as RING3 by Western blotting (data not shown). We showed the interaction between the two proteins *in vivo* (Fig. 1) and *in vitro* (Figs. 3 and 4), which indicates that Daxx and LANA directly bound to each other. Fluorescent immunostaining assay showed co-localization of LANA and Daxx in BCBL-1 cells, supporting LANA-Daxx interaction in cells (Fig. 2).

Daxx is reported to bind many cellular molecules, indicating its involvement in multiple cellular processes. Although Daxx could interact with proteins of cytoplasm or membrane, it also interacted with some transcription factors and localized sometimes in the nuclear matrix structure, PML NBs (promyelocytic leukemia nuclear bodies). PML NBs are thought to provide platforms for transcription regulation, DNA repair, apoptosis, DNA replication, RNA transport, and many viruses target PML NBs to pirate host functions (reviewed by Everett, Ref. 35). Ets-1 associates with a PML NBs protein, Sp100 (36). Therefore, it might be a strategy of KSHV that LANA targets Daxx of PML NBs to modulate the cellular function(s) of Ets-1.

Although most LANA-binding proteins are reported to interact through the C or N terminus of LANA, the critical domain for binding with Daxx seemed to be a central region, aa 321–344 of LANA (Fig. 3). The aa 320–431 of LANA consists mainly of aspartic acid and glutamic acid. It is reported that a transcriptional co-activator, CBP interacts through this acidic-rich region of LANA (30). This domain may have some roles in transcriptional regulation. On the other hand, although most Daxx-binding proteins interact around the C terminus of Daxx, a central domain containing the PAHs and the following region of Daxx appeared to be important for binding with LANA *in vivo* (Fig. 4). There was a discrepancy between *in vitro* and *in vivo* binding. Protein modification may be one possibility explaining *in vivo* binding activity. It is reported that Daxx is modified by hyperphosphorylation (13) and sumoylation (37). Because the sumoylation sites of Daxx are reported to be Lys^{630} and Lys^{631}, it is unlikely to affect the interaction. The hyperphosphorylation site on Daxx has not been identified, but it is possible to be related to the binding. There may be other possibilities, for example, constructive interference by fused GST protein. PAH is a characteristic domain that is involved in transcriptional co-repressors such as mSin3 (38). It is interesting that mSin3A binds to aa 1–340 of LANA (28). There is a report that acetylated histone H4 interacts through PAH1 within Daxx, but no report that any other host molecule binds through this region of Daxx. As Daxx interacts with Ets-1 through the C-terminal region of Daxx (12), there may be no direct competition for Daxx between LANA and Ets-1.

Based on the interaction between LANA and Daxx (Figs. 1–4), we found that LANA induced VEGF receptors in ECs (Fig. 6) in accordance with the results of reporter assays (Fig. 5). Although expression level changes were not consistent for Flt-1 and KDR in protein (Fig. 6*A*) and mRNA (Fig. 6*B*), it may be caused by time point difference. This is the first report of the function of LANA in angiogenesis. It is reported that KSHV ORF74 (viral G-protein coupled receptor, v-GPCR) contributes to expression of VEGF receptors (39). Because ORF74 is expressed in the viral lytic infection cycle, it is unlikely that ORF74 is the only gene of KHSV that induces angiogenesis in KS. It is likely that some other factors such as VEGF and hypoxia-inducible factor (HIF) additionally affect on expression of these receptors in KS (40) (41).

As to the mechanism of activation of the receptor expression by LANA, we propose a hypothesis that LANA sequesters Daxx from Ets-1 (Fig. 7*D*), based on the results of co-immunoprecipitation (Fig. 7*A*) and ChIP assay (Fig. 7*C*). LANA slightly activated Ets-1 dependent Flt-1 expression without exogenous Daxx in the reporter assay (Fig. 5*B*). It is thought that LANA sequestered endogenous Daxx. However it is possible that LANA activates Flt-1 expression through an unidentified mechanism(s). At least LANA did not activate Flt-1 expression through up-regulation of Ets-1 expression (Fig. 6*B*). In human Flt-1 promoter, there are five Ets motifs and a CRE (cAMP response element). It is reported that co-existence of the fourth Ets motif, and the CRE is necessary for Flt-1 expression (26). LANA is reported to modulate the expression of a reporter plasmid with CRE, but the effect of LANA on CRE is repression (29). There is no CRE in the promoter of KDR.

Given that LANA induces VEGF receptors in KS lesion, we propose this hypothesis: Daxx binds Ets-1 to repress expression of VEGF receptors in normal ECs, while in KSHV-infected cells, LANA binds to Daxx to inhibit Daxx-Ets-1 interaction, resulting in the activation of Ets-1-dependent VEGF receptors. Furthermore, LANA-Daxx interaction might contribute to not only VEGF receptor gene expression but also to other Daxx-mediated gene regulation related to the pathogenesis of KS, PEL, and MCD malignancy.

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Original article

Development of a rapid and convenient method for the quantification of HIV-1 budding

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Abstract

In cells, the expression of Gag protein, one of the major structural proteins of retroviruses, is sufficient for budding virus-like particles (VLPs) from the cell surface. We have previously reported that spheroplasts of *Saccharomyces cerevisiae* expressing HIV-1 Gag proteins from an episomal plasmid constitutively released a large amount of VLPs into culture media; however, commercially available ELISA kits which detect mature capsid of HIV-1 could not detect uncleaved 55-kDa Gag proteins released from budding yeast. We therefore developed a method to quantitate VLP levels released from budding yeast by using fusion protein from HIV-1 Gag and Firefly Luciferase. This system is useful for screening cellular factor(s) involved in retrovirus budding from *S. cerevisiae*.

Keywords: HIV-1; *Saccharomyces cerevisiae*; Luciferase

1. Introduction

All replication-competent retroviruses have three major structural proteins (i.e. Gag, Pol and Env), and some have regulatory proteins. In human immunodeficiency virus type 1 (HIV-1), Gag protein is synthesized as a 55-kDa precursor protein in the cytosol and cleaved to MA (matrix), CA (capsid), p2, NC (nucleocapsid), p1 and p6 by a viral encoded protease during budding from the cell surface. There is a myristoylation signal at the amino terminus of MA, so that precursor Gag proteins can target plasma membranes, where virus protein assembly and particle budding occur. The viral envelope is lined with MA, and CA forms a cone-shaped core encapsidating the RNA genome associated with NC. Pol proteins are initially synthesized as a 160-kDa precursor of Gag–Pol protein via a −1 translational frameshift, and the ratio of Gag and GAG–Pol proteins is about 20:1 [1]. Gag protein alone could be assembled into virus-like particles (VLPs) when expressed inside cells by recombinant vaccinia virus [2,3], baculovirus [4], and budding yeast [5]. These VLPs contain only the p55 Gag precursor because of the absence of protease encoded in the pol gene. When authentic *gag* and *pol* open reading frames were inserted in those expression vectors, VLPs with mature Gag and Pol proteins were produced by recombinant vaccinia virus vector, but not by baculovirus [4] or budding yeast (Sakuragi and Morikawa, unpublished data), probably due to higher expression levels of protease by baculovirus or budding yeast than by vaccinia virus. Significant protease expression would cleave Gag–Pol protein inside the cells [6], so that essential domains for virus assembly were lost before precursor proteins targeted the

Abbreviations: VLP, virus-like particle; MA, matrix; CA, capsid; NC, nucleocapsid; PEG, polyethylene glycol.

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plasma membrane. Similarly, an artificial Gag–Pol fusion protein generated by inserting four nucleotides at the natural frameshift region failed to produce VLPs even if expressed by a vaccinia virus vector [2]. When Gag–Pol fusion protein, whose protease was in an inactive form [7], or Gag protein fused with β-galactosidase at the C-terminus [8] was expressed in cells alone, VLP production was significantly reduced. These modified Gag fusion proteins could be incorporated into VLPs when co-expressed with authentic Gag proteins. [8,9]. In contrast, relatively short C-terminal extensions, such as the V3 loop of HIV-1 envelope protein (~100 bp long) [10] or green fluorescent protein (GFP; ~720 bp long) [11], on Gag proteins were tolerated for VLP formation.

It has been previously reported that several cellular proteins play important roles in transporting Gag protein to the plasma membrane and in budding from cells. For example, the MA region of Gag was shown to interact with AP-3 complex, which is involved in protein trafficking to certain cell compartments [12]. It has also been reported that Rab9, which mediates late endosome-to-*trans*-Golgi-network trafficking, plays an important role in viral assembly [13]. Moreover, Tsg101, which is localized in the multi-vesicular body (MVB), is involved in virus budding in a coordinated manner with the L domain, corresponding to the PTAP motif in the p6 region of HIV-1 Gag protein [14]. Proteasome inhibitors such as lactacystin or MG132 restricted virus budding from the cells [15], but they are not candidates for practical therapeutic use because the proteasome function is indispensable for cell survival. In addition, HP68, a cellular ATP binding protein, was essential for the assembly of immature HIV-1 capsids in vitro [16]. Furthermore, a peptide which bound to the C-terminal region of HIV-1 CA could inhibit the assembly of virion [17]. We speculate that other cellular factors may also be involved in the transport, assembly, and/or budding of HIV-1. We previously developed a yeast system which releases VLPs from cells when their cell walls were removed [5], and envisage that this yeast system could be applied to search for novel host factor(s) of retrovirus particle formation; however, commercially available ELISA kits to quantitate levels of mature CA protein in HIV-1 virion cannot detect precursor 55-kDa Gag protein, probably because the mature CA epitope used in this ELISA kit is absent or masked in the precursor 55-kDa Gag protein. We therefore developed a VLP system consisting of Gag–Firefly Luciferase fusion protein released from yeast for the rapid and convenient quantification of VLPs in culture media. The C-terminal extension to Gag protein in our system was approximately 1.65 kbp. In the expression system of vaccinia virus or baculovirus, it is not easy to completely remove infectious vector viruses from culture supernatant. In contrast, culture media of the budding yeast system do not contain any infectious vector viruses, because Gag proteins are expressed from episomal plasmids. Thus, this yeast system is safe to handle. We propose that this system would be useful to search for novel host factors involved in retrovirus budding by using the powerful genetics of budding yeast.

2. Materials and methods

2.1. Plasmid constructs

The entire *gag* coding sequence was amplified from an infectious proviral clone pNL-4-3 [18] by PCR with a primer pair of G5 (5′-GGCTAGAAGGAGAGCCATGGGTGCGAGAGC-3′) and G3 (5′-GCCGCTCACCAT*GGTACC*TTGTGACGAGGG-3′). The firefly luciferase gene was amplified from pGL3-basic (Promega Co., Madison, WI) by PCR using a primer pair of L5 (5′-CGG*GGTACC*ATGGAAGACGCCAAAAACATA-3′) and L3 (5′-CGGGGTACCTACCACATTTGTAGAGGTTTT-3′). Italicizing in G3 and L5 shows *Kpn*I restriction endonuclease recognition sites used for the ligation of *gag* and *luciferase* fragments. *gag* and a *firefly luciferase* fusion open reading frame were inserted into the poly-linker region of pGEM3Zf(+), resulting in pGEM3Zf(+)-NLgag-FL. In this construct, Gag and the Firefly Luciferase fusion protein were driven by the T7 promoter. A myristoylation signal mutant of the *gag* gene was generated using primer G5M (5′-TGCGGGATCCATGGCTG CGAGAGCGTCGG-3′) instead of G5, and the resultant plasmid was designated pGEM3Zf(+)-NLgagmyr(−)-FL. The wild type of the *gag–firefly luciferase* gene and its myristoylation signal mutant versions were also ligated to pRS425 [19] to be expressed in budding yeast.

2.2. Transfection and infection of mammalian cells

Human embryonic kidney 293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were inoculated with vaccinia virus expressing T7 polymerase (VTF7-3) at 10 PFUs/cell [20] and then transfected with pGEM3Zf(+), pGEM3Zf(+)-NLgag-FL or pGEM3Zf(+)-NLgagmyr(−)-FL by using DMRIE-C (Invitrogen, Carlsbad, CA, USA). Transfected cells were cultivated in the presence of 40 ng/ml of cytosine arabinonucleoside (AraC; Sigma, St. Louis, MO, USA). At 48–72 h after transfection, the cells were harvested and assayed for the levels of intracellular Gag–Firefly Luciferase protein by Western blot using anti-HIV-1 CA monoclonal antibody (Advanced Biotechnologies Inc., Columbia, MD, USA) and horseradish peroxidase-conjugated anti-mouse IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA, USA). Levels of bound antibody were measured using a chemiluminescence imager, LAS-1000plus (Fuji, Tokyo, Japan). The supernatant of transfected cells was clarified by centrifugation at 1600 × *g* for 20 min at 4 °C. VLPs were pelleted by ultracentrifugation at 35,000 rpm for 1 h at 4 °C on a Beckmann SW41Ti rotor through a 20% (wt/vol) sucrose cushion. The pellet lysates were analyzed by Western blot probing with anti-CA monoclonal antibody as described above.

2.3. Culture and transformation of yeast

S. cerevisiae BY4743 (*MATa/αhis3D1/his3D1 leu2DO/leu2DO MET15/met15DO LYS2/lys2DO ura3DO/ura3DO*) cells were transformed using the one-step transformation method [21] with some modifications. Briefly, BY4743 cells

were patched on a YPD plate (1% yeast extract, 2% polypeptone, 2% glucose and 2% agar) and incubated for 16–24 h at 30 °C. A loopful of cells was suspended in 100 μl of one-step buffer [40% PEG (polyethylene glycol) 3350, 0.2 M LiAc, 0.1 M β-mercapto-ethanol, 0.5 mg/ml RNAs from Trula Yeast (Nacalai Tesque, Kyoto, Japan), and ~1 μg of plasmid DNA], and incubated at 45 °C for 1 h. Some of the transformants were spread on a plate containing 2% agar and leucine drop-out synthetic media (0.67% yeast nitrogen base without amino acids, 2% glucose, and amino acid mixtures without leucine) and incubated at 30 °C to select transformants with leucine. Lysates of yeast transformants were prepared using acid-washed glass beads (Sigma, St. Louis, MO, USA) and subjected to Western blot with the serum of an HIV-1 patient and horseradish peroxidase anti-human IgG (H + L) (Vector Laboratories, Inc., Burlingame, CA, USA). A spheroplast of the yeast transformant was prepared as described previously [5].

2.4. Purification of Gag–Firefly Luciferase VLP budding from yeast

Culture media of spheroplasts were clarified by centrifugation at 1650 × *g* for 20 min at 4 °C, and the supernatants were layered onto a 30% (wt/vol) sucrose cushion and ultracentrifuged in SW41 rotor (Beckmann Coulter) at 35,000 rpm for 1 h at 4 °C. The pellets were resuspended with 200 μl of PBS(−) and applied onto a 20–70% (W/V) linear sucrose density gradient centrifugation in SW55 rotor (Beckmann Coulter) at 45,000 rpm for 16 h at 4 °C. Ten fractions were collected from the bottom and subjected to Western blot analysis using serum from an HIV-1 patient. The luciferase activity of each fraction was measured by luminometer (DIAYATORON, Tokyo, Japan) using the Bright Glo Luciferase assay system (Promega Co., Madison, WI, USA). The refractive index of each fraction was measured using a hand-held refractometer R-5000 (Atago Co., Tokyo, Japan) and converted to relative density.

2.5. Precipitation of VLPs by PEG

Culture media of spheroplasts were centrifuged at 1650 × *g* for 20 min at 4 °C, and supernatants were mixed with PEG 10,000 to a final concentration of 2.5, 5 or 10% (W/W) and put on ice for 16 h. After centrifugation at 1294 × *g* for 45 min at 4 °C, supernatants were removed, and the precipitated pellets were suspended with 100 μl of 1× Glo Lysis Buffer. Each suspension was reacted with an equal volume of Bright Glo System for 2 min at 25 °C, and the luciferase activity of each sample was measured using a luminometer (FLUOstar Optima, BMG LABTECH, Offenburg, Germany).

3. Results

3.1. VLP budding of HIV-1 Gag–Firefly Luciferase from higher eukaryotes

We first investigated whether VLPs consisting of Gag–Firefly Luciferase could be produced from higher eukaryotes. For this purpose, the entire *gag* gene derived from HIV-1 molecular clone NL4-3 and the *firefly luciferase* gene were fused and ligated to the poly-linker region of pGEM3Zf (+), and consequently, the *gag–firefly luciferase* fusion gene was driven by T7 promoter (Fig. 1A). A substitution mutant at the myristoylation signal of the *gag* gene was also fused to *firefly luciferase* [myr(−)-FL] and used as a negative control for VLP budding, because this mutation caused budding defects from the cellular membrane [22]. After inoculation with Vaccinia virus vTF7-3 expressing T7 RNA polymerase [20] for 1 h, 293T cells were transfected with pGEM3Zf(+)-NLgag-FL, pGEM3Zf(+)-NLgagmyr(−)-FL or pGEM3Zf(+) using cationic lipid reagent DMRIE-C. Transfected cells were incubated for 2 days to allow the release of VLPs into the culture media in the presence of cytosine arabinonucleoside to inhibit the DNA synthesis of vaccinia virus. Cells were lysed for the detection of Firefly Luciferase activity using the Bright Glo System. Both cells transfected with either pGEM3Zf(+)-NLgag-FL or pGEM3Zf(+)-NLgagmyr (−)-FL expressed high levels of Luciferase activity

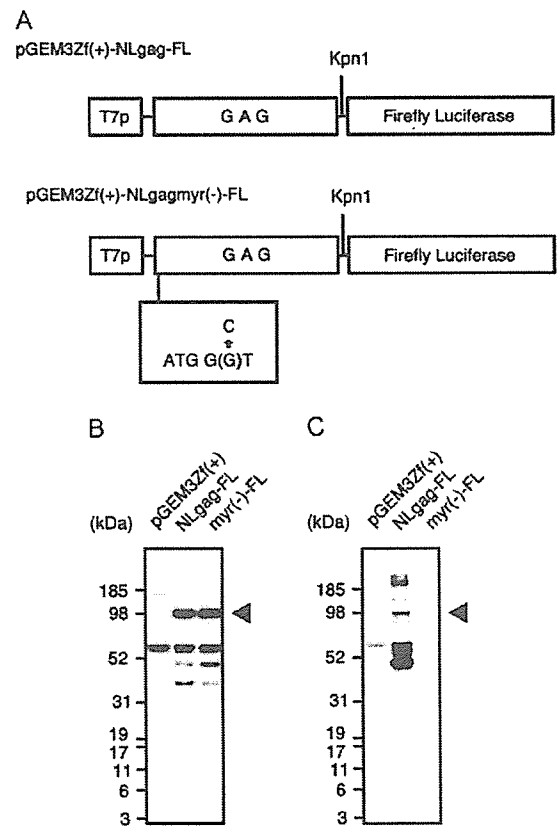

Fig. 1. Expression of Gag–Firefly Luciferase fusion protein in higher eukaryote cells. (A) Schematic representation of expression constructs for 293T cells. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of cell lysates of transfected cells. Twenty micrograms of each lysate was loaded on SDS-PAGE gel and probed with anti-CA mAb. (C) Western blotting of VLPs. Culture media pellets of transfected cells through a 20% sucrose cushion were probed with anti-CA mAb. An arrowhead indicates the positions of Gag–Firefly Luciferase fusion protein. Vector, WT, and myr(−) denote pGEM3Zf(+), pGEM3Zf(+)-NLgag-FL, and pGEM3Zf(+)-NLgagmyr(−)-FL, respectively.

[3×10^3–1.2×10^4-fold to pGEM3Zf(+)] (data not shown). Western blot analysis of the cell lysate using anti-CA monoclonal antibody (Fig. 1B) revealed the presence of approximately 100-kDa protein in cells transfected with pGEM3Zf(+)-NLgag-FL and pGEM3Zf(+)-NLgagmyr(−)-FL but not in cells transfected with empty vector pGEM3Zf(+). The size of this protein was in a good agreement with that of Gag–Firefly Luciferase.

We then examined whether VLPs consisting of Gag–Firefly Luciferase were produced from transfected cells. The culture media of transfected cells were collected and clarified by low-speed centrifugation 2 days after transfection. Ultracentrifugation was performed to remove soluble forms of Gag–Firefly Luciferase from the culture supernatants, and a sucrose cushion pellet was assayed for Gag–Firefly Luciferase fusion proteins by Western blotting probed with anti-CA monoclonal antibody. As shown in Fig. 1C, we could detect Gag–Firefly Luciferase fusion protein only from the culture supernatants of cells transfected with NLgag-FL, but not from those of cells transfected with NLgagmyr(−)-FL or empty vector. These results indicated that VLPs were released only from cells transfected with NLgag-FL, and no VLP was released from cells transfected with NLgagmyr(−)-FL. This result was consistent with the previous report on VLPs consisting of only Gag protein [22]. We also detected at least two discrete signals around 52 kDa with anti-CA monoclonal antibody only from the culture supernatants of NLgag-FL-transfected cells. Those are most likely the degraded products of Gag–Firefly Luciferase. Alternatively, cross-reactive cellular proteins (see Fig. 1B) may be incorporated into or co-purified with VLPs.

3.2. Expression of Gag–Firefly Luciferase protein in S. cerevisiae

We then constructed two expression vectors of *gag–firefly luciferase* in *S. cerevisiae*. The wild type and myristoylation signal mutant of *gag–firefly luciferase* fusion genes were driven under the control of TDH3 (glyceraldehyde-3-phosphate dehydrogenase) promoter derived from pKT10 [23] (Fig. 2A). These two constructs or parental plasmid, pRS425, were introduced into *S. cerevisiae* diploid strain BY4743 by the one-step transformation method. Cell lysates of the transformants were prepared with acid-washed glass beads with lysis buffer and then assayed for levels of luciferase activity. The luciferase activity of cells transformed with pRS425-NLgag-FL and pRS425–NLgagmyr(−)-FL was $5–6 \times 10^5$ times higher than that of transformants of empty vector pRS425 (data not shown). This result indicated that each transformant expressed an active form of luciferase. The cell lysates were then subjected to immunoblotting and probed with serum from an HIV-1 patient to identify whether intact Gag–Firefly Luciferase was expressed inside the cells. As shown in Fig. 2B, we could detect Gag–Firefly Luciferase fusion protein around the 98-kDa marker in cells with NLgag-FL and NLgagmyr(−)-FL. In order to compare the expression level of Gag–Firefly Luciferase fusion protein to Gag protein alone inside the cells, we also transformed budding yeast with

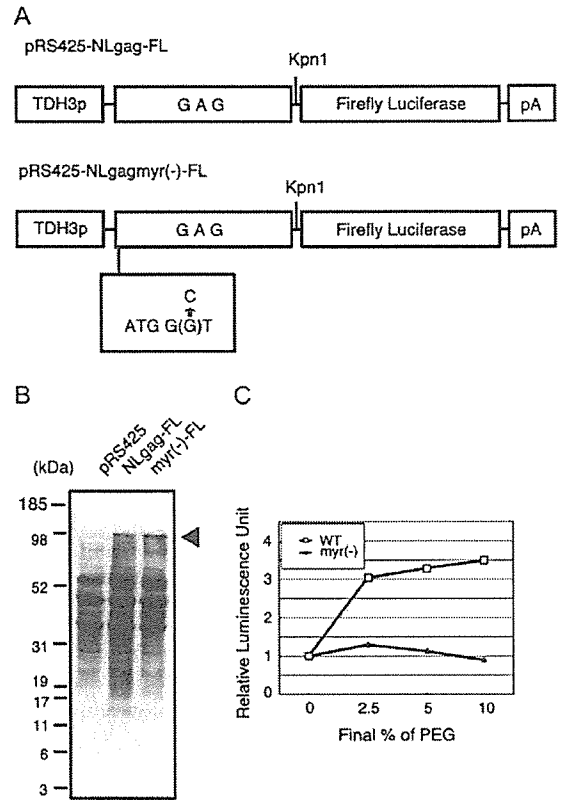

Fig. 2. Expression of Gag–Firefly Luciferase fusion protein in *S. cerevisiae*. (A) Schematic presentation of constructs for expression in *S. cerevisiae*. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of cell lysates of transformants. Forty micrograms of each lysate was loaded onto SDS-PAGE gel and probed with serum from an HIV-1 patient. The digitizing of band intensity corresponding to Gag–Firefly Luciferase fusion protein is shown below the blot. (C) Luciferase assay of PEG precipitants of VLPs in culture media. One millilitre of each culture medium of spheroplasts was mixed with PEG 10,000, and each precipitant was reacted with the Bright Glo System and measured with a luminometer. The value of each sample is divided by that of 0% PEG and shown as relative luminescence unit. Representative data of three independent experiments are shown. Vector, WT, and myr(−) denote pRS425, pRS425-NLgag-FL and pRS425-NLgagmyr(−)-FL, respectively.

pRS425-NLgag, an expression vector containing only *gag* open reading frame. Western blot analysis of cell lysate showed that expression of Gag protein alone was about 15-fold higher than Gag–Firefly Luciferase fusion protein (data not shown).

3.3. VLPs released from budding yeast

We then tried to purify VLPs carrying Firefly Luciferase from *S. cerevisiae*. Transformants of pRS425, pRS425-NLgag-FL, and pRS425-NLgagmyr(−)-FL were treated with Zymolyase-100T to remove their cell walls, and the resultant spheroplasts were cultured in YPD supplemented with 1 M sorbitol, used to optimize osmotic conditions for 16 h to allow the release of VLPs. The culture media were clarified by low-speed centrifugation, and VLPs in the supernatants were

precipitated with PEG 10,000. After low-speed centrifugation, pellets were assayed for luciferase activity levels using a luminometer. Raw values of luciferase activities of the pellets were divided by that of the pellets without PEG. As shown in Fig. 2C, we detected much higher luciferase activity of wild-type fusion protein in pellets with 2.5, 5, and 10% of PEG 10,000 than in those with 0% PEG 10,000. On the other hand, the level of luciferase activity of the myristoylation signal mutant in PEG 10,000 pellets was almost identical to that in 0% PEG 10,000. These results suggested that only the wild-type version of fusion protein could form VLPs in the culture media of spheroplasts, and that the majority of luciferase activity in the culture medium of the myristoylation signal mutant was a soluble form of fusion protein.

3.4. Properties of VLPs of Gag–Firefly Luciferase

We then measured the density of pelleted luciferase activity to identify whether pellets with luciferase activity really formed VLPs. The culture media of yeast spheroplasts were clarified by low-speed centrifugation and then ultracentrifuged through a 30% sucrose cushion to remove the soluble forms of Gag–Firefly Luciferase. The resultant pellets were resuspended with PBS and laid on the top of a 20–70% sucrose density gradient. After ultracentrifugation, 10 fractions were collected from the bottom of the tube and each fraction was assayed for VLP levels by Western blotting probed with serum from an HIV-1 patient. As shown in Fig. 3A (Western blotting) and Fig. 3B (quantification of Gag–Luciferase fusion protein), wild-type Gag–Firefly Luciferase fusion protein was distributed between densities of 1.2241 and 1.1764 (W/W), almost identical to that of VLPs composed of authentic Gag protein in budding yeast [5]. When we performed the same experiments on the myristoylation signal mutant or empty vector (Fig. 3B), no signal was detected on the immunoblotting membrane with serum from an HIV-1-positive patient (data not shown). We then measured the luciferase activity of each fraction of these three samples. The result showed that luciferase activity was detected only in fractions with Gag–Luciferase fusion proteins (Fig. 3C). These results clearly indicated that VLPs composed of Gag–Luciferase fusion proteins were successfully produced by spheroplasts of budding yeast.

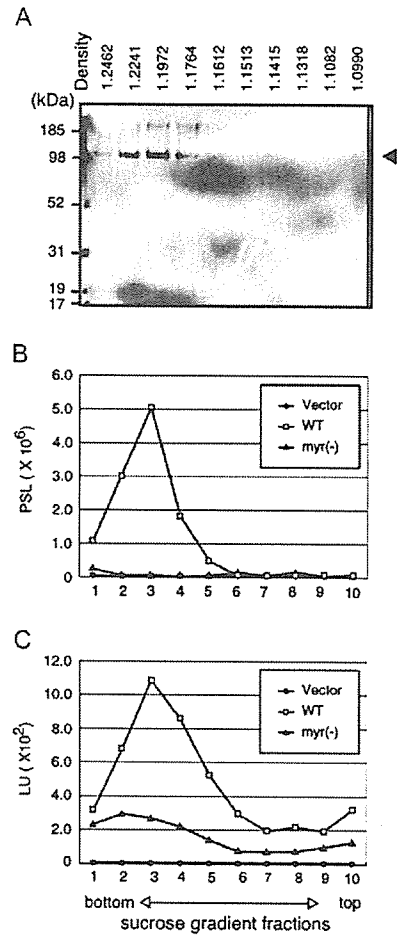

Fig. 3. Detection of VLPs released from spheroplasts of *S. cerevisiae*. (A) Western blotting of fractions of sucrose density gradient centrifugation with serum from an HIV-1 patient. (B) Digitizing of band intensity corresponding to Gag–Firefly Luciferase fusion protein on Western blotting. (C) Luciferase assay of each fraction of sucrose density gradients. Total luminescence counts detected in 10 s are shown as LU. Vector, WT, and myr(−) denote pRS425, pRS425-NLgag-FL and pRS425-NLgagmyr(−)-FL, respectively.

4. Discussion

Budding yeast was used in a model system of mammalian cells because it possesses approximately 6000 genes and the basic mechanisms of vital activity inside the cells are highly conserved from budding yeast to humans. The strong power of yeast genetics has unveiled the precise molecular mechanisms underlying several biological phenomena such as oncogenesis and signal transduction cascade [24]. Furthermore, budding yeast is easy and safe to handle.

We previously developed a budding yeast system releasing VLPs of HIV-1 Gag. We next wanted to apply this system to the exploration of cellular factor(s) involved in VLP budding. We initially used a commercially available ELISA kit which detects mature CA of HIV-1; however, this kit failed to detect precursor 55-kDa Gag proteins. It is possible, but would be expensive, to construct a new ELISA system by using the anti-Gag antibody which reacts to the precursor Gag protein. We then tried to produce VLPs composed of Gag–EGFP fusion protein; however, this was unsuccessful because of the autofluorescence of the culture medium. In this study, we developed a rapid and convenient method to quantitate HIV-1 VLPs by expressing Gag–Firefly Luciferase fusion protein in spheroplasts of *S. cerevisiae*.

In the culture supernatants of 293T cells and spheroplasts of budding yeast expressing wild-type Gag–Firefly Luciferase fusion protein, we could successfully detect Gag–Firefly Luciferase proteins which could be pelleted through a sucrose cushion by ultracentrifugation. When we used a myristoylation mutant, no Gag–Firefly Luciferase protein could be detected in the ultracentrifugation pellets. Furthermore, both Gag–Firefly Luciferase protein and luciferase activity were detected

in the density range of 1.2241–1.1764 mg/ml. These results clearly indicated that Gag protein could assemble into VLPs, although a 1.6 kbp-long *firefly luciferase* gene was fused at the 3′ end of the *gag* gene.

The expression levels of Gag–Firefly Luciferase fusion protein inside budding yeast was 15-fold lower than that of Gag protein expressed by a vector carrying *gag* open reading frame alone. This result suggested that Luciferase-tag might affect the expression in budding yeast. Nevertheless, the luciferase system has an apparent advantage in sensitivity. Furthermore, as shown in Fig. 2C, we successfully concentrated VLPs by PEG. The PEG sedimentation procedure could be performed by low-speed centrifugation and enabled us to use a high-throughput method using a 96-well deep-dish plate.

In Fig. 3B and C, the values for fraction 2 and 4 were inconsistent. This was most likely caused by a slight inhibitory effect of sucrose on luciferase activity, since the presence of 70% sucrose reduced luciferase activity to nearly 70% (data not shown).

There were weak luciferase activities in fractions 1–4 of myristoylation signal mutant. However, the peak of luciferase activity of myristoylation signal mutant was detected in the higher-density fraction than the peak of the wild type (Fig. 3C). So, it is possible that this myristoylation signal mutant of Gag–Firefly Luciferase fusion proteins with luciferase activity was an aggregate without lipid bilayer.

In summary, we showed that both higher eukaryotic cells and budding yeasts could release HIV-1 VLPs consisting of Gag–Firefly Luciferase fusion proteins. The property of VLPs consisting of Gag–Firefly Luciferase fusion protein examined here was consistent with the nature of the wild-type virion of HIV-1. Thus, we suggest that the VLP budding system described here can be applied in the search for novel host factors essential for the transport, assembly and/or budding of HIV-1 Gag protein. There are several precedents for such studies, in the viral RNA replication of Brome mosaic virus [25] or in the viral RNA recombination of Tomato Bushy Stunt Virus [26]. We also suggest that HIV VLPs produced by our system may be useful for screening inhibitors of HIV-1 virion budding.

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総　説

2. 酵母を用いた動物ウイルスの研究

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大腸菌が原核細胞のモデルであるように，酵母は真核細胞のモデルとしてしばしば用いられる．古くから酵母は詳細に解析され，近年そのゲノムも完全解読された．高等真核細胞との類似性が高くかつ分子遺伝学や分子生物学の解析手法が応用できることから，酵母は真核細胞のモデル細胞として多用されている．こうした酵母細胞で動物ウイルスの複製が再現できることが近年明らかとなってきた．本稿では，酵母の遺伝子発現と酵母における動物ウイルスの複製を概説する．特筆すべきは，酵母における完全なウイルス複製過程の成立と酵母を用いたウイルス様粒子ワクチンの製造である．酵母を用いた近年の研究は，ウイルス複製に関与する宿主因子や宿主機構の同定を中心に展開されており，それらは酵母遺伝学の利用や酵母の全遺伝子破壊株ライブラリを用いた網羅的解析によって推進されている．

1. はじめに

酵母 Yeast は単細胞性の fungi であり，厳密にいうと「出芽・分裂によって増殖し，性接合を行う ascomycetous あるいは basidiomycetous fungi」の微生物細胞である．出芽酵母の代表として Saccharomyces cerevisiae（パン酵母）が，分裂酵母の代表として Schizosaccharomyces pombe がよく知られている．酵母は大腸菌のように取り扱いが簡単な細胞であるにもかかわらず，高等真核細胞との類似性が高くかつ分子生物学・細胞生物学の解析手法が応用できることから，真核細胞のモデル細胞として多用されている．特筆すべきは遺伝子破壊や変異導入などの分子遺伝学（特に，逆遺伝学）である．また，酵母 Two-Hybrid Assay は結合する蛋白質をライブラリから探索する方法としてよく知られているところである．

出芽酵母には内在性 RNA ウイルス（L-A ウイルスやレトロエレメント Ty1-5）が存在することが古くから知られている．これらは細胞外感染経路をもたず，接合などの細胞質混合で伝達される．酵母の細胞外からのウイルス感染を最初に報告したのはタバコモザイクウイルスを用いてである[5]．下等真核細胞である酵母は細胞壁があるという点では植物細胞であり，植物ウイルスの報告があるのはなるほどと思われる．しかし近年，動物ウイルスの増殖も酵母で再現できることが明らかとなってきた．本稿では，酵母が動物ウイルスの研究にどう利用されているのか，またどう利用できるのか，実例と利用の利点・注意点などを概説する．

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2. 酵母における遺伝子発現

ウイルス研究者にとって，高等真核細胞はウイルスを増殖させるための宿主細胞であり，大腸菌はウイルス遺伝子をクローニングするための細胞である．しかし，酵母には馴染みが薄いと思う．酵母におけるウイルス遺伝子の発現を紹介する前に，酵母自体の遺伝子発現について少し解説する．

1）酵母の遺伝子発現系

① DNA 複製

出芽酵母は DNA 複製開始部位の構造がわかっている唯一の真核生物である．酵母細胞内で複製開始点となる DNA 配列を自律複製配列（Autonomously Replicating Sequence: ARS）と呼び，5'-(T/A)TTTA(C/T)(A/G)TTT(T/A)-3'-という共通配列が見出されている．ただし，分裂酵母の ARS とは一般的に交換できない．この ARS に加え，分極に関与するセントロメア CEN と染色体安定性に関与するテロメア TEL が染色体機能として必要である．酵母における DNA 複製機構の解析から同様の複製機構がヒト細胞で

も存在すると推測されている.

②転写

酵母から動物細胞まで，DNA依存性RNAポリメラーゼの基本的な構造や機能は共通であり，酵母RNAポリメラーゼはPol A, B, C（動物細胞のPol I, II, IIIに相当）と呼ばれている．mRNAの5'末端にはm^7GpppNのキャップ構造が付加され，モノシストロニックである．

動物細胞に比べ，出芽酵母の遺伝子にはイントロン-エクソン構造が少ない（約3％）．スプライシング機構は動物細胞の場合とほとんど同じであるが，スプライシングシグナルの配列がやや異なる．分裂酵母では遺伝子の3-4割がイントロンをもつ．

③翻訳

原核生物では翻訳開始点の上流にリボソーム結合部位が存在するのに対し，酵母を含め真核生物ではそれが認められない．また酵母では，転写開始点と翻訳開始コドンAUGの間の距離は長くても短くても翻訳レベルは変わらない．しかしながら，mRNAの2次構造（特に，Gと多く含む配列）は翻訳を強く阻害する．高等真核細胞では翻訳開始コドンAUGの周辺にはKozakのコンセンサス配列CCACCAUGGCが認められるが，酵母ではこれがAAAAAAAUGUCUである[13]．ただし，変異させても翻訳効率の低下はわずかである[3]．酵母のリボソームは一度翻訳を終結すると翻訳を再開できないといわれている．

2）酵母におけるウイルス遺伝子の発現

①ウイルス遺伝子の複製・転写

一般的にDNAウイルスの遺伝子の複製は，ウイルスゲノム長が短いウイルス（パポーバ科やパルボ科）では宿主細胞酵素系への依存性が高く，宿主のDNAポリメラーゼを利用する．転写はポックス科を除いてすべてのDNAウイルスが宿主細胞のRNA Pol IIに依存している．出芽酵母におけるDNAウイルスの複製・転写は，最も宿主細胞依存性が高いと思われるパポーバ科のパピローマウイルスを用いて成功している[2, 22, 49, 50]．酵母細胞内で安定して複製するには自律複製配列ARSなどが必要であるが，パピローマウイルスDNAのL1-LCR領域に酵母細胞内での自律複製に関与する配列[22]つまり弱いながらもARSとの塩基配列相同性のある部位が見出されている[49]．この酵母におけるウイルスmRNAの転写を直接証明した報告はないが，驚くべきことに，細胞壁を消化した酵母スフェロプラスト細胞にパピローマウイルス粒子を取り込ませると，ウイルスゲノムDNAがパッケージングされた感染性ウイルス粒子が回収されることから[50]，酵母細胞で完全なウイルス複製過程が進行すると思われる．

出芽酵母における（+）鎖RNAウイルスの複製・転写は，この分野の第1人者であるP. Ahlquistらが植物ウイルスのブロモモザイクウイルス（BMV）を用いて精力的に解析している．このウイルスの蛋白質1a（RNA helicaseとキャッピング機能をもつ）と蛋白質2a（RNA依存性RNA合成酵素）を共発現させた酵母細胞では，これら蛋白質がウイルスゲノムRNAと複合体を形成し，転写・複製がおこることが明らかになっている[12, 19, 20, 39, 46]．さらに，酵母細胞における完全なウイルス複製過程がノダ科の昆虫ウイルスを用いて示されており，感染性ウイルス粒子が回収されている[36, 37, 38]．これに対し，(-)鎖RNAウイルスの複製・転写ついて詳細な報告はないが，水泡性口炎ウイルス（VSV）粒子を取り込ませた酵母スフェロプラスト細胞ではウイルス蛋白の発現が観察されている．ただし，その著者らはウイルスゲノムが複製されたとはあまり考えていないようである[27]．

②ウイルスmRNAの翻訳

上記のように，酵母で感染性ウイルスが産生されることから，間接的ではあるが，多くのウイルスmRNAは正確に翻訳されると考えられる．気をつけねばならない点はInternal Ribosome Entry Site（IRES）である．上述したように，酵母では転写開始点と翻訳開始点の距離はさほど問題ではないが，mRNAの2次構造は翻訳に致命的である．そのせいなのか不明だが，酵母においてはヒトC型肝炎ウイルス（HCV）IRESの翻訳成功例[17, 40]とポリオウイルスIRESの翻訳不成功例[6]が報告されている．後者の不成功例では，酵母細胞からLa自己抗原のIRESへの結合を阻害する60塩基長RNAが見出されている[7, 8, 9]．筆者らもヒト免疫不全ウイルス1型（HIV-1）mRNAが酵母で翻訳されないことを見出している．ウイルスmRNAと酵母のRNA helicaseとの相性も指摘されている[33]．

3. 酵母を用いた蛋白質発現とその応用

酵母は真核細胞のモデルとして代用されるだけでなく，蛋白質産生の細胞としても利用されてきた．異種蛋白質発現のための酵母の遺伝子操作については多くの総説があると思うのでそれを参考にしてほしい．入門書としては柴垣と水本による総説「酵母発現系とその実際」がわかりやすい[42]．

蛋白質発現のための細胞として，大腸菌や高等真核細胞など様々な細胞が用いられる．**表1**にそれらの特徴をまとめた．酵母も大腸菌と同じで操作は簡単であり，寒天プレートで形質転換コロニーを形成させ，これを液体培地で大量に振盪培養する．ただし，酵母の生育は大腸菌より遅いので各ステップはovernightではなく2日はかかる．酵母の欠点として，①分厚い細胞壁をもつので細胞粉砕が困難である，②蛋白質分解酵素の活性が高く蛋白質を精製しづらい，③さほど発現量が高くないなどが挙げられる．昆虫細胞や哺乳類細胞での発現は，transfectionによる一過性発現（大量発現に不向き）か，transfectantの細胞株を樹立する（時間がかかる）かであり，そうでなければウイル

表1 蛋白質発現系の比較

	大腸菌	酵母	昆虫細胞	哺乳類細胞
培養形式	浮遊	浮遊	浮遊	接着
細胞倍加時間	30分	90-180分	24時間	24時間以上
蛋白質発現量	大	小	中	極小
蛋白質の翻訳後修飾				
糖鎖付加	なし	マンナン型	高マンノース型	複合型
リン酸化	His, Asp	Ser, Thr, Tyr	Ser, Thr, Tyr	Ser, Thr, Tyr
アシル化	なし	あり	あり	あり

スベクターに因らねばならないという難点があり，選択に迷うところである．

大腸菌とは異なる利点として，酵母では翻訳後修飾がおこることが挙げられる．ただし，酵母における糖鎖付加は問題で，その付加シグナル配列は高等真核細胞と同じであるが，マンナンと呼ばれる巨大なマンノースポリマー構造が形成される．例えば，HIV-1のエンベロープ蛋白gp120(高等真核細胞では120kDaの蛋白質として生成されこの分子量の約半分を糖鎖が占める）を酵母で発現させると600kDa以上になり抗体で認識されない[14]．しかし，糖鎖付加部位が少ない場合は活性あるエンベロープ蛋白となるようで，VSV G蛋白を発現させた酵母スフェロプラスト細胞では膜融合による多核巨細胞が形成される[27]．酵母における糖鎖付加のもう1つの問題は，複合型の糖鎖付加がおこらないことである（ただし，この点は昆虫細胞で発現させた場合も同じで，昆虫細胞でも複合型の糖鎖付加はおこらない)．こうした問題点を解決する目的で，糖鎖改変酵母株の分子育種がさかんに行われている．すなわち，マンナン型糖鎖ができないように酵母染色体遺伝子（OCH1, MNN1, MNN4遺伝子など)を破壊し，さらに複合型糖鎖が生成されるようヒト遺伝子(Mannosidase I, IIを含む7つの遺伝子)を導入した酵母株の作出が試みられている[4, 21, 32]．ヒト遺伝子をもたせた酵母株はまだ市販されていないが，酵母の糖鎖遺伝子破壊株はAmerican Type Culture Collection（ATCC）から入手できる．

酵母の蛋白質発現系が大変有用であると認識されたのは，酵母を用いたヒトB型肝炎ウイルス（HBV）ワクチンの製造によってである．当時はヒト感染血清から危険を冒しながらHBV粒子を精製しそれを不活化してワクチンとしていた．しかし，P. ValenzuelaらはHBVの表面抗原であるHBsAgを酵母で発現させたところ，球状のHBsAg粒子が形成されていることを見出した[31, 45]．さらに，その細胞破砕液から精製したウイルス様粒子（VLP）はヒト感染血清由来不活化ワクチンと同等の力価をもつことが明らかにされ[29]，現行のHBVワクチンとなっている．酵母細胞におけるVLP形成はパピローマウイルス[15, 16]やBMV[23]でも報告されている．また近年，HIV-1でも示され[41]，そのVLPの免疫原性が検討されている[43]．

3. 酵母における細胞生物学

1）細胞の構造と機能

酵母は核と細胞質が核膜で分離された真核細胞であるが，①有糸分裂の時も核膜（ラミンの裏打ち構造はない）が消失しないこと，②細胞は厚い細胞壁に覆われていることが，動物細胞との決定的な違いである．しかし，その他の細胞内構造はよく似ている．粗面小胞体は核膜と連続したものと，網状のネットワークが形質膜近くに存在する．ゴルジ装置はシス・ミディアム・トランスに分かれた層状構造として認められる．初期エンドソームや後期エンドソームの存在も知られており，液胞は動物細胞のリソソームに相当する．酵母オルガネラマーカーの抗体はMolecular Probesから市販されている．

2）酵母細胞におけるウイルス蛋白質の動態

ウイルスの吸着・侵入過程の解析に酵母を積極的に用いた研究は少ない．酵母には細胞壁があるからである．しかしながら，酵母は細胞壁を除去しても活発にエンドサイトーシスがおこる細胞であり，それによると思われる酵母スフェロプラスト細胞へのウイルス感染成立が，パピローマウイルス[49, 50]とVSV[26, 28]で報告されている．特にVSVでは，その感染成立が温度依存性・低pH依存性の膜融合によることが明らかにされている[28]．

ウイルス蛋白質の細胞内輸送や局在は，その蛋白質がエンベロープ蛋白か，キャプシド蛋白か，複製・転写酵素かによって様々だが，それぞれ酵母でも解析されている．例えば，一般的に(+)鎖RNAウイルスのRNA依存性RNA合

成酵素は細胞内の膜小器官に結合して複製・転写複合体を形成するが，どの膜小器官に targeting するかは個々のウイルスによって異なっている．この膜小器官に対する特異性は酵母細胞でも再現され，かつこの複製・転写複合体を人為的に本来とは異なる膜小器官に retargeting させても活性ある複製・転写複合体として維持されることから，この膜小器官特異性はゲノムの複製・転写に必要な現象ではなく，その他の段階(ゲノムのパッケージングやアッセンブリー)に必要な現象だと考えられるようになってきた[25,30]．

脂質二重膜をもたないウイルスでは，ウイルス粒子は細胞質内で形成され細胞破壊によって放出される．これに対し，脂質二重膜をもつウイルスでは，キャプシド蛋白が小胞体やゴルジ体あるいは形質膜に targeting し粒子を形成しながら出芽する．筆者らはこのようなウイルスキャプシド蛋白の膜 targeting を酵母で解析し，HIV-1 のキャプシド蛋白が形質膜に targeting し，細胞壁を除去すると VLP が出芽することを報告している[41]．

4. 酵母遺伝学の利用

酵母をウイルスの宿主細胞として使用する，その真価は酵母の分子遺伝学（特に，逆遺伝学）の利用にあるといっても過言でないと思う．ウイルスは細胞内寄生生物であり，その増殖過程において様々な宿主因子を利用する．こうした宿主因子を同定する目的で，ウイルスの研究に染色体遺伝子を破壊させた酵母株を宿主として用いる実験である．

1）遺伝子破壊株の利用

酵母は相同組換えの頻度が非常に高い生物であり，酵母の染色体遺伝子をクローニングしその遺伝子を改変（欠損や点変異を導入）して再び酵母の染色体に戻すと，遺伝子改変の酵母が作出できる．自分が調べたい宿主因子がある程度予想できるならば，こうした遺伝子改変酵母株を分与してもらうか（大抵もらえる）あるいは作成して実験に使ってみると良い．筆者らも細胞内輸送に関連する遺伝子破壊株を分与してもらい実験に使っている．

2）突然変異株の分離

しかし，調べたい宿主因子が予想できない場合にはもっと積極的なアプローチが必要となる．すなわち，エチルメタン硫酸やニトロソグアニジンあるいは紫外線照射によって突然変異を誘発し，ウイルス増殖を許容しなくなった酵母細胞を分離する試みである．知恵を絞らねばならないのはその突然変異株の選択方法である．最も理想的なのは，ウイルス増殖を許容しなくなった突然変異株だけが生育してくるといった条件設定であるが，そんなに都合良くいかない．ウイルス遺伝子あるいはウイルス蛋白を薬剤耐性や栄養要求性のマーカーで標識し，それを指標に突然変異株を分離するのが現実的である．ただし，こうした変異誘発で分離された突然変異株は，その表現型が単一遺伝子の変異によるものか，戻し交配と四分子解析が必要となることが多い．前者は突然変異株と親株の backcross でまだ簡単だが，後者は慣れないと難しい．次に，その遺伝子を同定する．酵母では突然変異株が得られた場合，その遺伝子を特定するのは比較的容易である．酵母の遺伝子ライブラリを導入してその突然変異を相補するのである．すなわち，その突然変異株に親株のゲノム DNA ライブラリを導入し突然変異の表現型が打ち消されたものを分離するか（その突然変異が劣性の場合）（**図 1**），逆に，親株に突然変異株

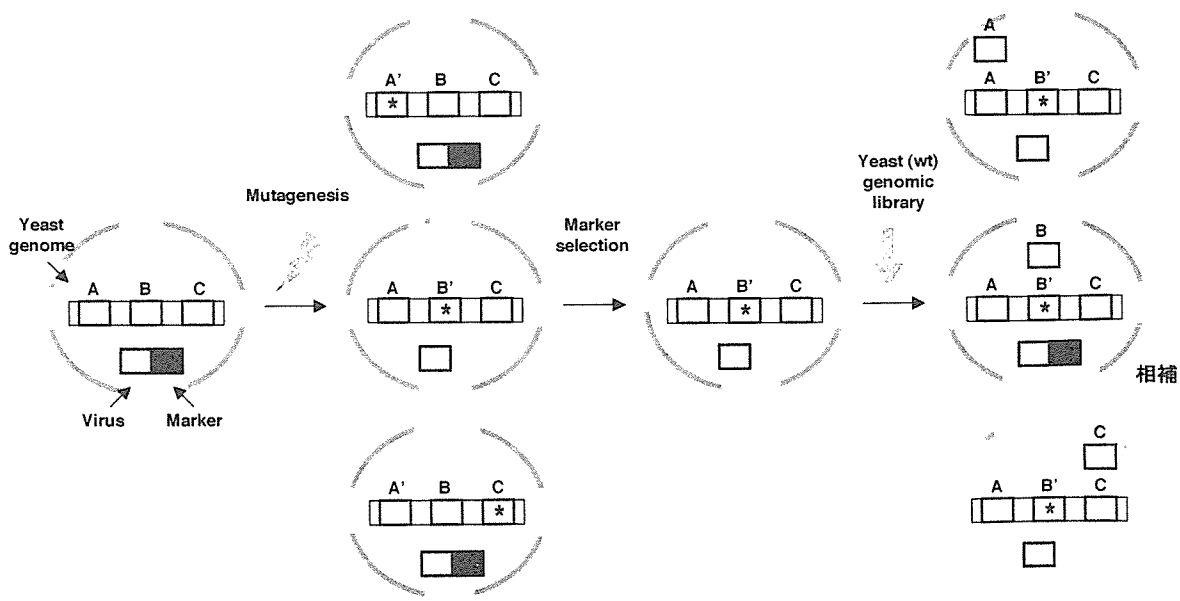

図 1 酵母相補性試験による遺伝子の同定

のゲノム DNA ライブラリを導入し突然変異の表現型が出現したものを分離する（その突然変異が優性の場合）．こうした解析方法により，BMV ではウイルス mRNA の安定性に関与する宿主因子 Sm 蛋白[10, 18]や転写・複製に関する分子シャペロン DNA J[43]が同定されている[1]．

3）全遺伝子破壊株ライブラリを用いた網羅的解析

もっと全遺伝子を網羅的に調べたいという要求が出てくるかもしれない．これを可能にするのがここに述べる全遺伝子破壊株ライブラリの利用である．

出芽酵母のゲノム解析は 1988 年より国際共同プロジェクトとして開始され 1996 年に全塩基配列データが公開された．出芽酵母のゲノム長はリボソーム DNA の反復配列の長さによって幅があるものの，12-16 Mbp で真核生物の中で最も短い．出芽酵母のゲノムは 16 本の染色体から構成されており，全 ORF 数は 6000 以上でそれぞれに規則的な ID 番号がついている．出芽酵母のゲノムのデータベースとして http://mips.gsf.de/genre/proj/yeast/（欧州マックスプランク研究所）と http://www.yeastgenome.org/（米国スタンフォード大学）がある．一方，分裂酵母のゲノムは 14 Mbp であり，3 本の染色体から成り立っている．代表的なデータベースとして http://www.sanger.ac.uk/Projects/S_pombe/（英国サンガーセンター）がある．

これらのポストゲノム研究はすべての遺伝子の体系的機能解析研究であり，出芽酵母では European Functional Analysis Network（EUROFAN）プロジェクトにより全遺伝子破壊株コレクションが作成された．この遺伝子破壊株作製には，PCR 法による遺伝子破壊アレルの作製をベースとしながら，全遺伝子共通配列（約 20 bp）と個別識別のための分子バーコード（約 20 bp）の挿入などの技術を駆使して作製された[11, 48]（**図 2**）．この破壊株ライブラリはゲノム解析によって明らかにされた約 6000 個の遺伝子 ORF をそれぞれ薬剤耐性遺伝子で破壊した 1 倍体あるいは 2 倍体のセット（96 穴フォーマット）で，Invitrogen やフナコシを通じて入手できる．この破壊株ライブラリを pooled で用いた場合には，遺伝子同定には蛍光標識分子バーコードをプローブとした DNA マイクロアレイが使用できる[48]．こうした遺伝子破壊株ライブラリを用いて，細胞形態[34]やヒト疾患[47]に関与する遺伝子の high-throughput な解析が始まっている．2003 年にはこの酵母遺伝子破壊株ライブラ

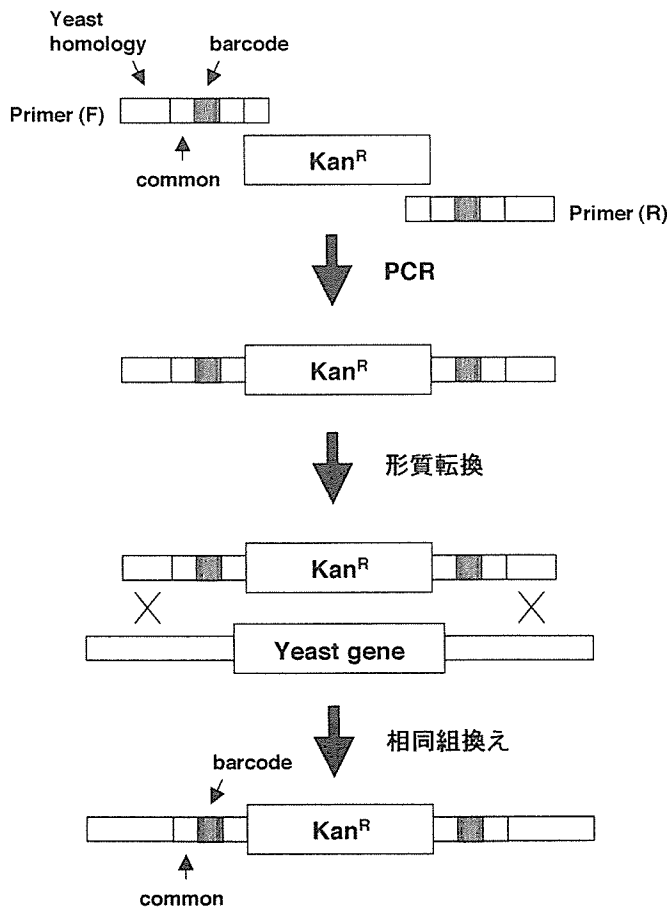

図 2　分子バーコードをもつ酵母遺伝子破壊株の作出法

リ（約4500株）を用いてBMV RNAの複製・転写に関与する宿主因子の網羅的解析が行われ，約100個の宿主因子が発表された[24]．

4）全遺伝子破壊株ライブラリVS siRNAライブラリ

上述のように，酵母は真核生物における遺伝学研究を担ってきた．それは酵母では相同組換え頻度が高くgene targetingが容易だったからである．哺乳類細胞における相同組換え頻度は残念ながら今なお低く，遺伝子破壊株ライブラリを作成できるまでに至っていない．しかし近年，RNA干渉法によるmRNAのknock-down法が確立され，この技術は哺乳類細胞における標的宿主因子のdepletionを可能にした．また，網羅的解析を目的としたsiRNAライブラリの構築も開始された．それを用いて，VSVによるクラスリン依存性エンドサイトーシスとSV40によるラフト/カベオラ依存性エンドサイトーシスに関与してくるキナーゼ群が解析されたところ，調べた590個のキナーゼのうち208個がこれらのウイルス感染によるエンドサイトーシスに関与していることが明らかとなった[35]．酵母の遺伝子破壊株かsiRNAか，どちらが良いか筆者にはまだ結論が出せない．

5. おわりに

筆者はフツウのウイルス研究者であり，酵母学者ではない．ただ，当時ラボに来てくれた櫻木小百合博士（現，阪大微研）と「酵母からHIV-1の粒子を出芽させる」といった実験を組んだことから酵母も扱うようになった．そうした経験から，ウイルス研究者が酵母に対して疑問に思うことや不安に思うことはできる限り書いたつもりであるが，書き漏らしはあると思うし，酵母学としては体系的に書けていないと思う．また，酵母Two-Hybrid Assayとその細胞質内Assayバージョンである酵母CytoTrap Two-Hybrid Assayの利用については紙面（時間か？）の関係から書けなかった．これらの点はどうぞお許しいただければと願う．足りない点などご指摘いただければ幸いである．

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