RANTES-28G. The analysis clearly showed a poorer survival in the group (ii) and a better survival in group (iii) when compared with group (i) ($P = 0.020$) (Fig. 1d). These results suggested that *RANTES-28G* had a protective effect against HIV-1 disease progression in Thai patients, and that *RANTES In1.1C* without *RANTES-28G* had an accelerating effect. We obtained virtually the same results as above when we used *RANTES-403A* instead of *RANTES In1.1C* (data not shown).

We then subtracted the numbers of days after patients started to receive ARV drugs from the observation period in order to adjust possible effects of ARV treatment, although the majority of our study subjects was ARV drug-free during the observation period. As shown in Fig. 1e–h, we obtained almost identical results to those shown in Figure 1a–d, except that difference between homozygous *IL4-589T* individuals and the others became statistically significant ($P = 0.034$, Fig. 1e). Thus, the risk of death for homozygous *IL4-589T* patients during the untreated period was lower than the other patients with heterozygous *IL4-589T* or homozygous *IL4-589C* (RR, 0.59 Cox model; 95% CI, 0.36–0.97; Fig. 1e). The RR of death for patients with *RANTES-28G* during the untreated period was also lower than those without *RANTES-28G* (RR, 0.43; 95% CI, 0.19–0.99; Fig. 1f). The RR of death for patients with *RANTES In1.1C* during the untreated period was not significantly higher than those without this allele (RR, 1.21; 95% CI, 0.74–1.98; Fig. 1g).

However, after adjustment for *RANTES-28G*, the RR of death for patients with *RANTES In1.1C* became significantly higher (adjusted RR, 1.75; 95% CI, 1.03–2.95). Similarly, the RR of death for patients with *RANTES-28G* became lower after adjustment for *RANTES In1.1C* (adjusted RR, 0.30; 95% CI, 0.12–0.74). These results were most likely caused by the fact that protective *RANTES-28G* was in strong linkage disequilibrium with deleterious *RANTES In1.1C* as shown in Table 2. Further adjustment for *IL4-589T* did not affect RR of death for patients with *RANTES-28G* (adjusted RR, 0.30; 95% CI, 0.12–0.74) or patients with *RANTES In1.1C* (adjusted RR, 1.79; 95% CI, 1.06–3.02). Similarly, adjustment for *RANTES-28G* and *RANTES Int1.1C* did not influence the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.59; 95% CI, 0.35–0.96). These results suggested that *IL4* and *RANTES* were independent factors involved in protection against HIV-1 disease progression.

Further adjustment for age at recruitment and previous ARV drug history did not affect the RR of death during the untreated period for homozygous *IL4-589T* patients (adjusted RR, 0.54; 95% CI, 0.32-0.89), patients with *RANTES-28G* (adjusted RR, 0.27; 95% CI, 0.11–0.68) or patients with *RANTES In1.1C* (adjusted RR, 1.94; 95% CI, 1.14–3.30). Adjustment for diagnosis of AIDS at the time of recruitment only slightly affected the RR of death for homozygous *IL4-589T* patients (adjusted RR, 0.61; 95% CI, 0.37–1.00; $P = 0.051$). However, the protective effect of homozygous *IL4-589T* became no longer significant after adjustment for serum virus load (adjusted RR, 0.76; 95% CI, 0.45–1.27; $P = 0.293$) or CD4 cell count (adjusted RR, 0.86; 95% CI, 0.51–1.44; $P = 0.563$). Adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment almost completely diminished the protective effect of homozygous *IL4-589T* (adjusted RR, 0.95; 95% CI, 0.56–1.62; $P = 0.854$). These results suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count. In a sharp contrast, the protective effect of *RANTES-28G* and the deleterious effect of *RANTES In1.1C* were not influenced by adjustment for virus load, CD4 cell count, and diagnosis of AIDS at the time of recruitment (adjusted RR of *RANTES-28G*, 0.27; 95% CI, 0.11–0.68; adjusted RR of *RANTES In1.1C*, 1.98; 95% CI, 1.15–3.41).

Discussion

This is the first host-genetic polymorphism study with a substantial sample size in the Southeast Asia, addressing an effect on HIV-1 disease progression. The *IL4-589T* allele was very common (0.78) in Thai patients as in other Asians – 0.69 in Japanese, 0.77 in Chinese – whereas it presents at a significantly lower frequency (0.15) in Caucasians [8,10,11]. For the *RANTES* gene, we observed more *RANTES-28G* (0.09) than in European and African–Americans [3,4,6]. Although our study group was not a sero-conversion cohort, we found a statistically significant association of the homozygous *IL4-589T* with a lower viral load, a higher CD4 cell count and a better survival during the untreated period. Individuals with *RANTES-28G* allele were also had a better survival rate.

The presence of *IL4-589T* allele was reported to be associated with an increased promoter activity [12]. Thus one expects the down-regulation of CCR5 and the up-regulation of CXCR4 in the CD4 T cell population in individuals with this allele [13]. Consequently, in individuals with *IL4-589T*, levels of replication of R5 viruses would be lower than in those without *IL4-589T*. It has been shown that R5 viruses were generally resistant to neutralizing antibodies whereas X4 viruses are sensitive, and X4 viruses are rarely detected until the advanced stage of HIV disease [14]. If it is the R5 viruses that are mainly responsible for driving immune deficiency in HIV infection, it is plausible that a higher expression of IL-4 associates with a better prognosis.

Two previous studies based on two independent cohorts in France showed a protective effect of *IL4-589T* on

HIV disease progression [8,9]. One paper based on the Amsterdam cohort showed an association of this allele with the delay of acquisition of X4 virus and the decline of CCR5 expressing memory CD4 T cell numbers, though this study did not show effects of *IL4-589T* on overall disease progression [10]. Our present study based on a cohort in Thailand is the third example to demonstrate the significant protective effect of *IL4-589T* on HIV disease progression. Only one previous report, which analyzed five USA natural history cohorts including several ethnic groups, failed to show such an association [11]. At present, the reason for the discrepancy among different studies is unclear, but may be due to the differences in study design (sero-conversion/cross-sectional, marker/endpoint of disease progression, duration of follow-up, etc.) or difference in the frequency of this allele. In addition, interactive effects may differ among different populations due to haplotype structure and allele frequency differences. As our study population was not a sero-conversion cohort, a considerable number of individuals had already progressed to end-stage HIV disease by the time of study enrollment. It is possible that individuals at the end stage of diseases might cause certain biases that we could not realize at present.

Compared with the most common *RANTES* haplotype (*RANTES In1.1T* and *RANTES-28C*), our study showed that individuals carrying *RANTES In1.1C* with *RANTES-28G* had better survival, while individuals carrying *RANTES In1.1C* without *RANTES-28G* had poorer survival. Our present finding on *RANTES-28G* was concordant with the previous study on Japanese patients [12], in whom the frequency of *RANTES-28G* was higher than in Thais. On the other hand, our result on *RANTES In.1C* was concordant with the previous results that *RANTES In1.1C* was associated with an increased rate of HIV-1 disease progression in African–Americans [3], in whom *RANTES-28G* is absent. Since most of *RANTES In1.1C* are associated with *RATNES-403A* in Thai patients (Table 2), our finding on *RANTES In1.1C* was also concordant with the previous results that *RANTES-403A* was associated with an increased rate of HIV-1 disease progression in European Americans [4], in whom the frequency of *RANTES-28G* was much lower than in Thais. Therefore, the discrepancy of the effect of *RANTES* polymorphisms in different studies may be due to the difference in *RANTES-28G* allele frequency in different ethnic groups. However, the differences in study design and effects of other genetic polymorphism background could not be excluded.

In contrast to the *IL4-589T* allele, the effect of *RANTES* polymorphisms on HIV-1 diseases was more clearly observed in the survival analysis (Fig. 1) than in baseline viral load and CD4 cell counts (Table 4). Analysis by the Cox proportional hazard models suggested that the protective effect of *IL4-589T* was mediated through a lower virus load and higher CD4 cell count, whereas effects of *RANTES* polymorphisms were totally independent from those factors. The reason for this discrepancy is not clear at present. One possible explanation for this discrepancy is that the *RANTES* polymorphisms affect HIV-1 disease progression through the mechanisms other than the direct suppressive effect of RANTES on HIV-1 entry. RANTES also affects trafficking and proliferation of cells expressing CCR1, CCR3 or CCR5 [15]. It is possible that, in individuals with different *RANTES* genotypes, certain types of cells expressing those chemokine receptors can be modulated to different extents, leading to the different degree of susceptibility to disease progression and/or opportunistic infections. It is noteworthy that numbers of CD8 cells, which are known to express more CCR5 than CD4 cells, also tended to be higher in patients with *RANTES-28G* than those without this allele in 246 drug-free Thai females (941 and 862 cells/μl, $P = 0.178$). Further studies are necessary to elucidate precise roles of RANTES in HIV-1 disease courses.

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Effects of human interleukin 7 on HIV-1 replication in monocyte-derived human macrophages

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Interleukin 7 (IL-7) contributes to development and proliferation of T cells. We investigated the effect of IL-7 on HIV-1 infected monocyte-derived human macrophages. IL-7 treatment of macrophages at a concentration of 10 ng/ml reduced replication of the R5 HIV-1 strain by approximately 50%. Meanwhile, HIV-1-infected macrophages themselves could excrete approximately 20% more IL-7 than uninfected macrophages. These results suggest that IL-7 could be used as a therapeutic modality to recover CD4 T cells.

Macrophages and CD4 T cells are the predominant cell types for HIV-1 infection [1,2]. Macrophages can be infected by HIV-1 in many tissues, including the brain, lung and lymph nodes [3]. Unlike infected CD4 T cells, which have a short half-life of 1–1.5 days, macrophages are quite resistant to the cytopathic effect of the virus, and may thus provide a reservoir for persistent infection and virus dissemination. It is known that cytokines play an important role in HIV-1 infection. Interleukin-7 (IL-7) is a cytokine produced by stromal cells of the thymus and bone marrow [4–6]. It has the capacity to induce growth of immature B lymphocytes [7], and contributes to development and proliferation of T cells [8–11]. With respect to HIV-1 infection, there is a reverse correlation between CD4 T cell number and IL-7 serum levels in HIV-1-infected patients [12–14]. After patients started to receive antiretroviral drugs, the elevated IL-7 in the serum decreased to normal levels [12]. On the other hand, IL-7 increases HIV-1 replication in thymic organ cultures [15–17] and induces latent HIV-1 in resting CD4 T cells [18]. However, effects of IL-7 on HIV-1 replication in macrophages remain unclear. In the present study, we evaluated levels of HIV-1 proliferation in monocyte-derived human macrophages treated with or without exogenous IL-7.

Peripheral blood mononuclear cells (PBMC) from blood buffy coats of healthy donors were isolated by centrifugation through Ficoll-Hypaque and plated on a 24-well MULTIWELL™ PRIMARIA™ plate (Becton Dickinson, Franklin Lakes, New Jersey, USA) with RPMI 1640 supplemented with 10% fetal calf serum (FCS). After incubation at 37°C for 1 day, the floating cells were removed by washing the plate with phosphate-buffered saline four times and the adherent cells were incubated at 37°C for 11 days with 0.5 ml of RPMI 1640 supplemented with 10% FCS plus 100 ng/ml of granulocyte macrophage colony stimulating factor (GM–CSF).

To determine whether or not differentiated macrophages express the IL-7 receptor, we analysed total RNA extracted from macrophages. PBMC stimulated with phytohaemagglutinin (PHA) and 10 ng/ml of IL-2 for 3 days and monkey kidney CV-1 cells served as positive and negative controls, respectively. RNA was reverse-transcribed into cDNA, and levels of IL-7 receptor cDNA were measured by an ABI 7500 Real-time PCR System. As shown in Fig. 1a, IL-7 receptor mRNA in macrophages was clearly detected. It was also detected in PHA and IL-2-stimulated PBMC. Levels of IL-7 receptor mRNA in macrophages were approximately one-tenth of those in PHA and IL-2 stimulated PBMC, while CV-1 cells were totally negative for expression of the IL-7 receptor.

Macrophages were infected with 6.5 ng of p24 of HIV-1 SF162 strain for 2 h on day 12. Cells were then incubated with 1 ml of RPMI 1640 supplemented with 10% FCS plus 100 ng/ml of GM–CSF and 0, 3 or 10 ng/ml of IL-7 (Techne, Minneapolis, Minnesota, USA). Infected macrophages were fed on days 3, 6, 9 and 12 (after infection) by exchanging one-half of the culture supernatant with fresh media containing the same amount of cytokines. Levels of HIV-1 p24 antigen in culture supernatants on days 1, 3, 6, 9 and 12 were measured by using an HIV-1 P24 Antigen ELISA Kit (Fig. 1b). The p24 antigen level in the culture supernatant of macrophages treated with 3 ng/ml of IL-7 was apparently less than that of macrophages without IL-7 treatment (Fig. 1b). This difference was evident on day 3 and became greater with time. On day 12, the p24 level of macrophages treated with 3 ng/ml of IL-7 was 2220 pg/ml while that of untreated macrophages was 3542 pg/ml. That is, the suppression with 3 ng/ml of IL-7 on HIV-1 replication was 37.4% on day 12. Treatment of macrophages with 10 ng/ml of IL-7 showed a greater suppressive effect on HIV-1 replication (Fig. 1b). On day 12, the p24 level of macrophages treated with 10 ng/ml of IL-7 was 1760 pg/ml. That is, suppression with 10 ng/ml of IL-7 on HIV-1 replication was 50.3% on day 12.

Monocyte-derived human macrophages were also infected with the Z strain of the Sendai virus. Hemagglutination assay (HA) titres were measured on days 1, 3 and 6 after infection. As shown in Fig. 1c, there

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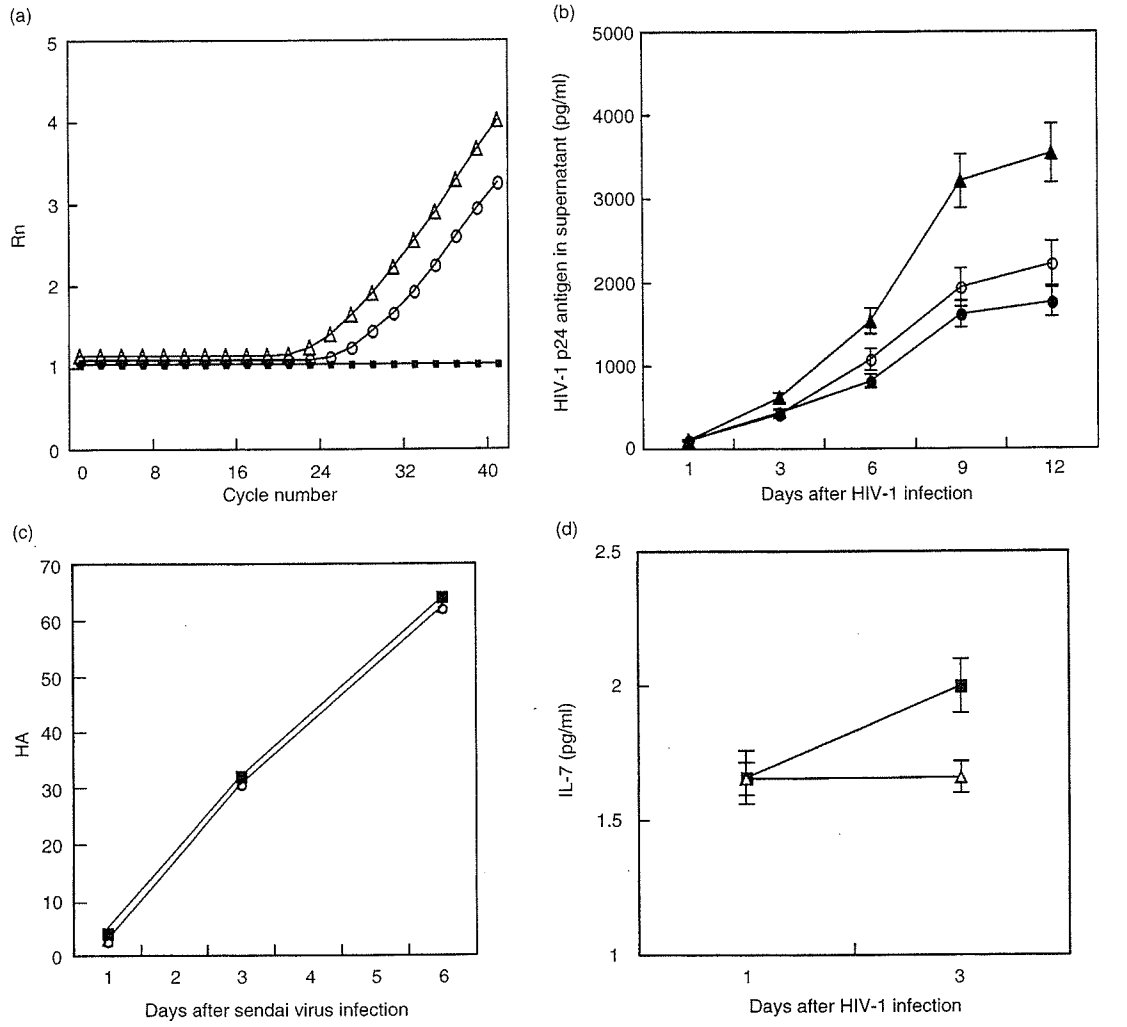

Fig. 1. (a) Expression of IL-7 receptor mRNA in macrophages measured by real-time monitoring of fluorescence signals. IL-7 receptor mRNA in macrophages (open circles), PHA-stimulated PBMC (open triangles) as the positive control and CV-1 cells (closed squares) as the negative control are shown. Rn is the ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye. Data shown are representative of two independent experiments with similar results. (b) The HIV-1 p24 antigen in the supernatant of HIV-1 infected macrophages treated with 0 (closed triangles), 3 (open circles) or 10 ng/ml (closed circles) of IL-7 was measured on days 1, 3, 6, 9 and 12 after HIV-1 infection. Data show the mean of duplicate samples from the same blood donor with bars indicating the actual fluctuation between two individual measurements. Data shown are the representative of three independent experiments with similar results. (c) Growth of the Sendai virus in monocyte-derived macrophages treated with 10 ng/ml of IL-7 (open circles) or untreated with IL-7 (closed squares). HA titres in culture supernatants were measured on days 1, 3 and 6 after infection. Data shown are representative of two independent experiments with similar results. (d) Levels of IL-7 in the supernatants of HIV-1 infected macrophages (closed squares) or uninfected macrophages (open triangles) were measured on days 1 and 3 after HIV-1 infection. Data show the mean of duplicate samples from the same blood donor with bars indicating the actual fluctuation between two individual measurements. Data shown are representative of two independent experiments with similar results.

was no difference in HA titres between IL-7-treated and untreated macrophages, indicating no apparent effect of IL-7 on Sendai virus replication in macrophages.

Levels of IL-7 in supernatants of macrophages were measured on days 1 and 3 after HIV-1 infection (Fig. 1d). On day 1, there was no difference in levels of IL-7 between HIV-1 infected and uninfected macrophages. On day 3, IL-7 levels in HIV-1-infected macrophages slightly increased, while those in uninfected macrophages did not.

The mechanisms of the suppressive effect of IL-7 on R5 HIV-1 replication in macrophages are still not clear. A previous study showed that IL-7 treatment for PBMC enhanced excretion of β chemokines including MIP-1β, which can suppress R5 HIV-1 replication [19]. We pretreated macrophages with 10 ng/ml of IL-7 for 1 day,

and then inoculated them with HIV-1. However, we failed to detect any further suppression of HIV-1 replication compared with macrophages treated with IL-7 only after HIV-1 infection (data not shown). Therefore, it is unlikely that IL-7 could suppress HIV-1 replication by upregulation of MIP-1β. It is possible that IL-7 could affect HIV-1 replication after HIV-1 enters macrophages.

We have shown that HIV-1-infected macrophages could excrete more IL-7 than uninfected ones. Therefore, it is likely that IL-7 is a self-defence system for macrophages against HIV-1 infection. Furthermore, our finding that IL-7 could moderately suppress HIV-1 replication in macrophages suggested the advantage of IL-7 as an immune modulator which could be used to recover CD4 T-cell numbers in HIV-1-infected individuals.

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Interferon-γ decreases replication of primary R5 HIV-1 isolates in thymocytes

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As interferon-γ (IFN-γ) is produced at lower levels by neonatal than adult T cells, we determined whether IFN-γ influences HIV-1 replication in thymocytes. IFN-γ significantly decreased replication of R5 but not X4 viruses, and reduced depletion of $CD3^{hi}CD27$ (mature) thymocytes, the preferential targets for R5 HIV-1. Thus infection and

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A dominant-negative effect of cynomolgus monkey tripartite motif protein TRIM5α on anti-simian immunodeficiency virus SIVmac activity of an African green monkey orthologue

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Abstract

African green monkey (AGM) tripartite motif protein (TRIM) 5α can inhibit both human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIVmac, whereas cynomolgus monkey (CM) TRIM5α can inhibit HIV-1, but not SIVmac. We previously reported that the 17-amino-acid region and an adjacent 20-amino-acid duplication in the SPRY(B30.2) domain of AGM TRIM5α determined the species specificity. In the present study, we demonstrated that CM TRIM5α had a dominant-negative effect on the anti-SIVmac activity of AGM TRIM5α. In contrast, mutant TRIM5αs lacking the 20-amino-acid duplication did not have the dominant-negative effect, even though they failed to restrict SIVmac. These results indicated that oligomerization of the SPRY domain is required for anti-SIVmac activity and suggest that tight interaction between the viral capsid and all three molecules in one TRIM5α trimer may not be necessary for restriction activity.

Keywords: TRIM5α; Human immunodeficiency virus; Simian immunodeficiency virus; SPRY domain; Dominant-negative; African green monkey; Cynomolgus monkey

Introduction

Human immunodeficiency virus type 1 (HIV-1) efficiently enters cells of Old World monkeys but encounters a block before reverse transcription (Himathongkham and Luciw, 1996; Hofmann et al., 1999; Shibata et al., 1995). This restriction is mediated by a dominant-repressive factor, tripartite motif protein (TRIM) 5α (Stremlau et al., 2004). Rhesus and cynomolgus monkey (CM) TRIM5α restricted HIV-1 infection (Nakayama et al., 2005), whereas human TRIM5α restricts N-tropic murine leukemia virus (N-MLV) infection (Hatziioannou et al., 2004; Keckesova et al., 2004; Perron et al., 2004; Yap et al., 2004). African green monkey (AGM) TRIM5α restricts simian immunodeficiency virus SIVmac, human immunodeficiency virus type 2 (HIV-2), and equine infectious anemia virus in addition to HIV-1 infection (Hatziioannou et al., 2004; Keckesova et al., 2004; Nakayama et al., 2005). TRIM5α shares a common amino-terminal TRIM motif, comprising RING, B-box, and coiled-coil domains, with other splicing variants, and encodes a unique SPRY (B30.2) domain (Reymond et al., 2001). Several recombinant studies between human and rhesus monkey TRIM5α revealed that the determinant of the species specificity lies in the SPRY (B30.2) domain of TRIM5α (Perez-Caballero et al., 2005; Sawyer et al., 2005; Stremlau et al., 2005; Yap et al., 2005). We also previously demonstrated that 17-amino-acid residues and the adjacent AGM-specific 20-amino-acid duplication in the SPRY domain determined species-specific restriction of SIVmac (Nakayama et al., 2005). Owl monkey TRIM5 is fused with cyclophilin A (cypA) within the SPRY domain and has strong anti-HIV-1 activity (Nisole et al., 2004; Sayah et al., 2004). Structural predictions suggested that the coiled-coil domain exhibits a propensity to form oligomers. It was first reported that rhesus TRIM5γ, a splice variant lacking SPRY (B30.2) domain, had a dominant-negative effect on HIV-1 restriction activity of rhesus TRIM5α (Stremlau et al., 2004).

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Recently, Berthoux et al. reported that expression of rhesus monkey TRIM5α or owl monkey TRIM5-cypA in human cells interferes with the anti-N-MLV activity of endogenous human TRIM5α (Berthoux et al., 2005). Here, we show that CM TRIM5α lacking anti-SIVmac activity had a dominant-negative effect on the anti-SIVmac activity of AGM TRIM5α.

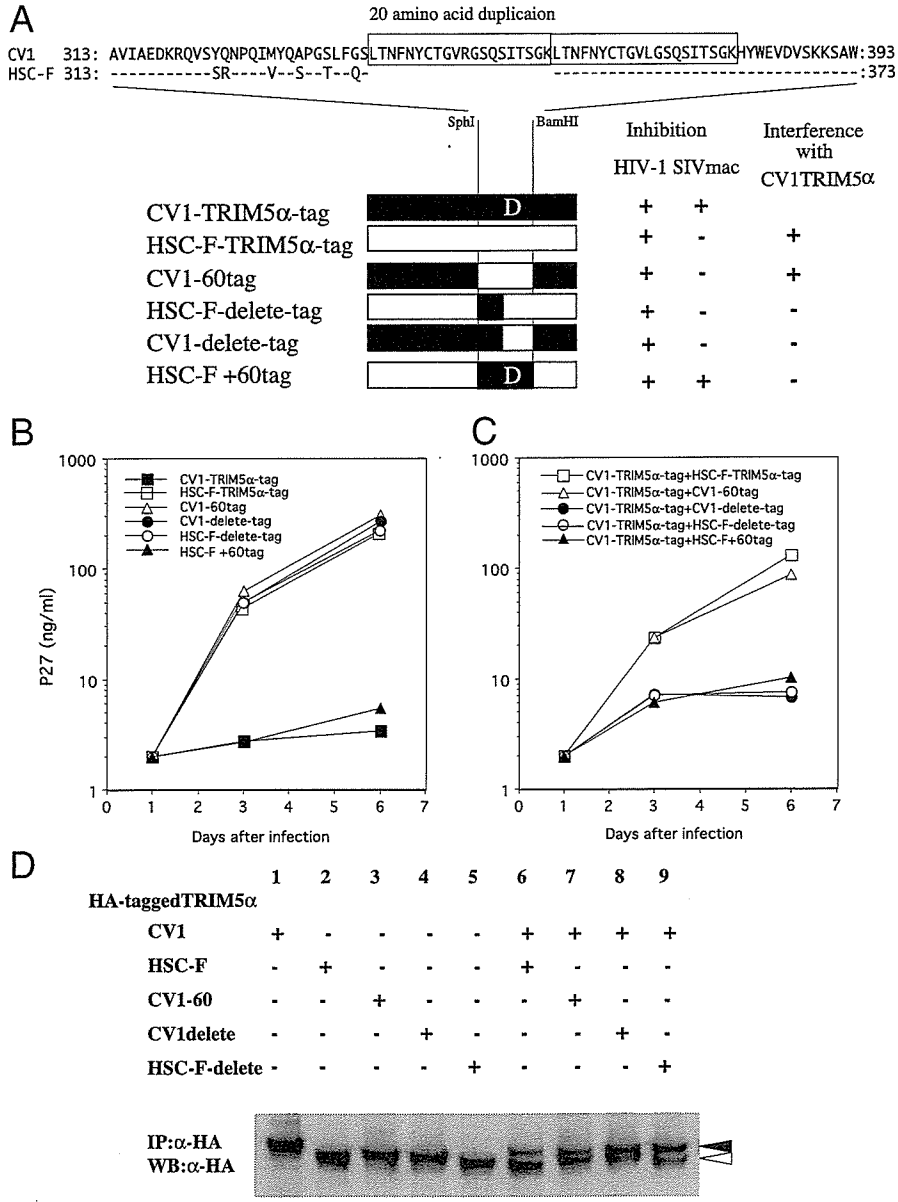

Fig. 1. (A) Schematic representation of chimeric tripartite motif protein (TRIM) 5α and summary of the results. Alignment of amino-acid sequences of the highly variable region of the SPRY (B30.2) domain of CV1 and HSC-F TRIM5αs is shown on the top. Boxes in amino-acid sequence denote the CV1-TRIM5α-specific 20-amino-acid duplication. Filled and open bars denote CV1 and HSC-F sequences, respectively. "D" denotes the CV1-TRIM5α-specific 20-amino-acid duplication. + and − on the column of inhibition denote full and no suppression, respectively. + and − on the column of interference denote presence and absence of the dominant-negative effect on anti-SIVmac activity of CV1-TRIM5α, respectively. Anti-human immunodeficiency virus type 1 (HIV-1) activity of those TRIM5αs was reported previously (Nakayama et al., 2005). (B) MT4 cells infected with 10 plaque-forming units per cell of a recombinant Sendai virus (SeV) expressing hemagglutinin (HA)-tagged version of CV1-TRIM5α-tag (closed squares), HSC-F-TRIM5α-tag (open squares), CV1-60tag (open triangles), CV1-delete-tag (closed circles), HSC-F-delete-tag (open circles), or HSC-F +60tag (closed triangles) were challenged with simian immunodeficiency virus SIVmac239, and culture supernatants were assayed for levels of p27. The representative results of three independent experiments with similar results are shown. (C) MT4 cells co-infected with 5 plaque-forming unit per cell of an SeV expressing CV1-TRIMα-tag and 5 plaque-forming unit per cell of an SeV expressing HSC-F-TRIM5α-tag (open squares), CV1-60tag (open triangles), CV1-delete-tag (closed circles), HSC-delete-tag (open circles), or HSC-F +60tag (closed triangles) were challenged with SIVmac239, and culture supernatants were assayed for levels of p27. The representative result of three independent experiments with similar results is shown. (D) Comparison of expression levels of HA-tagged TRIM5α proteins. MT4 cells were infected with SeVs expressing the indicated TRIM5α protein at a multiplicity of infection of 10 plaque-forming unit per cell (lanes 1 to 5). In cases 2 recombinant SeVs were simultaneously infected, 5 plaque-forming units per cell of each SeV were inoculated (lanes 6 to 9). Twelve hours after SeV infection, cells were lysed and immunoprecipitated by an anti-HA antibody. A closed triangle indicates HA-tagged CV1-TRIM5α, which contained the 20-amino-acid duplication. An open triangle indicates an HA-tagged HSC-F-TRIM5α (lanes 2 and 6), CV1-60 (lanes 3 and 7), CV1-delete (lanes 4 and 8), and HSC-F-delete (lanes 5 and 9), lacking the 20-amino-acid duplication.

Results

Among Old World monkey cells, an AGM kidney cell line CV1 is resistant to not only HIV-1, but also simian immunodeficiency virus SIVmac. TRIM5α cDNA of CV1 and HSC-F cells, a T cell line from a CM, were cloned into Sendai virus vectors (SeVs). As described previously (Nakayama et al., 2005), both CV1- and HSC-F-TRIM5αs inhibited HIV-1 infection (Fig. 1A). CV1-TRIM5α could also inhibit SIVmac infection, whereas HSC-F-TRIM5α could not (Figs. 1A, B). A comparison of the CV1 and HSC-F TRIM5α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain. In this region, CV1-TRIM5α had a 20-amino-acid duplication, which was totally absent in HSC-F-TRIM5α (Fig. 1A). We constructed chimeric TRIM5αs from hemagglutinin (HA)-tagged version of HSC-F-TRIM5α and CV1-TRIM5α by using *Sph*I and *Bam*HI restriction enzyme digestion (Fig. 1A). HSC-F +60tag contained the 242 bp *Sph*I–*Bam*HI fragment of CV1-TRIM5α in the background of HA-tagged version of HSC-F-TRIM5α. The reciprocal chimera, CV1-60tag, contained a corresponding 182 bp fragment of HSC-F-TRIM5α in the background of HA-tagged version of CV1-TRIM5α. In this *Sph*I–*Bam*HI fragment, the differences between CV1 and HSC-F TRIM5α were located in a small region of 37-amino-acid residues including CV1-specific 20-amino-acid duplication and adjacent 17-amino-acid residues. In CV1-delete-tag, CV1-specific 20-amino-acid duplication was deleted by PCR mutagenesis. In HSC-F-delete-tag, the *Sph*I–*Bam*HI fragment of HSC-F-TRIM5α was replaced with the corresponding fragment of CV1-delete-tag. As shown in Fig. 1B, HSC-F +60tag, the chimeric TRIM5α containing 37-amino-acid residues from CV1-TRIM5α in the background of HSC-F-TRIM5α fully acquired the ability to inhibit SIVmac infection. Conversely, all the chimeric TRIM5αs lacking the CV1-specific 20-amino-acid duplication (CV1-60tag, CV1-delete-tag, and HSC-F-delete-tag) failed to restrict SIVmac infection (Figs. 1A, B). All the mutant TRIM5αs described above showed potent anti-HIV-1 activity as described previously (Fig. 1A) (Nakayama et al., 2005).

In the present study, we examined whether or not simultaneous expression of HSC-F-TRIM5α or TRIM5α SPRY domain mutants that failed to inhibit SIVmac infection altered the anti-SIVmac activity of CV1-TRIM5α. Human T cell line MT4 cells were first infected with an SeV expressing CV1-TRIM5α-tag and an SeV expressing HSC-F-TRIM5α-tag, CV1-60tag, CV1-delete-tag, HSC-F-delete-tag, or HSC-F +60tag, incubated at 37 °C for 9 h, and then challenged with SIVmac239. As shown in Fig. 1C, HSC-F-TRIM5α and CV1-60tag strongly interfered with the anti-SIVmac activity of CV1-TRIM5α-tag. The HSC-F-TRIM5α and CV1-60tag shared the 17-amino-acid region of the HSC-F-TRIM5α SPRY domain and lacked the CV1-specific 20-amino-acid duplication.

Contrary to our expectation, however, the CV1-delete-tag and HSC-F-delete-tag, which failed to restrict SIVmac infection, also failed to show the dominant-negative effect on anti-SIVmac activity of CV1-TRIM5α-tag. Both of these mutant TRIM5αs lacked the CV1-specific 20 amino-acid duplication but shared the 17-amino-acid region of the SPRY domain with the parental CV1-TRIM5α. These results indicated that the ability of TRIM5α to interfere with the anti-SIVmac activity of CV1-TRIM5α was not only determined by its lack of restriction activity against SIVmac. On the other hand, the HSC-F +60-tag that inhibited SIVmac infection (Fig. 1B) did not interfere with the restricting activity of CV1-TRIM5α-tag (Fig. 1C).

The levels of expression of those TRIM5αs were comparable to each other (Fig. 1D lanes 1 to 5). Co-expression of CV1-TRIM5α-tag did not alter the levels of expression of those TRIM5αs (Fig. 1D, lanes 6 to 9). Conversely, the expression levels of co-expressed CV1-TRIM5α-tag were also comparable to each other (Fig. 1D, lanes 6 to 9).

A single round replication assay further confirmed the above findings since co-expression of HSC-F-TRIM5α-tag or CV1-60tag with CV1-TRIM5α-tag strongly interfered with anti-SIVmac activity of CV1-TRIM5α-tag in CD4 negative HeLa cells infected with vesicular stomatitis virus G-protein-pseudotyped SIVmac. Similarly, co-expression of CV1-delete-tag or HSC-F-delete-tag with CV1-TRIM5α-tag did not interfere with anti-SIVmac activity of CV1-TRIM5α-tag in a single round replication assay (Fig. 2).

To assess whether or not these parental and chimeric TRIM5αs really form hetero-oligomer, we performed immunoprecipitation followed by Western blot analysis. We used hamster kidney cell line TK-ts13 since it lacks primate TRIM5α proteins. Cells were transfected with plasmid expressing various HA-tagged version of chimeric TRIM5αs with a plasmid expressing myc-tagged version of CV1-TRIM5α. Sixty hours after transfection, cells were lysed and TRIM5α proteins were immunoprecipitated with an antibody against HA. Resultant immunoprecipitants were subjected to Western blot analysis using

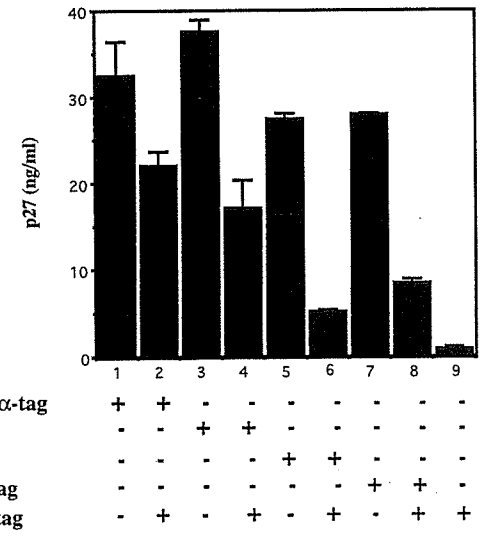

Fig. 2. A single round replication assay. CD4-negative human cell line HeLa was infected with an SeV expressing indicated TRIM5α at a multiplicity of infection of 10 plaque-forming unit per cell (columns 1, 3, 5, 7, and 9) and challenged with vesicular stomatitis virus glycoprotein-pseudotyped SIVmac239. In cases 2 recombinant SeVs were simultaneously infected, 5 plaque-forming units per cell of each SeV were inoculated (columns 2, 4, 6, and 8). Three days after infection, culture supernatants were assayed for levels of p27. Bars show actual fluctuations of duplicate samples.

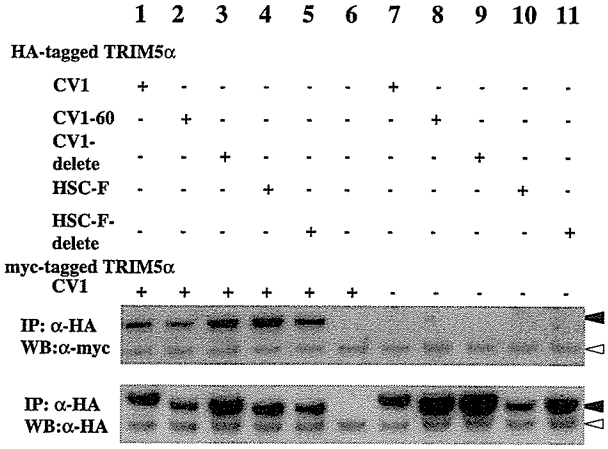

Fig. 3. Co-precipitation analysis of TRIM5α multimerization. For lanes 1 to 5, hamster TK-ts13 cells were transiently co-transfected with a plasmid expressing myc-tagged version of CV1-TRIM5α protein along with a plasmid expressing an indicated HA-tagged TRIM5α. For lanes 6 to 11, cells were co-transfected with an empty vector pcDNA3.1(−) along with a plasmid expressing an indicated HA-tagged version of TRIM5α protein. Cell lysates were subjected for immunoprecipitation by using an anti-HA antibody, and immunoprecipitates were analyzed by Western blot by using antibody indicated. Closed triangles denote TRIM5α proteins. Open triangles denote non-specifically reacted protein.

anti-myc or anti-HA antibodies as probe. As shown in Fig. 3B, myc-tagged CV1-TRIM5αs were clearly co-immunoprecipitated with HA-tagged version of CV1-, CV1-60, CV1-delete, HSC-F, and HSC-F-delete TRIM5α proteins. These results suggested that not only homologous but also heterologous oligomers of TRIM5α examined above were formed in cells. A heat shock protein inhibitor geldanamysin was reported to inhibit cytoplasmic body formation (Song et al., 2005). Addition of 10 μM of geldanamysin in the culture media did not affect the homo- or hetero-oligomerization of TRIM5αs (data not shown), excluding the possibility that co-immunoprecipitation of CV-TRIM5α with various TRIM5αs was caused by non-specific aggregation of TRIM5α proteins in cytoplasmic bodies. Therefore, the lack of the dominant-negative effect of chimeric TRIM5α on anti-SIVmac activity of CV-1-TRIM5α was not due to a lack of oligomer formation between CV1 and chimeric TRIM5αs.

Discussion

It was first reported that rhesus monkey TRIM5γ, a splice variant lacking SPRY (B30.2) domain, had a dominant-negative effect on HIV-1 restriction activity of rhesus monkey TRIM5α (Stremlau et al., 2004). Subsequently, Berthoux et al. reported that expression of rhesus monkey TRIM5α or owl monkey TRIM5-cypA in human cells interferes with the anti-N-MLV activity of endogenous human TRIM5α (Berthoux et al., 2005). Perez-Caballero et al. reported that truncated human TRIM5α proteins lacking the B30.2/SPRY domain form heteromultimers with full-length human TRIM5α and are dominant inhibitors of its N-MLV restriction (Perez-Caballero et al., 2005). Furthermore, Mishe et al. clearly showed that the TRIM5α protein oligomerizes into trimers and proposed that trimerization potentially allows SPRY domain to interact with threefold pseudosymmetrical structures on retroviral capsids (Mishe et al., 2005).

In the present study, we demonstrated that the HSC-F-TRIM5α had a dominant-negative effect on the anti-SIVmac activity of CV1-TRIM5α. A chimeric TRIM5α carrying the HSC-F-derived 17-amino-acid residues without the CV1-specific 20-amino-acid duplication (CV1-60tag) also showed a dominant-negative effect. These results emphasized the importance of the 17-amino-acid residue as well as the 20-amino-acid duplication of CV1-TRIM5α in the restriction of SIVmac infection and further suggested that the oligomeric structure of SPRY domain is required for the restriction of SIVmac infection by CV1-TRIM5α. Nevertheless, two mutant TRIM5αs carrying the CV1-derived 17-amino-acid region without the 20-amino-acid duplication (HSC-F-delete-tag and CV1-delete-tag) did not show the dominant-negative effect, even though all the chimeric and mutant TRIM5αs mentioned above failed to restrict SIVmac as the parental HSC-F-TRIM5α-tag and CV1-60tag. Immunoprecipitation and Western blot analysis of cells co-transfected with myc-tagged version of CV1-TRIM5α and HA-tagged versions of those chimeric and mutant TRIM5αs confirmed their heterologous oligomers formation regardless of the presence or absence of a dominant-negative effect on anti-SIVmac activity of CV1-TRIM5α. These results indicated that it is not necessary for all the TRIM5α molecules in one trimer to carry the 20-amino-acid duplication for anti-SIVmac activity. It is possible that tight interaction between the viral capsid and all three TRIM5α molecules in one trimer may not be necessary for restriction activity. Alternatively, a certain capsid modification, which is not observed on all the capsid molecules in the virions, may mimic the CV1-specific 20-amino-acid duplication.

It is expected that four types of trimer form in heterologous TRIM5α expressing cells. In cells expressing equal amount of CV1-TRIM5α and HSC-F-TRIM5α, (1) one out of eight trimers would be a homo-trimer of CV1-TRIM5α molecules, (2) three out of eight trimers would be hetero-trimers composed of two molecules of CV1-TRIM5α and one molecule of HSC-F-TRIM5α, (3) three out of eight trimers would be hetero-trimers composed of one molecules of CV1-TRIM5α and two molecules of HSC-F-TRIM5α, and (4) one out of eight trimers would be a homo-trimer of HSC-F-TRIM5α molecules. The CV1-TRIM5α homo-trimer would completely inhibit SIVmac infection, whereas HSC-F-TRIM5α homo-trimer would fail to inhibit SIVmac. We showed that cells expressing CV1-TRIM5α-tag and HSC-F-TRIM5α-tag produced approximately two third of p27 antigen of the cells expressing HSC-F-TRIM5α-tag alone (Fig. 2). The slight reduction of p27 antigen production is most likely caused by the presence of at least one out of eight TRIM5α trimers possessing anti-SIVmac activity. Conversely, cells co-expressing CV1-delete-tag or HSC-F-delete-tag with CV1-TRIM5α-tag showed slight but significant increase in p27 production upon SIVmac infection compared with those expressing CV1-TRIM5α-tag alone. This is again explained by the presence of one out of eight TRIM5α trimers lacking anti-SIVmac activity. Therefore, determination of the exact numbers of molecules carrying the 20-amino-acid duplication within an

active TRIM5α trimer against SIVmac would be important to help understand the precise molecular mechanisms of anti-viral activity of TRIM5α.

Materials and methods

Construction of chimeric TRIM5α expressing plasmid

Construction of parental and chimeric TRIM5αs carrying an HA tag (YPYDVPDYAA) at their C-termini was described previously (Nakayama et al., 2005). Briefly, cloned CV1-TRIM5α and HSC-F-TRIM5α cDNAs in pcDNA3.1 were used as templates for PCR amplification with a primer containing a nucleotide sequence corresponding to the HA-tag fused with the C-terminal portion of TRIM5α. The C-terminal portion of TRIM5α fused with the HA-tag (*Bam*HI to *Not*I) and the N-terminal portion of TRIM5α (*Not*I to *Bam*HI) were assembled in a pCEP4 vector (Invitrogen). To generate chimeric TRIM5α HSC-F +60tag, the 182 bp *Sph*I–*Bam*HI fragment of HSC-F-TRIM5α-tag was replaced with the corresponding 242 bp *Sph*I–*Bam*HI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Conversely, the 242 bp *Sph*I–*Bam*HI fragment of CV1-TRIM5α was replaced with the 182 bp *Sph*I–*Bam*HI fragment of HSC-F-TRIM5α-tag in the background of CV1-TRIM5α-tag to generate CV1-60tag. CV1-delete-tag, which possessed the 3′ proximal 98 bp of the *Sph*I–*Bam*HI fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, was generated by a PCR-based mutagenesis of CV1-TRIM5α-tag. The *Sph*I–*Bam*HI fragment of HSC-F-TRIM5α was replaced with the corresponding fragment of CV1-delete-tag to generate HSC-F-delete-tag.

SIVmac infection

Construction of recombinant SeVs expressing various TRIM5αs was described previously (Nakayama et al., 2005). For CD4-dependent infection assays, 2.5×10^5 MT4 cells were infected with SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, CV1-60tag, HSC-F-delete-tag, CV1-delete-tag, or HSC-F +60tag at a multiplicity of infection of five plaque-forming units per cell for each SeV and incubated at 37 °C for 9 h. Cells were then super-infected with 30 ng of p27 of SIVmac239. The culture supernatants were collected periodically, and the level of p27 was measured with a RETROtek antigen ELISA kit (ZeptoMetrix). For a single round replication assay, CD4-negative HeLa cells were infected with SeV expressing various TRIM5αs at a multiplicity of infection of 5 plaque-forming unit per cell. Cells were then super-infected with 30 ng of p27 of vesicular stomatitis virus G-protein-pseudotyped SIVmac239.

Immunoprecipitation and Western blot analysis

For co-immunoprecipitation analysis, we constructed CV1-TRIM5α carrying myc-tag (EQKLISEEDL) at its C-termini and cloned into pcDNA3.1(−) vector (Invitrogen). Hamster kidney TK-ts13 cells were co-transfected with 10 μg of a plasmid expressing myc-tagged version of CV1-TRIM5α and 10 μg of a plasmid expressing HA-tagged TRIM5α by using calcium phosphate method. Sixty hours after transfection, cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonident P40, 0.5% sodium deoxycholate). TRIM5α proteins in the lysates were precipitated with anti-HA High Affinity rat monoclonal antibody (Roche) using a Protein G Immunoprecipitation Kit (Roche). Precipitated materials were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins in the gel were then electronically transferred to a membrane (Immobilon, Millipore). Blots were blocked and probed with anti-myc mouse monoclonal antibody (9B11, Cell Signaling) or anti-HA High Affinity rat monoclonal antibody (Roche) overnight at 4 °C. Blots were then incubated with peroxidase-conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories) or anti-rat IgG (American Qualex), and bound antibodies were visualized with a Chemi-lumi-one chemiluminescent kit (Nacalai tesque, Kyoto, Japan).

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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 dropout 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

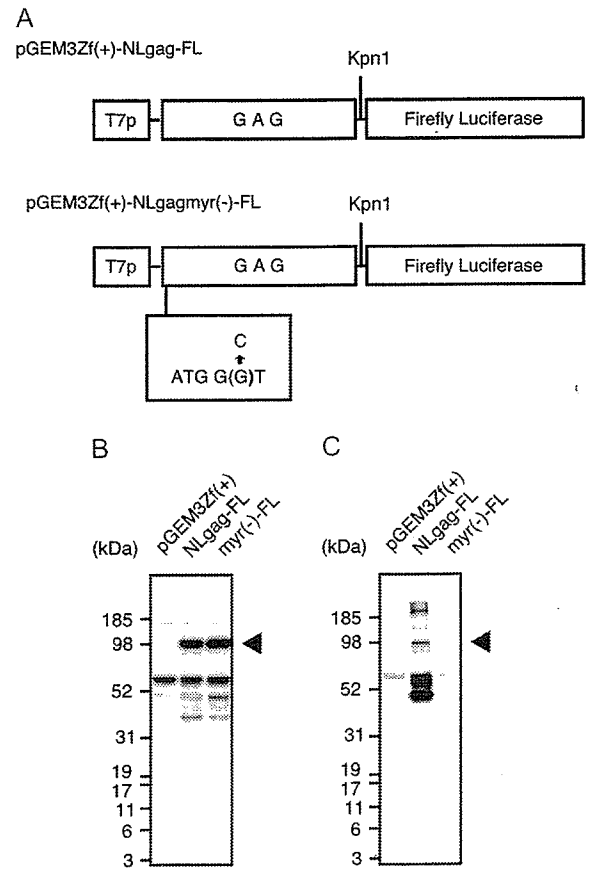

Fig. 1. Expression of Gag–Firefly Luciferase fusion protein in higher eukaryote cells. (A) Schematic representation of expression constructs for 293T cells. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of 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

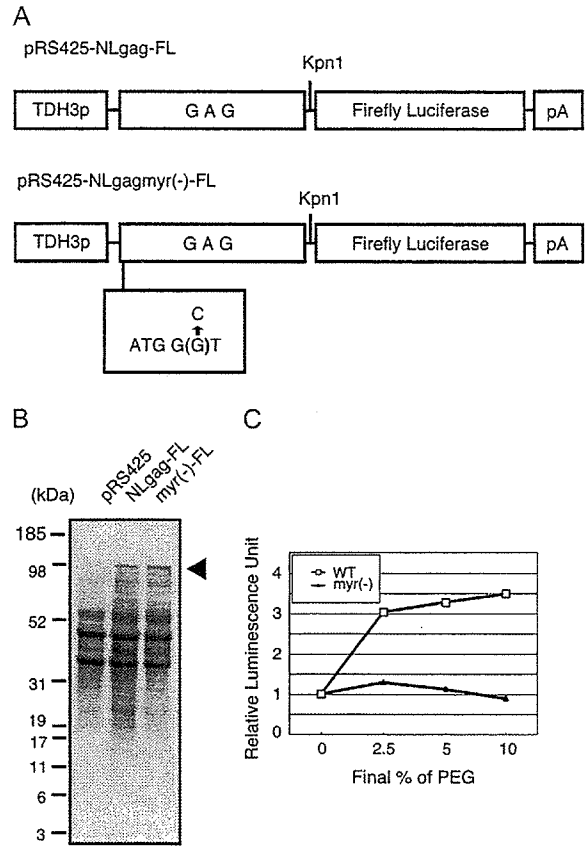

Fig. 2. Expression of Gag–Firefly Luciferase fusion protein in *S. cerevisiae*. (A) Schematic presentation of constructs for expression in *S. cerevisiae*. In the case of myristoylation signal mutants of Gag protein, the second amino acid glycine was substituted with alanine. (B) Western blotting of 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.

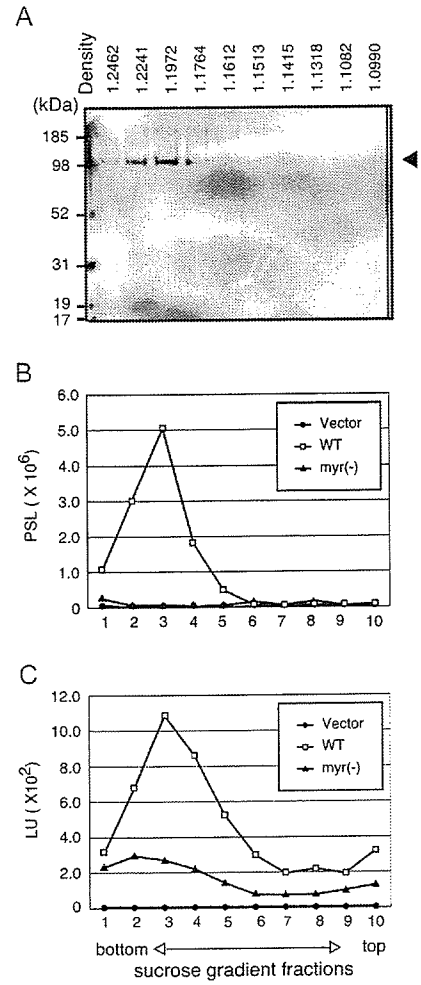

Fig. 3. Detection of VLPs released from spheroplasts of *S. cerevisiae*. (A) Western blotting of fractions of sucrose density gradient centrifugation with serum from an HIV-1 patient. (B) Digitizing of band intensity corresponding to Gag–Firefly Luciferase fusion protein on Western blotting. (C) Luciferase 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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Human Genetic Polymorphisms Affecting HIV-1 Diseases

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Abstract

Human immunodeficiency virus type 1 (HIV-1) infection is generally characterized by a long-term, chronic disease course that gradually progresses to acquired immunodeficiency syndrome (AIDS). However, a small fraction of HIV-1–infected individuals remain both clinically and immunologically healthy for 10 years or more after seroconversion. Conversely, the disease of another significant fraction is characterized by an extremely rapid progression to AIDS within 1 year. There are also individuals not infected with HIV-1 who have had repetitive sexual exposure to HIV-1 in extremely high-risk situations. Determining the host factors involved in these different susceptibility and disease courses would be helpful for better understanding AIDS and its control. Today, a worldwide scientific endeavor called the Human Genome Project has completed the production of a full-length sequence of the 3 billion base pairs that make up the human genome. The Human Genome Project has also revealed the presence of variation in human genomes. Relating these genetic differences to human phenotypes offers a very attractive prospect for a genetic understanding of the different sensitivities to various human diseases, including cancer, brain and heart diseases, allergy, and infectious diseases. In this review, we present examples of human genetic variation that can affect HIV-1 infection and disease progression.

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Key words: HIV-1; AIDS; SNP; Chemokine; Cytokine

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) infection is generally characterized by a long-term, chronic disease course that gradually progresses to acquired immunodeficiency syndrome (AIDS). However, there are a few strikingly different scenarios. A small fraction of HIV-1–infected individuals remain clinically and immunologically healthy for 10 years or more after seroconversion. Conversely, the disease of another significant fraction of patients is characterized by extremely rapid progression within a period as short as 1 year. Determining the host factors involved in these different disease courses would be extremely helpful for better understanding AIDS and its control.

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2. Host Factors Involved in HIV-1 Entry into Target Cells

Figure 1 shows host factors involved in HIV-1 entry. CD4 is the main HIV-1 receptor for attachment. However, HIV-1 also needs a coreceptor for entry into target cells. In 1996, chemokine receptors CCR5 and CXCR4 were reported to be HIV-1 coreceptors. CCR5 is a coreceptor used by R5 strains of HIV-1, which are more likely to be transmitted through sexual contact and are present early after infection in nearly all HIV-1–infected individuals. CC chemokines CCL5 (or RANTES [regulated on activation normal T-cell expressed and secreted]), CCL3 (or MIP-1α [macrophage inflammatory peptide 1α]), and CCL4 (MIP-1β) are natural ligands of CCR5 and competitively inhibit the replication of R5 viruses. In contrast, CXCR4 is a coreceptor used by X4 variants of HIV-1, which are usually present at later stages of infection, although the precise role of X4 variants in AIDS pathogenesis remains to be elucidated. Similar to CCR5 ligands, CXCR4 ligands CXCL12α (or SDF-1α [stromal cell-derived factor 1α]) and CXCL12β (SDF-1β) competitively inhibit the replication of X4 variants.