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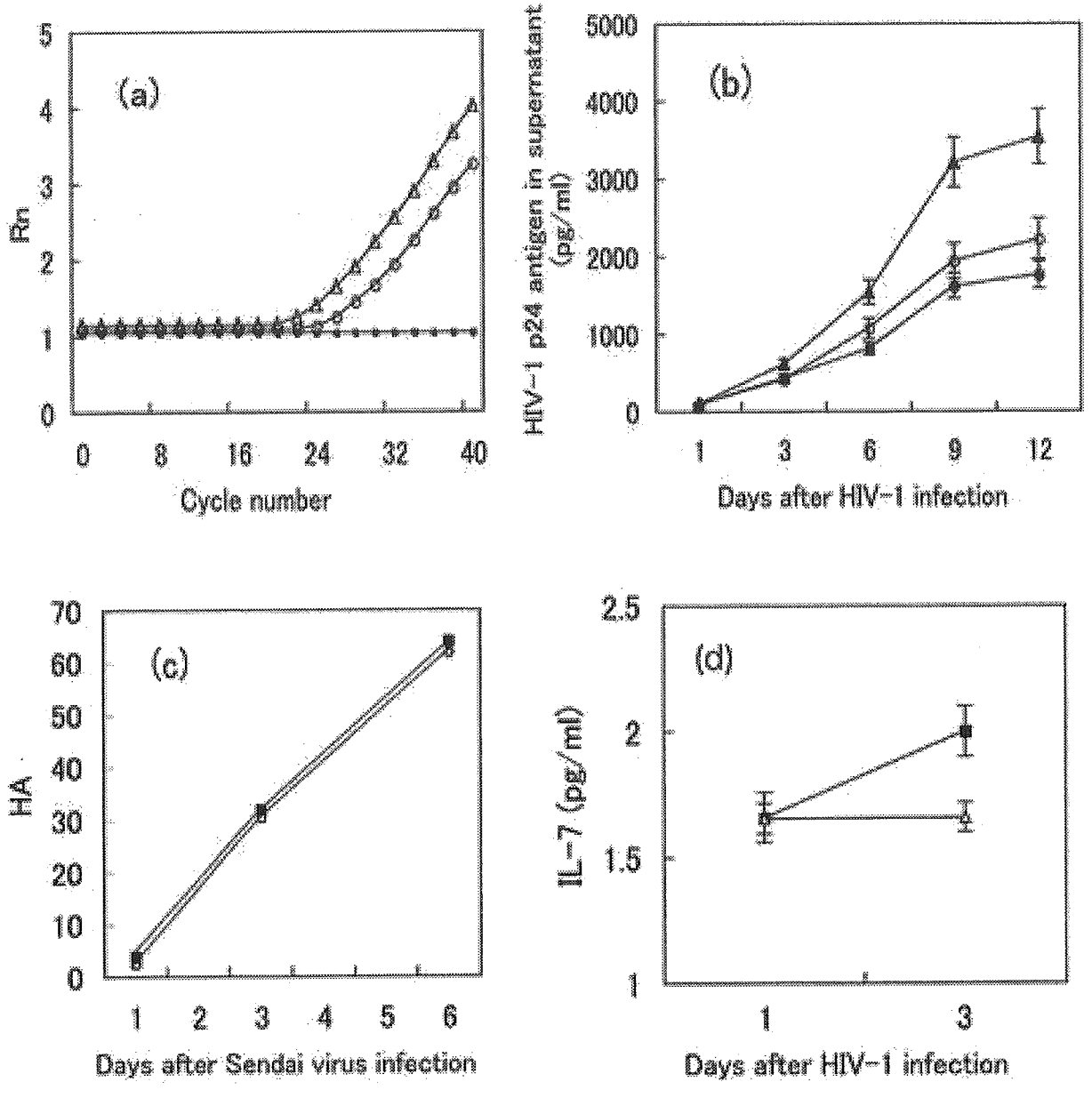
FIG. 1. (a) Expression of IL-7 receptor mRNA in macrophages measured by real-time monitoring of fluorescence signals. IL-7 receptor mRNA in macrophages (open circles), PHA-stimulated PBMC (open triangles) as the positive control and CV-1 cells (closed squares) as the negative control are shown. Rn is the ratio of the fluorescence intensity of the target cDNA to the fluorescence intensity of water instead of cDNA. 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 10ng/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 10ng/ml of IL-7 (open circles) or un-treated with IL-7 (closed squares). HA titers 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.


(a)
Rn
Cycle number
(b)
HIV-1 p24 antigen in supernatant (pg/ml)
Days after HIV-1 infection
(c)
HA
Days after Sendai virus infection
(d)
IL-7 (pg/ml)
Days after HIV-1 infection

Protective Effects of *IL4-589T* and *RANTES*-28G on HIV-1 disease progression in infected Thai females

Nuanjun Wichukchinda[a,b], Emi E. Nakayama[b], Archawin Rojanawiwat[a], Panita Pathipvanich[c], Wattana Auwanit[a], Suthon Vongsheree[a], Koya Ariyoshi[d], Pathom Sawanpanyalert[a] and Tatsuo Shioda[b]

Objective: To evaluate the effect of polymorphisms in interleukin-4 (*IL4*) and *RANTES* promoters on disease progression in HIV-1 infected Thais.

Design: Antiretroviral (ARV) drug-free HIV-1 infected females from the prospective cohort.

Methods: A total of 246 DNA samples were genotyped for *IL4* and *RANTES* promoter polymorphisms by PCR–RFLP. Associations of genotype with HIV-1 disease progression were assessed with respect to baseline clinical data including plasma HIV-1 load, CD4 cell counts, and proportion of symptomatic/AIDS, and survival status during 3 years of follow-up.

Results: Patients with homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and a higher CD4 cell count ($P = 0.003$) than the other patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* allele. Kaplan–Meier analysis demonstrated an apparent but insignificant trend towards better survival in homozygous *IL4-589T* patients. On the other hand, patients with *RANTES-28G* allele showed a significantly better survival while those with *RANTES In1.1C* allele without *RANTES-28G* showed a significantly poorer survival compared with those who did not possess either *RANTES In1.1C* or *RANTES-28G* ($P = 0.02$), although those polymorphisms only weakly associated with baseline viral load and CD4 cell counts.

Conclusions: Our results implicate the significant protective effect of *IL4-589T* and *RANTES-28G* on HIV disease progression in Thais. In contrast, *RANTES In1.1C* without *RANTES-28G* had an accelerating effect on HIV disease progression.

AIDS 2006, **20**:189–196

Keywords: *IL4-589T*, *RANTES* promoter polymorphisms, HIV disease progression, survival, Thailand, viral load, CD4 cells

Introduction

HIV-1 infected individuals have widely different rates of disease progression. Some infected individuals become symptomatic within 2–3 years while others remain asymptomatic for more than 10–15 years [1]. It is important to investigate factors modulating rates of disease progression for designing novel therapies and vaccines.

RANTES is a natural CCR5 ligand and potently inhibits cell entry of HIV-1 that uses CCR5 as a coreceptor

From the [a]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, the [b]Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, the [c]Day Care Center, Lampang Hospital, Lampang, Thailand, and the [d]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan.

Correspondence to T. Shioda, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, Osaka, 565-0871 Japan.

Tel: +81 6 6879 8346; fax: +81 6 6879 8347; e-mail: shioda@biken.osaka-u.ac.jp

Received: 26 May 2005; revised: 9 September 2005; accepted: 27 October 2005.

ISSN 0269-9370 © 2006 Lippincott Williams & Wilkins

(R5 virus). Two single nucleotide polymorphisms (SNP) in *RANTES* promoter; *-403G/A* and *-28C/G*, were identified [2]. The *RANTES-28G* variant was shown to elevate promoter activity *in vitro* [2,3] and is associated with a slower rate of CD4 cell depletion in HIV-1 infected Japanese patients [2]. Although this result was not confirmed in subsequent studies in other ethnic groups in which the *RANTES-28G* frequency was lower than in the Japanese [3–5], McDermott *et al.* reported that patients with *RANTES-28G* showed a clear trend towards slower rate of CD4 cell depletion in HIV-1 infected European Americans [6].

With respect to *RANTES-403A* variants, HIV-1 infected European Americans with *RANTES-403A* were initially reported to progress more slowly to AIDS [6]. However, subsequent studies with a larger sample size showed that *RANTES-403A* or *RANTES In1.1C*, a *RANTES* intron polymorphism which is in a strong linkage disequilibrium with *RANTES-403A* and which negatively affects *RANTES* transcription *in vitro*, was reported to be associated with an increased rate of disease progression [3,4].

A variant of the interleukin-4 (*IL4*) promoter, *-589T* was found to be associated with elevated levels of serum IgE and to accelerate phenotypic switches of HIV-1 from non-syncytium inducing (NSI) to syncytium-inducing (SI) type in HIV-infected Japanese patients probably due to the suppressive effect of IL4 on CCR5 expression together with its enhancing effect on CXCR4 expression [7]. This allele was subsequently found to be associated with a lower viral load and a slower rate of disease progression in HIV-infected French patients [8,9]. However, studies in other ethnic groups did not show consistent results [10,11].

There is a considerable variation in allele frequencies at these loci among different ethnic groups. Furthermore, diverse strains of HIV-1 circulate in different geographical areas and effects of host genetic polymorphisms may differ in individuals infected with different HIV-1 subtypes. Therefore, it is important to study influences of host genetic polymorphisms in different populations; however, such data in Asian populations remain sparse. In this paper, we report that polymorphisms in *IL4* and *RANTES* influence HIV load, CD4 cell count and survival time in Thai patients infected with HIV-1 subtype E.

Materials and methods

Patients and samples

We conducted a prospective cohort study at the HIV clinic in the Day Care Center of Lampang Hospital in northern Thailand. The Lampang Hospital is a government referral hospital with approximately 800 beds. It is located in the center of Lampang province, a province 600 km to the north of Bangkok. We approached all HIV-1 infected individuals who attended the clinic from 6 July 2000 to 12 July 2001 and asked them to participate in this cohort study. After giving written informed consent, all participants were interviewed by trained study coordinators, and clinically examined by two designated doctors. EDTA-treated blood was taken from each individual. Plasma and buffy coat fractions were separated and stored at −80°C until use. For a control group we collected blood samples from 119 female blood donors at the blood bank of the Lampang Hospital.

Plasma HIV-1 RNA copy number was measured using a commercial kit (Amplicor HIV-1 Monitor Test, Roche Molecular System, Inc. Branchburg, New Jersey, USA), which has a lower limit of detection of 400 copies/ml. CD4 cell count was measured by FACScan (BD Biosciences, California, USA) using fresh EDTA-treated whole blood samples at the time of recruitment. The survival status of participants until 1 October 2003 was ascertained from the cohort database, mailing letters, and death certificates at the Lampang Provincial Health Office. Data were double entered and validated using the access program. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand in January 2000.

Polymorphism genotyping

Genomic DNA was extracted and purified from the buffy coat fraction using QIAamp mini blood kit (QIAGEN GmbH, Hiden, Germany). Each specimen was analyzed for *IL4-589C/T*, *RANTES-403G/A*, *RANTES-28C/G*, *RANTES In1.1T/C*, and *RANTES 3'222T/C* by PCR–RFLP as described elsewhere [2,3,7].

Sample analysis

We conducted this study strictly in blinded manner so that field investigators were masked from any host genetic polymorphism data and laboratory investigators were masked from any clinical data. The data set was analyzed only after deleting sample and patient's identification number. Continuous variables of two groups with different genetic background were compared by a non-parametric Kruskal–Wallis test. Qualitative variables of two groups were compared by the Chi-square test. Significance in Kaplan–Meier analysis was determined by the log-rank test. Statistical analyses were carried out using Epi Info version 3.01 (US-CDC). *RANTES* haplotypes were constructed and calculated for their frequencies by an Expectation-Maximization algorithm using software (Arlequin version 2.01, Genetica and Biometry Laboratory, Geneva, Switzerland). D′ measure for linkage disequilibrium was also calculated by using Arlequin. Incidence rate ratios were calculated by STATA (Stata Corp., Texas, USA). Crude and adjusted relative risks (RR) and their 95% confidence intervals (CI) were calculated by Cox proportional hazard models using StatView (SAS Institute Inc. North Carolina, USA).

Results

Baseline characteristics of study subjects

During the study period, 487 HIV-infected individuals participated in this study. There were 187 males and 300 females; baseline CD4 cell counts and antiretroviral (ARV) drug use information was available in 185 males and 297 females. We found that HIV-disease was significantly more advanced in men than in women. Median [interquartile range (IQR)] CD4 cell count at the time of recruitment in men [66 (15–258) cells/μl) was significantly lower ($P < 0.0001$) than that in women [262 (90–418)]. The proportion of patients diagnosed as AIDS was also higher ($P < 0.0001$) in men (77/185; 41.6%) than in women (44/297; 14.8%). Because the aim of this study was to investigate the influence of host genetic polymorphisms on disease progression and the majority of male individuals were already in the advanced stage of HIV disease, we analyzed the data of men and women separately and in this paper we present our analysis on the 297 females.

Among these females, 186 were ARV drug naive and 111 were ARV drug experienced: 51 were receiving ARV drugs and 60 reported prior ARV treatment but were not on ARV at the time of recruitment. The latter group included 18 females who were given zidovudine (ZDV) as prophylaxis for HIV mother-to-child transmission. The remaining 42 subjects stopped ARV drugs because of financial constraints.

All females reported that they had contracted HIV-1 through heterosexual contact. Baseline characteristics of ARV drug naive and ARV drug interrupted groups were similar (Table 1). In this analysis, we excluded the 51 individuals who were on ARV drug treatment, because on-going ARV drug treatment considerably affects CD4 cell count and viral load so that an association between these parameters and genetic polymorphisms may be obscured.

Allele frequencies of *IL4-589T* and *RANTES-403A, RANTES-28G, RANTES In1.1C*, and *RANTES 3'222C*

We genotyped 246 ARV drug-free females and 119 blood donors for *IL4-589C/T, RANTES-403G/A, RANTES-28C/G, RANTES In1.1T/C*, and *RANTES 3'222T/C*. Allele frequency of *IL4-589T* was 0.774 in 246 ARV drug-free females and 0.798 in 119 blood donors. Frequencies of four *RANTES* SNP in ARV drug-free females and blood donors are shown at the bottom of Table 2. There was no difference in frequency of those five SNP among subgroups, including the ARV-treated group (data not shown). All of the observed genotypes were in Hardy–Weinberg equilibrium (data not shown).

Table 2 shows estimated *RANTES* haplotype distribution in 246 ARV drug-free females and 119 blood donors. As reported in European- and African-Americans, the haplotype R1 was the most common. In European- and African-Americans, approximately 75% and 55% of *RANTES-403A* was associated with *RANTES In1.1C*, respectively. *RANTES-403A* was more closely associated with *RANTES In1.1C* in Thai patients than in European- and African-Americans, since 93.4% of *RANTES-403A* were associated with *RANTES In1.1C* in 246 ARV drug-free female patients ($D'=1.0$, Table 2). Therefore, we focused on *RANTES-28G* and *RANTES In1.1C* in the subsequent analysis among four *RANTES* polymorphisms, as *RANTES-28G* and *RANTES In1.1C* were shown to be functional and actually affected RANTES promoter activity [2,3], while roles of *RANTES-403A* and *RANTES 3'222T/C* as functional alleles were not yet confirmed.

Genotype variation and HIV disease status (clinical, CD4 cell count, viral load)

We analyzed viral load, CD4 cell count, and clinical status among ARV drug-free females at recruitment by stratifying individuals according to their host genetic background (Table 3). We found that patients with the homozygous *IL4-589T* allele showed a significantly lower HIV-1 viral load ($P = 0.005$) and higher CD4 cell count ($P = 0.003$) than patients with heterozygous *IL4-589C/T* or homozygous *IL4-589C* alleles. Furthermore the proportion of symptomatic individuals was lower among females with homozygous *IL4-589T* allele than among others, although this trend did not reach statistical significance ($P = 0.183$).

Patients with *RANTES-28G* allele (*C/G* or *G/G*) showed a weak trend toward a higher CD4 cell count

Table 1. Baseline data of ARV drug-free HIV-1 infected females.

Characteristics	ARV drug naive (n = 186)	ARV treatment interrupted (n = 60)	Combined (ARV drug free) (n = 246)
Age at recruitment: median years (IQR)	31 (28–36)	31 (27.5–38)	31 (28–36)
CD4 cell count [median cells/μl (IQR)]	269 (71–422)	259.5 (63–400.5)	262 (67–421)
HIV-1 load [median $\log_{10}$ HIV RNA copies/ml (IQR)]	5.084 (4.424–5.549)	5.0194 (3.897–5.592)	5.078 (4.331–5.571)
AIDS- related symptoms [% (n)]	37.1(69)	45(27)	39 (96)
95% CI	30.1–44.5	32.1–58.4	32.9–45.4
Diagnose of AIDS [%(n)]	15.6 (29)	20 (12)	16.7 (41)
95% CI	10.7–21.6	10.8–32.3	12.3–21.9

ARV, Antiretroviral; IQR, interquartile range; CI, confidence interval.

Table 2. *RANTES* haplotype frequencies in ARV drug free HIV-1 infected females and female blood donors.

RANTES Haplotype	*RANTES* polymorphism site				ARV drug free[a] (n = 246)	Blood donor[b] (n = 119)
	−403	−28	In1.1	3′222		
R1	*G*	*C*	*T*	*T*	0.697	0.731
R2	*A*	*C*	*T*	*T*	0.020	0.034
R3	*A*	*C*	*C*	*T*	0.002	0.000
R4	*A*	*C*	*C*	*C*	0.191	0.160
R5	*A*	*G*	*C*	*T*	0.089	0.076

[a]Allele frequencies of *RANTES-403A* = 0.303, *RANTES-28G* = 0.089, *RANTES In1.1C* = 0.283, and *RANTES 3′222C* = 0.191.
[b]Allele frequencies of *RANTES-403A* = 0.269, *RANTES-28G* = 0.076, *RANTES In1.1C* = 0.235, and *RANTES 3′222C* = 0.160. ARV, Antiretroviral.

($P = 0.165$), although the differences were not statistically significant. There was no significant difference in HIV-1 viral load or proportion of symptomatic individuals between patients with and without the *RANTES-28G* allele. Patients with *RANTES In.1C* showed no difference from those without this allele in HIV-1 viral load, CD4 cell count, or proportion of symptomatic cases.

Genotype variation and survival status

Of 246 ARV drug-free females, we obtained follow-up information from 238 (96.7%): the median (IQR) of follow-up was 964 (495–1072) days. During the follow-up period, 55 patients had started ARV drug treatment. Among 524.05 persons-years of follow up, 54.16 persons-years were ARV drug treatment positive. Therefore, the majority (89.7%) of our study subjects was ARV drug-free. There was no difference in proportion of patients who started ARV drugs during the observation period among each genotype group (Table 3). During the follow up period, 65 deaths occurred (including one suicide). Death was then used as the marker for HIV-1 disease progression. We found that the mortality rate among homozygous *IL4-589T* individuals was slightly lower than for the other groups, but this difference did not reach statistical significance [incidence rate ratio (IRR), 0.68; 95% CI, 0.40–1.14; $P = 0.121$) (Table 3). For *RANTES* promoter, there was significantly lower mortality rate among individuals with *RANTES-28G* allele when compared to those without this allele (IRR, 0.41; 95%CI, 0.15–0.95; $P = 0.024$) (Table 3). In contrast, there was no difference in the mortality rate between individuals with *RANTES In1.1C* and those without this allele (IRR. 1.11; 95%CI, 0.66–1.84; $P = 0.67$).

Table 3. Median HIV-1 load and CD4 cell count, HIV-1 related symptoms, diagnosis of AIDS and number of deaths/person years of observation (PYO) stratified by *IL4-589C/T, RANTES-28C/G,* and *RANTES In1.1T/C* genotype group.

	IL4-589		*RANTES-28*		RANTES In1.1	
	C/C and C/T (n = 99)	T/T (n = 147)	C/C (n = 205)	C/G and G/G (n = 41)	T/T (n = 133)	T/C and C/C (n = 113)
Median viral load [$\log_{10}$ copies/ml (IQR)]	5.381 (4.519–5.650)	4.908 (4.214–5.441)	5.107 (4.406–5.578)	4.843 (4.177–5.491)	5.098 (4.287–5.615)	5.053 (4.433–5.507)
P (Kruskal–Wallis test)		0.005		0.275		0.929
Median CD4 count [(cells/μl (IQR)]	171 (46–354)	303 (144–433)	261 (58–412)	297 (144–451)	285 (66–414)	244 (71–421)
P (Kruskal–Wallis test)		0.003		0.165		0.684
HIV-1 related symptoms [%(n)]	44.4(44)	35.4(52)	40.5(83)	31.7(13)	37.6(50)	40.7(46)
(95% CI)	(34.5–54.8)	(27.7–43.7)	(33.7–47.5)	(18.1–48.1)	(29.3–46.4)	(31.6–50.4)
P (Chi^2 test)		0.153		0.293		0.618
Diagnosis of AIDS [% (n)]	19.2(19)	15(22)	17.1 (35)	14.6 (6)	16.5(22)	16.8(19)
(95% CI)	(12.0–28.3)	(9.6–21.8)	(12.2–22.09)	(5.6–29.2)	(10.7–24.0)	(10.4–25.0)
P (Chi^2 test)		0.383		0.702		0.954
Deaths (n)	30	35	59	6	34	31
PYO	192.59	331.90	420.84	103.66	288.17	236.33
Mortility rate (%)	15.58	10.54	14.02	5.79	11.8	13.12
(95% CI)	(10.01–21.15)	(7.05–14.03)	(10.44–17.60)	(1.16–10.42)	(7.83–15.77)	(8.5–17.74)
Incidence rate ratio of death (95% CI)		0.68 (0.40–1.14)		0.41 (0.15–0.95)		1.11 (0.66–1.84)
P (Chi^2 test)		0.121		0.024		0.67
ARV treatment during follow-up	22	33	44	11	32	23
Observed (n)	94	144	197	41	129	109
P (Chi^2 test)		0.930		0.534		0.499

IQR, Interquartile range; CI, confidence interval.

Consistent with the results on mortality rate, the Kaplan–Meier analysis demonstrated an insignificant better survival in homozygous *IL-589T* ($P = 0.125$) (Fig. 1a), a significant better survival in individuals carrying *RANTES-28G* ($P = 0.037$) (Fig. 1b), and no difference between individuals carrying *RANTES In1.1C* and those without this allele ($P = 0.668$) (Fig. 1c). Since *RANTES-28G* was associated with *RANTES In1.1C* (Table 3), we then grouped the patients as: (i) individuals who posses sed neither *RANTES-28G* nor *RANTES In1.1C*; (ii) individuals who possessed *RANTES In1.1C* but not *RANTES-28G*; and (iii) individuals who possessed

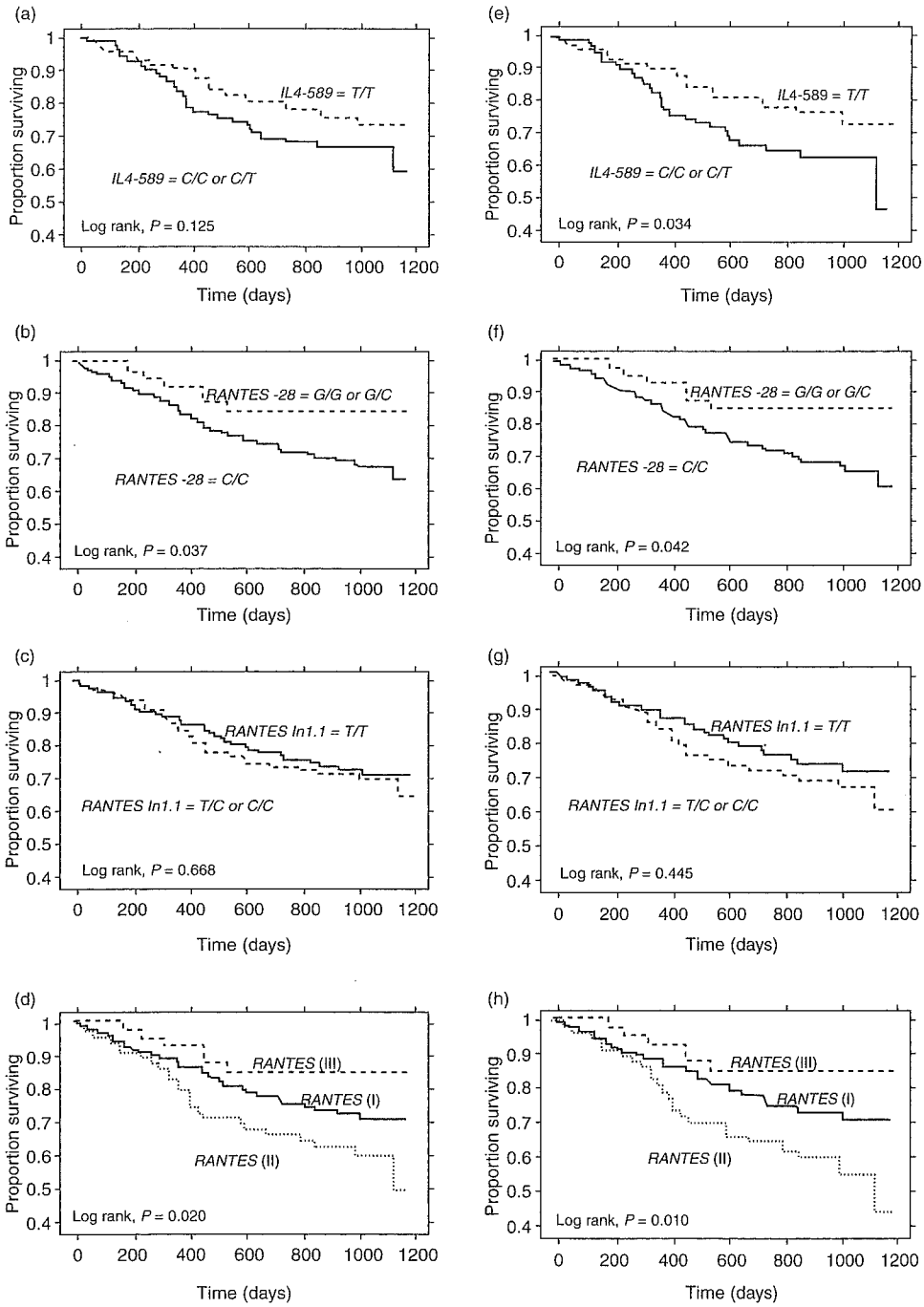


Fig. 1. Kaplan–Meier analysis of the effect of promoter polymorphism of *IL4* and *RANTES* on survival in ARV drug-free HIV-1 infected Thai females during the total observation period (a–d) or untreated period (e–h). (a and e) *IL4-589T*; (b and f) *RANTES-28*; (c and g) *RANTES-403*; and (d and h): grouped *RANTES* as (I) individuals who possessed neither *RANTES In1.1C* nor *RANTES-28G*, (II) individuals who possessed *RANTES in1.1C* but not *RANTES-28G*, and (III) individuals who possessed *RANTES-28G*.

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.

Acknowledgements

We are grateful to all the patients participated in the Lampang cohort and blood donors for their allowing us using their blood in this study. We also thank medical staff in Lampang Hospital for their cooperation.

Sponsorship: Supported by the Department of Medical Sciences, Ministry of Public Health Thailand; Japan International Cooperation Agency (JICA), Japanese Foundation for AIDS Prevention (JFAP), the Ministry of Health, Labour and Welfare, Japan, and the Ministry of Education, Science, Sports and Culture, Japan.

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JOURNAL OF VIROLOGY, July 2005, p. 8870–8877 Vol. 79, No. 14
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.14.8870–8877.2005

A Specific Region of 37 Amino Acid Residues in the SPRY (B30.2) Domain of African Green Monkey TRIM5α Determines Species-Specific Restriction of Simian Immunodeficiency Virus SIVmac Infection

Emi E. Nakayama,[1] Hiroyuki Miyoshi,[2] Yoshiyuki Nagai,[3] and Tatsuo Shioda[1]*

Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka,[1] BioResource Center, RIKEN Tsukuba Institute, Tsukuba,[2] and Toyama Institute of Health, Toyama,[3] Japan

Received 2 December 2004/Accepted 30 March 2005

Human immunodeficiency virus type 1 (HIV-1) efficiently enters cells of Old World monkeys but encounters a block before reverse transcription. This restriction is mediated by a dominant repressive factor. Recently, a member of the tripartite motif (TRIM) family proteins, TRIM5α, was identified as a blocking factor in a rhesus macaque cDNA library. Among Old World monkey cell lines, the African green monkey kidney cell line CV1 is highly resistant to not only HIV-1 but also simian immunodeficiency virus SIVmac infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and found that both CV1- and HSC-F-TRIM5αs could inhibit CD4-dependent HIV-1 infection, as well as vesicular stomatitis virus glycoprotein-mediated infection. CV1-TRIM5α could also inhibit SIVmac infection, whereas HSC-F-TRIM5α could not. In the SPRY (B30.2) domain of CV1-TRIM5α, there was a 20-amino-acid duplication that was not present in HSC-F-TRIM5α. A chimeric TRIM5α containing 37 amino acid residues from CV1-TRIM5α, which spanned the 20-amino-acid duplication, in the background of HSC-F-TRIM5α fully gained the ability to inhibit SIVmac infection. Conversely, the mutant CV1-TRIM5α lacking the 20-amino-acid duplication completely lost the ability to restrict SIVmac infection. These findings clearly indicated that a specific region of 37 amino acid residues in the SPRY domain of CV1-TRIM5α contained a determinant of species-specific restriction of SIVmac.

Human immunodeficiency virus type 1 (HIV-1) is thought to have been introduced into the human population from chimpanzees (9) and shows a very narrow host range limited only to humans and chimpanzees. HIV-1 does not experimentally infect Old World monkeys, such as rhesus and cynomolgus monkeys, and fails to replicate in activated CD4-positive T lymphocytes obtained from these monkeys (13, 31). In contrast, simian immunodeficiency virus (SIV) isolated from a macaque monkey (SIVmac) can replicate well in rhesus (13, 31) and cynomolgus monkeys (2, 3). The restricted host range of HIV-1 has greatly hampered its use in animal experiments and, hence, caused difficulty in developing prophylactic vaccines against HIV-1 infection.

Several studies have suggested that the block of HIV-1 replication in Old World monkey cells occurred at a postentry step (7, 13, 31) and appeared to result from a failure to initiate reverse transcription (13). The block was still observed when CD4-negative monkey cells were infected with HIV-1 pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) and was overridden by a high multiplicity of infection (MOI) with VSV-G-pseudotyped virus or virus-like particles lacking genomic RNA (5, 10, 16, 19). Importantly, resistance against HIV-1 infection was shown to be dominant in heterokaryons between human and Old World monkey cells, suggesting the presence of inhibitory factor(s) against HIV-1 infection in Old World monkey cells (19). Studies on chimera of HIV-1 and SIVmac have suggested that restriction determinants lie within the HIV-1 P24 capsid protein (CA) (11, 23, 24, 29).

Recently, the screening of a rhesus monkey cDNA library identified tripartite motif 5α (TRIM5α), a component of cytoplasmic bodies, as a factor that confers resistance to HIV-1 infection (33). Shortly after, TRIM5α of African green monkey, another Old World monkey, was also shown to restrict HIV-1 infection, whereas human TRIM5α was reported to restrict N-tropic murine leukemia virus (12, 14, 25, 36).

An African green monkey kidney cell line, CV1, was shown to be highly resistant to SIVmac infection, as well as to HIV-1 infection. We analyzed TRIM5α of CV1 cells and HSC-F cells, a T-cell line from a cynomolgus monkey, and report here that the ability of CV1-TRIM5α to suppress SIVmac infection was determined by a small region composed of 37 amino acid residues in the SPRY (B30.2) domain of CV1-TRIM5α.

MATERIALS AND METHODS

Cloning and expression of TRIM5α. TRIM5α cDNA was amplified by reverse transcription-PCR from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F (2, 3), and African green monkey cell lines CV1 and Vero by using 5′-GCGGCCGCTACTATGGCTTCTGG-3′ as a forward primer and 5′-GAA TTCTCAAGAGCTTGGTGA-3′ as a reverse primer. Amplified products were then cloned into the vector pCR-2.1TOPO (Invitrogen), and the nucleotide sequence of 10 clones for each TRIM5α was determined.

The entire coding regions of selected clones were transferred to pcDNA3.1 (Invitrogen) by using NotI and EcoRI sites, which were introduced by primers used in the PCR step. Hamster TK-ts13 cells (4) were transfected with pcDNA3.1 carrying TRIM5α cDNA and cultured in the presence of 0.75 mg of

* Corresponding author. Mailing address: Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan. Phone: 81-6-6879-8346. Fax: 81-6-6879-8347. E-mail: shioda@biken.osaka-u.ac.jp.

G418 (Gibco)/ml for 14 days. The colonies obtained were examined for the expression of TRIM5α by using the TaqMan PCR method according to the manufacturer's instructions (Applied Biosystems). Sequences of the probe and primers used to specifically detect each TRIM5α were as follows: MT4-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAGGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCAGTTTCAGAG TTCG-TAMRA-3′); HSC-F-TRIM5, forward primer (5′-AACCTGGAGAAG GAGAAAGAAGAC-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TTCGTTTCAGACTTTG-TAMRA-3′); and CV1-TRIM5, forward primer (5′-AACCTGGAGAAGGAGGAAGAAGA-3′), reverse primer (5′-CTGGGTCTGCTGCACCAT-3′), and probe (5′-FAM-TCCGTTTCAGAC TTCG-TAMRA-3′). These primers amplify the coiled-coil region of TRIM5 genes. Plasmid DNA used for transfection served as a standard to determine the number of copies of TRIM5α transcripts. The parental TK-ts13 cells were totally negative for the primate TRIM5α expression. Clones expressing each TRIM5α at comparable levels (ca. 4 to 6 × 10^7 copies/μg of total RNA) were used for subsequent experiments.

To generate CV1-TRIM5α and HSC-F-TRIM5α cDNAs carrying a hemagglutinin (HA) tag (YPYDVPDYAA) at their C termini, 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 (BamHI to NotI) and the N-terminal portion of TRIM5α (NotI to BamHI) were assembled on a pCEP4 vector (Invitrogen). To generate chimeric TRIM5α HSC-F+60tag, the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag was replaced with the corresponding 242-bp SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Conversely, the 242-bp SphI-BamHI fragment of CV1-TRIM5α was replaced with the 182-bp SphI-BamHI fragment of HSC-F-TRIM5α-tag in the background of CV1-TRIM5α-tag to generate CV1-60tag. PCR-based mutagenesis of HSC-F-TRIM5α-tag was performed to generate HSC-delete-tag, which possessed the 5′-proximal 84 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, and HSC-insert-tag, which possessed 3′-proximal 158 bp of the SphI-BamHI fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. Similarly, CV1-delete-tag, which possessed the 3′-proximal 98 bp of the SphI-BamHI fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, was generated by a PCR-based mutagenesis of CV1-TRIM5α-tag. The entire coding sequences of these TRIM5α-tags were then transferred to the NotI site of pSeV18+b(+). Recombinant Sendai viruses (SeVs) carrying various TRIM5α-tags were recovered according to a previously described method (32). The viruses passaged a second time in embryonated chicken eggs were used as stock for all experiments. The wild-type Z strain of SeV served as a control in all of the experiments.

To establish human cell lines which constitutively express primate TRIM5αs or their chimeras, human osteosarcoma C143 cells were transfected with pCEP4 containing cDNA of CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, CV1-60tag, or HSC-F+60tag, and cells were cultured in the presence of 0.3 mg of hygromycin B (Gibco)/ml for 14 days.

Immunoprecipitation and Western blot analysis. When we performed Western blot analysis of cells expressing HA-tagged TRIM5α proteins, we consistently observed nonspecific binding of anti-HA antibody to a protein that comigrated with HSC-F-TRIM5α. Therefore, we analyzed the expression of each HA-tagged TRIM5α protein in the hygromycin B-resistant C143 cells or MT4 cells infected with recombinant SeVs by immunoprecipitation, followed by Western blot analysis as described previously (20) to reduce nonspecific background. Briefly, cell lysate was first adsorbed with protein A-agarose before the addition of anti-HA antibody to avoid nonspecific protein binding to protein A-agarose. TRIM5α proteins in the cell lysate were then precipitated with anti-HA high-affinity rat monoclonal antibody (Roche) by using a protein A-immunoprecipitation kit (Roche). Precipitated materials were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 12% NuPAGE Bis-Tris gel (Invitrogen). Proteins in the gel were then electronically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore). Blots were blocked and probed with anti-HA antibody overnight at 4°C and then incubated with peroxidase-conjugated anti-rat immunoglobulin G (American Qualex) and developed by using the Immun-Star HRP chemiluminescence kit (Bio-Rad). Visualized image was recorded by LAS1000 (Fuji) and quantified by ImageGauge (Fuji). At least three independent experiments were performed, and the means and standard deviations (SD) for the data were calculated.

Viruses and HIV-1 lentivirus vector. VSV-G-pseudo typed HIV-1-NL43, SIVmac239, or HIV-2-GH123 was prepared by transfection of 293T cells with a combination of pMD.G (17, 18) and pNL432 (1), pBRmac239 (15), or pGH123 (30), respectively. HIV-1 vector expressing green fluorescence protein (GFP) was prepared as described previously (17, 18). Two days after transfection, culture supernatants of 293T cells were collected and assayed for reverse transcriptase activity using a reverse transcriptase colorimetric assay (Roche).

Viral infection. Assays for the HIV-1 vector expressing GFP were performed in 24-well plates containing 4 × 10^4 Tk-ts13-derived target cells. Serially diluted VSV-pseudotyped HIV-1 vectors encoding GFP were inoculated, and infected cells were enumerated by using a flow cytometer (FACScan; Becton Dickinson) 40 h after infection. For VSV-pseudotyped HIV-1, SIVmac239, and HIV-2 infection assays, we inoculated viruses containing 1 ng of reverse transcriptase into 4 × 10^4 C143 cells. For CD4-dependent infection assays, 2.5 × 10^5 MT4 cells were infected with SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain of SeV at a MOI of 10 PFU per cell, followed by incubation at 37°C for 9 h. Cells were then superinfected with 30 ng of p24 of an X4 HIV-1 strain, NL43, or 30 ng of p27 of SIVmac239. The culture supernatants were collected periodically, and the level of p24 or p27 was measured by using a RETROtek antigen ELISA kit (ZeptoMetrix).

Data deposition. The sequences described here have been deposited in the GenBank database under accession numbers AB210050 to AB210052.

RESULTS

Variation in TRIM5α. We cloned TRIM5α cDNA from the human T-cell line MT4, cynomolgus monkey T-cell line HSC-F, and African green monkey kidney cell lines CV1 and Vero. The predicted amino acid sequences of TRIM5αs are compared in Fig. 1A and B.

Human TRIM5α from MT4 cells differed at an amino acid position 249 (G249D) from the previously published sequence (33) and was designated MT4-TRIM5α. The cynomolgus monkey TRIM5α from HSC-F (HSC-F-TRIM5α) was two amino acids shorter than the rhesus monkey TRIM5α (33) and two amino acids longer than the human TRIM5α. All 10 clones derived from Vero cells had the same sequence as the previously published one (36). On the other hand, we found at least two distinct TRIM5α sequences in CV1 cells. The two major TRIM5α sequences obtained from CV1 were designated CV1-TRIM5α-type1 and CV1-TRIM5α-type2, and five amino acids were found to differ between the two sequences (I259V, L337S, R351L, G359R, and G438S). Among 10 cDNA clones obtained from CV1, there were four type 1 clones and four type 2 clones. The remaining two clones were most likely chimeric artifacts. Two recently published sequences of TRIM5α from CV1 cells (AY593973 and AY625002) showed differences at three positions—L7V, I259V, and G438S—and have S, L, and R at positions 337, 351, and 359, respectively (14, 36), whereas both the type 1 and 2 clones had leucines at the seventh position. All sequences obtained from Vero and CV1 contained a 20-amino-acid duplication within the SPRY domain, which was not observed in human MT4 and cynomolgus monkey HSC-F (Fig. 1B).

The phylogenetic tree of various TRIM5α sequences showed that cynomolgus and rhesus monkey TRIM5αs are similar to each other, a finding consistent with the fact that these two monkeys belong to the genus *Macaca* (Fig. 1C).

African green monkey and cynomolgus monkey TRIM5α inhibit HIV-1 infection in nonprimate cells. We first sought to determine whether or not each TRIM5α can inhibit HIV-1 infection in the context of nonprimate cells because human and primate cells express endogenous TRIM5α that could complicate a functional analysis of TRIM5α-mediated restriction. The hamster cell line TK-ts13 was used, because it is very susceptible to a VSV-G-pseudotyped, HIV-1-based GFP-expressing lentivirus vector, HIV-1-GFP. Cell clones stably ex-

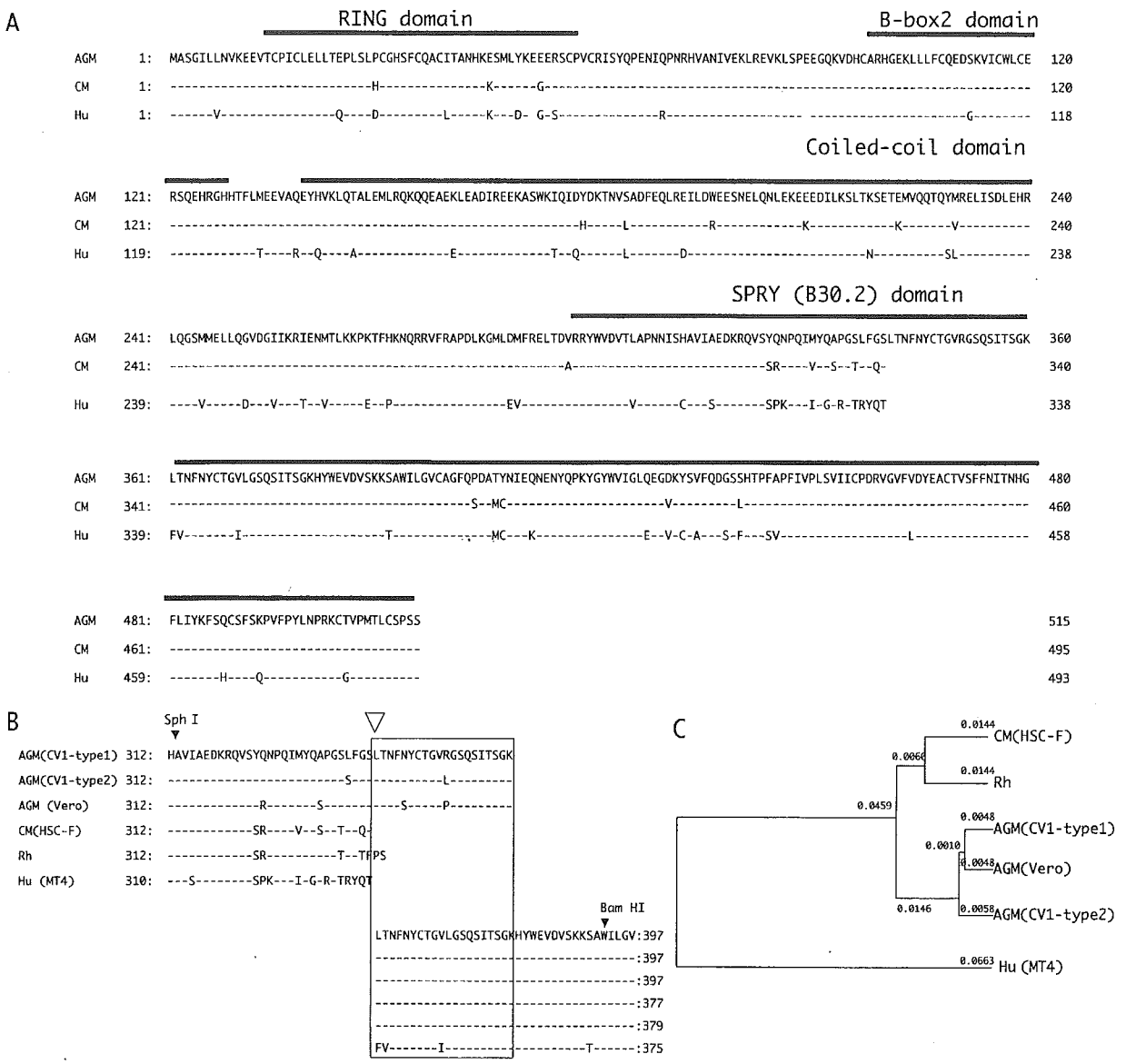

FIG. 1. (A) Alignment of amino acid sequences of African green monkey (AGM, CV1-TRIM5α-type1), cynomolgus monkey (CM, HSC-F-TRIM5α), and human TRIM5α (Hu, MT4-TRIM5α) predicted from the sequences of the cDNAs used in the present study with key domains indicated. (B) Alignment of amino acid sequences of the highly variable region within the SPRY (B30.2) domain of TRIM5α. The rhesus monkey TRIM5α (Rh) sequence published by Stremlau et al. (33) was added. A box indicates the 20-amino-acid duplication within African green monkey TRIM5α. An open arrowhead denotes the recombination point in chimeric TRIM5αs, HSC-delete-tag, HSC-insert-tag, and CV1-delete-tag (see Fig. 4A). (C) Phylogenetic tree of various TRIM5α sequences produced by the UPGMA (unweighted pair-group method with arithmetic averages) method.

pressing MT4-, CV1-, and HSC-F-TRIM5α were selected according to the method described in Materials and Methods. The levels of expression of TRIM5α were determined by using a real-time PCR, and cells expressing comparable amounts of TRIM5α (4×10^6 to 6×10^6 copies/μg of total RNA) were used for subsequent study. Restriction can be quantified by comparing the percentage of GFP-positive cells with or without TRIM5α.

As can be seen in Fig. 2A, MT4-TRIM5α had a very weak anti-HIV-1 effect (~1.2-fold), a finding consistent with a previous study (33). In contrast, restriction of HIV-1 was clearly evident over a wide range of initial MOIs in cells expressing HSC-F-TRIM5α, CV1-TRIM5α-type1, and CV1-TRIM5α-type2 (ca. 5- to 10-fold). There was no significant difference between CV1-TRIM5α-type1 and type2; therefore, we chose CV1-TRIM5α-type1 to be representative of CV1-TRIM5α in the subsequent experiments. We obtained the same results as described above when we used an HA-tagged version of TRIM5α (data not shown).

African green monkey and cynomolgus monkey TRIM5α inhibit CD4-dependent HIV-1 infection in human cells. To test the restriction properties of TRIM5α in CD4-dependent

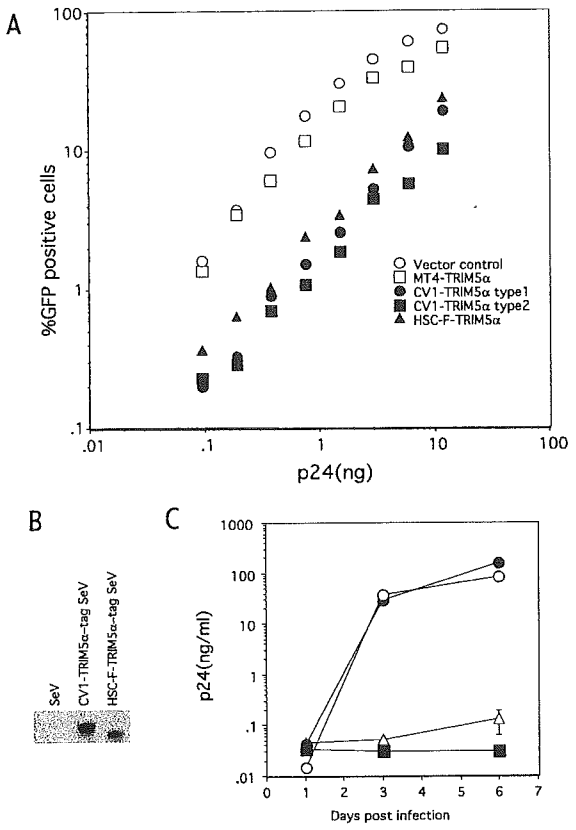

FIG. 2. (A) TK-ts13 cell clones expressing MT4-TRIM5α (□), CV1-TRIM5α-type1 (●), CV1-TRIM5α-type2 (■), HSC-F-TRIM5α (▲), or empty vector (○) were exposed to the indicated GFP-expressing HIV-1 vector. GFP-positive cells were counted with a flow cytometer. Data typical of at least three independent clones for each TRIM5α are shown. (B) Lysates of MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, HSC-F-TRIM5α-tag, or the parental Z strain were immunoprecipitated by anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of four independent experiments is shown. (C) MT4 cells were mock infected (○), or infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (△), or the parental Z strain (●). At 9 h after infection, cells were inoculated with an HIV-1 strain, NL43, and culture supernatants were periodically assayed for levels of p24. The datum points are means for triplicate samples with the SD.

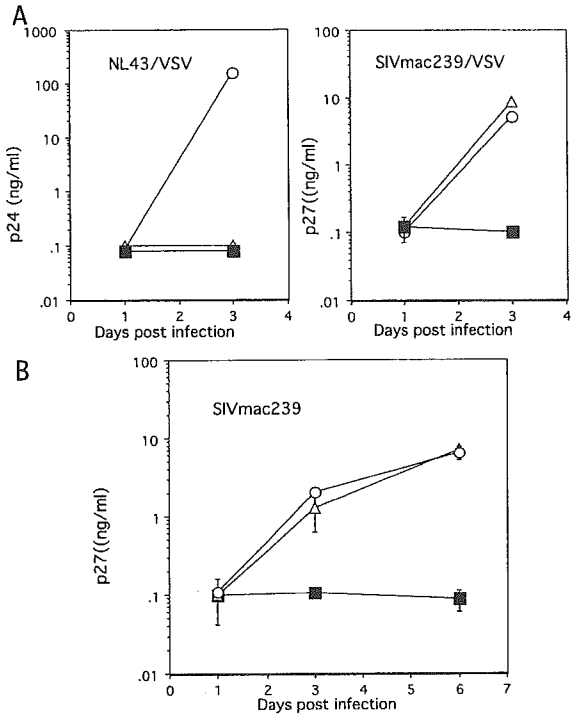

FIG. 3. (A) MT4 (○), HSC-F (△), or CV1 (■) cells were infected with VSV-pseudotyped NL43 or VSV-pseudotyped SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (B) MT4 cells were mock infected (○) or infected with SeV expressing CV1-TRIM5α-tag (■) or HSC-F-TRIM5α-tag (△). At 9 h after infection, cells were inoculated with SIVmac239, and culture supernatants were periodically assayed for levels of p27. The datum points are means for triplicate samples with the SD.

HIV-1 infection, we constructed a recombinant SeV expressing TRIM5α fused with the HA tag in the C-terminal of HSC-F-TRIM5α or CV1-TRIM5α (HSC-F-TRIM5α-tag SeV or CV1-TRIM5α-tag SeV). Human T-cell line MT4 cells were first infected with the SeV expressing TRIM5α-tag (Fig. 2B), incubated at 37°C for 9 h, and then infected with an X4-tropic HIV-1 strain NL43. As can be seen in Fig. 2C, both HSC-F-TRIM5α-tag and CV1-TRIM5α-tag completely inhibited HIV-1 replication, whereas MT4 cells infected with SeV empty vector fully supported HIV-1 replication.

Distinct patterns of restriction for SIVmac among nonhuman primate TRIM5α. In African green monkey CV1 cells, both HIV-1 and SIVmac239 were restricted, whereas only HIV-1 was restricted in cynomolgus monkey HSC-F cells (Fig. 3A). Therefore, we examined whether or not CV1-TRIM5α also could inhibit the replication of SIVmac. In MT4 cells infected with recombinant SeV expressing HSC-F-TRIM5α-tag, the replication of SIVmac239 was not suppressed at all (Fig. 3B), indicating that the HSC-F-TRIM5α showed a similar specificity to rhesus monkey TRIM5α (33). In MT4 cells infected with recombinant SeV expressing CV1-TRIM5α-tag, in contrast, the replication of SIVmac239 was completely suppressed. These results suggested that the distinct sensitivity of African green monkey and cynomolgus monkey cells to HIV-1 and SIVmac infection was, at least partly, determined by TRIM5α.

A small region of 37 amino acid residues in the SPRY domain of CV1-TRIM5α determines SIVmac restriction. A comparison of the human and nonhuman primate TRIM5α sequences showed the presence of a highly variable region in the N-terminal portion of the SPRY domain (Fig. 1A and B). In this region, CV1 and Vero TRIM5α had a 20-amino-acid repetition that was totally absent in HSC-F-TRIM5α. These findings prompted us to test whether this highly variable region of TRIM5α determined the species-specific inhibition of SIVmac infection. We constructed chimeric TRIM5αs from HSC-F-TRIM5α-tag and CV1-TRIM5α-tag by using SphI and BamHI restriction enzyme digestion (Fig. 4A). HSC-F+60tag contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag. The reciprocal chimera, CV1-60tag, contained a 182-bp fragment of HSC-F-TRIM5α in the

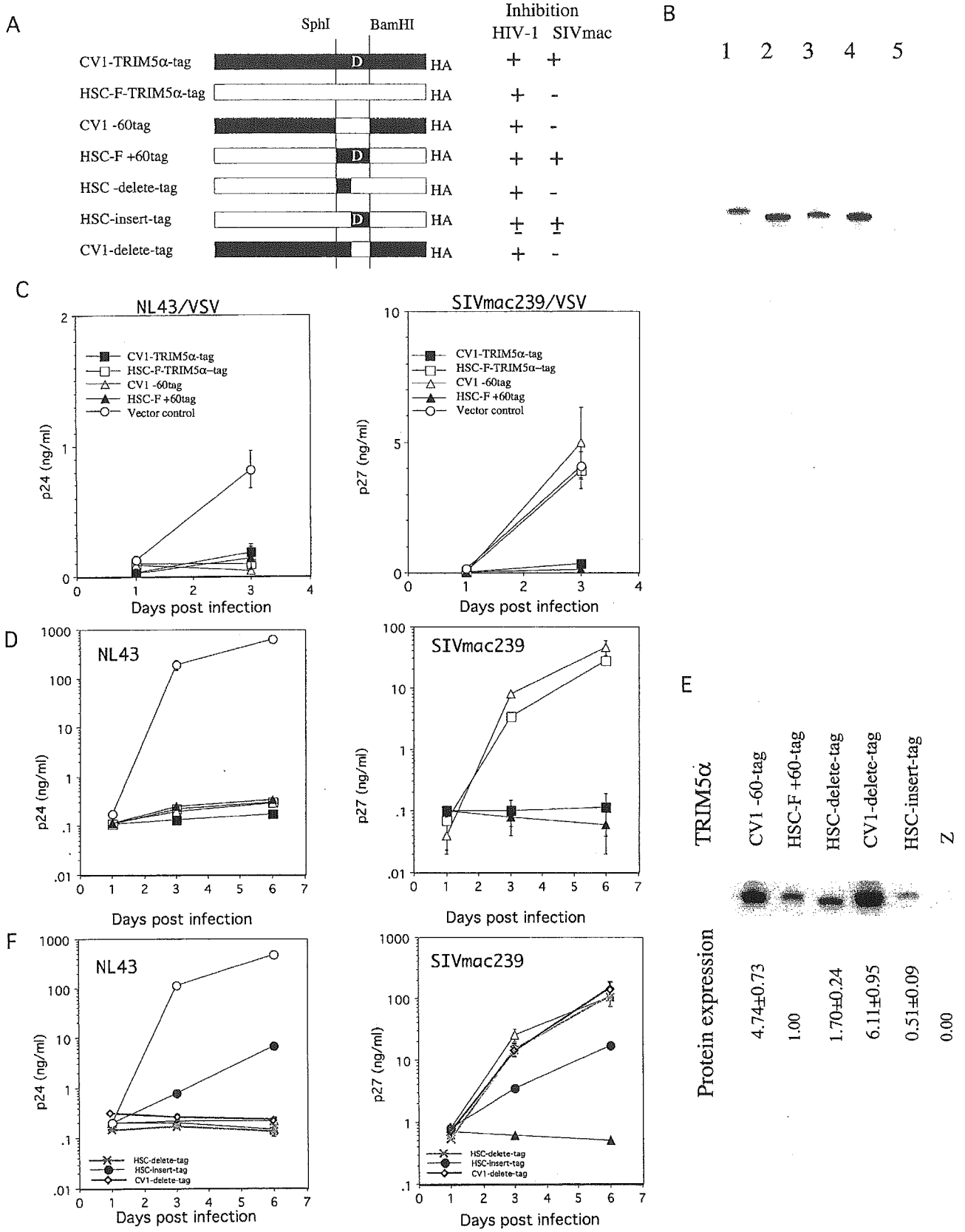

FIG. 4. (A) Schematic representation of chimeric TRIM5α and summary of the results. Filled and open bars denote CV1 and HSC-F sequences, respectively. D, the CV-1-TRIM5α-specific 20-amino-acid duplication. The +, ±, and − symbols denote full, partial, and no

background of the CV1-TRIM5α-tag. In this fragment, the differences between CV1 and HSC-F TRIM5α, including the 20-amino-acid repetition, were located in a small region of 37 amino acid residues (Fig. 1B). Human osteosarcoma C143 cells stably expressing various TRIM5αs (Fig. 4B) were infected with VSV-G-pseudotyped HIV-1 NL43, and levels of P24 in the culture supernatants were assayed periodically. As expected, both chimeric TRIM5αs and parental TRIM5αs clearly inhibited the replication of HIV-1 NL43 (Fig. 4C). When these cells were infected with VSV-G-pseudotyped SIVmac239, the parental CV1-TRIM5α-tag could also inhibit the replication of SIVmac239, whereas CV1-60tag, which contained the 182-bp fragment of HSC-F-TRIM5α in the background of CV1-TRIM5α-tag, could not. Conversely, the parental HSC-F-TRIM5α-tag did not inhibit the replication of SIVmac239 at all, whereas HSC-F+60tag, which contained the 242-bp fragment of CV1-TRIM5α in the background of HSC-F-TRIM5α-tag, clearly inhibited SIVmac239 (Fig. 4C). We obtained the same results as described above when we used SeVs to express parental TRIM5α-tag or chimeras (Fig. 4D). These results indicated that the determinant of the species-specific inhibition of SIVmac239 replication is located in 37 amino acid residues in the SPRY domain of CV1-TRIM5α.

To narrow the SIVmac restriction determinant more precisely, we generated two more chimeric TRIM5αs (Fig. 4A). HSC-delete-tag contained a CV1-derived 17-amino-acid fragment without the 20-amino-acid duplication in the background of the HSC-TRIM5α-tag. HSC-insert-tag contained the HSC-F-derived 17-amino-acid fragment with the CV-1 derived 20-amino-acid duplication in the background of HSC-TRIM5α-tag. Recombinant SeVs expressing these chimeric TRIM5αs were generated and used in the subsequent experiments. Although the expression levels of mRNA of each chimericTRIM5α was virtually identical to each other (data not shown), we observed considerable variations in the levels of TRIM5α protein expression among chimeras (Fig. 4E). The HSC-delete-tag showed slightly higher levels of protein expression than those of HSC-F+60tag. However, HSC-delete-tag failed to inhibit SIVmac replication, whereas it restricted HIV-1 replication as completely as HSC-F+60tag did (Fig. 4F). These results clearly indicated that the CV1-derived 17-amino-acid region alone was not sufficient for SIVmac restriction. On the other hand, HSC-insert-tag partially restricted both HIV-1 and SIVmac, although this chimera showed lower levels of protein expression than other chimeras did (Fig. 4E and F). These results indicated that the CV1-specific 20-amino-acid duplication was important in SIVmac restriction.

To determine whether the CV1-specific 20-amino-acid duplication was indispensable for SIVmac restriction, we generated CV1-delete-tag, which lacked the 20-amino-acid duplication in the CV1-TRIM5α-tag (Fig. 4A). The protein expression level of CV1-delete-tag was comparable to that of CV1-60tag (Fig. 4E), and CV1-delete-tag inhibited HIV-1 replication as completely as CV1-60tag did. However, CV1-delete-tag was shown to lose the ability to inhibit SIVmac infection (Fig. 4F). Taken together, our data clearly indicated that the 20-amino-acid duplication of CV1-TRIM5α was necessary for SIVmac restriction and suggested that the adjacent 17-amino-acid region of CV1-TRIM5α was also necessary to fully restrict SIVmac infection.

HIV-2 GH123 is sensitive to cynomolgus monkey TRIM5α, as well as African green monkey TRIM5α. HIV-2 is closely related to SIVmac (9). We tested whether or not the sensitivity of HIV-2 to various TRIM5αs was similar to that of SIVmac239. C143 cells expressing CV1, HSC-F, and their chimeric TRIM5αs were infected with the VSV-G-pseudotyped HIV-2 strain GH123. Surprisingly, HSC-F-TRIM5α-tag inhibited HIV-2 replication as CV1-TRIM5α-tag had done (Fig. 5). Both chimeric TRIM5αs, CV1-60tag and HSC-F+60tag, also inhibited HIV-2 replication to a similar extent (Fig. 5). These results indicated that HIV-2 strain GH123 was sensitive to cynomolgus monkey TRIM5α despite its high level of sequence homology to SIVmac239.

DISCUSSION

In the present study, we showed that both cynomolgus and African green monkey TRIM5αs could inhibit HIV-1 infection. African green monkey TRIM5α could also inhibit SIVmac infection, whereas cynomolgus monkey TRIM5α could not. Experiments on chimeras of the cynomolgus and African green monkey TRIM5αs unequivocally demonstrated that a small region composed of 37 amino acid residues in the SPRY domain of African green monkey TRIM5α was responsible for restricting the SIVmac infection.

A previous study showed that rhesus monkey TRIM5γ, a splicing variant lacking the SPRY domain, did not suppress HIV-1 infection (33). In the case of TRIM7, the SPRY domain alone was sufficient for binding to its ligand glycogenin (38). Deletion of the entire SPRY domain from TRIM11 also abol-

suppression, respectively. (B) Lysates of C143 cells expressing CV1-TRIM5α-tag (lane 1), HSC-F-TRIM5α-tag (lane 2), HSC-F+60tag (lane 3), CV1-60tag (lane 4), or empty vector (lane 5) were immunoprecipitated by using anti-HA antibody. The resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. (C) C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped NL43 or SIVmac239, and culture supernatants were periodically assayed for levels of p24 or p27. The datum points are means for triplicate samples with the SD. (D) MT4 cells infected with SeV expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD. (E) Lysates of MT4 cells infected with recombinant SeVs expressing CV1-60tag, HSC-F+60tag, HSC-delete-tag, CV1-delete-tag, HSC-insert-tag, or parental Z strain were immunoprecipitated by using anti-HA antibody. Resultant immunoprecipitates were visualized by Western blotting with an antibody to HA. A representative result of three independent experiments is shown. The relative amounts of TRIM5α protein to that of HSC-F+60tag were calculated, and means and SD values of three independent experiments are shown. (F) MT4 cells infected with SeV expressing CV1-60tag (△), HSC-F+60tag (▲), HSC-delete-tag (asterisks with blue lines), HSC-insert-tag (●), CV1-delete-tag (diamonds with red lines), or empty vector (○) were infected with NL43 or SIVmac239, and culture supernatants were assayed for levels of p24 and p27. The datum points are means for triplicate samples with the SD.

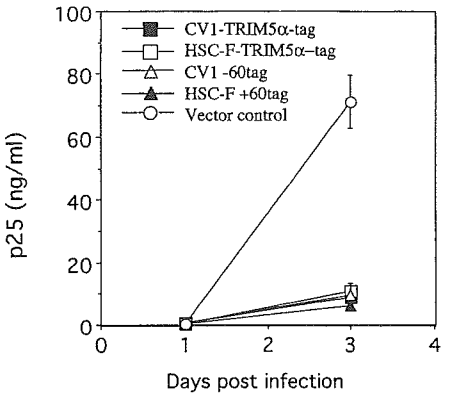

FIG. 5. C143 cells expressing CV1-TRIM5α-tag (■), HSC-F-TRIM5α-tag (□), CV1-60tag (△), HSC-F+60tag (▲), or empty vector (○) were infected with VSV-pseudotyped GH123, and culture supernatants were periodically assayed for levels of p25. The datum points are means for triplicate samples with the SD.

ished its ability to bind Humanin (21). Therefore, it is reasonable to assume that the variable N-terminal region of the SPRY domain of TRIM5α binds to HIV-1 or SIVmac CA protein. This assumption is consistent with the recent findings that in owl monkey cells, HIV-1 infection was restricted by a TRIM5-cyclophilin A fusion protein in which the SPRY domain was replaced with cyclophilin A, since cyclophilin A is a well-known ligand of HIV-1 CA protein (22, 28).

In the attempt to further narrow the SIVmac restriction determinant more precisely, we were able to demonstrate that the African green monkey-specific 20-amino-acid duplication was indispensable for SIVmac restriction and that the adjacent 17-amino-acid region of African green monkey alone was not sufficient. However, HSC-insert-tag carrying the cynomolgus monkey-derived 17-amino acid region with African green monkey-specific 20-amino-acid duplication showed low levels of protein expression and only partial suppression of HIV-1 and SIVmac replication. It is possible that an artificial combination of African green monkey-specific 20-amino-acid duplication with the cynomolgus monkey-derived 17-amino-acid region made TRIM5α molecules unstable. Further studies, including mutational analysis of the African green monkey-specific 17-amino-acid region, are necessary to determine the precise role of this region in SIVmac restriction.

Despite its close similarity to SIVmac, HIV-2 strain GH123 was restricted by cynomolgus monkey TRIM5α, as well as by African green monkey TRIM5α. Although both HIV-2 and SIVmac were considered to come from SIVsm (9), it is possible that HIV-2 has been replicating in the human population in the absence of TRIM5α restriction for a certain period and has lost its ability to escape from cynomolgus monkey TRIM5α. However, it has also been reported that there was a considerable degree of variation in the ability to grow in monkey cells among HIV-2 strains (6, 8, 26). Therefore, it is necessary to examine various HIV-2 strains for their sensitivity to human and monkey TRIM5αs before we can draw a definite conclusion. It would also be interesting to identify specific amino acid changes determining the sensitivity to cynomolgus monkey TRIM5α in viral CA proteins, since nearly 90% of the amino acid residues in SIVmac239 CA protein are conserved in HIV-2GH123.

In CV1 cells, the level of TRIM5 gene expression was ca. 3×10^6 copies/μg of total RNA, a level similar to that observed in other human cell lines examined (data not shown). Although HIV-1 infection was suppressed in hamster TK-ts13 or human C143 cells expressing CV1-TRIM5α, relatively high levels of TRIM5α (nearly 5×10^7 copies/μg of total RNA) appeared to be required for a level of suppression similar to that observed in CV1 cells. One possible explanation for this discrepancy is that certain molecules cooperating with TRIM5α also showed species specificity, and CV1-TRIM5α was not fully supported in hamster and human cells. Because TRIM5 gene products are suspected to be an E3 ubiquitin ligase (35), it is important to identify the E2 ubiquitin-conjugating enzyme interacting with TRIM5α. Alternatively, restriction factors other than TRIM5α may exist in CV1 cells, or certain molecules required for efficient lentivirus infection may be absent in CV1 cells.

After we submitted these findings for publication, small amino acid differences in the SPRY domain between human and rhesus monkey TRIM5αs were reported to determine HIV-1 restriction (27, 34, 37). Our findings are in good agreement with the results of these studies.

ACKNOWLEDGMENTS

We thank Setsuko Bando for skillful technical assistance. HSC-F cells were kindly supplied by Hirofumi Akari. pGH123 was a gift from Akio Adachi.

This study was supported by grants from the Human Science Foundation; the Ministry of Education, Culture, Sports, Science, and Technology; and the Ministry of Health, Labour, and Welfare of Japan.

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