population, a high seroprevalence of KSHV has been shown in African countries; a medium seroprevalence in countries around the Mediterranean Sea; and a low seroprevalence in other regions, such as North America, Europe, and Asia, suggesting that the KSHV infections are spreading globally [Ganem, 2005]. Although serum antibodies to KSHV are detected in healthy individuals at various rates around the world, including 1.4% in the general Japanese population [Katano et al., 2000], they have been detected more frequently in men who have sex with men than in the general population in the United States and other countries. In previous studies, the seropositivity of KSHV in men who have sex with men ranged from 8% to 24% [Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007]. Furthermore, there is a higher rate of KSHV seropositivity (i.e., >50%) in men who have sex with men and who are infected with HIV-1 [Katano et al., 2000; Casper et al., 2002]. These studies have argued that KSHV infection spreads effectively among men who have sex with men.

In Japan, the incidence of AIDS-KS has been increasing for several years. KS was found in 2.5% of AIDS patients in 1998, and increased to 5.6% in 2008. Similarly, the prevalence of individuals infected with HIV-1 has been increasing, with 70% of the total affected Japanese population being comprised of men who have sex with men (AIDS Surveillance Committee 2011, http://api-net.jfap.or.jp/status/index.html, Japanese). An earlier study reported that 60% of Japanese men who have sex with men infected with HIV-1 were also seropositive for KSHV [Katano et al., 2000]. However, the incidence of KSHV seropositivity among the total population of Japanese men who have sex with men is unknown. Despite the 1997 introduction of highly active antiretroviral therapy (HAART) in Japan, the number of KS cases has not decreased, due to the increasing number of men who have sex with men infected with HIV-1. In the present study, the seroprevalence of KSHV was measured and compared between Japanese men who have sex with men and age- and area of residence-matched control men; the investigation was conducted using enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs).

MATERIALS AND METHODS

Study Subjects

The study protocol was approved by the Institutional Review Board of the National Institute of Infectious Diseases (Approval Nos. 228 and 303). Sera were obtained during KSHV testing from participants at a free and anonymous HIV-1 test clinic for men who have sex with men. All participants in this study were also participants in the 2011 annual Nagoya Lesbian & Gay Revolution festival, one of the largest annual events for Japanese sexual minorities, held on June 4–5, 2011. The HIV-1 test was organized especially for the participants of the festival at a nearby public health center. A total of 257 individuals visited the public health center for the HIV-1 test; 237 agreed to provide informed consent and participate in the study. All participants completed questionnaires, including data on age, gender, area of residence, and sexual behavior. For the purposes of this study, men who have sex with men were defined as men who have insertive anal or oral sex with other men. Individuals who practiced both homosexual and heterosexual activities were also classified as men who have sex with men. Seven participants were excluded from the analysis: four were women, and three were men who described themselves as heterosexual in the questionnaire. Thus, 230 men who have sex with men were included in the study (Fig. 1).

Sera from 400 age-, gender-, and area of residence-matched individuals were collected as controls (Table I). The control sera were obtained from the World Health Organization and the National Serum Reference Bank/Tokyo, the National Institute of Infectious Diseases (http://idsc.nih.go.jp/yosoku/index-E.html). These sera were collected from healthy donors across all districts of Japan and across all age groups in order to survey the prevalence of various infectious diseases. Blood samples were collected in serum-separating tubes from individuals who visited public health centers for medical checks between 2008 and 2010. Collected sera were frozen, shipped to the serum bank, and stored at −80°C until use. There is no information regarding the sexual orientation of the control sera donors.

KSHV Serology

Serum KSHV antibodies were detected using both mixed-antigen ELISAs and IFAs, with a positive

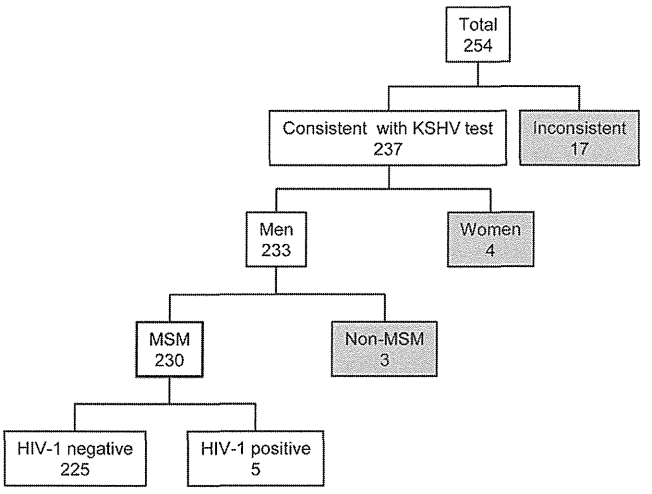

Fig. 1. Study flow diagram. Of the 257 individuals attending the free and anonymous HIV-1 test clinic, 237 agreed to participate in the study. According to the participants' responses to a questionnaire, three men who described themselves as heterosexuals and four women were excluded. Thus, 230 men who have sex with men were enrolled in the study. Five of them were HIV-1-positive.

TABLE I. Kaposi's Sarcoma-Associated Herpesvirus Seropositivity Among Men Who Have Sex With Men and Controls

	Men who have sex with men[a]	Control[a]	OR	(95% CI)	P^*
Total	27/230 (11.70%)	20/400 (5.00%)	2.52	1.38–4.61	0.003
ELISA	6/230 (2.61%)	2/400 (0.50%)	5.33	1.07–26.63	0.057**
IFA	26/230 (11.3%)	18/400 (4.50%)	2.70	1.45–5.05	0.001
Both	5/230 (2.17%)	0/400 (0.00%)	—		0.013**
Age					
18–29	5/75 (6.67%)	8/150 (5.33%)	1.23	0.39–3.90	0.957
30–39	11/81 (13.58%)	9/150 (6.00%)	2.46	0.97–6.22	0.087
40–60	6/46 (13.04%)	3/100 (3.00%)	4.85	1.16–20.35	0.048**
No answer	5/25 (20.00%)	—	—	—	—
Area					
Chubu	22/200 (11.00%)	16/319 (5.02%)	2.34	1.20–4.57	0.018
Other	5/30 (16.67%)	4/81 (4.94%)	3.85	0.96–15.46	0.105**

KSHV, Kaposi's sarcoma-associated herpesvirus; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; OR, odds ratio; CI, confidence interval.
[a]n/N (%): where n is the number KSHV seropositives, N is the total number of participants, and (%) is the percent of KSHV seropositive individuals in each category.
*Chi-square test for comparison of KSHV positivity between men who have sex with men and controls.
**Chi-square test with Yates correction was used because of sparse data.

result from either test indicating a positive serum sample. The mixed-antigen ELISA and IFA were performed as reported previously [Katano et al., 2000]. All of the serum samples were heat-incubated at 55°C for 30 min to inactivate any viruses in the serum. Mixed antigens, including K8.1 and open reading frames 59, 65, and 73 proteins, were employed as the immunogens in the ELISA. These proteins were identified as antigenic proteins encoded by KSHV using an expression library-based analysis [Katano et al., 2000]. These recombinant proteins were produced as glutathione *S*-transferase fusion proteins in *Escherichia coli*, as described previously [Smith and Johnson, 1988]. The cut-off value for the mixed-antigen ELISA was determined as the mean value plus 5× SD for 43 normal serum samples. The ELISA was validated by 100% (24/24) positivity in KS patients and 1.4% (14/1,004) in the general Japanese population [Katano et al., 2000]. Sera, diluted at 1:100, were used in the assay and all positive sera were tested in duplicate to confirm their positivity.

In the IFA, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced TY-1 cells, a KSHV-infected cell line, were initially used as antigen cells. Positive sera were then examined in TPA-induced BCBL-1, a KSHV-infected PEL cell line, BJAB, a KSHV-negative B-cell line, and Raji, a KSHV-negative, EBV-positive B-cell line [Renne et al., 1996; Katano et al., 1999]. Sera, positive in BCBL-1 and TY-1 but negative in BJAB and Raji cells, were categorized as positive.

Human Immunodeficiency Virus, *Treponema pallidum*, and Hepatitis B (HBV) and C (HCV) Virus Infections

Serum HIV-1 RNA was measured by reverse transcription-polymerase chain reactions (COBAS AmpliPrep/COBAS TaqMan HIV-1 Test; Roche Diagnostics, Boehringer Mannheim, Germany). The presence of *T. pallidum* (TP) infection was determined using a Latex suspension (a rapid plasma regain, Sekisui Medical, Tokyo, Japan). HBV and HCV antigens were identified using Architect HBsAg QT and HCV (Abbott, Abbott Park, IL).

Statistical Analysis

Chi-square tests, with Yates correction, were used to compare KSHV seropositivity between men who have sex with men and controls. A multivariable logistic regression analysis, with a forced entry method, was performed to determine the independent role of the variables (answers in the participants' questionnaires). All of the statistical analyses were conducted using SPSS (IBM, Armonk, NY).

RESULTS

The median ages (mean, range) of the men who have sex with men and controls were 33.0 (33.1, 18–60) and 32.0 (33.4, 20–49) years, respectively. Twenty-seven (11.7%) of the 230 Japanese men who have sex with men were seropositive for KSHV, as determined by ELISA or IFA (Figs. 2 and 3, and Table I). Five serum samples were found to be positive by both ELISA and IFA, and one serum sample, positive by ELISA in the men who have sex with men group, was negative by IFA. In the control group, 20 (5%) of the 400 age- and area of residence-matched Japanese men were seropositive by ELISA or IFA; none of the ELISA-positive control sera were positive by IFA. Compared to the controls, the seroprevalence among men who have sex with men was significantly higher (odds ratio [OR] = 2.52, 95% confidence intervals (CI) = 1.38–4.61, $P = 0.003$, Chi-square test) than among the control men. In an examination of seroprevalence by age groups, 40–60 year-old men who have sex with men showed significantly higher positivity for KSHV than did the age-matched

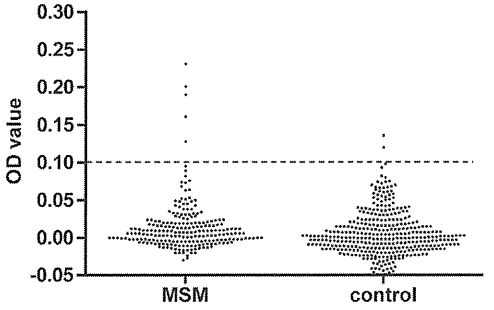

Fig. 2. Group scatter diagrams for enzyme-linked immunosorbent assay (ELISA) results. The scatter diagrams show the results of reactions of sera from men who have sex with men and controls in the mixed Kaposi's sarcoma-associated herpesvirus antigen ELISA. Optical density (OD) values were calculated as follows: (sample OD – negative control OD)/(positive control OD – negative control OD) [Katano et al., 2000]. A horizontal broken line indicates the cut off value.

controls ($P = 0.048$, Chi-square test with Yates correction), indicating a higher seroprevalence of KSHV among older men who have sex with men. Furthermore, men who have sex with men from the Chubu area showed significantly more prevalent KSHV positivity than was observed in controls ($P = 0.018$, Chi-square test), but did not in any other area. This may have been due to the small number of samples from other areas.

The presence of serum antibodies against HIV-1, TP, HBV, and HCV was also tested in all samples from men who have sex with men. Of the five men who have sex with men and who were also HIV-1-positive, KSHV antibodies were detected in one. HIV-1 positivity among KSHV seropositive men who have sex with men (1/27, 3.7%) was 1.91 (95% CI: 0.21–17.78) times higher than among KSHV seronegative men who have sex with men (4/203, 2.0%). Of the 12 test subjects with TP antibodies, three were KSHV seropositive. The rate of TP positivity among KSHV seropositive men who have sex with men (3/27, 11.1%) was 2.69 (95% CI: 0.68–10.64) times higher than that among KSHV seronegative men who have sex with men (9/203, 4.4%). However, there was no significant difference between HIV-1 or TP infection rates and KSHV seropositivity ($P = 0.14$ and 0.56, respectively, Chi-square test). Two HBV-positive and 1 HCV-positive men who have sex with men were negative for KSHV; there was no association between KSHV infection and the presence of these antibodies.

The association between the infections and sexual behaviors, determined using the participants' questionnaires, is shown in Table II. KSHV seropositivity was not correlated with the possibility of HIV-1 infection (subjects' perceived potential HIV-1 infection status) or with their sexual behaviors during the previous 6 months. There were no statistical differences between the use of condoms during anal sex and the rate of KSHV seropositivity, regardless of whether the subjects were performing or receiving

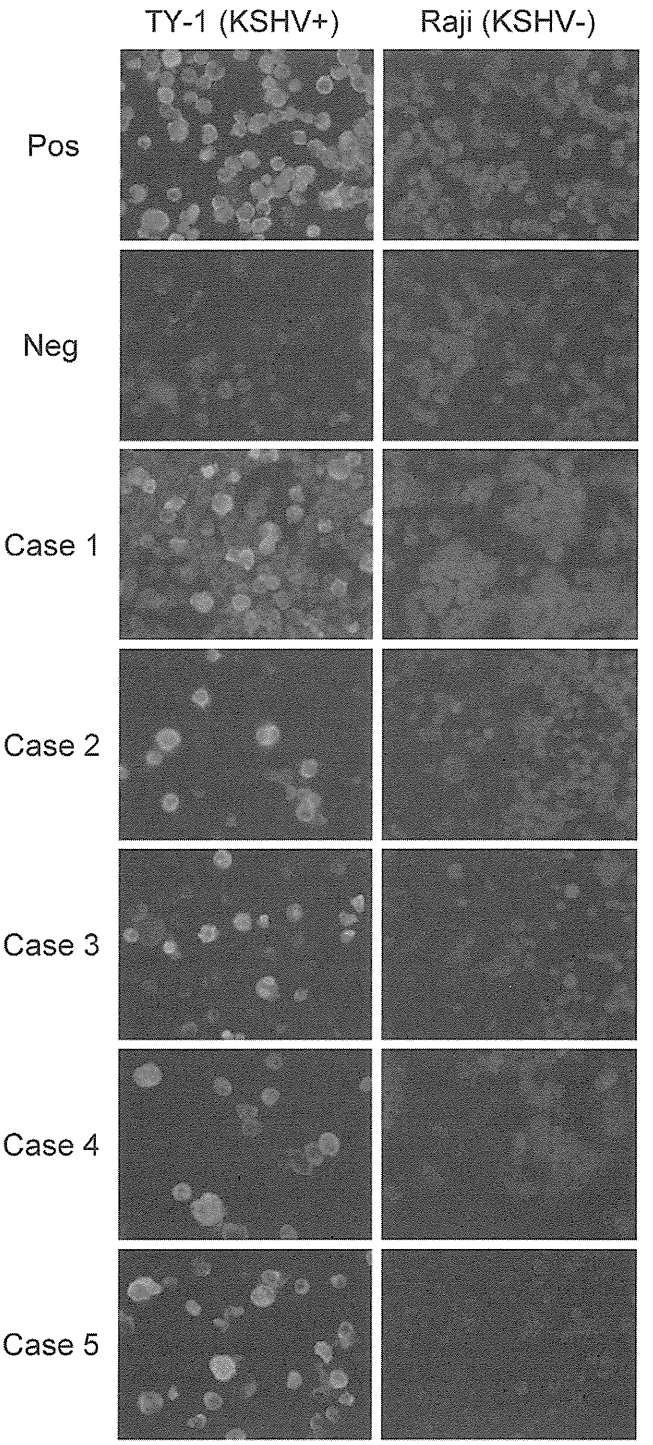

Fig. 3. Immunofluorescence images for Kaposi's sarcoma-associated herpesvirus (KSHV) immunofluorescence assay (IFA). Five positive samples from men who have sex with men are shown. The positive sera reacted with antigens in TY-1 (KSHV-positive, Epstein-Barr virus-negative lymphoma cell line), but not in Raji (KSHV-negative, Epstein-Barr virus-positive lymphoma cell line). Positive control serum from a Kaposi's sarcoma patient and negative control serum from a healthy individual are also shown.

TABLE II. Multivariate Model of Predictors of Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Seropositivity in Sexual Behaviors

Question	Answer	KSHV+	Total	%	AOR (95% CI)*	P
Sexual orientation	Homosexual	25	196	12.76	Reference	
	Bisexual	2	34	5.88	0.431 (0.088–2.117)	0.300
Possibility of HIV infection	No	14	144	9.70	Reference	
	Yes	13	86	15.10	1.867 (0.767–4.544)	0.169
Sexual behaviors in last 6 months	No	2	10	20.00	Reference	
	Yes	25	216	11.60	0.356 (0.59–2.144)	0.260
Performance of insertive anal sex with main partner	Not wearing condom	4	30	13.30	Reference	
	Sometimes wearing condom	4	39	10.30	1.077 (0.141–8.224)	0.943
	While wearing condom	6	56	10.70	0.737 (0.095–5.724)	0.771
Receipt of anal sex with main partner	Partner not wearing condom	3	29	10.30	Reference	
	Partner sometimes wearing condom	2	30	6.70	1.467(0.123–17.574)	0.762
	Partner wearing condom	8	50	16.00	3.676 (0.365–36.975)	0.269
Performance of insertive anal sex with casual partner(s)	Not wearing condom	5	20	25.00	Reference	
	Sometimes wearing condom	1	31	3.20	0.117 (0.008–1.786)	0.123
	While wearing condom	8	68	11.80	0.346 (0.049–2.419)	0.285
Receipt of anal sex with casual partner(s)	Partner not wearing condom	4	14	28.60	Reference	
	Partner sometimes wearing condom	1	31	3.20	0.093 (0.005–1.699)	0.109
	Partner wearing condom	10	48	20.80	0.737 (0.085–6.400)	0.782

*AOR, adjusted odds ratio; CI, confidence interval.

anal sex or whether the anal sex was performed with the subject's main partner or with casual partners. However, condom use was associated with decreased (0.3–0.7 times less) KSHV positivity among subjects performing or receiving anal sex with casual partners than among those who did not use condoms.

DISCUSSION

This study showed that KSHV seroprevalence in Japanese men who have sex with men is 11.7%, which is similar to the seroprevalence among a similar population of men in the USA and Europe. The higher seroprevalence of KSHV among men who have sex with men, compared with controls, suggests that the circulation of KSHV infection among Japanese men who have sex with men is more efficient than among heterosexual males, as previously reported [Goudsmit et al., 2000; Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007; Giuliani et al., 2007]. Although the transmission route of KSHV remains unclear, the higher seroprevalence of KSHV between men who have sex with men than that among the general population suggests that transmission likely occurs through homosexual behaviors in non-endemic areas, such as in the USA, Europe, and Asia [Goudsmit et al., 2000; Diamond et al., 2001]. In contrast, in KSHV endemic areas, such as Africa, a high seroprevalence of KSHV has been found even among children [Bourboulia et al., 1998; Butler et al., 2009]. Since high copy numbers of KSHV have been detected in the saliva of those infected with KSHV, vertical mother-to-child transmission may occur through saliva [Pauk et al., 2000; Mbulaiteye et al., 2006]. In addition, in KSHV endemic areas, sexual transmission has not been associated with KSHV infection [Shebl et al., 2011].

Of the 230 subjects in this study, 12 (5.2%) were positive for TP, suggesting that these were individuals with high levels of sexual activity. There were no significant associations between HIV-1, HBV, HCV, or TP and KSHV infections in Japanese men who have sex with men in the present study. A previous study with a large sample size, on individuals without HIV-1 infection but at high risk for sexually transmitted infections, demonstrated that the incidence of KSHV infection was different from that for HIV-1 and other sexually transmitted infections [Giuliani et al., 2007], suggesting that the routes of KSHV transmission and the opportunity for KSHV infection are different from other infections. The present study showed that the seroprevalence of KSHV is higher than that of the aforementioned sexually transmitted diseases in Japanese men who have sex with men, implying that KSHV infection can be an early marker of sexually transmitted infections in a certain proportion of study subjects.

Japanese men who have sex with men tend to use condoms less frequently for oral sex than for anal sex [Inoue et al., 2006]. Considering that the saliva of KSHV-infected persons contains high loads of KSHV, oral sex is possibly a transmission route of KSHV [Pauk et al., 2000]. There was no statistical difference in the incidence of KSHV positivity between those who did and those who did not use condoms during anal sex with their main partners (Table II). However, in subjects performing or receiving anal sex with

casual partners, the incidence of KSHV positivity was 0.3–0.7 times less among those who used condoms, compared with those who did not use condoms (Table II); this finding suggests that the risk of KSHV infection through anal sex can be reduced by condom use.

A gold standard for KSHV serology testing does not currently exist [Corchero et al., 2001; Pellett et al., 2003]. However, a combination of ELISA and IFA has been found to be more accurate for the detection of serum KSHV antibodies than any individual method. In the present study, 5% of the control sera were positive for KSHV in ELISA or IFA. A previous study demonstrated that by ELISA, alone, 1.4% of the Japanese general population was found to be positive for the KSHV serum antibody [Katano et al., 2000]. However, the findings in the present study are not directly comparable with those in that study as different serological assays were used in the present study and the control sera was obtained predominantly from 30- to 40-year-old men, most of whom resided in the Chubu area. Data, from the current study, using a combination of ELISA and IFA suggests that the seroprevalence of KSHV antibodies among the general, Japanese population is between 2% and 5%. Although information was not available on the sexual habits of those providing the control sera, 2% of adult Japanese men are estimated to have had sex with other men [Ichikawa et al., 2011]. Thus, in the present study involving 400 control subjects, there may have been up to eight participants who have engaged in homosexual sexual activity. If eight are excluded from 380 KSHV-negative controls, the seroprevalence of KSHV among men who have sex with men (11.7%) remains statistically higher than that among controls (OR 2.47, 95% CI 1.35–4.52, $P = 0.002$, Chi-square test), suggesting that the potential inclusion of a small number of men who have sex with men in the control group did not affect the conclusions. However, a more focused investigation, examining sexual orientation-matched samples, would be required to more accurately state the KSHV positivity among men in the control group.

In conclusion, this study revealed that the seroprevalence of KSHV between Japanese men who have sex with men is 11.7%, which is higher than that among controls, suggesting that the circulation of KSHV infection among men who have sex with men in Japan is more efficient than among heterosexual males. In addition, the higher prevalence of KSHV antibodies than those for other infectious diseases that may be sexually transmitted suggests that the KSHV test may be an early maker for sexually transmitted diseases. Nonetheless, the transmission route of KSHV remains unclear. Further detailed studies on sexual behaviors and virus shedding in the saliva will be required to clarify the mechanism of KSHV infection among men who have sex with men.

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Prevalence of Transmitted HIV Drug Resistance in Iran between 2010 and 2011

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Abstract

Objective

Drug-resistant (DR) HIV emerges during combined antiretroviral treatment (cART), creating concern about widespread transmission of DR-HIV as cART is expanded in resource-limited countries. The aim of this study was to determine the predominant HIV-1 subtypes and prevalence of transmitted DR mutations among antiretroviral-naïve patients in Iran.

Design

To monitor transmission of DR HIV, a threshold surveillance based on the world health organization (WHO) guidelines was implemented in Iran.

Methods

For this HIVDR threshold surveillance study, blood samples were collected from 50 antiretroviral-naïve HIV-1-infected patients. Antiretroviral-resistant mutations were determined by sequencing HIV-1 protease, reverse transcriptase and integrase regions. The HIV-1 subtype was determined by sequencing the p17 and C2-V5 regions of the *gag* and *env* genes, respectively.

Results

Phylogenetic analyses of the sequenced regions revealed that 45 (95.7%) of 47 samples that were successfully obtained were CRF35_AD. The remaining two cases were subtype B (2.1%) and CRF01_AE (2.1%). Consistent results were obtained also from Env and Gag sequences. Regarding prevalence of transmitted DR viruses, two cases were found to harbor reverse transcriptase-inhibitor-resistant mutations (4.3%). In addition, although not in the WHO list for surveillance of transmitted mutations, 13 minor protease-inhibitor-resistant mutations listed in the International AIDS Society-USA panel of drug resistance mutations were found. No DR mutations were detected in the integrase region.

Conclusions

Our study clarified that CRF35_AD is the major subtype among HIV-1-infected patients in Iran. According to the WHO categorization method of HIVDR threshold survey, the prevalence of transmitted drug resistant HIV in Iran was estimated as moderate (5–15%).

Figures

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Introduction

Selection and acquisition of HIV-1 drug resistance is inevitably associated with combined antiretroviral treatment (cART) of HIV-1-infected patients. Thus, the emergence of drug-resistant (DR) HIV is a major concern as a potential consequence of scaling up cART [1]. Indeed, expansion of cART might be jeopardized by widespread transmission of DR-HIV [2], particularly in countries where antiretroviral options are limited.

The first HIV-infected case in Iran was a hemophilic patient identified in 1986, and the first cases of HIV transmission through drug injections were reported in 1989 [3]. As of March 2012, 24290 HIV-1-infected individuals have been reported in Iran [4]. The transmission routes were attributed to injected drugs (69.6%), sexual contact (10.5%), transfusion of blood products (1.0%) and mother-to-child transmission (0.1%) [4]. Iran is experiencing a concentrated HIV-1 epidemic among injecting drug users (IDUs) [5], [6].

In Iran, cART was introduced in 1997 when the antiretrovirals zidovudine, lamivudine, and indinavir became available as a part of the country's healthcare system, making it possible to scale up cART in Tehran and other cities such as Kermanshah and Shiraz [7]. Subsequently, indinavir was replaced with nelfinavir from the list

of Iranian generic drugs. Later, stavudine, nevirapine, and didanosine in 2005, efavirenz, lopinavir/ritonavir, tenofovir, and abacavir in 2006, atazanavir in 2011 became available for HIV/AIDS patients [3], [7]. cART is in line with Iran's guidelines on clinical care for HIV/AIDS patients, which state the 14 possible three-drug combinations from the antiretrovirals mentioned above [3]. cART is supplied to HIV/AIDS patients for free at counseling and behavioral centers (triangular clinics) [7].

In addition to receiving cART, all HIV/AIDS patients in Iran are monitored for $CD4^+$ T cell counts periodically by the government at no cost for patients. On the other hand, the Iranian guidelines do not include measurement of viral load. Similarly, drug-resistance monitoring is available on a limited basis, and it is not tested on every Iranian patient for drug resistance. Therefore, to address concerns about the emergence and transmission of DR-HIV in Iran, we conducted a threshold survey among drug-naïve individuals by following recommendations of the World Health Organization (WHO) drug-resistance network. In this study, we report the estimated prevalence of DR-HIV transmission in Iran.

Methods

Eligibility Criteria

Samples were collected according to the HIV Drug Resistance Threshold Survey (HIVDR-TS) recommended by the WHO for the surveillance of DR-HIV in resource-limited countries [8]. Individuals were recruited from all counseling and behavioral centers in Tehran from January 2010 through February 2011 if they met these eligibility criteria: under age 25 years at HIV diagnosis and no previous pregnancy for females [8]. However, after these 9 months, only 15 newly diagnosed cases were recruited. Thus, after consultation with WHO experts, to reduce sample collection period, the inclusion criteria were expanded to less than 30 years of age, $CD4^+$ T cell counts >500/µl, without previous pregnancies for females and no previous exposure to antiretroviral drugs. In addition to Tehran, samples were collected from two other areas in Iran, Kermanshah in the west and Shiraz in the south of Iran, where antiretroviral therapy was started at the same time as Tehran.

Sample

Newly diagnosed, antiretroviral-naïve HIV-1 patients (n = 50) were enrolled in this study. They visited counseling and behavioral centers in Tehran (n = 30), Kermanshah (n = 10), or Shiraz (n = 10) between January 2010 and February 2011. After patients signed informed consent, 10 ml of their peripheral blood was collected into EDTA-containing vacutainer tubes. Plasma samples were obtained by centrifugation and aliquots were stored at -70°C until use. If possible, each patient was asked to complete a questionnaire regarding patient's basic information, including age, sex, risk behavior, marital status, and status of hepatitis B virus (HBV) or hepatitis C virus (HCV) co-infection.

Ethics Statement

This study was approved by the Ethics Committees in Medical Sciences Research at the Tehran University of Medical Sciences and Ministry of Health and Medical Education.

Determination of Drug-Resistance Mutations by Genotyping

We used the drug resistance genotypic testing protocol described previously by Sugiura, et al. with some modification [9]. Briefly, viral RNA was extracted from 140 µl of plasma by the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Nucleotide sequences of whole HIV-1 protease (PR, HXB2 position 2253–2549), the N-terminal portion of reverse transcriptase (RT, 2550–3269), and full-length integrase (INT, 4230–5093) were amplified using region-specific primer pairs by reverse transcription (RT)-polymerase chain reaction (PCR) using SuperScript III one-step RT-PCR system with platinum Taq high-fidelity kit (Life Technologies Corp., Tokyo, Japan), and amplified further in nested PCR using LA Taq (Takara Bio Inc., Shiga, Japan). After gel electrophoresis, the amplified PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan). Sequencing reaction was performed using the BigDye terminator cycle sequencing kit v3.1 (Applied Biosystems, Tokyo, Japan). Nucleotide sequences were determined on the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The sequenced data were aligned against HXB2 and amino acid mutations were detected by SeqScape 2.5 software (Applied Biosystems). Presence of transmitted HIV-1 drug-resistance mutations was determined using the WHO mutation list [10].

HIV-1 Subtype Determination

To determine the subtype of each sample, the nucleotide sequences including p17 of the *gag* gene (Gag, HXB2 position 708–1230) and the C2-V5 region of the *env* gene (Env, 6934–7651) were determined using the same protocol as for the drug-resistant genotypic test described above. The nucleotide sequences obtained for Gag and Env as well as PR, RT, and INT were aligned by ClustalW, and phylogenetic trees were constructed using neighbor-joining analysis with 1000 bootstraps as implemented in MEGA 5 [11].

Nucleotide Sequence Accession Numbers

The nucleotide sequences obtained in this study were deposited in the DNA Data Bank of Japan, and are available under the following accession numbers: AB716095–AB716141 for PR and RT, AB716142–AB716188 for INT, AB716189–AB716232 for Gag, and AB716233–AB716276 for Env. To be noted, one of the *env* sequences contained ambiguous regions. Thus, it was submitted in two parts: AB716271 and AB716272.

Results

The Major HIV-1 Transmission Route in Iranian Cases is Injecting Drug Use

Background information on the 50 newly diagnosed HIV-1-infected cases is summarized in Table 1. More than a half of study cases were male (64.0%) and in the 25–30 year-old age group (56.9%). Their average age was 26.0 years. Among 35 participants who completed the questionnaire, 51.4% were infected by drug injections. Together with 9 individuals who acquired HIV through heterosexual contact with IDU partners, IDU-related transmission accounts for 77.1%. Co-infection with hepatitis C virus and hepatitis B virus was identified among 46.7% and 7.1% of 30 and 28 tested participants, respectively.

Characteristic	*n* (%)	
Men[1]	32	(64.0)
Age (years) [1]		
<20	2	(4.0)
20–24	13	(26.0)
25–30	35	(70.0)
Residence[1]		
Tehran	30	(60.0)
Kermanshah	10	(20.0)
Shiraz	10	(20.0)
Marital status[2]		
Single	20	(57.1)
Married	11	(31.4)
Divorced	3	(8.6)
Widowed	1	(2.9)
Transmission route[2]		
Injecting drugs	18	(51.4)
Heterosexual contact	6	(17.1)
From injecting drug-using husband	8	(22.9)
From injecting drug-using wife	1	(2.9)
Unknown	2	(5.7)
Coinfection[3]		
Hepatitis C virus	14	(46.7)
Hepatitis B virus	2	(7.1)

[1]Information on gender, age, and city of residence were obtained from all 50 cases who completed the questionnaire.
[2]Information on marital status and transmission route were obtained from 35 cases who completed questionnaires.
[3]Infection with hepatitis C virus and hepatitis B virus were tested in 30 and 28 cases, respectively.
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Table 1. Baseline characteristics of newly diagnosed HIV-1 infected patients in Iran.
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The Predominant HIV-1 Subtype is CRF35_AD

Among 50 collected specimens, 47 were successfully sequenced in the PR, RT and INT regions (94.0%). Phylogenetic analyses of these PR, RT and INT sequences clarified that 45 of 47 samples were CRF35_AD (95.7%), one was subtype B (2.1%), and one was CRF01_AE (2.1%) (Figures 1A–C). Amplification and nucleotide sequences of Gag and Env were successfully obtained from 44 (88.0%) and 43 (86.0%) specimens, respectively. Consistent results were obtained from phylogenetic analyses of Gag and Env sequences, and no discrepant case was found (Figures 1D, E).

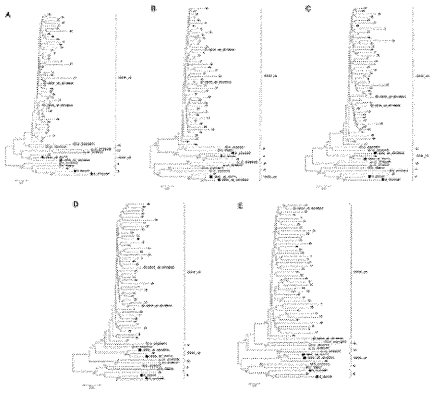

Figure 1. Phylogenetic trees of sequenced Iranian samples.
(A) protease, (B) reverse transcriptase, (C) integrase, (D) gag, and (E) env. Trees were constructed using neighbour joining method with 1000 replicates. Two sequences each of 6 HIV-1 subtypes retrieved from the GenBank are included as references. Open circles (○) indicate the reference sequence of CRF35_AD, open squares (□) indicate subtype A1, open triangles (△) indicate A2, open diamonds (◊) indicate subtype D, closed circles (•) indicate subtype B, and closed squares (▪) indicate CRF01_AE. Bootstrap values over 70% are shown.
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Prevalence of Transmitted Drug-resistant Mutations of Our Study Population is Moderate

Surveillance drug-resistant mutations (SDRMs) associated with transmitted HIV-1 drug resistance [10] were detected in the RT gene of two specimens. Both SDRMs were related to nucleotide reverse transcriptase inhibitor (NRTI) resistance mutations: T215D found in a patient from Kermanshah and K219Q in a patient from Tehran (Table 2).

SDRMs	n	Frequency (%)
NRTI-resistant mutations		
T215D	1	2.1
K219Q	1	2.1
IAS-USA		
PI-resistant mutations		
L10I/V	5	10.6
V11I	2	4.3
G16E	3	6.4
K20R	46	97.9
L33V	1	2.1
M36I	47	100.0
D60E	2	4.3
I62V	5	10.6
L63P	1	2.1
I64L/V	3	6.4
H69K	46	97.9
L89M	46	97.9
I93L	1	2.1

IAS-USA, the international Antiviral society-USA; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; SDRMs, surveillance drug-resistant mutations.
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Table 2. Prevalence of drug resistance-associated mutations among 47 sequenced samples.
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Other than the SDRMs in the WHO list [10], 13 minor mutations in the PR gene listed in the IAS-USA panel [12] were detected (Table 2). M36I was found in all cases (100%), H69K and L89M were found in all except one subtype B sample (97.9%), and K20R was found in 46 samples (97.9%), and they are considered as polymorphic to non-B HIV. In addition, L10V, V11I, G16E, L33V, D60E, I62V, L63P, I64L and I93L were detected, but were less common. Within the INT gene, we found no drug resistance-associated mutation listed in among the IAS-USA panel [12].

Discussion

Here we report the circulating HIV-1 subtype and prevalence of DR-HIV among 50 newly diagnosed HIV-1-infected cases in three different cities of Iran. Among 47 sequenced samples, 45 samples (95.7%) were determined as CRF35_AD, one sample (2.1%) as subtype B and another (2.1%) as CRF01_AE. Our results are consistent with two recent reports on the predominance of CRF35_AD in Iran [13], [14]. Before 2007, subtype A1 had been reported as the predominant HIV-1 subtype in Iran [15]–[17]. However, when we obtained these HIV-1 Env and Gag sequences registered as subtype A1 before 2007 and conducted phylogenetic analysis, we found that they make an evident cluster with CRF35_AD (data not shown). This result suggests that CRF35_AD has been circulating in Iran even before 2007. Taken together, these results indicate that the predominant HIV-1 in Iran is CRF35_AD.

Based on the WHO surveillance list for transmitted resistance [10], only two SDRMs, K219Q and T215D, were found in this study. K219Q predict potential low level resistance to zidovudine and stavudine, and T215D is a revertant mutation associated with an increased risk of virologic failure to TAMs. The prevalence rate calculated from our sample was 4.3% (2/47). Applying the WHO HIVDR-TS formula [18], the prevalence of SDRMs in the RT gene in Iran can be classified as moderate (5–15%). This finding is not surprising considering that NRTIs have been available since 1997 and are widely distributed in Iran as main part of cART. Mutations conferring resistance to reverse transcriptase inhibitors (NRTI and NNRTI) are most common form of transmitted drug resistance detected worldwide, while protease inhibitor resistance is generally less frequent. Two TDR mutations observed in this study were associated with the NRTI drug class. The transmission of NRTI resistance is worrisome since first-line antiretroviral therapy In Iran is based on NRTI+NNRTI combination since 2008. The low prevalence of transmitted drug resistance to PIs was expected giving that the access to these drugs has been limited in Iran [7]. In fact, within the PR gene, we found no mutations associated with SDRMs in our studied cases. However, these specimens had 13 minor drug-resistance mutations based on the IAS HIV-DR list of mutations [12]. Four mutations, K20R, M36I, H69K, and L89M were found in high prevalence (97.9%, 100%, 97.9%, and 97.9%, respectively), consistent with previous reports [13], and can be considered polymorphisms for the predominant subtype CRF35_AD in Iran. Thus, the prevalence of SDRMs in the PR gene was classified as low (<5%). It is likely that transmission of HIV-DR in PR is still uncommon.

A recent pilot surveillance study of HIV drug-resistance transmission in Iran found SDRMs in the RT gene among 39 sequenced specimens; D67D/G was found in a specimen from Esfahan, and V75A/V was found in a 5-year-old female from Tehran both of which confer resistance to NRTIs [13]. Although the sample for this pilot threshold survey was too small to estimate the prevalence of SDRMs, finding two SDRMs in the RT gene is remarkable. Besides CRF35_AD, a subtype G and an A HIV-1 were found in pilot study, whereas in this study, one subtype B and one CRF01_AE cases were found, which suggest that these minor strain are not spreading rapidly.

Although increasing number of individuals is receiving ART in Iran, the coverage rate was still low amoung those in need of therapy in December 2011 [7]. It is even lower among IDU because physicians are concerned that injecting drugs is considered as a risk factor for poor adherence which results in development of DR HIV, and they hesitate to prescribe antiretrovirals [19], [20]. Considering low cART coverage among IDU, who comprised the majority of our major study sample, the moderate (5–15%) prevalence of SDRMs estimated by applying the WHO HIVDR-TS leads us to suspect that frequent and efficient DR transmission is taking place within the IDU population. As more eligible patients actually start to receive cART, it is assumed that the prevalence of DR HIV may increase. Thus, it is important to review monitoring data on antiretroviral therapy programs for the relevant geographical area and investigate potential problems with regard to several factors: continuous access to services, drug supply, drug quality, prescribing practice toxicity, adverse events, drug sharing, and treatment failures.

In conclusion, according to the WHO categorization method of HIV-drug resistance threshold survey, the prevalence of transmitted drug resistant HIV in Iran was estimated as moderate. This indicates that transmitted drug resistance surveillance should be repeated in 1-year period, according to the WHO guidelines.

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Author Contributions

Interpreted the data: M. Mohraz. Designed the study: EM HJ AM. Collected samples and interpreted the data: EM HK HJ KK AM. Conceived and designed the experiments: FJ KA WS JH SRM AM MRA. Performed the experiments: FJ JH M. Matsuda. Contributed reagents/materials/analysis tools: M. Mohraz SI. Wrote the paper: FJ JH.

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RETROVIROLOGY

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The phosphorylation of HIV-1 Gag by atypical protein kinase C facilitates viral infectivity by promoting Vpr incorporation into virions

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Abstract

Background: Human immunodeficiency virus type 1 (HIV-1) Gag is the main structural protein that mediates the assembly and release of virus-like particles (VLPs) from an infected cell membrane. The Gag C-terminal p6 domain contains short sequence motifs that facilitate virus release from the plasma membrane and mediate incorporation of the viral Vpr protein. Gag p6 has also been found to be phosphorylated during HIV-1 infection and this event may affect virus replication. However, the kinase that directs the phosphorylation of Gag p6 toward virus replication remains to be identified. In our present study, we identified this kinase using a proteomic approach and further delineate its role in HIV-1 replication.

Results: A proteomic approach was designed to systematically identify human protein kinases that potently interact with HIV-1 Gag and successfully identified 22 candidates. Among this panel, atypical protein kinase C (aPKC) was found to phosphorylate HIV-1 Gag p6. Subsequent LC-MS/MS and immunoblotting analysis with a phospho-specific antibody confirmed both in vitro and in vivo that aPKC phosphorylates HIV-1 Gag at Ser487. Computer-assisted structural modeling and a subsequent cell-based assay revealed that this phosphorylation event is necessary for the interaction between Gag and Vpr and results in the incorporation of Vpr into virions. Moreover, the inhibition of aPKC activity reduced the Vpr levels in virions and impaired HIV-1 infectivity of human primary macrophages.

Conclusion: Our current results indicate for the first time that HIV-1 Gag phosphorylation on Ser487 is mediated by aPKC and that this kinase may regulate the incorporation of Vpr into HIV-1 virions and thereby supports virus infectivity. Furthermore, aPKC inhibition efficiently suppresses HIV-1 infectivity in macrophages. aPKC may therefore be an intriguing therapeutic target for HIV-1 infection.

Keywords: HIV-1 infection, Phosphorylation, Vpr, aPKC

Background

Human immunodeficiency virus type 1 (HIV-1), a causative agent of AIDS, is an intracellular parasite that has evolved to invade complex human systems and utilize its host machinery for its proliferation. A dynamic interplay between HIV-1 and its human host systems plays a crucial role in promoting virus replication. The identification of the host factors required for viral infection can provide further insights into the nature of HIV-1 replication pathways and assist with identifying new targets for anti-viral therapies. Recent studies have revealed that host factors are involved in the post-translational modification of viral proteins, such as phosphorylation and ubiquitination, thereby regulating HIV-1 replication and pathogenicity [1-3].

The *gag* gene of HIV-1 encodes both structural and functional proteins essential for the assembly and release of enveloped virus-like particles [4]. In the infected cell, Gag is synthesized as a 55-kDa polyprotein and assembled into spherical immature particles at plasma membrane.

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Concomitant with, or after these viral particles pinch off and are released from the host cell via budding, the virus-encoded protease becomes activated and cleaves Gag into its functional subdomains, matrix (MA, p17), capsid (CA, p24), and nucleocapsid (NC, p7), as well as several shorter segments: SP1 (spacer peptide 1), SP2, and p6. This proteolytic maturation in tandem with the incorporation of viral enzymes and accessory proteins into virions results in the acquisition of HIV-1 infectivity [5-8].

Retroviral assembly can be subdivided into distinct stages of Gag membrane targeting, virus bud formation and induction of membrane curvature, and release of the newly assembled virus bud through a membrane fission event. HIV-1 budding from the cell surface depends on viral late domains within Gag p6 [9]. Two late domains have been identified within p6, the PTAP and $LYPX_nL$ motifs. The PTAP motif binds the cellular protein Tsg101 [10,11], whereas the $LYPX_nL$ motif is the docking site for Alix/AIP-1 [12,13]. Tsg101 functions in HIV-1 budding as a member of the Endosomal Sorting Complex Required for Transport-1 (ESCRT-I), which initiates the sorting of surface proteins into late endosomal compartments known as multivesicular bodies (MVB) [14,15]. Alix, ALG-2 interacting protein, functions in endosomal metabolism, promotes viral budding by interconnecting HIV-1 Gag with the ESCRT-III CHMP4 proteins [16,17].

Another important domain within Gag p6 is the C-terminal LXXLF domain. Interestingly, both the Leu486 and Leu491 residues in this motif are highly conserved and together with the downstream Phe492, comprise the LXXLF binding domain for the HIV-1 accessory viral protein R (Vpr) [18,19]. The substitution of residues in this domain causes a decrease in the Vpr incorporation levels compared with full-length HIV-1 Gag protein, indicating that this conserved region is essential for this process.

HIV-1 Vpr is a non-structural protein that is incorporated into the viral particles and possesses several characteristic features that are known to play important roles in HIV-1 replication and disease progression. Vpr mediates multiple functions, including the nuclear import of the HIV-1 pre-integration complex, G2 cell cycle arrest, the transactivation of both viral replication and host genes, and the induction of apoptosis [20]. Vpr interacts with the LXXLF binding domain of Gag p6 and is thereby packaged into the virus particles. Virion-incorporated Vpr is known to positively regulate the infection of non-dividing cells and enhance virus production in macrophages and in resting T cells. However, it remains elusive whether and how Vpr incorporation is indeed regulated. Furthermore, although p6 has been shown to be post-translationally modified by phosphorylation [2,21,22], it is unknown whether this phosphorylation event has any functional relevance to Vpr incorporation and HIV-1 infectivity.

In our current study, we utilized an in vitro high-throughput protein-protein interaction assay using full-length HIV-1 Gag and host protein kinases synthesized by the wheat germ cell-free protein production system in an attempt to identify the kinase (s) that directs the phosphorylation of Gag p6 to promote virus replication. We here report that atypical protein kinase C (aPKC) is a functional interactor of HIV-1 Gag and facilitates viral infectivity by promoting the incorporation of Vpr into virions. We provide evidence that Gag Ser487 (p6 Ser40) is phosphorylated by aPKC, and that this phosphorylation is essential for p6-Vpr interactions and the resultant Vpr incorporation within viral particles. Using computer-assisted structural modeling, we further explore the biological significance of the phosphorylation of Gag-p6 Ser487 by aPKC for the physiological interaction between Gag and Vpr. Our current study sheds new light on the molecular link between Gag phosphorylation and viral infectivity through the incorporation of Vpr into virions.

Results

aPKC binds and phosphorylates HIV-1 Gag

Our initial goal was to identify host kinases that phosphorylate the HIV-1 Gag protein. Because Gag phosphorylation is important for its functional role, we focused on human protein kinases as potential Gag regulators. We synthesized more than 287 full-length protein kinases using a wheat germ cell-free protein production system, and screened them for their association with Gag with the amplified luminescent proximity homogenous assay (AlphaScreen) [23]. In this method, the extent of the protein-protein interaction was measured by assaying the luminescence intensity (Figure 1A). Full-length Gag and human protein kinases were synthesized using a wheat germ cell-free system and subjected to an AlphaScreen assessment. The binding efficiency of HIV-1 Gag with each kinase was normalized relative to the luminescent activity of a control DHFR protein (Figure 1B). When a relative light unit per cutoff (RLU/Co) ratio of ≥ 3.0 was used as the threshold, we found that 22 host kinases could selectively interact with HIV-1 Gag and thus were identified as primary kinase candidates for the phosphorylation of HIV-1 Gag (Figure 1B).

Our assay detected Erk2 and PKCβ as Gag interactors (S/N = 1.76 and 1.17, respectively), both of which have been already reported to phosphorylate Gag during HIV-1 infection [2,22,24,25]. This validated our screening approach. Interestingly, we further found that the aPKC family kinases, PKCζ and PKCι, could interact with HIV-1 Gag at a relatively high score (S/N = 3.57 and 3.19, respectively). PKCζ and PKCι share a more than 70% amino acid identity in entire protein sequence and 84% in the catalytic domain, and an almost identical

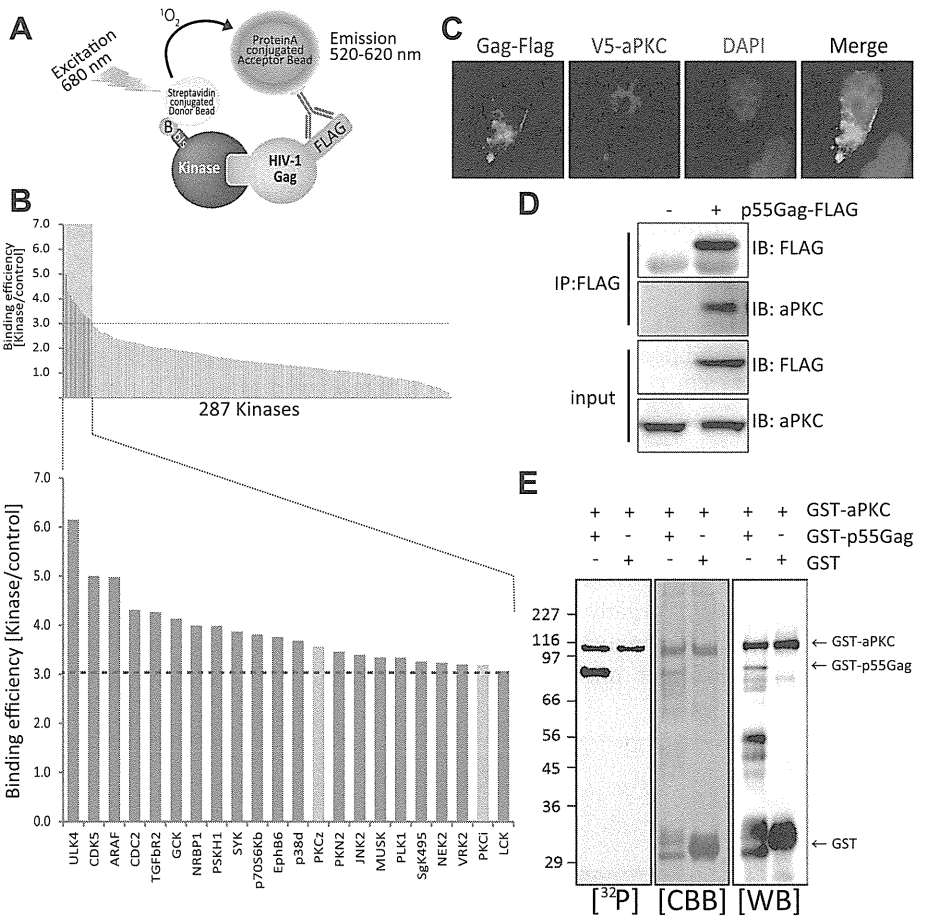


Figure 1 aPKC phosphorylates HIV-1 Gag. (A) Schematic representation of the AlphaScreen based luminescent system used to screen for human kinases that interact with HIV-1 Gag. Briefly, HIV-1 Gag was incubated with each human protein kinase and protein A-conjugated acceptor beads with anti-FLAG antibody and streptavidin coated donor beads were added and bound to the tagged substrate. Upon laser excitation, the donor beads convert ambient oxygen to singlet oxygen. When a molecular interaction occurs between HIV-1 Gag and a particular kinase, singlet oxygen transfers across to activate the acceptor beads and subsequently emit light at 520–620 nm. **(B)** HIV-1 Gag interacting kinases identified from a human kinase protein library. **(C)** Confocal microscopy analysis of V5-aPKC (Alexa 568) and Gag-Flag (Alexa 488) in 293T cells. **(D)** Immunoprecipitation of Gag with aPKC. 293T cells were transfected with Flag tagged Gag and cell lysates were immunoprecipitated with anti-Flag antibody 48 hours later. Samples were then probed with anti-aPKC and anti-Flag antibodies. Bottom panel shows input for pull-down assay. **(E)** *In vitro* phosphorylation of HIV-1 Gag by aPKC. GST, GST tagged aPKC and Gag proteins were expressed and affinity-purified from wheat germ cell-free extract. aPKC-mediated phosphorylation was assessed by incubating recombinant GST or GST-Gag with recombinant active GST-aPKC in the presence of [γ-^{32}P] ATP. The reaction products were analyzed by autoradiography, Coomassie Brilliant Blue (CBB) stain and blotted with GST antibody.

substrate specificity [26]. We thus focused on aPKC as a previously uncharacterized Gag-interacting factor for further in depth functional analysis.

To better understand the functional relevance of aPKC in HIV-1 infection, we first examined the subcellular localization of both HIV-1 Gag protein and aPKC protein in 293T cells by immunofluorescent analysis. 293T cells were transfected with Flag tagged HIV-1 Gag and V5-aPKC expression vector. Gag-Flag displayed a punctate expression pattern in the cytoplasm and a partial co-localization with aPKC in cytoplasm and plasma membrane (Figure 1C).

We performed immunoprecipitation analysis and found that aPKC could bind Gag in cells (Figure 1D). We next examined whether aPKC can directly phosphorylate HIV-1 Gag protein in vitro. Recombinant GST-Gag or GST proteins were expressed and purified from wheat germ cell-free extract by glutathione sepharose beads and used as substrates for in vitro kinase assays. aPKC was found to phosphorylate GST-Gag but not GST, with a prominent auto-phosphorylation of aPKC also observed (Figure 1E). These data together indicate that aPKC binds and phosphorylates HIV-1 Gag.

aPKC phosphorylates the Ser487 residue of HIV-1 Gag

We next sought to determine the sites of aPKC phosphorylation in HIV-1 Gag. GST-Gag was incubated with recombinant aPKC for their phosphorylation and this

mixture was then processed for proteomic analysis. Initial phosphorylation site analysis was performed using the data dependent of tandem matrix-assisted laser desorption Ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS), followed by in depth analysis with selected peptides through data collection. Fragmentation of this peptide by MS/MS produced a spectrum through which we identified one of the b-ions and 10 of the y-ions matching the sequence QEPIDKELYPLTpSLR. Tandem mass spectra of the signals at m/z 1881.95, m/z 1783.95(neutral loss) and m/z 1801.97 revealed sequences corresponding to the unmodified, mono- phospho peptide of Gag-p6 (QEPIDKELYPLTpSLR; Figure 2). Furthermore, a Mascot search result identified the sequence QEPIDKELYPLTpSLR (score 73). The Ser487 site was found to be located at Ser40 of Gag-p6 domain in close proximity to both $LYPX_nL$ and LXXLF motif.

Based on our MS analysis, we constructed a GST-tagged p6 and its site-directed mutant GST-p6-Ser487Ala (S487A) and GST-p6-Ser461Ala (S461A) as a negative control. Subsequent in vitro kinase assay results demonstrated that GST-p6 is phosphorylated by aPKC, but not GST-p6-S487A (Figure 3A). These results suggested that aPKC indeed phosphorylates the Ser487 residue of HIV-1 Gag *in vitro*.

To further assess the phosphorylation of Gag at Ser487, we generated a polyclonal antibody against phosphoryated-Ser487 (pS487). We initially confirmed the specificity and sensitivity of the antibody using the AlphaScreen system. We found that our antibody recognized only Ser487 phosphorylated peptides but neither a non-phosphorylated peptide nor a peptide harboring a Ser487 to Ala substitution (Figure 3B). We then used this antibody for in depth cell culture study. 293T cells were transfected with V5 tagged wild type aPKC or a kinase-negative mutant (aPKC-Kn), together with wild type Gag-Pol. A marked increase in the level of Gag phosphorylation at Ser487 was observed in cells expressing the wild type aPKC, whereas there was no obvious increase in the amounts of phosphorylation in either aPKC-Kn or mock transfected cells (Figure 3C). These observations clearly indicate that the expression of aPKC leads to the phosphorylation of HIV-1 Gag at Ser487 in cells, and that this phosphorylation is dependent of the kinase activity of aPKC.

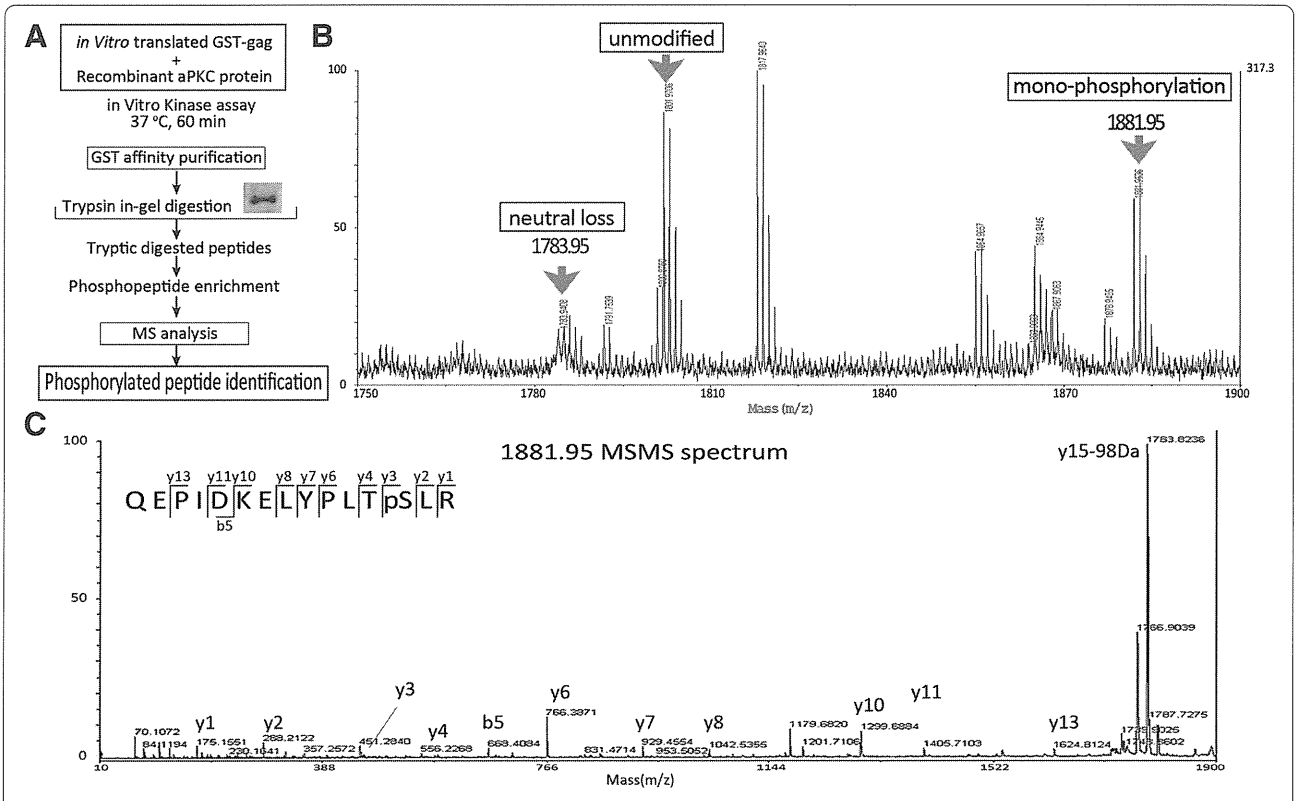


Figure 2 Identification of the phosphorylated Gag-p6 site. (A) Schematic overview of the experimental workflow. **(B)** MS peptide map of phosphorylated GST-Gag after trypsin digestion. The red arrow indicates the 1881.95 Da peak which is unique to the sample treated with active aPKC. This is consistent with the mass of the theoretical phosphorylated peptides and the map shows a neutral loss peak at 1783.95. **(C)** The resulting phosphopeptide was analyzed using MALDI-TOF/TOF MS. Fragment ions of the b and y-series identified in the tandem mass spectra from the peptide are shown. Tandem mass spectra of m/z 1881.95 signals revealed that each sequence corresponded to the phosphopeptide (QEPIDKELYPLTpSLR) of Gag-P6.

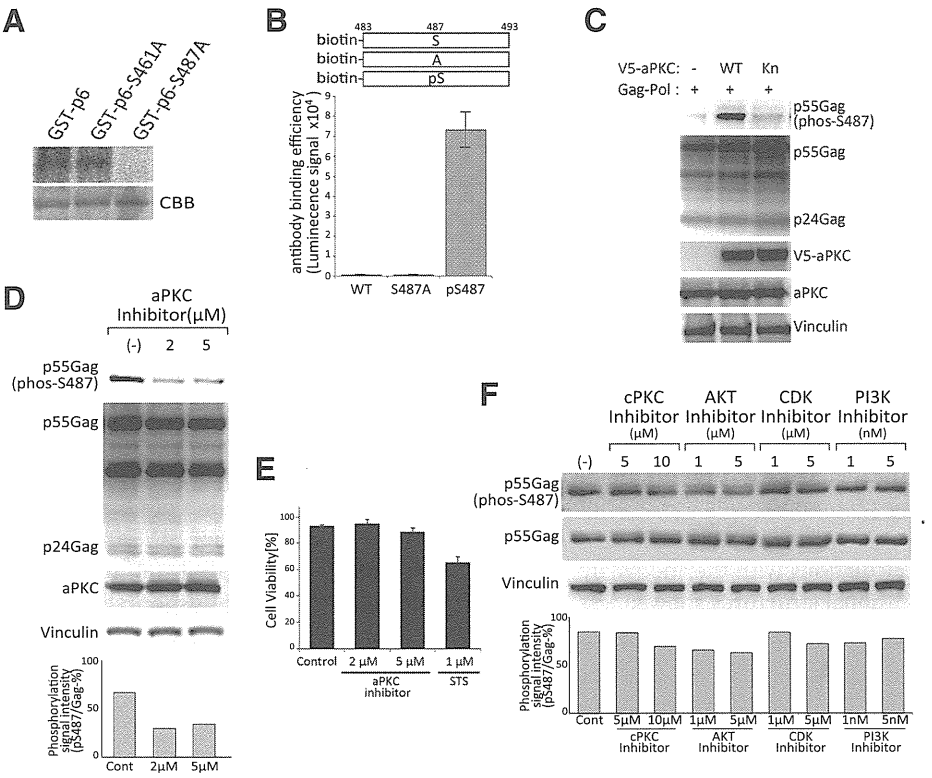


Figure 3 aPKC phosphorylates HIV-1 Gag on Ser487. (A) GST-p6, GST-p6S461A or GST-p6S487A proteins were expressed and affinity-purified from wheat germ cell-free extracts. aPKC-mediated phosphorylation was assessed by incubating recombinant GST-p6, GST-p6S461A or GST-p6S487A with recombinant active GST-aPKC in the presence of [γ-^{32}P] ATP for 60 min at 37°C. The reaction products were analyzed by autoradiography and Coomassie Brilliant Blue (CBB) staining. **(B)** Assessment of phospho-Ser487 antibody specificity and binding efficiency using the AlphaScreen system. Biotin tagged peptides were incubated with phospho-Ser487 specific antibodies for. The graph shows the luminescence signal intensity which represents antibody binding efficiency. Each value is the mean of three independent experiments. **(C)** 293T cells were transfected with wild type Gag-pol and either V5 tagged wild type aPKC (WT) or kinase-negative aPKC (Kn). Whole-cell extracts were prepared, and equal amounts of proteins for each sample were separated by SDS-PAGE and subjected to western blot analysis using indicated antibodies. **(D)** Effects of aPKC inhibitor on the phosphorylation of Gag-Ser487. 293T cells were transfected with Gag-pol expression vector and cultured in the presence of the indicated concentrations of aPKC inhibitor. Clarified cell lysates were prepared and equal amounts of proteins were subjected to SDS-PAGE, followed by western blot analysis using indicated antibodies. Densitometry analysis of the p55Gag (pS487)/p55Gag bands in the western blot is shown in the bottom panel. **(E)** Effects of aPKC inhibitor on viability of 293T cells. 293T cells were treated with aPKC inhibitor (2 or 5 μM) or 1 μM Staurosporine (STS) and analysed for cell viability using trypan blue exclusion at 48 hours. **(F)** Effects of kinase specific inhibitors on the phosphorylation of Gag-Ser487. 293T cells were transfected with Gag-pol expression vector and treated with inhibitors against cPKC, AKT, CDK and PI3K. Cell lysates were prepared and analyzed as described in **(D)**.

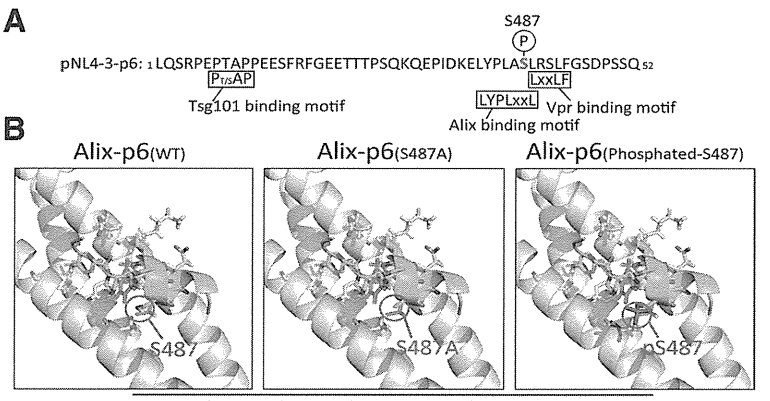
To further investigate whether the phosphorylation of HIV-1 Gag at Ser487 is mediated by endogenous aPKC activity, we employed a myristoylated PKCζ pseudosubstrate peptide as an aPKC inhibitor. This PKCζ pseudosubstrate peptide mimics the substrate binding site in PKCζ (113–125) and PKCι (114–126), and suppresses the activity of endogenous PKCι and PKCζ. HIV-1 Gag-Pol expression plasmids were transfected into 293T cells with or without aPKC inhibitor treatment. Immunoblot analysis revealed that the aPKC inhibitor suppressed Gag phosphorylation at Ser487. Subsequent titration analysis demonstrated a dose-dependent inhibitory effect of the PKCζ pseudosubstrate peptide by showing an 74.9% and 70.4% decrease in Gag phosphorylation at 2 μM and 5 μM doses, respectively (Figure 3D). Note that at these concentrations the aPKC inhibitor did not affect the expression levels of endogenous aPKC as well as a house-keeping protein Vinculin (Figure 3D). Furthermore, cell viability was not prominently affected by aPKC inhibitor when cells were assessed by trypan blue exclusion (Figure 3E). Conventional PKC (PKCα, PKCβ), Akt, CDK and PI3 kinases have been reported previously to affect HIV-1 replication through their phosphorylation of HIV-1 or of host proteins [3,24,27-30]. We thus also investigated using specific inhibitors whether these kinases could mediate the phosphorylation of HIV-1 Gag at Ser487. Our results show that neither PKCα nor PKCβ specific pseudosubstrates affect Gag phosphorylation at Ser487 (Figure 3F). Similarly, neither Akt inhibitor, the CDK inhibitor roscovitine nor the PI3K inhibitor wortmannin blocked Gag phosphorylation at Ser487 (Figure 3F). Taken together, these observations

indicate that aPKC specifically phosphorylates HIV-1 Gag at Ser487 both *in vitro* and *in vivo*.

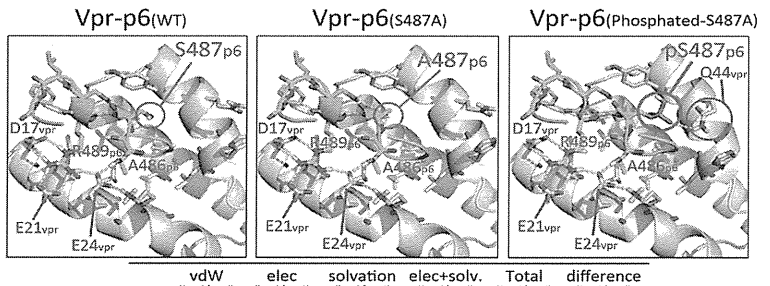
The phosphorylation of Gag Ser487 facilitates the interaction between Gag and Vpr

HIV-1 Gag p6 contains a late domain consisting of three protein binding motifs, PTAP (Tsg101 binding), LYPXnL (Alix binding) and C-terminal Vpr. Ser487 is located in the Alix binding motif and is also adjacent to the Vpr binding motif spanning amino acids 488–492 (Figure 4A). To obtain structural-based information on Gag phosphorylation on Ser487 and how it affects the interaction of Gag with Alix or Vpr, we conducted computer-assisted molecular modeling of the Gag p6 domain coupled with peptides derived from either Alix or Vpr. The models constructed in this study included unphosphorylated and phosphorylated Gag-p6 (amino acids Tyr483-Ser494), and its Ser/Ala substituted mutant on Ser487 (S487A). Molecular modeling calculations with thermodynamically optimized three dimensional structures showed less than 1 Å of positional shifts of Cα atoms of Gag-p6 by phosphorylation, suggesting no obvious difference in the basic structure of Gag-p6 irrespective of the phosphorylation status. Furthermore, binding interface between Gag-p6 and Alix was not affected by the phosphorylation (Figure 4B, Upper panels) or Ser/Ala substitution of Gag Ser487 [31-33]. On the other hands, the binding of Gag-p6 with Vpr was facilitated since the phosphorylation of Ser487 can create another hydrogen bond between Gag-p6 and Vpr (Figure 4B, Lower panels). The Ser487 was predicted to form no hydrogen bonds with Vpr in non-phosphorylated state, whereas the phosphorylated Ser487 could form the hydrogen bond with Gln44 of Vpr. Consequently, binding energy calculated with Molecular Operating Environment (MOE) was significantly increased by phosphorylation of Ser487 only for the Gag-p6-Vpr complex (Figure 4B, tables). These data suggest that the phosphorylation of Gag-p6 on Ser487 could indeed affect the binding affinity of Gag-p6 with Vpr but not Alix.

Based on our structural modeling results, we next asked whether the phosphorylation of Gag at Ser487 has



	vdW (kcal/mol)	elec (kcal/mol)	solvation (kcal/mol)	elec+solv. (kcal/mol)	Total (kcal/mol)	difference (kcal/mol)
WT	-55.1	-196.9	187.8	-9.2	-64.3	-
S487A	-56.3	-117.3	109.3	-8.1	-64.4	0.0
pS487	-54.8	-197.8	188.6	-9.2	-64.0	0.3



	vdW (kcal/mol)	elec (kcal/mol)	solvation (kcal/mol)	elec+solv. (kcal/mol)	Total (kcal/mol)	difference (kcal/mol)
WT	-80.0	-303.0	278.9	-24.1	-104.2	-
S487A	-82.4	-294.1	271.7	-22.4	-104.8	-0.6
pS487	-86.9	-246.2	221.3	-24.9	-111.8	-7.6

Figure 4 The phosphorylation of Gag at Ser487 facilitates the interaction of Vpr and Gag-p6. (A) Schematic representation of the HIV-1 Gag-p6 sequence indicating the binding motifs, Tsg101, Alix and Vpr, located around the Ser487 site. **(B)** Complex structure models of Gag-p6 with Alix or Vpr. The structural calculations were undertaken using MOE. Upper panel shows structural models of the interaction of Alix with wild-type Gag-p6, Gag-p6Ser487A or phosphorylated Gag-p6-Ser487. Bottom panel shows structural models of the interaction of Vpr with wild-type Gag-p6, Gag-p6Ser487A or phosphorylated Gag-p6-Ser487. Binding energies of p6 with Alix or Vpr calculated from the structural models were shown in the bottom tables, where the highly negative value indicates the stable binding.

any effect on the interaction between Vpr and Gag. We have selected Bimolecular Fluorescence Complementation (BiFC) system to quantify the Vpr-Gag interaction in live cells as previously reported [34]. Plasmids encoding C-terminally KGC-tagged Gag (Gag-KGC) and N-terminally KGN-tagged Vpr (KGN-Vpr) were transfected and evaluated for BiFC signal by flow cytometry (Figure 5A). Flow cytometry analysis revealed that the interaction of Vpr with Gag-Ser487Ala mutant was reduced as compared with wild-type Gag (Figure 5A). To further assess whether the phosphorylation of Gag at Ser487 provides another hydrogen bond with Vpr Gln44 to facilitate Gag-

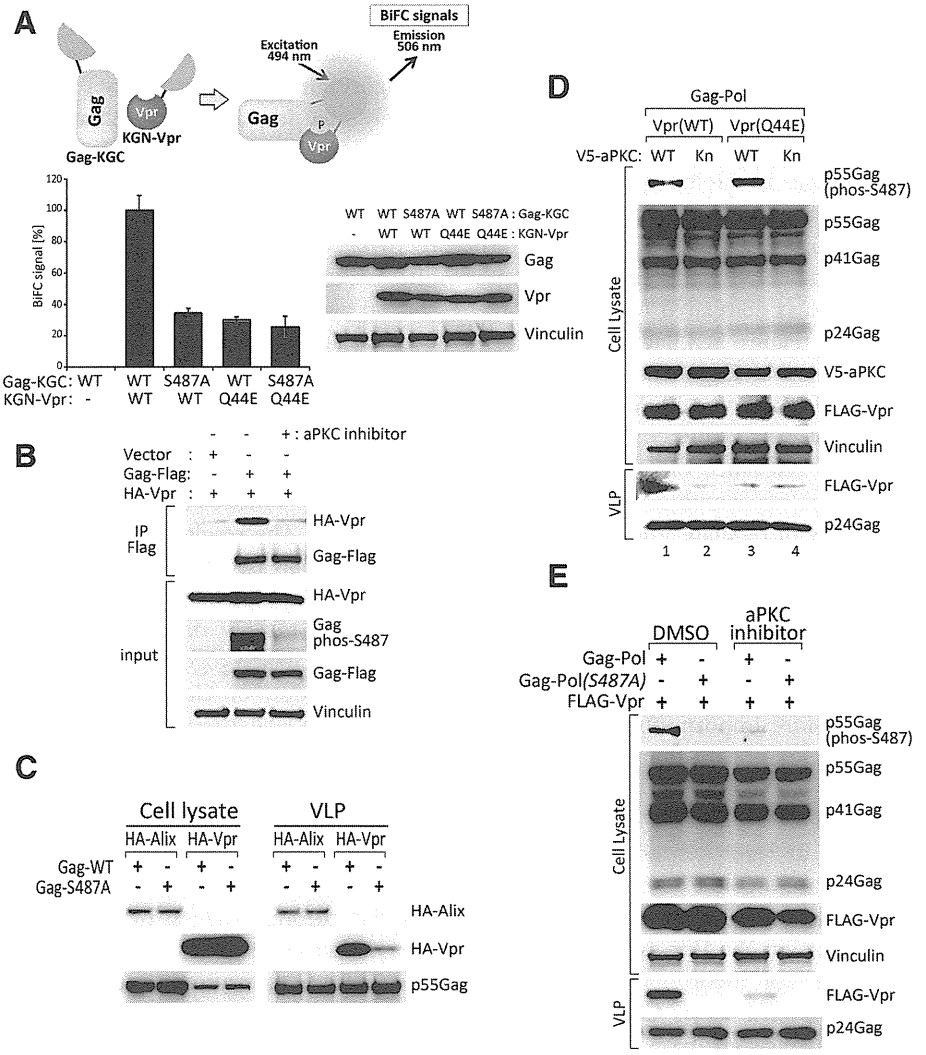


Figure 5 The phosphorylation of Gag at Ser487 promotes the incorporation of Vpr into HIV-1 virions. (A) Bimolecular fluorescence complementation (BiFC) of 293T cells expressing Gag-KGC and KGN-Vpr (upper panel). 293T cells were transfected with KGN-Vpr (WT or Q44E) and Gag-KGC or Gag (S487A)-KGC, were harvested and analyzed by flow cytometry to determine the BiFC signal at 48 hours post-transfection. Results represent the means of three independent experiments. The cells were harvested and subjected to western blot analysis. **(B)** The phosphorylation of Gag at Ser487 promotes interaction of Vpr with Gag. HA-tagged Vpr was co-transfected with Flag tagged Gag into 293T cells in presence of 5 μM aPKC inhibitor or DMSO, followed by pulldown with Flag-beads and immunoblotting with indicated antibodies. Bottom panel shows input for pulldown assay. **(C)** Differential effect of Gag Ser487 phosphorylation on Alix and Vpr incorporation into VLPs. 293T cells were transfected with GFP-tagged Gag (Gag-WT) or with the Ser487 point mutated Gag (Gag-S487A), with either HA-tagged Alix, or HA-tagged Vpr expression vectors. Cells and VLPs were collected at 24 hours post-transfection, and their protein extracts were resolved using SDS-PAGE and subjected to western blot analysis. **(D)** aPKC-mediated Gag phosphorylation at Ser487 facilitates Vpr incorporation into virions. 293T cells were transiently transfected with Gag-Pol, either Flag-tagged Vpr (WT) or Gln44 point mutated Vpr (Q44E), and with either V5-tagged aPKC (WT) or kinase-negative aPKC (Kn). Cell lysates and VLPs were collected 24 hours post-transfection and subjected to western blot analysis. **(E)** The phosphorylation of Gag-pol-Ser487 is required for Vpr incorporation into HIV-1 virions. 293T cells were transfected with Flag-tagged Vpr and either with Gag-Pol (WT) or with the Ser487 point mutated Gag-Pol (S487A). The transfected 293T cells were treated with DMSO or aPKC inhibitor for 24 hours. Cell lysates and VLPs were collected and subjected to western blot analysis.

Vpr interaction, we constructed Vpr Q44E mutant for BiFC analysis. Results demonstrated that Vpr Q44E mutant exhibited weker interactions to Gag and Gag S487A as compared with wild type Vpr (Figure 5A). We further found that aPKC inhibitor suppressed the interaction between Gag-Flag and HA-Vpr in imunoprecipitation analysis (Figure 5B).

The phosphorylation of Gag at Ser487 affects Vpr incorporation into virions and viral infectivity

We next examined whether the phosphorylation of Gag at Ser487 has any effects on the incorporation of Vpr into HIV-1 virus like particles (VLP). As shown in Figure 4B, we found no distinct changes in the incorporation of Alix into VLPs regardless of a Ser/Ala substitution at Gag Ser487 in 293T cells. However, Vpr incorporation into VLP was significantly decreased in cells transfected with the Gag-Ser487Ala mutant as compared with cells transfected with wild-type Gag (Figure 5C). Hence, it is plausible that the phosphorylation of Gag at Ser487 may have an important role in its interaction with Vpr thereby affecting the Vpr incorporation into VLPs.

To further explore the relevance of Gag phosphorylation to HIV-1 replication, we examined whether aPKC kinase activity is necessary to regulate Vpr incorporation into HIV-1 virions. Gag phosphorylation at Ser487 was prominently enhanced by wild type aPKC but not kinase negative mutant aPKC (Kn) (Figure 5D). Concomitantly, the level of Vpr incorporation into virions was shown to be paralleled with the Gag phosphorylation status (lane 1 and 2 in Figure 5D). More importantly, virion incorporation of Vpr Q44E mutant was much lesser than wild-type Vpr irrespective of Gag phosphorylation at Ser487 (lane3 and 4 in Figure 5D). These results suggest that Gag phosphorylation at Ser487 is indeed affect Vpr incorporation and this process could be mediated by the Gln44 residue of Vpr. Although no significant effect of the Gag-pol S487A mutant on the Vpr expression levels in cells was evident, the Vpr incorporation level into VLPs was significantly reduced upon Gag-pol-S487Ala transfection (Figure 5E). Consistent with this result, the incorporation of Vpr into VLPs was significantly reduced in cells treated with the aPKC inhibitor peptide; the Vpr incorporation efficiency was reduced in aPKC inhibitor treated cells (Figure 5E). These data indicate that aPKC can enhance the incorporation of Vpr into HIV-1 virions.

It has been well established that Vpr incorporation into HIV-1 virions augments viral infectivity in macrophages [5,6,35-37]. We thus assessed whether aPKC affects HIV-1 infectivity by increasing Vpr incorporation into virions. We hypothesized that if the Gag phosphorylation at Ser487 by aPKC was beneficial for HIV-1 infection in this way, aPKC activity would affect wild type HIV-1 but not a Vpr-null virus. To test this, we employed pNL4-3ΔEnv-luc (WT) or pNL4-3ΔEnvΔVpr-luc (Vpr-null) strains. We then produced the corresponding viruses with a fusiogenic envelope G glycoprotein of the vesicular stomatitis virus (VSV-G) in the presence or absence of aPKC inhibitor in 293T cells (Figure 6A). Immunoblotting analysis of VLP demonstrated that the level of Vpr incorporation was prominently reduced by treatment with the aPKC peptide inhibitor (Figure 6B). The infectivity of the generated viruses was tested using the human monocyte/macrophage cell line MonoMac6. The aPKC inhibitor-treated WT virus exhibited approximately 50% less infectivity than the control WT virus (Figure 6C). The Vpr-null virus showed a 35% reduction in infectivity compared with the WT virus in the MonoMac6 cells (Figure 6C). However, the primarily low infectivity of the Vpr-null virus was not significantly affected by the aPKC inhibitor (Figure 6C). aPKC inhibitor did not exhibit obvious cytotoxic effect to MonoMac 6 cells (Figure 6D).

To assess the role of aPKC in multi-round HIV-1 replication in primary monocyte-derived macrophages (MDMs), we infected these cells with HIV-$1_{89.6}$, a dual tropic virus, or HIV-1_{NLAD8}, an R5 tropic virus, in conjunction with treatments of various concentrations of the aPKC inhibitor (Figure 7A). The results revealed that the aPKC inhibitor strongly suppressed the replication of both viruses in a dose-dependent manner (Figure 7B, C), although there was no obvious toxicity or growth inhibition in these cells (Figure 7D). Taken together, these results indicate that the phosphorylation of Gag by aPKC regulates Vpr incorporation and HIV-1 replication in macrophages.

Discussion

We here demonstrate that aPKC is a crucial regulator of HIV-1 infection via the phosphorylation of Gag-p6 which enhances the incorporation of Vpr into virions. Our current data strongly suggest that Ser487 is the specific phosphorylation site on HIV-1 Gag for aPKC and is crucial for the Gag p6-Vpr interaction that leads to Vpr incorporation into viral particles. Furthermore, our current data demonstrate that an aPKC inhibitor prominently inhibits HIV-1 replication in primary human macrophages. Hence, the phosphorylation of Gag by aPKC may well be an important mechanism through which HIV-1 efficiently infects macrophages and by which an excessive accumulation of the cytotoxic Vpr protein in the host infected cells is prevented.

The Gag-p6 domain has been identified as the predominant site of phosphorylation in HIV-1 particles [22]. Ser487 is a highly conserved residue in this p6 domain among various HIV-1 strains, suggesting that the phosphorylation of this residue is of fundamental functional importance. Votteler et al. have demonstrated that a HIV-1 Gag mutant with a deleted PTAP region

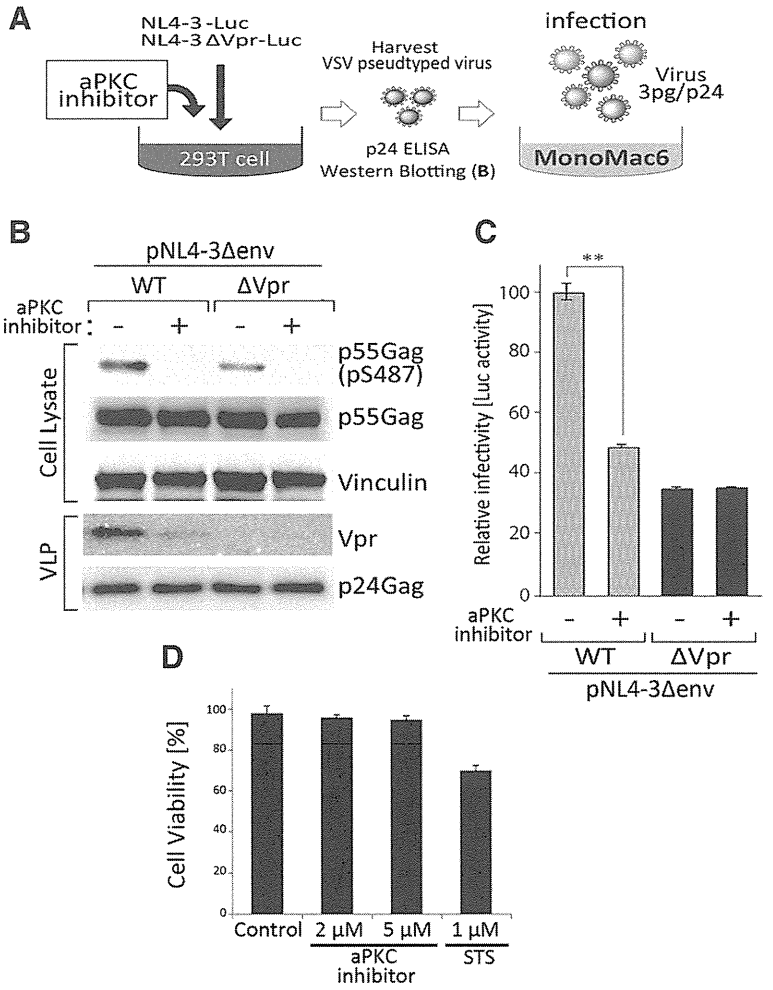


Figure 6 The inhibition of aPKC significantly decreases single-round HIV-1 infection. (A) Schematic representation of the experimental system. Briefly, 293T cells were either mock treated, or treated with aPKC inhibitor. After 4 hours, these cells were co-transfected with pNL4-3Δenv-luc or pNL4-3ΔenvΔVpr-luc and with pVSV-G. Viral release was measured through the quantification of p24CA antigen concentration in the culture supernatants at 48 hours post-transfection. MonoMac6 cells were infected with VSV-G pseudotyped WT and ΔVpr viruses for 48 hours. **(B)** The VSV-G pseudotyped WT and ΔVpr stocks generated from pNL4-3Δenv-luc and pNL4-3ΔenvΔVpr-luc with/without aPKC inhibitor treatment were analyzed by immunoblotting for the incorporation of Vpr into virion particles. **(C)** Viral infectivity was detected by measuring the luciferase activity in the cell lysates. Data are mean ± s.e.m. of three independent experiments. **(D)** Effects of aPKC inhibitors on viability of MonoMac6 cells. MonoMac6 cells were treated with aPKC inhibitor (2 or 5 μM) or 1 μM Staurosporine (STS) and analyzed for cell viability using trypan blue exclusion at 72 hours. Data are mean ± s.e.m. of three independent experiments: **p < 0.01, Student's t-test.

and a phenylalanine substitution at Ser487 (ΔPTAP/S487F) shows aberrant core formation and reduced viral infectivity in TZM-b1 cells [33]. More recently, steady state affinity analysis using a surface plasmon resonance sensorgram has revealed that the phosphorylated form of p6 at Ser487 has a stable binding affinity for cytoplasmic membranes [38]. These reports have therefore revealed that Gag Ser487 is a highly conserved phosphorylation site of likely crucial importance for HIV-1 infection. On the other hand, Radestock et al. recently reported in tissue culture experiments that the phosphorylation of Gag-p6 including Ser487 is dispensable for HIV-1 infectivity. These authors showed that asparagine substitutions at five serine residues (Ser487, Ser490, Ser494, Ser497 and Ser498) within the C-terminus of Gag p6 produced no impairment of Gag assembly or virus release and caused only very subtle deficiencies in viral infectivity in T-cell lines and in primary lymphocytes [39]. These discrepancies may be due to different experimental approaches using different Gag substitution mutants as well as different cell types. In contrast, our present approach is distinct from these earlier studies as we initially attempted to identify the kinases responsible for Gag-p6 phosphorylation and then explore their role in HIV-1 replication. Our current results clearly demonstrate that aPKC phosphorylates Gag-p6 and regulates the interaction of Gag with Vpr for