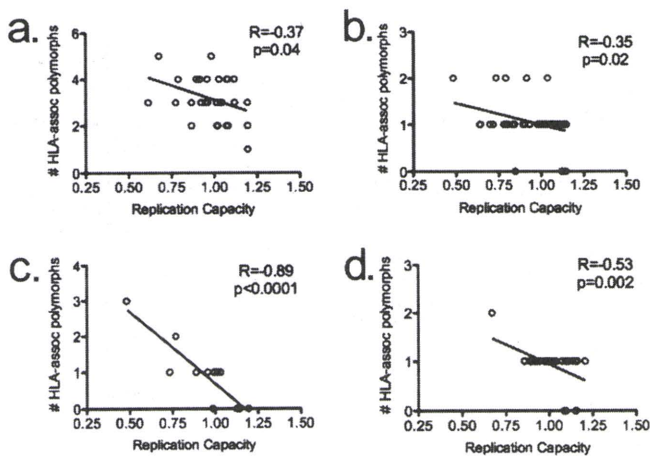

FIG. 4. Cumulative effects of HLA-associated polymorphisms on viral RC during chronic infection. RC measurements were compared to the total number of Gag mutations present in each virus that could be attributed to the host's HLA, as defined previously (14). Significant associations were observed between the number of HLA-associated polymorphisms specific for A*25 (Pearson $R = -0.37$; $P = 0.04$) (a), A*26 ($R = -0.35$; $P = 0.02$) (b), B*41 ($R = -0.89$; $P < 0.0001$) (c), and B*55 ($R = -0.53$; $P = 0.002$) (d) and lower RCs in individuals who expressed these alleles, suggesting a dose-dependent impact of new Gag polymorphisms selected for by these HLA alleles. Associations for HLA-A*26, -B*41, and -B*55 remained significant after correction for multiple comparisons ($q < 0.2$). The slope of each association is indicated by a solid line.

amined. Consistent with results from acute/early infection studies, no associations were observed between the viral RC and the total number of HLA-A-, HLA-B-, or HLA-C-associated polymorphisms for alleles that were not expressed by the host (not shown). This result suggests that putative transmitted mutations could be observed for chronic viruses but that these polymorphisms were not a major determinant of RC for this cohort. However, the possibility that fitness-reducing mutations were transmitted and subsequently reverted cannot be ruled out.

Associations between Gag sequence polymorphisms and viral RC in chronic infection. A systematic analysis of viral amino acids associated with the RC in chronic infection was also undertaken. A total of 63 polymorphisms located at 40 codons in Gag and two polymorphisms located at codon 61 in protease that were associated with viral RC at a q value of <0.2 were identified (see Table S2 in the supplemental material). Of the Gag codons associated with viral RC, 22 (55%) are under selection pressure by at least one HLA allele (14). Polymorphisms correlating with lower viral RCs in chronic infection included Gag E12Q (associated with B*49), K28T (A*03, A*24, and C*17), R30K (B*15), R58G (B*49), S67A (C*03), T122I (A*33), A146P (B*13, B*39, B*57, and C*08), S148T (B*53, C*02, C*06), V218Q (B*40), V223L (A*25, B*55, B*56), F383T (A*31 and B*27), T389I (B*13, B*27, B*39, B*42, and B*44), T427P (A*26, B*40, and B*58), and E482D (B*40 and B*78). Of note, changes at Gag codons 67, 218, 479, and 486 were identified as being associated with RC in both the acute/early and chronic infection data sets.

Impact of secondary mutations in Gag on the B*57-associated T242N mutation in chronic infection. In contrast to acute/early infection, B*57-derived recombinant viruses did not display significantly reduced RCs in chronic infection. In order to further investigate this observation, we examined chronic viral sequences for the presence of HLA B*57-associated polymorphisms. As expected, we observed a significant enrichment of known Gag CTL escape mutations, A146P, I147L, T242N, and others, in chronically infected B*57-expressing individuals; however, we failed to observe a significant correlation between the overall number of B*57-associated polymorphisms in these sequences and viral RC (not shown). Taken together, these observations indicate that during chronic infection, the relationship between viral sequence and RC is more complex than the raw number of primary escape mutations.

We therefore hypothesized that the lack of a B*57 association with RC at the chronic stage was due to the accumulation of compensatory mutations during the natural course of infection. To assess the potential relationship between compensatory mutations and viral RC in chronic infection, we examined the sequences of 50 viruses derived from B*57-expressing individuals harboring the T242N mutation for evidence of secondary mutations at Gag H219Q, I223V, M228I, and G248A. These mutations have been shown to restore the *in vitro* RC of T242N variants, presumably by altering the ability of capsid to interact with the host cyclophilin A protein (10, 41, 42). In viruses encoding T242N, we observed a significant positive correlation between RC and the number of compensatory mutations in the recombinant viral sequence ($R = 0.36$; $P = 0.01$) (Fig. 5a). Similar results were obtained if all T242N mutation-containing viruses (regardless of the host HLA type) were analyzed ($n = 72$; $R = 0.33$; $P = 0.004$) (not shown). Together, these data indicate that the effects of the T242N escape mutation on RC may be restored by secondary mutations in a cumulative manner.

Limited experimental data exist to show the consequence of secondary mutations following CTL escape on HIV-1 fitness. We hypothesized that other polymorphisms in Gag might alter the impact of the T242N mutation, and therefore, we examined our chronic data set for evidence of additional secondary mutations that were associated with either an enhanced or reduced RC in context of T242N. Polymorphisms at Gag codons 12 ($P = 0.027$), 127 ($P = 0.027$), 146 ($P = 0.003$), 315 ($P = 0.015$), 483 ($P = 0.015$), and 488 ($P = 0.016$) were associated with lower RCs, indicating a deleterious effect of these mutations in combination with T242N (Fig. 5b). Polymorphisms at residues 146 and 315 are consistent with escape from HLA B*57-restricted CTL responses to the IW9 ($Gag_{147-155}$) and QW9 ($Gag_{308-316}$) epitopes, but only Gag codon 146 remained significant after correction for multiple comparisons ($q < 0.2$). These results support previous work indicating an additive effect of B*57-mediated escape mutations on viral fitness (9, 19).

In addition, we observed that polymorphisms at Gag codons 84 ($P = 0.005$), 132 ($P = 0.008$), 218 ($P = 0.030$), 219 ($P = 0.015$), 228 ($P = 0.0006$), 248 ($P = 0.002$), 370 ($P = 0.031$), and 487 ($P = 0.014$) were associated with higher RCs in T242N mutation-containing viruses (Fig. 5c). Of these polymorphisms, those present at codons 219, 228, and 248 were described previously to function as compensatory mutations for the T242N mutation (10, 41, 42). Note that only codons 228 and 248 remained significant after correction for multiple compar-

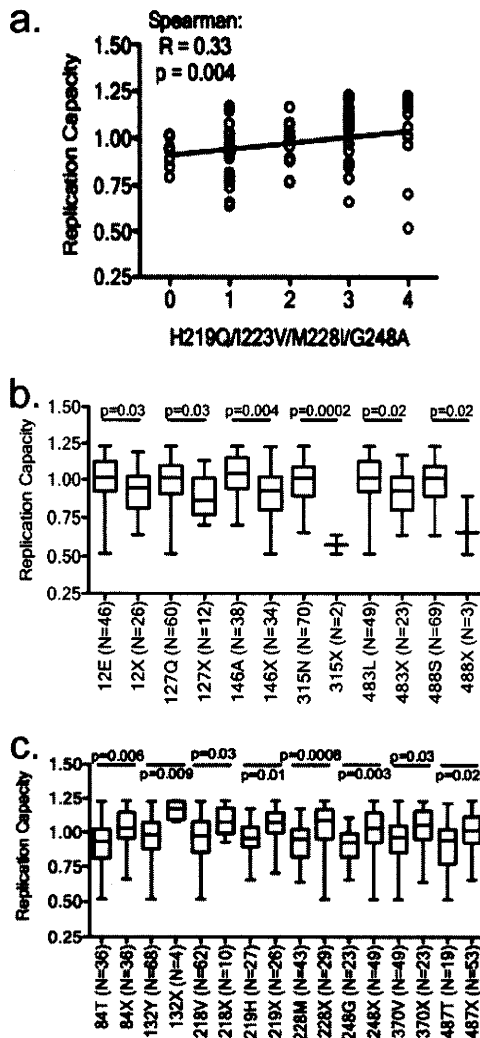

FIG. 5. Impact of secondary mutations in Gag on RC of chronic viruses carrying the T242N mutation. The presence of Gag H219Q, I223V, M228I, and G248A mutations was examined for recombinant viruses derived from B*57-expressing hosts that carried the T242N mutation (n = 50). (a) A significant correlation was observed between the RC and the total number of sites harboring previously described compensatory mutations (Pearson $R = 0.36$; $P = 0.01$). The slope of the association is indicated by a solid line. To identify novel secondary mutations that might alter the fitness of T242N mutation-carrying viruses, pairwise Mann-Whitney U tests were conducted to compare the RCs of 72 chronic viruses from all patients that harbored the T242N mutation with all variable residues in Gag. (b and c) Results indicated significant associations between mutations at Gag codons 12, 127, 146, 315, 483, and 488 with lower RCs (b) and between mutations at Gag codons 84, 132, 218, 219, 228, 248, 370, and 487 with higher RCs (c) (all $P < 0.05$). Associations at codons 146, 228, and 248 remained significant after correction for multiple comparisons ($q < 0.2$). Median RC values (lines), interquartile ranges (boxes), and extreme values (whiskers) are indicated for each group.

isons ($q < 0.2$). Altogether, our results confirm and extend the list of secondary mutations in Gag that might enhance or compensate for fitness costs associated with the T242N mutation.

Clinical associations with viral RC in chronic infection. Finally, we wished to examine associations between Gag-Protease RC and clinical parameters (Fig. 6). A modest positive correlation was observed between RC and plasma viral load (pVL) ($R = 0.12$; $P = 0.0007$), while an inverse correlation was observed between RC and the CD4 cell count ($R = -0.17$; $P < 0.0001$) in chronic infection. Restricting this analysis to individuals expressing protective HLA alleles associated with lower acute/early fitness in our study (B*13, B*57, or B*5801) (n = 110) revealed stronger associations between the RC and pVL ($R = 0.27$; $P = 0.005$) and CD4 cell counts ($R = -0.33$; $P = 0.0005$). Although these analyses are derived from cross-sectional data, results suggest that Gag fitness may increase over the course of chronic infection. The stronger Rho obtained from the analysis of protective alleles suggests that the increase in viral RC over the infection course may be greater for these alleles than for others, which is consistent with our observation of reduced RCs in acute/early infection with recombinant viruses derived from individuals expressing protective alleles.

DISCUSSION

An effective AIDS vaccine must overcome the extreme genetic diversity of HIV-1 (4, 26, 61). Targeting of the most conserved regions of the viral proteome has been proposed as a means to elicit robust, long-lasting CD8 T-cell responses. Indeed, highly conserved HIV-1 epitopes that escape very slowly during natural infection have been identified, for example, the B*57-restricted KF11 ($Gag_{162-172}$) (20) and B*27-restricted KK10 ($Gag_{263-272}$) (25, 29) epitopes. Structural constraints on these regions of the viral capsid likely require compensatory mutations to occur concurrently in order for viruses encoding these altered epitopes to remain viable (20, 58, 59, 65). A vaccine approach that targets these and other regions of Gag may also force HIV-1 to adopt an attenuated phenotype through the selection of detrimental CTL escape mutations. This seems plausible, since some conserved epitopes escape relatively rapidly in the presence of CTL pressure but revert to the wild-type sequence upon transmission to an HLA-mismatched recipient. The B*57-restricted TW10 ($Gag_{240-249}$) epitope is an example of one such case where escape mutations reduced viral fitness; however, this mutation also appears to be restored through the subsequent acquisition of compensatory mutations (8, 10, 42). The functional consequences of escape and potential pathways for compensation have been described for only a limited number of selected HIV-1 mutations, and data remain biased toward certain protective HLA alleles. Therefore, it is not known whether escape mutations selected in the presence of other alleles will display similar functional limitations that might be exploited for vaccine design.

Here, we have examined clinical Gag and protease sequences from individuals during acute/early infection and relatively advanced chronic infection to investigate the impact of HLA-associated immune selection pressures on HIV-1 fitness. We observed that Gag-Protease function, as indicated by the *in vitro* RC, was significantly lower for acute/early viruses derived from individuals who expressed a protective HLA class I allele, either B*13, B*57, or B*5801. This was associated with the presence of known HLA-associated polymorphisms in Gag that were expected in the context of each host's HLA class I

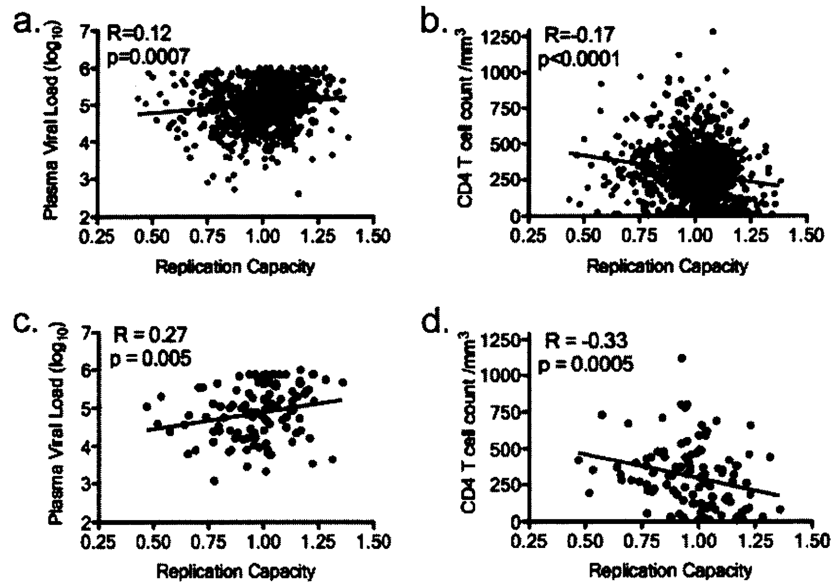

FIG. 6. Clinical associations with Gag-Protease RC in chronic infection. RC was compared to clinical pVL and peripheral blood CD4 T-cell counts at the time of collection for all chronic samples (a and b) and for the subset of samples collected from individuals who expressed a protective HLA allele, either B*13, B*57, or B*5801 (n = 110) (c and d). (a and b) Among all subjects, modest statistically significant correlations were observed between RC and pVL (a) (Pearson $R = 0.14$; $P < 0.001$) and CD4 cell count (b) ($R = -0.21$; $P < 0.0001$). (c and d) For individuals who expressed protective HLA alleles, we observed stronger associations between RC and pVL (c) ($R = 0.27$; $P = 0.005$) as well as CD4 cell count (d) ($R = -0.33$; $P = 0.0005$). The slope of each association is indicated by a solid line.

genotype. Notably, for viruses derived from acute/early-infected, B*57-expressing individuals, we observed that the RC correlated inversely with the number of days elapsed since the estimated infection date. Although based on a cross-sectional analysis, this result is consistent with the appearance of escape mutations in Gag early following infection (2, 7, 11, 21, 28, 44, 54) and their continued selection over time (29), leading to cumulative reductions in the viral replication capacity during the early stages of HIV-1 infection (8, 19). Our observation that the RC of viruses from acute/early infection correlated inversely with the total number of host HLA-B-associated polymorphisms present in Gag also supports this model. Taken together, these results suggest that an accumulation of primary escape mutations, selected predominantly by HLA-B alleles, contributes to reduced Gag function in a cumulative manner during acute/early infection. More detailed longitudinal analyses of Gag protein function will be necessary to evaluate this association further.

In an exploratory analysis of acute/early viruses, we identified polymorphisms at 14 codons that were significantly associated with viral RC (see Table S1 in the supplemental material). A majority of these sites (8 of 14 sites) were associated previously with HLA pressure (14), and all of them lay within known CTL epitopes. However, we were surprised to observe an overall lack of significant associations between RC and specific escape mutations restricted by protective HLA alleles. Trends were observed for several epitopes targeted by HLA-B*57/*5801, suggesting that the effect of these HLA alleles on RC may require multiple mutations to act in concert. The significant inverse association identified between the RC and the total number of host-specific HLA-B polymorphisms in Gag further suggests that the observed functional defects result from an additive effect of mutations, perhaps selected by more than one HLA-B allele during acute/early infection. It should be noted that our data set of 66 acute/early viruses was insufficiently powered to comprehensively identify specific amino acids associated with viral RC and to fully investigate potential correlations between RC, HLA, and clinical parameters.

Using a panel of 803 recombinant viruses generated from patients with chronic infection, we failed to observe an association between these same protective HLA alleles and Gag-Protease RC. However, a number of significant associations between RC and host HLA alleles were apparent. Our results indicated that host expression of HLA-A*26, -A*31, -B*48, -B*53, and -B*5801 may be associated with lower Gag function during chronic HIV-1 infection. Of these alleles, A*26, A*31, and B*5801 have been associated with a lower relative hazard for progression to AIDS in natural-history studies (51). In this study, only the host expression of HLA B*5801 correlated with a lower Gag-Protease RC in both acute/early infection and chronic infection.

The lack of a major association between HLA-B*57 and viral fitness in our chronic cohort may be due to the relatively late-stage infection of these individuals. In a previous study, we observed a modest association between HLA-B*57/B*5801 and a reduced Gag-Protease RC in a smaller cohort of chronically infected individuals with higher CD4 cell counts (48). Indeed, it is possible that the functional impact of early immune pressure by protective HLA class I alleles on Gag wanes over the disease course. This outcome is consistent with the ability of HIV-1 to evolve and to develop compensatory mutations, which appeared to be particularly relevant for the chronic infection samples analyzed here. Notably, we observed that nearly all viruses derived from chronically infected B*57-

and B*5801-expressing hosts harbored the Gag T242N escape mutation in the TW10 epitope and that the RCs of these strains correlated significantly with the accumulation of known and putative compensatory mutations in the capsid protein. Together with previously reported data from mutagenesis studies (10), these findings indicate that the accumulation of secondary mutations restores the fitness defects associated with primary escape mutations in a dose-dependent manner.

Despite a complex mixture of CTL escape and compensatory mutations present in chronic viral sequences, we observed a modest negative correlation between Gag-Protease function and CD4 T-cell count, which was strengthened upon restriction to protective HLA alleles only. Although these analyses are derived from cross-sectional data, results suggest that viral RC increases concomitantly as CD4 cell counts decline over the course of chronic infection. Indeed, the stronger correlations observed when analyses were restricted to protective HLA alleles suggest that changes in RC are greater over the course of chronic disease in the context of these alleles. This interpretation is consistent with our observation of a reduced RC in acute/early infection of individuals expressing protective HLA alleles, which is followed by an accumulation of compensatory changes that rescue the fitness costs of primary escape mutations. Unfortunately, we cannot fully explore this hypothesis, since infection dates are unknown for the chronic infection cohort. Efforts to examine RCs using longitudinal samples from well-characterized seroconverter cohorts will be necessary to address these issues further. Importantly, however, the fact that individuals with protective alleles exhibit reduced viral loads in chronic infection compared to those of their non-protective-allele-expressing counterparts strongly suggests that these early fitness hits, although no longer directly detectable at later time points due to the accumulation of compensatory mutations, may provide long-lasting beneficial effects.

The larger data set of recombinant viruses from chronically infected patients provided greater power to uncover specific amino acids in Gag and protease associated with RC. We identified 63 polymorphisms located at 40 codons in Gag and two polymorphisms located at codon 61 in protease that were independently associated with RC after correction for multiple comparisons ($q < 0.2$) (see Table S2 in the supplemental material). As seen for acute/early infection samples, the majority of these sites (22 of 42 sites) were previously associated with HLA-mediated selection (14), and many others lie within known CTL epitopes. However, few of these mutations matched the predominant HLA-associated polymorphisms identified in the published literature, possibly suggesting that less-common mutations at these sites are more likely to result in severe RC defects. Of interest, the strongest HLA associations with lower RCs in chronic infection were observed for HLA-A*31 and -B*40, and polymorphisms targeted by these alleles were also identified, namely, F383T (A*31) and E482D (B*40). Future studies will be necessary to examine the impact of these specific mutations on Gag RC and to assess their potential relevance for disease progression.

Polymorphisms at Gag codons 67, 218, 479, and 486 were associated with RC in both acute/early and chronic viruses. Of these polymorphisms, the S67A, V218X (acute/early) or V218Q (chronic), and L486X (acute/early) or L486S (chronic) mutations appeared to attenuate Gag RC, but interpretation of these results proved complex. For example, Gag position 67 appears to be under opposing selection pressures by different HLA alleles at the population level: while HLA-C*03 selects for S67A, HLA-A*02 selects for the wild-type serine at this residue (14). Mutations at codon 218 are associated with HLA-B*40, but this allele typically selects for alanine (rather than glutamine) at this residue (14). To our knowledge, mutations at codon 486 have not been identified as HLA footprints, although this residue lies within the known B*40-restricted KL9 epitope. Future site-directed mutagenesis studies will confirm these and other associations with viral RC so that they may be considered potential regions for inclusion or exclusion from Gag vaccine antigens designed to attenuate viral RC.

Several limitations of this study should be noted. First, we focused this work only on the Gag-Protease region of HIV-1. While we believe that the approach used provides a robust analysis of the impact of mutations in Gag and protease on the viral RC, we have not assessed important potential roles of polymorphisms located elsewhere in the viral genome. Second, due to the very large number of samples tested, we used bulk PCR products to construct recombinant viruses. The presence of amino acid mixtures in resulting stocks may have reduced our ability to detect minor differences in RC. Third, our acute/early infection study was limited to 66 individuals. This number was sufficient to observe a strong effect of protective HLA alleles on acute/early RC, but substantially larger acute/early infection cohorts may yield the ability to investigate RC associations with individual alleles and/or viral polymorphisms in greater detail.

In conclusion, this study and our previous report (64) focusing on subtype C infection represent the first systematic, population-based investigations of the contribution of HLA class I selection pressure on HIV-1 RC. Taken together, our findings are consistent with a model whereby HLA-associated CTL responses select for primary escape mutations in Gag during acute/early infection, some of which occur at a substantial fitness cost. Negative consequences for RC may be cumulative as additional escape mutations are selected; however, compensatory mutations that restore Gag function may also arise over extended periods of time. By late chronic infection, due to a balance of escape and compensatory mutations, many HLA-associated fitness defects observed during early infection are no longer detectable. A significant positive correlation between RC and the presence of compensatory mutations in chronic viruses illustrates the profound ability of continued *in vivo* viral evolution to rescue fitness defects. Nevertheless, the fact that individuals with protective alleles maintain lower pVLs than their non-protective-allele-expressing counterparts well into chronic infection strongly suggests that these early fitness hits may provide long-lasting benefits. These data provide important new information to better understand the complex interactions between HLA-mediated immune pressure and HIV-1 sequence evolution, the impact of escape and compensatory mutations on HIV-1 RC, and the potential utility of targeting attenuation-inducing sites in Gag for the rational design of an effective vaccine.

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M.A.B., Z.L.B., T.M., B.D.W., and T.M.A. designed this study. M.A.B, Z.L.B., J.S., P.C.R, and T.J.M. conducted experiments and analyzed data. C.J.B., C.M.K., J.M.C., and D.H. developed statistical methods and/or contributed to data analysis. H.S., A.D.K., H.J., E.R., M.M., M.A., and P.R.H. contributed specimens and/or clinical data. The manuscript was written by M.A.B. and Z.L.B. and critically reviewed by C.J.B., T.M., T.J.M., H.S., A.D.K., M.M., H.J., E.R., M.A., P.R.H., B.D.W., and T.M.A.

REFERENCES

1. **Allen, T. M., and M. Altfeld.** 2008. Crippling HIV one mutation at a time. J. Exp. Med. **205:**1003–1007.
2. **Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins.** 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **407:** 386–390.
3. **Avila-Rios, S., C. E. Ormsby, J. M. Carlson, H. Valenzuela-Ponce, J. Blanco-Heredia, D. Garrido-Rodriguez, C. Garcia-Morales, D. Heckerman, Z. L. Brumme, S. Mallal, M. John, E. Espinosa, and G. Reyes-Teran.** 2009. Unique features of HLA-mediated HIV evolution in a Mexican cohort: a comparative study. Retrovirology **6:**72.
4. **Barouch, D. H., K. L. O'Brien, N. L. Simmons, S. L. King, P. Abbink, L. F. Maxfield, Y. H. Sun, A. La Porte, A. M. Riggs, D. M. Lynch, S. L. Clark, K. Backus, J. R. Perry, M. S. Seaman, A. Carville, K. G. Mansfield, J. J. Szinger, W. Fischer, M. Muldoon, and B. Korber.** 2010. Mosaic HIV-1 vaccines expand the breadth and depth of cellular immune responses in rhesus monkeys. Nat. Med. **16:**319–323.
5. **Barrie, K. A., E. E. Perez, S. L. Lamers, W. G. Farmerie, B. M. Dunn, J. W. Sleasman, and M. M. Goodenow.** 1996. Natural variation in HIV-1 protease, Gag p7 and p6, and protease cleavage sites within gag/pol polyproteins: amino acid substitutions in the absence of protease inhibitors in mothers and children infected by human immunodeficiency virus type 1. Virology **219:** 407–416.
6. **Borrow, P., H. Lewicki, B. H. Hahn, G. M. Shaw, and M. B. Oldstone.** 1994. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J. Virol. **68:**6103–6110.
7. **Borrow, P., H. Lewicki, X. Wei, M. S. Horwitz, N. Peffer, H. Meyers, J. A. Nelson, J. E. Gairin, B. H. Hahn, M. B. Oldstone, and G. M. Shaw.** 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. **3:**205–211.
8. **Boutwell, C. L., and M. Essex.** 2007. Identification of HLA class I-associated amino acid polymorphisms in the HIV-1C proteome. AIDS Res. Hum. Retroviruses **23:**165–174.
9. **Boutwell, C. L., C. F. Rowley, and M. Essex.** 2009. Reduced viral replication capacity of human immunodeficiency virus type 1 subtype C caused by cytotoxic-T-lymphocyte escape mutations in HLA-B57 epitopes of capsid protein. J. Virol. **83:**2460–2468.
10. **Brockman, M. A., A. Schneidewind, M. Lahaie, A. Schmidt, T. Miura, I. Desouza, F. Ryvkin, C. A. Derdeyn, S. Allen, E. Hunter, J. Mulenga, P. A. Goepfert, B. D. Walker, and T. M. Allen.** 2007. Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. J. Virol. **81:**12608–12618.
11. **Brumme, Z. L., C. J. Brumme, J. Carlson, H. Streeck, M. John, Q. Eichbaum, B. L. Block, B. Baker, C. Kadie, M. Markowitz, H. Jessen, A. D. Kelleher, E. Rosenberg, J. Kaldor, Y. Yuki, M. Carrington, T. M. Allen, S. Mallal, M. Altfeld, D. Heckerman, and B. D. Walker.** 2008. Marked epitope and allele-specific differences in rates of mutation in HIV-1 Gag, Pol and Nef CTL epitopes in acute/early HIV-1 infection. J. Virol. **82:**9216–9227.
12. **Brumme, Z. L., C. J. Brumme, C. Chui, T. Mo, B. Wynhoven, C. K. Woods, B. M. Henrick, R. S. Hogg, J. S. Montaner, and P. R. Harrigan.** 2007. Effects of human leukocyte antigen class I genetic parameters on clinical outcomes and survival after initiation of highly active antiretroviral therapy. J. Infect. Dis. **195:**1694–1704.
13. **Brumme, Z. L., C. J. Brumme, D. Heckerman, B. T. Korber, M. Daniels, J. Carlson, C. Kadie, T. Bhattacharya, C. Chui, J. Szinger, T. Mo, R. S. Hogg, J. S. Montaner, N. Frahm, C. Brander, B. D. Walker, and P. R. Harrigan.** 2007. Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. PLoS Pathog. **3:**e94.
14. **Brumme, Z. L., M. John, J. M. Carlson, C. J. Brumme, D. Chan, M. A. Brockman, L. C. Swenson, I. Tao, S. Szeto, P. Rosato, J. Sela, C. M. Kadie, N. Frahm, C. Brander, D. W. Haas, S. Riddler, R. Haubrich, B. D. Walker, P. R. Harrigan, D. Heckerman, and S. Mallal.** 2009. HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. PLoS One **4:**e6687.
15. **Carlson, J. M., Z. L. Brumme, C. M. Rousseau, C. J. Brumme, P. Matthews, C. Kadie, J. I. Mullins, B. D. Walker, P. R. Harrigan, P. J. Goulder, and D. Heckerman.** 2008. Phylogenetic dependency networks: inferring patterns of CTL escape and codon covariation in HIV-1 Gag. PLoS Comput. Biol. **4:**e1000225.
16. **Carrington, M., and S. J. O'Brien.** 2003. The influence of HLA genotype on AIDS. Annu. Rev. Med. **54:**535–551.
17. **Chopera, D. R., Z. Woodman, K. Mlisana, M. Mlotshwa, D. P. Martin, C. Seoighe, F. Treurnicht, D. A. de Rosa, W. Hide, S. A. Karim, C. M. Gray, and C. Williamson.** 2008. Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. PLoS Pathog. **4:**e1000033.
18. **Couillin, I., B. Culmann-Penciolelli, E. Gomard, J. Choppin, J. P. Levy, J. G. Guillet, and S. Saragosti.** 1994. Impaired cytotoxic T lymphocyte recognition due to genetic variations in the main immunogenic region of the human immunodeficiency virus 1 NEF protein. J. Exp. Med. **180:**1129–1134.
19. **Crawford, H., W. Lumm, A. Leslie, M. Schaefer, D. Boeras, J. G. Prado, J. Tang, P. Farmer, T. Ndung'u, S. Lakhi, J. Gilmour, P. Goepfert, B. D. Walker, R. Kaslow, J. Mulenga, S. Allen, P. J. Goulder, and E. Hunter.** 2009. Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. J. Exp. Med. **206:**909–921.
20. **Crawford, H., J. G. Prado, A. Leslie, S. Hue, I. Honeyborne, S. Reddy, M. van der Stok, Z. Mncube, C. Brander, C. Rousseau, J. I. Mullins, R. Kaslow, P. Goepfert, S. Allen, E. Hunter, J. Mulenga, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2007. Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. J. Virol. **81:**8346–8351.
21. **Duda, A., L. Lee-Turner, J. Fox, N. Robinson, S. Dustan, S. Kaye, H. Fryer, M. Carrington, M. McClure, A. R. McLean, S. Fidler, J. Weber, R. E. Phillips, and A. J. Frater.** 2009. HLA-associated clinical progression correlates with epitope reversion rates in early human immunodeficiency virus infection. J. Virol. **83:**1228–1239.
22. **Edwards, B. H., A. Bansal, S. Sabbaj, J. Bakari, M. J. Mulligan, and P. A. Goepfert.** 2002. Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J. Virol. **76:**2298–2305.
23. **Fiebig, E. W., D. J. Wright, B. D. Rawal, P. E. Garrett, R. T. Schumacher, L. Peddada, C. Heldebrant, R. Smith, A. Conrad, S. H. Kleinman, and M. P. Busch.** 2003. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. AIDS **17:**1871–1879.
24. **Friedrich, T. C., E. J. Dodds, L. J. Yant, L. Vojnov, R. Rudersdorf, C. Cullen, D. T. Evans, R. C. Desrosiers, B. R. Mothe, J. Sidney, A. Sette, K. Kunstman, S. Wolinsky, M. Piatak, J. Lifson, A. L. Hughes, N. Wilson, D. H. O'Connor, and D. I. Watkins.** 2004. Reversion of CTL escape-variant immunodeficiency viruses in vivo. Nat. Med. **10:**275–281.
25. **Gao, X., A. Bashirova, A. K. Iversen, J. Phair, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, M. Altfeld, S. J. O'Brien, and M. Carrington.** 2005. AIDS restriction HLA allotypes target distinct intervals of HIV-1 pathogenesis. Nat. Med. **11:**1290–1292.
26. **Gaschen, B., J. Taylor, K. Yusim, B. Foley, F. Gao, D. Lang, V. Novitsky, B. Haynes, B. H. Hahn, T. Bhattacharya, and B. Korber.** 2002. Diversity considerations in HIV-1 vaccine selection. Science **296:**2354–2360.
27. **Goepfert, P. A., W. Lumm, P. Farmer, P. Matthews, A. Prendergast, J. M. Carlson, C. A. Derdeyn, J. Tang, R. A. Kaslow, A. Bansal, K. Yusim, D.**

Heckerman, J. Mulenga, S. Allen, P. J. Goulder, and E. Hunter. 2008. Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. J. Exp. Med. **205:**1009–1017.

28. **Goonetilleke, N., M. K. Liu, J. F. Salazar-Gonzalez, G. Ferrari, E. Giorgi, V. V. Ganusov, B. F. Keele, G. H. Learn, E. L. Turnbull, M. G. Salazar, K. J. Weinhold, S. Moore, N. Letvin, B. F. Haynes, M. S. Cohen, P. Hraber, T. Bhattacharya, P. Borrow, A. S. Perelson, B. H. Hahn, G. M. Shaw, B. T. Korber, and A. J. McMichael.** 2009. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. J. Exp. Med. **206:**1253–1272.
29. **Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones.** 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212–217.
30. **Guindon, S., and O. Gascuel.** 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. **52:**696–704.
31. **Honeyborne, I., A. Prendergast, F. Pereyra, A. Leslie, H. Crawford, R. Payne, S. Reddy, K. Bishop, E. Moodley, K. Nair, M. van der Stok, N. McCarthy, C. M. Rousseau, M. Addo, J. I. Mullins, C. Brander, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2007. Control of human immunodeficiency virus type 1 is associated with HLA-B*13 and targeting of multiple gag-specific CD8+ T-cell epitopes. J. Virol. **81:**3667–3672.
32. **Huang, X., and J. Zhang.** 1996. Methods for comparing a DNA sequence with a protein sequence. Comput. Appl. Biosci. **12:**497–506.
33. **Janssen, R. S., G. A. Satten, S. L. Stramer, B. D. Rawal, T. R. O'Brien, B. J. Weiblen, F. M. Hecht, N. Jack, F. R. Cleghorn, J. O. Kahn, M. A. Chesney, and M. P. Busch.** 1998. New testing strategy to detect early HIV-1 infection for use in incidence estimates and for clinical and prevention purposes. JAMA **280:**42–48.
34. **John, M., D. Heckerman, I. James, L. P. Park, J. M. Carlson, A. Chopra, S. Gaudieri, D. Nolan, D. Haas, S. A. Riddler, D. Haubrich, and S. Mallal.** 2010. Adaptive interactions between HLA and HIV-1: highly divergent selection imposed by HLA class I molecules with common supertype motifs. J. Immunol. **184:**4368–4377.
35. **Kelleher, A. D., C. Long, E. C. Holmes, R. L. Allen, J. Wilson, C. Conlon, C. Workman, S. Shaunak, K. Olson, P. Goulder, C. Brander, G. Ogg, J. S. Sullivan, W. Dyer, I. Jones, A. J. McMichael, S. Rowland-Jones, and R. E. Phillips.** 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T lymphocyte responses. J. Exp. Med. **193:**375–386.
36. **Kiepiela, P., K. Ngumbela, C. Thobakgale, D. Ramduth, I. Honeyborne, E. Moodley, S. Reddy, C. de Pierres, Z. Mncube, N. Mkhwanazi, K. Bishop, M. van der Stok, K. Nair, N. Khan, H. Crawford, R. Payne, A. Leslie, J. Prado, A. Prendergast, J. Frater, N. McCarthy, C. Brander, G. H. Learn, D. Nickle, C. Rousseau, H. Coovadia, J. I. Mullins, D. Heckerman, B. D. Walker, and P. Goulder.** 2007. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat. Med. **13:**46–53.
37. **Koch, N., N. Yahi, J. Fantini, and C. Tamalet.** 2001. Mutations in HIV-1 gag cleavage sites and their association with protease mutations. AIDS **15:**526–528.
38. **Korber, B. T., N. L. Letvin, and B. F. Haynes.** 2009. T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces. J. Virol. **83:**8300–8314.
39. **Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho.** 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J. Virol. **68:**4650–4655.
40. **Leslie, A. J., and P. J. Goulder.** 2006. HIV escape and attenuation by cytotoxic T lymphocytes. Curr. Opin. HIV AIDS **1:**34–39.
41. **Leslie, A. J., K. J. Pfafferott, P. Chetty, R. Draenert, M. M. Addo, M. Feeney, Y. Tang, E. C. Holmes, T. Allen, J. G. Prado, M. Altfeld, C. Brander, C. Dixon, D. Ramduth, P. Jeena, S. A. Thomas, A. St. John, T. A. Roach, B. Kupfer, G. Luzzi, A. Edwards, G. Taylor, H. Lyall, G. Tudor-Williams, V. Novelli, J. Martinez-Picado, P. Kiepiela, B. D. Walker, and P. J. Goulder.** 2004. HIV evolution: CTL escape mutation and reversion after transmission. Nat. Med. **10:**282–289.
42. **Martinez-Picado, J., J. G. Prado, E. E. Fry, K. Pfafferott, A. Leslie, S. Chetty, C. Thobakgale, I. Honeyborne, H. Crawford, P. Matthews, T. Pillay, C. Rousseau, J. I. Mullins, C. Brander, B. D. Walker, D. I. Stuart, P. Kiepiela, and P. Goulder.** 2006. Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J. Virol. **80:**3617–3623.
43. **Matthews, P. C., A. Prendergast, A. Leslie, H. Crawford, R. Payne, C. Rousseau, M. Rolland, I. Honeyborne, J. Carlson, C. Kadie, C. Brander, K. Bishop, N. Mlotshwa, J. D. Mullins, H. Coovadia, T. Ndung'u, B. D. Walker, D. Heckerman, and P. J. Goulder.** 2008. Central role of reverting mutations in HLA associations with human immunodeficiency virus set point. J. Virol. **82:**8548–8559.
44. **McMichael, A. J., P. Borrow, G. D. Tomaras, N. Goonetilleke, and B. F. Haynes.** 2010. The immune response during acute HIV-1 infection: clues for vaccine development. Nat. Rev. Immunol. **10:**11–23.
45. **McMichael, A. J., and R. E. Phillips.** 1997. Escape of human immunodeficiency virus from immune control. Annu. Rev. Immunol. **15:**271–296.
46. **Miura, T., M. A. Brockman, C. J. Brumme, Z. L. Brumme, J. M. Carlson, F. Pereyra, A. Trocha, M. M. Addo, B. L. Block, A. C. Rothchild, B. M. Baker, T. Flynn, A. Schneidewind, B. Li, Y. E. Wang, D. Heckerman, T. M. Allen, and B. D. Walker.** 2008. Genetic characterization of human immunodeficiency virus type 1 in elite controllers: lack of gross genetic defects or common amino acid changes. J. Virol. **82:**8422–8430.
47. **Miura, T., M. A. Brockman, Z. L. Brumme, C. J. Brumme, F. Pereyra, A. Trocha, B. L. Block, A. Schneidewind, T. M. Allen, D. Heckerman, and B. D. Walker.** 2009. HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-Protease from elite controllers of human immunodeficiency virus type 1. J. Virol. **83:**140–149.
48. **Miura, T., M. A. Brockman, A. Schneidewind, M. Lobritz, F. Pereyra, A. Rathod, B. L. Block, Z. L. Brumme, C. J. Brumme, B. Baker, A. C. Rothchild, B. Li, A. Trocha, E. Cutrell, N. Frahm, C. Brander, I. Toth, E. J. Arts, T. M. Allen, and B. D. Walker.** 2009. HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J. Virol. **83:**2743–2755.
49. **Miura, T., Z. L. Brumme, M. Brockman, P. Rosato, J. Sela, C. J. Brumme, F. Pereyra, D. E. Kaufmann, A. Trocha, B. L. Block, E. S. Daar, E. Connick, H. Jessen, A. D. Kelleher, E. Rosenberg, M. Markowitz, K. Schafer, F. Vaida, A. Iwamoto, S. Little, and B. D. Walker.** 2010. Impaired replication capacity of acute/early viruses in persons who become HIV controllers. J. Virol. **84:**7581–7591.
50. **Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal.** 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science **296:**1439–1443.
51. **O'Brien, S. J., X. Gao, and M. Carrington.** 2001. HLA and AIDS: a cautionary tale. Trends Mol. Med. **7:**379–381.
52. **Peyerl, F. W., H. S. Bazick, M. H. Newberg, D. H. Barouch, J. Sodroski, and N. L. Letvin.** 2004. Fitness costs limit viral escape from cytotoxic T lymphocytes at a structurally constrained epitope. J. Virol. **78:**13901–13910.
53. **Prado, J. G., I. Honeyborne, I. Brierley, M. C. Puertas, J. Martinez-Picado, and P. J. Goulder.** 2009. Functional consequences of human immunodeficiency virus escape from an HLA-B*13-restricted CD8+ T-cell epitope in p1 Gag protein. J. Virol. **83:**1018–1025.
54. **Price, D. A., P. J. Goulder, P. Klenerman, A. K. Sewell, P. J. Easterbrook, M. Troop, C. R. Bangham, and R. E. Phillips.** 1997. Positive selection of HIV-1 cytotoxic T lymphocyte escape variants during primary infection. Proc. Natl. Acad. Sci. U. S. A. **94:**1890–1895.
55. **Rolland, M., D. C. Nickle, and J. I. Mullins.** 2007. HIV-1 group M conserved elements vaccine. PLoS Pathog. **3:**e157.
56. **Rousseau, C. M., M. G. Daniels, J. M. Carlson, C. Kadie, H. Crawford, A. Prendergast, P. Matthews, R. Payne, M. Rolland, D. N. Raugi, B. S. Maust, G. H. Learn, D. C. Nickle, H. Coovadia, T. Ndung'u, N. Frahm, C. Brander, B. D. Walker, P. J. Goulder, T. Bhattacharya, D. E. Heckerman, B. T. Korber, and J. I. Mullins.** 2008. HLA class I-driven evolution of human immunodeficiency virus type 1 subtype C proteome: immune escape and viral load. J. Virol. **82:**6434–6446.
57. **Rowland-Jones, S. L., R. E. Phillips, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, et al.** 1992. Human immunodeficiency virus variants that escape cytotoxic T-cell recognition. AIDS Res. Hum. Retroviruses **8:**1353–1354.
58. **Schneidewind, A., M. A. Brockman, J. Sidney, Y. E. Wang, H. Chen, T. J. Suscovich, B. Li, R. I. Adam, R. L. Allgaier, B. R. Mothe, T. Kuntzen, C. Oniangue-Ndza, A. Trocha, X. G. Yu, C. Brander, A. Sette, B. D. Walker, and T. M. Allen.** 2008. Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid. J. Virol. **82:**5594–5605.
59. **Schneidewind, A., M. A. Brockman, R. Yang, R. I. Adam, B. Li, S. Le Gall, C. R. Rinaldo, S. L. Craggs, R. L. Allgaier, K. A. Power, T. Kuntzen, C. S. Tung, M. X. LaBute, S. M. Mueller, T. Harrer, A. J. McMichael, P. J. Goulder, C. Aiken, C. Brander, A. D. Kelleher, and T. M. Allen.** 2007. Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. J. Virol. **81:**12382–12393.
60. **Storey, J. D., and R. Tibshirani.** 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. U. S. A. **100:**9440–9445.
61. **Taylor, B. S., and S. M. Hammer.** 2008. The challenge of HIV-1 subtype diversity. N. Engl. J. Med. **359:**1965–1966.
62. **Troyer, R. M., J. McNevin, Y. Liu, S. C. Zhang, R. W. Krizan, A. Abraha, D. M. Tebit, H. Zhao, S. Avila, M. A. Lobritz, M. J. McElrath, S. Le Gall, J. I. Mullins, and E. J. Arts.** 2009. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. PLoS Pathog. **5:**e1000365.
63. **Wang, Y. E., B. Li, J. M. Carlson, H. Streeck, A. D. Gladden, R. Goodman, A. Schneidewind, K. A. Power, I. Toth, N. Frahm, G. Alter, C. Brander, M. Carrington, B. D. Walker, M. Altfeld, D. Heckerman, and T. M. Allen.** 2009. Protective HLA class I alleles that restrict acute-phase CD8+ T-cell responses

are associated with viral escape mutations located in highly conserved regions of human immunodeficiency virus type 1. J. Virol. **83:**1845–1855.

64. **Wright, J. K., Z. L. Brumme, J. M. Carlson, D. Heckerman, C. M. Kadie, C. J. Brumme, B. Wang, E. Losina, T. Miura, F. Chonco, M. van der Stok, Z. Mncube, K. Bishop, P. J. R. Goulder, B. D. Walker, M. A. Brockman, and T. Ndung'u.** 2010. Gag-Protease-mediated replication capacity in HIV-1 subtype C chronic infection: associations with HLA type and clinical parameters. J. Virol. **84:**10820–10831.
65. **Yeh, W. W., E. M. Cale, P. Jaru-Ampornpan, C. I. Lord, F. W. Peyerl, and N. L. Letvin.** 2006. Compensatory substitutions restore normal core assembly in simian immunodeficiency virus isolates with Gag epitope cytotoxic T-lymphocyte escape mutations. J. Virol. **80:**8168–8177.
66. **Zuniga, R., A. Lucchetti, P. Galvan, S. Sanchez, C. Sanchez, A. Hernandez, H. Sanchez, N. Frahm, C. H. Linde, H. S. Hewitt, W. Hildebrand, M. Altfeld, T. M. Allen, B. D. Walker, B. T. Korber, T. Leitner, J. Sanchez, and C. Brander.** 2006. Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J. Virol. **80:**3122–3125.

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T cell-based functional cDNA library screening identified SEC14-like 1a carboxy-terminal domain as a negative regulator of human immunodeficiency virus replication

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ABSTRACT

Genome-wide screening of host factors that regulate HIV-1 replication has been attempted using numerous experimental approaches. However, there has been limited success using T cell-based cDNA library screening to identify genes that regulate HIV-1 replication. We have established a genetic screening strategy using the human T cell line MT-4 and a replication-competent HIV-1. With this system, we identified the C-terminal domain (CTD) of SEC14-like 1a (SEC14L1a) as a novel inhibitor of HIV-1 replication. Our T cell-based cDNA screening system provides an alternative tool for identifying novel regulators of HIV-1 replication.

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1. Introduction

The molecular interaction between HIV-1 and the host is not fully understood. A systematic genome-wide approach provides the critical information for the completion of the HIV-1-host interactome. Many experimental genome-wide screening systems have been established to identify the cellular genes required for HIV-1 replication (Table 1, [1–8]). More than a hundred genes have been identified as being cellular factors that regulate HIV-1 replication. However, different screening systems do not identify the same set of genes, and many systems yielded non-overlapping candidates. These discrepancies are assumed to be due to differences in the experimental approaches, such as the virus, the cell line, or the genetic materials used.

For viruses, the wild-type HIV-1 [1,3–6] or a replication-incompetent HIV-1 pseudotyped with vesicular stomatitis virus (VSV)-G is used [2,7,8]. The VSV-G-pseudotyped "HIV-1-based vector" has been used to identify factors associated with the viral entry processes. However, in reality, it covers the events from post-membrane fusion to translation. One of the potential caveats in the use of the VSV-G-pseudotyped vector is that it enters cells via the VSV-G-restricted route, which is fundamentally different from the HIV-1 *Env*-mediated entry pathway [9–12]. The replication-competent HIV-1 should be ideal to cover the entire viral replication cycle; however, this may raise biosafety concerns.

For cells, non-T cells, such as a genetically engineered HeLa cells that ectopically express luciferase or beta-galactosidase (TZM-bl cells), are often used, since they are efficiently transduced with genetic materials [2,5–8]. Not many studies employ a T cell-based system, partly because genetic materials are not efficiently transduced into T cells [1,3,4]. To identify HIV-1 replication regulatory factors, it is preferable to perform the functional analysis in the natural targets of HIV-1 including T cells. The gene expression profile of non-T cells is apparently different from that of T cells as exemplified by the absence of T cell specific markers on non-T cells such as CD4. It is possible that a candidate gene isolated in the non-T cell-based system might not be expressed in T cells. It is impossible to identify T cell-specific factors in the non-T cell-based screening using the siRNA library or in the screening using cDNA libraries derived from non-T cells. Also, the effect or functions of some genes may not be identical in distinct cell types. The potential risk of a non-T cell-based assay is that we may falsely score a gene as a regulator of HIV-1 replication, although many genes have been discovered using non-T cell-based screening systems including the viral receptors. Ideally, the primary CD4-positive T cells, dendritic cells, macrophages, or NK/T cells should be used.

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Table 1
Summary of genome-wide screening strategies to identify regulatory factors of HIV-1 replication.

Genetic material	Transduction approach	Cell line	Replication competency of HIV-1	Reference
cDNA library	Retroviral, stable	TE671	Incompetent	[2,8]
siRNA library	Transfection, transient	HeLa or 293T	Competent or incompetent	[5,6,7]
cDNA library	Lenti- or retroviral, stable	MT-4	Competent	[1,3,4]

Given technical limitations, this is currently unrealistic for genetic screening experiments.

As for the genetic material, cDNA libraries are often used [1–4,8]. Recent studies utilized siRNA libraries [5–7]. The cDNA approach is advantageous for providing genetic diversity. Expression of the full-length open reading frame of a gene can upregulate the function of the gene, whereas cDNA fragments can function in a diverse fashion. The gene silencing approach downregulates gene expression; however, the silencing efficiency of a gene varies in different cell types and at different time points in the assay (reviewed in [13]). As mentioned above, the gene silencing approach is unable to score the contribution of genes that are not expressed in the cells used in the assay.

The screening can be performed in cells that are either transiently [5–7] or stably [1–4,8] transduced with genetic materials. In the transient transfection assays, it is possible that the dysregulation of a gene function can damage the physiology of the cells. In such a case, the inhibition of HIV-1 replication can be observed, but may not be a direct inhibitory effect of the gene of interest. Such a risk can be minimized by using cells stably transduced with the genetic materials.

We conducted a phenotype cDNA screen using a T cell line-based assay to identify cellular genes that render cells resistant to HIV-1 replication [3]. The advantage of our functional screening system is that cDNA libraries are stably transduced into cells, and that a replication-competent HIV-1 and a human T cell line MT-4 are used. With this system, we have successfully identified the SEC14-like 1a (SEC14L1a) C-terminal domain (CTD) as an inhibitor of HIV-1 replication that targets the late phase of the viral life cycle.

2. Materials and methods

2.1. Cells, transfection, cDNA selection

Cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MA) supplemented with 10% fetal bovine serum (Japan Bioserum, Tokyo, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Tokyo, Japan). Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were transfected with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The method of selecting human cDNAs that confer resistance to HIV-1 has been described previously in detail [1,3].

2.2. Plasmids

The SEC14L1a CTD1 was amplified from MT-4 polyA RNA by reverse transcriptase PCR (RT-PCR) using the primers 5′-GCACCGG-TCTCGAGCCACCATGGACTACAAAGACGATGACGACCCTGCGTGCGC-CGCCAGCAGC-3′ and 5′-CCAATTGCTACCTGGAGATCATGGAGCTG-3′. The SEC14L1a CTD2 was amplified by PCR from human lymph node cDNA library (Takara, Otsu, Japan) using the primers 5′-GCAC-CGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACTGCGAAG-TGCCAGAGGGTGGAC-3′ and 5′-CCAATTGCTACCTGGAGATCAT-GGAGCTG-3′. Full length (FL) SEC14L1a was amplified by PCR from a plasmid containing the SEC14L1a open reading frame (ORF, CSODL004YN18, Invitrogen), using the primers 5′-GCA-CCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACGTGCAG-AAATACCAGTCCCCAG-3′ and 5′-CCAATTGCTACCTGGAGATCATGG-AGCTG-3′. The AgeI-MfeI fragments of the PCR products were cloned into the XmaI-MfeI sites of the pEGFP-C3 plasmid (Clontech, Palo Alto, CA), generating pEGFP-SEC14L1a-CTD1, -CTD2, and -FL. The XhoI-MfeI fragments from the resulting plasmids were cloned into the corresponding restriction sites of the pCMMP KRAB vector, creating pCMMP GFP-SEC14L1a-CTD1, -CTD2, and -FL. The HIV-1 *tat* was amplified by PCR using the primers 5′-AACCGGTCTCGAGCCACCATGGAGCCAGTAGATCCTAGAC-3′ and 5′-GGATCCTCAGTCGTCATCGTCTTTGTAGTCTTCCTTCGGGCCTGTCGG-GTC-3′. A Tat expression vector pCMMP Tat was constructed by cloning the AgeI-BamHI fragment of the PCR product into the corresponding restriction sites of the pCMMP KRAB vector. The HIV-1 *Env* and GFP expression vectors (pIIIex and pCMMP GFP, respectively) are described previously [3,12,14]. To construct the pCMMP GFP-FLAG (GFPf), pCMMP CXCR4 d-10 [15] was digested with AgeI and XhoI to remove CXCR4 d-10 ORF and self-ligated after blunting with T4 DNA polymerase. The HIV-1 *gag-pol*, *tat*, and *rev* expressing plasmid pCMVR8.91 was a generous gift from Dr. Trono's group [16].

2.3. Western blotting

Western blotting was performed according to techniques described previously [17]. The following reagents were used: anti-FLAG (rabbit polyconal, 600-401-383, Rockland, Gilbertsville, PA), anti-p24 (183-H12-5C, NIH AIDS Research and Reference Reagent Program), anti-gp120 (vA-20 and vT-21 antibodies, Santa Cruz Biotech, Santa Cruz, CA), biotinylated anti-goat antibody (GE Healthcare Bio-Sciences, Piscataway, NJ), horseradish peroxidase-conjugated streptoavidin (GE Healthcare Bio-Sciences), and EnVision+ system (Dako, Glostrup, Denmark). Signals were visualized with an LAS3000 imager (Fujifilm, Tokyo, Japan) and quantified by Multi Gauge ver 3.0 software (Fujifilm).

2.4. Confocal microscopy

293T cells transiently transfected with expression vectors for SEC14L1a derivatives were grown on glass plates, fixed in 4% formaldehyde in phosphate buffer saline (PBS) for 5 min at 24 h post-transfection, stained with Hoechst 33258 (Sigma), mounted (Vectorshield, Vector Laboratories, Burlingame, CA), and imaged using a confocal microscope META 510 (Carl Zeiss, Tokyo, Japan). For MT-4 cells, live cells were incubated with Hoechst 33258 and imaged unfixed. Image brightness and contrast were processed by META510 software (Carl Zeiss).

2.5. Immunoprecipitation

Cells expressing FLAG-tagged proteins were harvested and washed twice with PBS and then lysed in the lysis buffer (50 mM Tris–HCl, pH 8.0, 0.5% IGEPAL CA630, protease inhibitor cocktail from Sigma) on ice for 30 min. The soluble fraction was obtained by centrifugation at 15,000 rpm for 30 min at 4 °C, and was incubated with 20 μl of Red-Anti-FLAG M2 Affinity Gel (Sigma) with gentle mixing overnight at 4 °C. After washing the agarose beads for five times with the lysis buffer, the bound complexes were eluted with the FLAG peptide, and analyzed by Western blotting.

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2.6. Flow cytometry

Cells were labeled with PE-Cy5-conjugated anti-CD4 antibody or PE-conjugated anti-CXCR4 antibody (Beckton Dickinson, San Jose, Calif.) for 30 min at 4 °C. Cells were washed once with PBS supplemented with 1% FBS and analyzed by FACS Aria (Beckton Dickinson). The GFP-positive cells were sorted using FACS Aria.

2.7. Monitoring HIV-1 replication

For HIV-1 infection, 1×10^5 cells were incubated at the room temperature with the HIV-1_{HXB2}-containing culture supernatant, which had approximately 1.0 ng of $p24^{CA}$, for approximately 30 min. The culture supernatants were collected at 4 d post-infection and subjected to ELISA to measure the p24 ^{CA} antigen, using a Retro TEK p24 Antigen ELISA Kit according to the manufacturer's protocol (Zepto Metrix, Buffalo, NY). The signals were measured with an ELx808 microplate photometer (BIO-TEK®, Winooski, VT).

2.8. PCR analysis

The cellular DNA and RNA were extracted from cells infected with VSV-G-pseudotyped HIV-1 vector produced by using pNL-Luc plasmid, as described previously [17]. The Alu-LTR PCR and RT-PCR were performed as described previously [3,17] using the following primers: for the first Alu-LTR PCR reaction, 5'-AACTAGGGAACCCACTGCTTAAG-3' and 5'-TGCTGGGATTACAGGC-GTGAG-3'; and for the second Alu-LTR PCR reaction, 5'-AACT-AGGGAACCCACTGCTTAAG-3' and 5'-CTGCTAGAGATTTTCCACA-CTGAC-3'. For amplification of HIV-1 mRNA, 5′-ATGGAGCCAGTAG-ATCCTAGAC-3′ and 5′-CTATTCCTTCGGGCCTGTCGGG-3′ primers were used. For the control, we amplified beta-globin and cyclophilin A using the following primers: beta-globin, 5'-TATTGGTCT-CCTTAAACCTGTCTTG-3' and 5'-CTGACACAACTGTGTTCACTAGC-3'; and cyclophilin A, 5′- CACCGCCACCATGGTCAACCCCACCGTGTTCT-TCGAC-3′ and 5′- CCCGGGCCTCGAGCTTTCGAGTTGTCCACAGTCA-GCAATGG-3′. The amplicons were separated in a 2% agarose gel, stained with ethidium bromide, and imaged with a Typhoon scanner (GE Healthcare Bio-Sciences).

2.9. Collection of virus-like particle

Tissue culture supernatants containing virus-like particles (VLP) were passed through nitrocellulose filters (0.45 μm, Millipore, Tokyo, Japan) and the virions were collected by centrifugation (Optima™ TL, TLA 100.3 rotor, 541 k × g for 1 h; Beckman Coulter, Miami, FL).

3. Results

3.1. Identification of SEC14L1a as a potential regulator of HIV-1 replication

We prepared MT-4 cells that constitutively express cDNA transduced by a lentiviral vector or an MLV-based retroviral vector (Fig. 1A). The cDNAs were derived from human peripheral blood mononuclear cells (PBL) and *Oryctolagus cuniculus* (European rabbit) kidney-derived cell line RK13 cells. MT-4 cells transduced with cDNA were collected by FACS sorter using the green fluorescence as a marker since viral vectors encoded the GFP expression cassette. Then, cells were infected with HIV-1. Surviving cells were propagated and the genomic DNA was extracted to recover the transduced cDNA by PCR as previously described [3]. We isolated two clones encoding the carboxy terminal domain (CTD) of SEC14L1a (Gene ID 6397, Fig. 1B and C); one from the PBL cDNA library (1/65 independent clones, 1.5%), and one from the RK13 cDNA library (1/42 independent clones, 2.4%). The fact that the SEC14L1a CTD was successfully identified from two independent cDNA libraries strongly suggests that it is a negative regulator of HIV-1 replication. It is important to note that previous genome-wide screenings for HIV-1 regulators have not identified SEC14L1a CTD. This clearly suggests that our T cell-based cDNA screening system is unique, and should be able to complement the other genome-wide screening systems.

SEC14L1a belongs to the widely-expressed SEC14-superfamily that is involved in membrane trafficking and phospholipid metabolism [18–21]. The function of SEC14L1a is not well understood. The C-terminus of SEC14L1a encodes a Golgi dynamics (GOLD) domain (amino acids (aa) 523–674; Fig. 1C) that mediates the protein-protein interaction possibly involved in the maintenance of Golgi apparatus function and vesicular trafficking [22]. The only reported biological activity of SEC14L1a is to interact with cholinergic receptors AchT and CHT1 [23]. The GOLD domain is responsible for the physical interaction between SEC14L1a and cholinergic receptors. However, the functional significance of these interactions remains to be clarified. The conserved SEC14 domain directly interacts with lipid molecules [17–21]. However, the lipid ligand of SEC14L1a (aa 319–490, Fig. 1C) has yet to be identified.

3.2. Construction of expression vectors for SEC14L1a derivatives

The longest SEC14L1a cDNA recovered from the PBL cDNA library spanned nucleotides (nt) 2045–2492 of SEC14L1a mRNA (NM_003003.3), covering the CTD of the SEC14L1a open reading frame (ORF; Fig. 1B). We detected a potential translational start codon at nt 2188-2190 within the GOLD domain (asterisk, Fig. 1B). We speculated that the isolated cDNA might have expressed the carboxy half of the GOLD domain (aa 641–715) in MT-4 cells, leading to the inhibition of HIV-1 replication.

To test this, we constructed an expression plasmid for FLAG-tagged CTD (aa 642-715) fused to the carboxy terminus of GFP (CTD1; Fig. 1C). We also constructed GFP fusion proteins spanning the GOLD domain (CTD2, aa 493–715) or the full-length SEC14L1a (FL; Fig. 1C). Expression of these proteins was verified by Western blotting of transient transfected 293T cells (Fig. 1D). The confocal microscopy analysis indicated that the FL localized mainly in the cytoplasm, with some accumulation in the perinuclear regions (Fig. 1E), consistent with a previous report [23]. CTD1 was distributed in the cytoplasm and the nucleus, with a slight preference for the cytoplasm. CTD2 was evenly distributed to the nucleus and cytoplasm. When MT-4 cells constitutively expressing FL, CTD1, and CTD2 were analyzed, the subcellular distribution was less clear, due to the small cytoplasm (Fig. 1F). However, FL was distributed evenly to the nucleus and cytoplasm in MT-4 cells. In contrast, CTD1 was excluded from the nucleus in MT-4 cells (Fig. 1F). The distribution of CTD2 in MT-4 cells was similar to that in 293T cells (Fig. 1F). The differences of protein distribution in two cell types may be caused by the cell type-dependent regulation of protein trafficking and/or the effect of protein expression levels.

3.3. Verification of anti-HIV-1 activity associated with SEC14L1a CTD1

We introduced FL, CTD1, or CTD2 into MT-4 cells using the MLV vector, and isolated cells constitutively expressing FL, CTD1, or CTD2. Expression of SEC14L1a derivatives in MT-4 cells was verified by Western blotting (Fig. 2A). FL expression was verified by immuno-precipitation assay (Fig. 2A). The detection of FL by Western blotting was inefficient considering the fact that all the SEC14L1a derivatives are GFP-tagged, and the GFP intensity of FL-expressing MT-4 cells was not lower than that of CTD1-expressing

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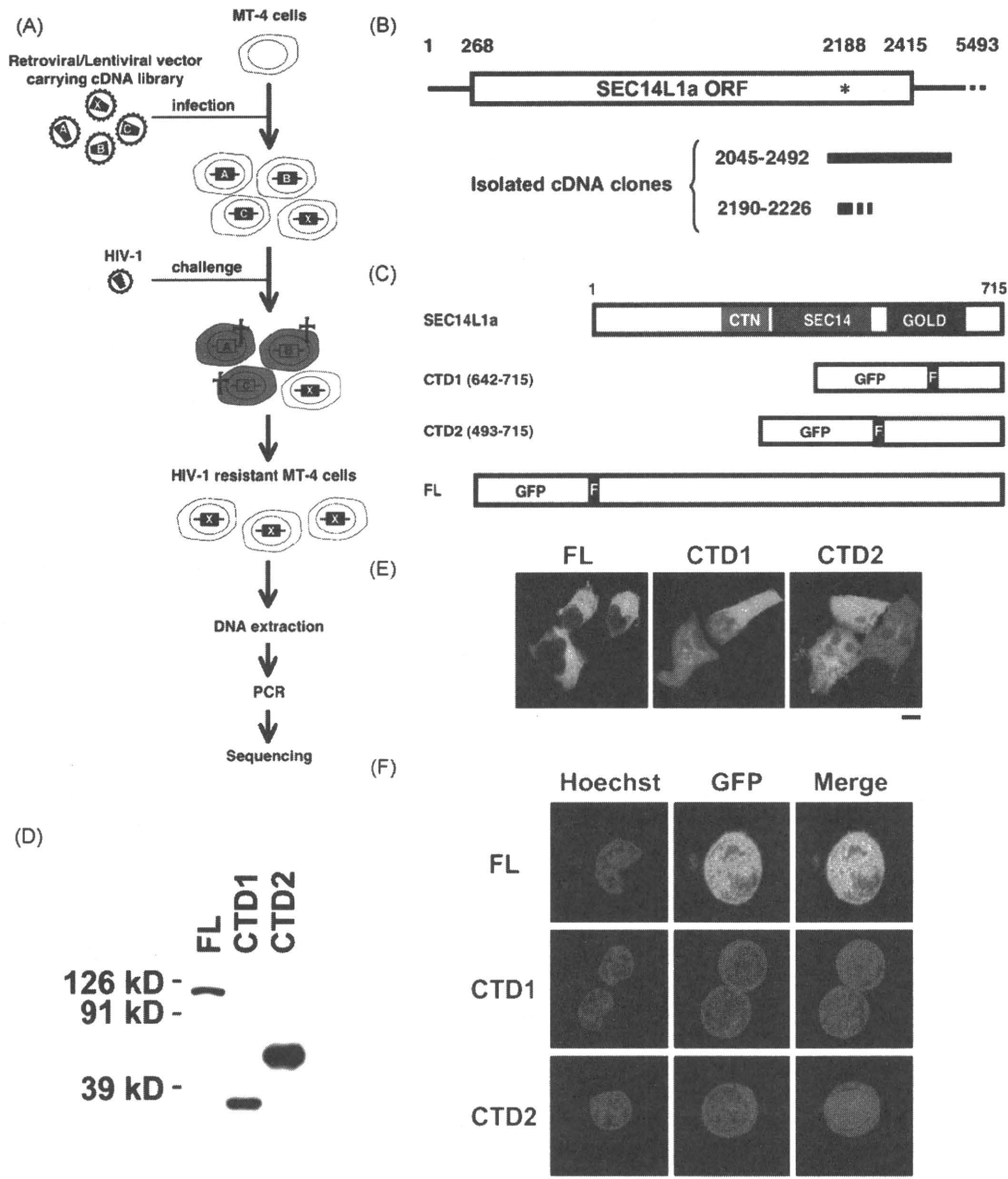

Fig. 1. Identification of SEC14L1a CTD as a potential regulator of HIV-1 replication. (A) The experimental strategy used to screen a cDNA library for genes rendering cells resistant to HIV-1. MT-4 cells were infected with a retroviral or lentiviral vector carrying cDNA libraries and were challenged with wild-type HIV-1$_{HXB2}$. The HIV-1-infected cells (gray with cross) quickly undergo cell death. The surviving cells were propagated, collected, and the transduced cDNA labeled X was determined. (B) Schematic representation of SEC14L1a mRNA (NM_00303.3) and the isolated gene fragments. The open reading frame (ORF) is assigned from nucleotides (nt) 268 to 2415. The potential internal translational initiation codon is marked with an asterisk. (C) Schematic representation of the SEC14L1a protein (NP_002994). SEC14L1a has a CRAL_TRIO_N domain (CTN, amino acids 241–313), a SEC14p-like lipid-binding domain (SEC14, amino acids 319–490), and a Golgi dynamics domain (GOLD, amino acids 523–674). The cloned fragments (CTD1 and CTD2) and full-length (FL) gene were tagged with a FLAG epitope (indicated with an "F") on their N-termini, and fused to the C-terminus of GFP. (D) Verification of FL, CTD1, and CTD2 expression in 293T cells by Western blotting using anti-FLAG antibody. (E) Confocal microscopy images of 293T cells expressing FL, CTD1, or CTD2. The green signal represents GFP fluorescence. Magnification, 630×; scale bar, 10 μm. (F) Confocal microscopy images of MT-4 cells constitutively expressing FL, CTD1, or CTD2. The blue signal represents the Hoechst-stained nucleus, and green represents GFP fluorescence. Magnification, 630×; scale bar, 5 μm.

cells (Fig. 1F). The MLV vector expressing GFP alone was used as a control. The cell proliferation, morphology, and cell surface levels of HIV-1 receptors were unaltered by any of the SEC14L1a derivatives (Fig. 1F, 2B, and data not shown). HIV-1 replication was tested in these cells. The level of HIV-1 replication was significantly inhibited in CTD1- and CTD2-expressing cells (69.1% and 69.8% on the average from seven independent experiments, respectively, $P < 0.05$, two-tailed Student's t-test), but was hardly inhibited in FL-expressing cells (86.4%, not statistically significant; Fig. 2C). This observation was reproducible in independently established MT-4 cells and SupT1 cells (data not shown). These data verified the original screening results, and suggest that the C-terminal half of GOLD domain of SEC14L1a serves as an inhibitor of HIV-1 replication. In contrast, it is suggested that FL is not a potent negative regulator of HIV-1 replication.

3.4. SEC14L1a CTD1 and CTD2 target the late phase of the HIV-1 life cycle

We analyzed the viral entry and production phases to determine which step of the HIV-1 life cycle CTD1 and CTD2 target.

The Alu-LTR PCR assay was performed to examine the effect of SEC14L1a derivatives on the viral entry phase. The MT-4 cells stably expressing GFP, FL, CTD1, or CTD2 were infected with VSV-

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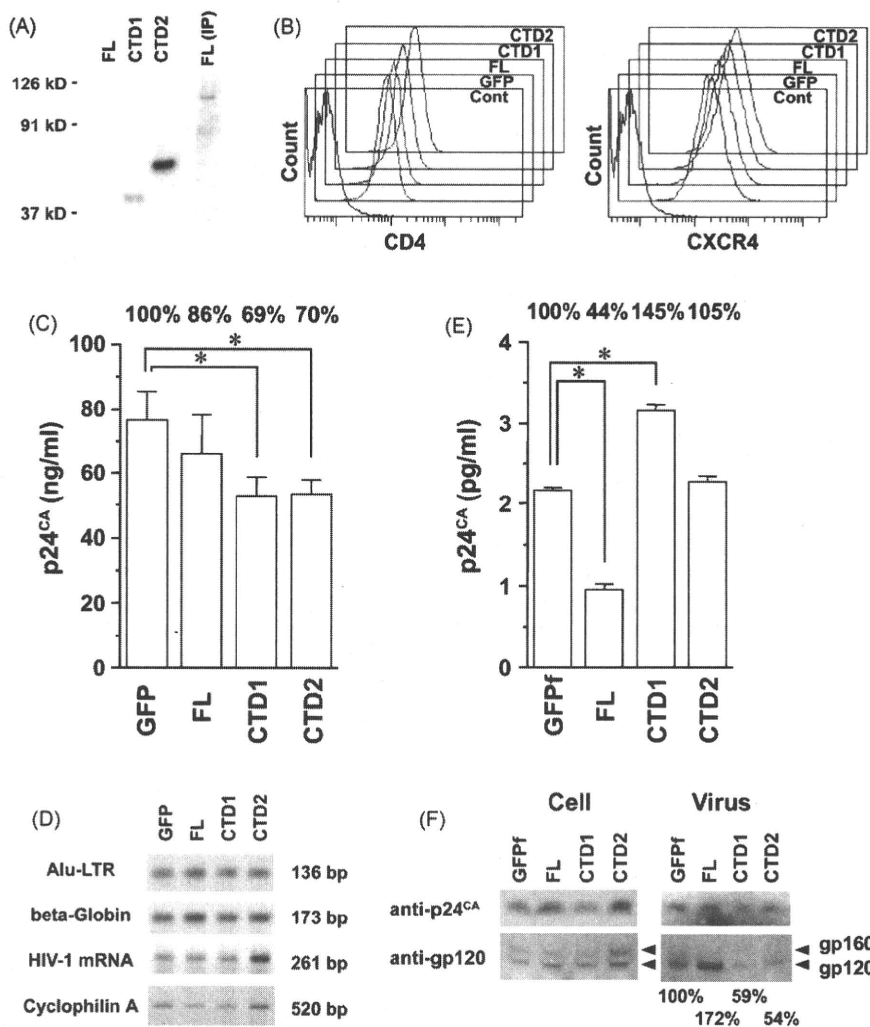

Fig. 2. Functional characterization of the SEC14L1a derivatives. (A) Detection of stable expression of FL, CTD1, and CTD2 in MT-4 cells by Western blotting using anti-FLAG antibody. FL was detected by the immunoprecipitation (IP) assay using agarose beads conjugated with anti-FLAG antibody. The flow cytometric analysis of the cell surface expression of HIV-1 receptors CD4 and CXCR4 in MT-4 cells stably expressing GFP, FL, CTD1, and CTD2. (C) Constitutive expression of CTD1 and CTD2 limited the replication of HIV-1 in MT-4 cells. The concentration of viral $p24^{CA}$ antigen in the culture supernatant was measured at 4 d post-infection. The results represent the average of seven independent experiments ± the standard error of the mean. The reduction of viral $p24^{CA}$ concentration relative to GFP was shown on the top. Asterisks indicate the statistical significance compared to GFP ($P<0.05$ by two-tailed Student's *t*-test). (D) The PCR-based assay to examine the effect of SEC14L1a derivatives on the early phase of viral life cycle (top two panels) and the transcription from LTR promoter (bottom two panels). The HIV-1 entry efficiency was examined by Alu-LTR PCR. Beta globin was used as an internal control. The HIV-1 transcription efficiency was examined by RT-PCR targeting spliced viral mRNA. Cyclophilin A was used as a control. The expected length of each PCR amplicon was indicated. (E) The effect of SEC14L1a derivatives on the HIV-1 production. The 293T cells grown in a well of a 6-well plate were transfected with 200 ng of HIV-1 proviral DNA and 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The culture supernatant was recovered at 2 d post-transfection and the $p24^{CA}$ concentration was measured. The representative data from five independent experiments was shown. The results indicate the average ± the standard deviation. The relative $p24^{CA}$ concentration compared to GFPf was shown on the top. Asterisks indicate the statistical significance compared to GFPf ($P<0.001$ by two-tailed Student's *t*-test). The *Env* incorporation onto the virus-like particles (VLP) produced by 293T cells expressing SEC14L1a derivatives. The 293T cells grown in a well of a 6-well plate were transfected with 1 μg of *gag-pol* (pCMVR8.91) and *Env* (pIIIex) expression vectors along with 2 μg of expression vector for GFPf, FL, CTD1, or CTD2. The cell lysates (Cell) and VLP fractions (Virus) were subjected to Western blot analysis detecting gp120 and $p24^{CA}$ harvested at 2 d post-transfection. The *Env* incorporation levels normalized to $p24^{CA}$ relative to GFPf were shown at the bottom.

G-pseudotyped HIV-1 vector, and the cellular genomic DNA was recovered at 4 d post-infection. The amount of Alu-LTR PCR products from FL-, CTD1-, or CTD2-expressing MT-4 cells was almost equal to that from GFP-expressing cells, suggesting that the early phase of the viral life cycle is not inhibited by any of the SEC14L1a derivatives (Fig. 2D). To examine the viral production phase, we examined the LTR-driven viral gene transcription by RT-PCR. Cellular RNA was extracted from the same MT-4 cells infected with VSV-G-pseudotyped HIV-1 vector, and RT-PCR was conducted to amplify LTR promoter-driven spliced HIV-1 mRNA. The amount of viral RNA expressed in FL-, CTD1-, or CTD2-expressing cells was not lower than that in GFP-expressing cells when the levels of the internal control was taken into account (Fig. 2D). Given that the similar number of viral genome was integrated as indicated by the Alu-LTR PCR, these data suggest that viral transcription is not inhibited by any of the SEC14L1a derivatives, and that the action point of CTD1 and CTD2 should be at post-transcriptional levels of the viral production phase.

Next, the FL, CTD1, or CTD2 expression vector was co-transfected with HIV-1 proviral DNA into 293T cells, and viral production was quantified by $p24^{CA}$ ELISA. The FLAG-tagged GFP (GFPf) was used as a control hereafter. We found that the FL expression significantly reduced the production of HIV-1 (44.2%, $P<0.001$, two-tailed Student's *t*-test) compared to the GFPf control (Fig. 2E). In contrast, the CTD1 enhanced the production of HIV-1 (145.9%, $P<0.001$, two-tailed Student's *t*-test; Fig. 2E). However, CTD2 did not measurably affect the HIV-1 production (105.1%, not statistically significant; Fig. 2E). As the ELISA assay examines the effect

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of CTDs on *Gag* functions, we next tested the functional interaction between CTDs and *Env*. The *Env* incorporation onto the virion was examined by tripartite-transfection of expression vectors for *Env*, *gag-pol*, and SEC14L1a derivatives into 293T cells, and the VLP was collected by centrifugation. The immunoblotting against gp120 was performed on the cell lysate and the VLP fraction. The cellular *Env* and *Gag* expressions were not detectably affected by any of the SEC14L1a derivatives (Fig. 2F, left panel). The *Env* incorporation onto the VLP was slightly enhanced by FL (157%; Fig. 2F, right panel). In contrast, the VLP produced from CTD1- or CTD2-expressing cells incorporated substantially fewer *Env* than those from GFPf-expressing cells (59% or 54%, respectively; Fig. 2F, right panel). These data were reproducible in independently performed experiments. The densitometric analysis of Western blot image showed that the average ± the standard error of the mean of Env incorporation onto the virion was 129.7 ± 39.9%, 54.8 ± 24.7%, and 25.5 ± 10.3% for FL, CTD1, and CTD2 compared to GFPf, respectively (3–4 independent experiments). The *Env*-mediated cell-to-cell fusion assay indicated that SEC14L1a derivatives did not limit the cell surface targeting and function of *Env* (data not shown). In addition, the *Gag* processing in virion was unaffected by any of the SEC14L1a derivatives (data not shown). Collectively, these data suggest that the HIV-1 replication is inhibited by CTD1 and CTD2 due to the inefficient *Env* incorporation onto the virion. To test this possibility, we infected fresh MT-4 cells with the equal amount of HIV-1 propagated in CTD1- or CTD2-expressing MT-4 cells (1–2 ng $p24^{CA}$), and the viral replication was monitored at 3–4 days post-infection by measuring the $p24^{CA}$ concentration. The infectivity of HIV-1 propagated in CTD1- or CTD2-expressing cells was attenuated to 83.1 ± 17.9% or 82.4 ± 5.5% relative to the virus recovered from GFP-expressing cells, respectively (the average ± the standard error of the mean of 3 independent experiments). Altogether, these data suggest that the inhibition of HIV-1 replication by CTD1 and CTD2 is attributed to the attenuation of viral infectivity by lowering the *Env* incorporation onto the virion.

4. Discussion

In the present study, we provide the first evidence that the C-terminal fragment of SEC14L1a functions as an inhibitor of HIV-1 replication. The advantage of this system is that, since MT-4 cells are stably transduced with a cDNA library, the anti-HIV-1 function of a candidate gene is not due to a perturbed cell physiology. This system has been successful in identifying CD14, CD63, and Brd4-CTD as regulators of HIV-1 replication [1,3,4], and more candidates are being analyzed. Among the candidates, SEC14L1a CTD appeared to be one of the relatively modest inhibitors of HIV-1 replication. However, of note, the SEC14L1a derivatives have not been identified in other genetic screening systems. These facts point that our T cell-based system is sensitive in detecting the modest anti-HIV-1 activity of a gene, and is a unique tool in the pursuit of HIV-1 regulatory factors to complete the HIV-1-host interactome.

SEC14L1a may affect the Golgi-mediated vesicular trafficking since SEC14L1a lowers the cell surface levels of cholinergic transporters [23]. However, we do not have any data to suggest that SEC14L1a and its derivatives affect the cell surface targeting of membrane proteins including CD4, CXCR4 and *Env*. These data suggest that SEC14L1a's effect on cholinergic receptor expression is specific, and that the CTD's ability to inhibit HIV-1 replication is independent from SEC14L1a's regulatory functions on vesicular trafficking. The action point of CTD1 and CTD2 was shown to be the late phase of the viral life cycle. Given that CTD1 and CTD2 did not inhibit the biogenesis and the cell surface targeting of *Gag* and *Env*, the major mechanism of CTD1 and CTD2 to inhibit HIV-1 replication was to reduce the infectivity of HIV-1 by limiting the *Env* incorporation onto the virion. Consistent with this idea, the viral infectivity of virions produced in CTDs-expressing cells was attenuated. Then, how do CTDs block the *Env* incorporation onto the virion? We detected a weak interaction between *Gag* and CTD1 or CTD2 by immuno-coprecipitation analysis. Thus, we speculate that the interaction between *Env* and *Gag* at the plasma membrane is interfered by *Gag*-CTDs interaction, resulting in the reduction of *Env* incorporation onto the virion.

The CTD1 was an inhibitor of HIV-1 replication. While the CTD1 negatively affected the *Env* incorporation onto the virion, it positively affected the HIV-1 production. These observations may be seemingly controversial. However, the SEC14L1a derivatives' effect on HIV-1 replication is a summation of their effects of on each step of the viral life cycle. Therefore, it is conceivable that CTD1 can serve as a negative regulator of HIV-1 replication as well as a positive and negative factor on distinct steps of the viral life cycle. These seemingly controversial findings may be in part due to the cells in which the biological functions of SEC14L1a derivatives were examined. The effect of SEC14L1a derivatives on HIV-1 replication was investigated in MT-4 cells, whereas those on the HIV-1 production and *Env* incorporation onto the virion were examined in 293T cells. Although the basic biological features are largely shared among different cell types, it is possible that the SEC14L1a derivatives may function slightly differently in MT-4 cells from 293T cells given that the intracellular distribution of SEC14L1a derivatives in MT-4 cells was not identical to that in 293T cells (Fig. 1E and 1F).

Elucidating the molecular mechanism underlying CTDs' activity not only provides a hint to understand how the HIV-1 virion actively uptakes *Env* through the *Gag-Env* interaction, but also leads to the development of a novel anti-retroviral drug that lowers the infectivity of the virus by preventing *Env* incorporation onto the virion. This is the strength of our T cell-based assay since CTDs inhibit HIV-1 replication specifically. In the previous study, we proposed that a small portion of Brd4 may serve as a therapeutic molecular target for HIV-1 infection, since the constitutive expression of Brd4-CTD limited HIV-1 replication specifically [3], akin to the SEC14L1a CTDs. However, it remains to be examined whether the SEC14L1a and Brd4 derivatives inhibit HIV-1 replication in primary HIV-1 target cells.

The genome-wide screening has potential caveats, including a cDNA bias and a cell line bias. A cDNA library is not a perfect representation of mRNA expressed in the cells from which the library is constructed. For example, the longer the mRNA, the less efficiently the full-length cDNA is synthesized. In fact, we isolated Brd4-CTD from the PBL cDNA library as a potent inhibitor of HIV-1 replication [3]. However, although Brd4 (approximately 5000 nt mRNA in length) is expressed in MT-4 cells, we were unable to recover Brd4-CTD from the MT-4 cDNA library [3]. This clearly demonstrates the cDNA bias in the genetic screening. A cDNA library derived from non-T cells does not contain genes specifically expressed in T cells. Thus, we have to explore many more cDNA libraries to completely cover the genetic diversity of human cells. The cDNA libraries isolated from long-term non-progressors of HIV-1-seropositive individuals or from elite controllers might be of particular interest, considering that a dominant innate HIV-1 resistance gene, such as CCR5 delta 32, may partly account for the slow progression of AIDS. Similarly, use of a particular cell line and/or virus strain may bias the results. MT-4 cells are positive for HTLV-1, and are able to support robust HIV-1 replication. MT-4 cells do not express CCR5, and are unable to support R5-tropic HIV-1 strains. What if other T cell lines and R5-tropic viral strains are used? What if we assay the same cDNA library in TZM-bl cells? We plan to address these issues in the future studies.

In conclusion, genome-wide genetic screening is a powerful tool for identifying the regulatory factors of HIV-1 replication and innate HIV-1 resistance factors that limit HIV-1 infection and AIDS progression. The HIV-1-host interactome should also reveal poten-

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tial therapeutic molecular targets that may be used to develop novel anti-AIDS drugs to tackle the emerging drug resistant viruses. However, the fact that different experimental systems often yield non-overlapping candidates suggests that we have to explore more experimental systems to fully understand the HIV-1-host interactome. Our T cell-based system provides an alternative tool for identifying novel HIV-1 regulatory factors, and should help us understand the HIV-1-host interaction in more detail.

Acknowledgements

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Conflict of interest statement

The authors state that they have no conflict of interest.

References

[1] Kawano Y, Yoshida T, Hieda K, Aoki J, Miyoshi H, Koyanagi Y. A lentiviral cDNA library employing lambda recombination used to clone an inhibitor of human immunodeficiency virus type 1-induced cell death. J Virol 2004;78(20):11352–9.
[2] Valente ST, Goff SP. Inhibition of HIV-1 gene expression by a fragment of hnRNP U. Mol Cell 2006;23(4):597–605.
[3] Urano E, Kariya Y, Futahashi Y, Ichikawa R, Hamatake M, Fukazawa H, et al. Identification of the P-TEFb complex-interacting domain of Brd4 as an inhibitor of HIV-1 replication by functional cDNA library screening in MT-4 cells. FEBS Lett 2008;582(29):4053–8.
[4] Yoshida T, Kawano Y, Sato K, Ando Y, Aoki J, Miura Y, et al. A CD63 mutant inhibits T-cell tropic human immunodeficiency virus type 1 entry by disrupting CXCR4 trafficking to the plasma membrane. Traffic 2008;9(4):540–58.
[5] Zhou H, Xu M, Huang Q, Gates AT, Zhang XD, Castle JC, et al. Genome-scale RNAi screen for host factors required for HIV replication. Cell Host Microbe 2008;4(5):495–504.
[6] Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science 2008;319(5865):921–6.
[7] Konig R, Zhou Y, Elleder D, Diamond TL, Bonamy GM, Irelan JT, et al. Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. Cell 2008;135(1):49–60.
[8] Valente ST, Gilmartin GM, Mott C, Falkard B, Goff SP. Inhibition of HIV-1 replication by eIF3f. Proc Natl Acad Sci USA 2009;106(11):4071–8.
[9] Aiken C. Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. J Virol 1997;71(8):5871–7.
[10] Akari H, Uchiyama T, Fukumori T, Iida S, Koyama AH, Adachi A. Pseudotyping human immunodeficiency virus type 1 by vesicular stomatitis virus G protein does not reduce the cell-dependent requirement of vif for optimal infectivity: functional difference between Vif and Nef. J Gen Virol 1999;80(Pt 11): 2945–9.
[11] Chazal N, Singer G, Aiken C, Hammarskjold ML, Rekosh D. Human immunodeficiency virus type 1 particles pseudotyped with envelope proteins that fuse at low pH no longer require Nef for optimal infectivity. J Virol 2001;75(8): 4014–8.
[12] Komano J, Miyauchi K, Matsuda Z, Yamamoto N. Inhibiting the Arp2/3 complex limits infection of both intracellular mature vaccinia virus and primate lentiviruses. Mol Biol Cell 2004;15(12):5197–207.
[13] Goff SP. Knockdown screens to knockout HIV-1. Cell 2008;135(3):417–20.
[14] Urano E, Aoki T, Futahashi Y, Murakami T, Morikawa Y, Yamamoto N, et al. Substitution of the myristoylation signal of human immunodeficiency virus type 1 Pr55Gag with the phospholipase C-delta1 pleckstrin homology domain results in infectious pseudovirion production. J Gen Virol 2008;89(Pt 12): 3144–9.
[15] Futahashi Y, Komano J, Urano E, Aoki T, Hamatake M, Miyauchi K, et al. Separate elements are required for ligand-dependent and-independent internalization of metastatic potentiator CXCR4. Cancer Sci 2007;98(3):373–9.
[16] Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, et al. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J Virol 1998;72(12):9873–80.
[17] Shimizu S, Urano E, Futahashi Y, Miyauchi K, Isogai M, Matsuda Z, et al. Inhibiting lentiviral replication by HEXIM1, a cellular negative regulator of the CDK9/cyclin T complex. AIDS 2007;21(5):575–82.
[18] Chinen K, Takahashi E, Nakamura Y. Isolation and mapping of a human gene (SEC14L), partially homologous to yeast SEC14, that contains a variable number of tandem repeats (VNTR) site in its 3' untranslated region. Cytogenet Cell Genet 1996;73(3):218–23.
[19] Howe AG, McMaster CR. Regulation of phosphatidylcholine homeostasis by Sec14. Can J Physiol Pharmacol 2006;84(1):29–38.
[20] Saito K, Tautz L, Mustelin T. The lipid-binding SEC14 domain. Biochim Biophys Acta 2007;1771(6):719–26.
[21] Mousley CJ, Tyeryar KR, Vincent-Pope P, Bankaitis VA. The Sec14-superfamily and the regulatory interface between phospholipid metabolism and membrane trafficking. Biochim Biophys Acta 2007;1771(6):727–36.
[22] Anantharaman V, Aravind L. The GOLD domain, a novel protein module involved in Golgi function and secretion. Genome Biol 2002;3(5), research0023.0021-0023.0027.
[23] Ribeiro FM, Ferreira LT, Marion S, Fontes S, Gomez M, Ferguson SS, et al. SEC14-like protein 1 interacts with cholinergic transporters. Neurochem Int 2007;50(2):356–64.

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ORIGINAL ARTICLE

Intracellular localization of human immunodeficiency virus type 1 Gag and GagPol products and virus particle release: relationship with the Gag-to-GagPol ratio

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ABSTRACT

Human immunodeficiency virus (HIV) Gag precursor protein is cleaved by viral protease (PR) within GagPol precursor protein to produce the mature matrix (MA), capsid, nucleocapsid, and p6 domains. This processing is termed maturation and required for HIV infectivity. In order to understand the intracellular sites and mechanisms of HIV maturation, HIV molecular clones in which Gag and GagPol were tagged with FLAG and hemagglutinin epitope sequences at the C-termini, respectively were made. When coexpressed, both Gag and GagPol were incorporated into virus particles. Temporal analysis by confocal microscopy showed that Gag and GagPol were relocated from the cytoplasm to the plasma membrane. Mature cleaved MA was observed only at sites on the plasma membrane where both Gag and GagPol had accumulated, indicating that Gag processing occurs during Gag/GagPol assembly at the plasma membrane, but not during membrane trafficking. Fluorescence resonance energy transfer imaging suggested that these were the primary sites of GagPol dimerization. In contrast, with overexpression of GagPol alone an absence of particle release was observed, and this was associated with diffuse distribution of mature cleaved MA throughout the cytoplasm. Alteration of the Gag-to-GagPol ratio similarly impaired virus particle release with aberrant distributions of mature MA in the cytoplasm. However, when PR was inactive, it seemed that the Gag-to-GagPol ratio was not critical for virus particle release but virus particles encasing unusually large numbers of GagPol molecules were produced, these particles displaying aberrant virion morphology. Taken together, it was concluded that the Gag-to-GagPol ratio has significant impacts on either intracellular distributions of mature cleaved MA or the morphology of virus particles produced.

Key words GagPol, HIV-1, plasma membrane, ratio.

Human immunodeficiency virus (HIV) contains three viral structural proteins: Gag, GagPol, and Env. Gag protein, the main structural component of HIV particles, is synthesized in the cytosol as a 55 kDa precursor protein and is targeted to the cellular membrane, where virus particle budding occurs. During translation of Gag mRNA, ribosomal frameshifting occurs at an efficiency of 5–10% and generates a 160 kDa GagPol fusion protein at a 10–20:1 ratio of Gag to GagPol (1). Following protein synthesis, Gag and GagPol proteins are relocated to the membrane

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List of Abbreviations: CA, capsid; EGFP, enhanced green fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HA, hemagglutinin; HIV, Human immunodeficiency virus; IN, integrase; MA, matrix; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; WT, wild-type.

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and co-assembled into virus particles. Although HIV particle assembly is essentially directed by Gag protein (2, 3) and GagPol protein is only incorporated into virus particles through co-assembly with Gag (4, 5), the incorporation of GagPol into virus particles is crucial for virion infectivity, since the Pol region harbors virus-specific enzymes, PR, RT, and IN, all of which are essential for HIV replication.

Concomitant with the incorporation of GagPol protein, Gag protein undergoes a process termed maturation in which it is cleaved by PR located in GagPol to produce mature p17/MA, p24/CA, p7/NC, and p6 domains, and p1 and p2 spacer peptides (6). Expression of the intact GagPol region produces a mature form of virus particle, which displays an electron-dense core encased in a conical capsid, whereas expression of Gag without the Pol region results in an immature form of virus particle, with a characteristic doughnut-like morphology (2, 5). A number of studies have shown that Gag expression with genetically engineered inactive PR fails to cleave Gag protein and results in production of virus particles that have been arrested at the immature stage (4, 7, 8). Since two forms of virus particles (immature and mature) have traditionally been observed for many retroviruses, retroviral maturation has been thought to take place at a very late stage of particle assembly, such as particle budding, or even after particle release (9). However, it is difficult to know when and where Gag processing takes place because mature virion morphology is only seen extracellularly, whereas Gag processing products are often observed in the cytoplasm.

Despite the obligatory role of active PR in HIV maturation, overexpression of GagPol fusion protein or an active PR domain induces enhancement of intracellular Gag processing, leading to a failure of particle production (10–12). These findings suggest that premature Gag processing in the cytoplasm can occur when the Gag-to-GagPol ratio is altered, and that the products of such premature processing may not participate in virus particle formation. Recent studies have also indicated that the Gag-to-GagPol ratio is important for virion RNA dimerization (13). Since the enzymatically active form of PR is a dimer, and the appearance of PR activity is accompanied by autoprocessing of PR from a GagPol precursor (14, 15), it is plausible that dimerization of GagPol precursors is a prerequisite for autoprocessing of PR. Timing of autoprocessing of the putative precursor dimer must be regulated for efficient particle production to occur in HIV infection. However, so far no studies have revealed the primary intracellular sites of Gag/GagPol processing or GagPol dimerization. The difficulties for such studies are: (i) discrimination between Gag and GagPol, because the N-terminal half of GagPol is identical with Gag, and (ii) discrimination between Gag/GagPol precursors and their processing products. To overcome these difficulties, we here added two distinct epitope tags (FLAG and HA) to the C-termini of Gag and GagPol, respectively, and immunostained with anti-HIV-1 p17/MA antibody, an antibody which recognizes the mature form of p17/MA but not uncleaved Gag precursors (16). Our confocal data demonstrated that Gag and GagPol were relocated from the cytoplasm to the plasma membrane and that Gag processing occurred only at sites in the plasma membrane where both Gag and GagPol had accumulated. Furthermore, we confirmed that these were the sites of GagPol dimerization by FRET imaging. Importantly, our data indicated that the failure of particle release caused by overexpression of GagPol could be explained by aberrant distribution of the mature p17/MA in the cytoplasm. Our study provides clues toward understanding the overall process of HIV maturation.

MATERIALS AND METHODS

Construction of HIV-1 molecular clones

In this study HIV-1 molecular clone pNL43 was used to construct its derivatives (17). An HIV molecular clone expressing an inactive form of PR has also been described previously (18). The FLAG and HA epitope sequences were added to the C-termini of Gag and GagPol proteins, respectively. For in-frame insertion of the FLAG sequence into the p6 region, overlapping PCR was carried out using a forward primer 5′-GATTACAAGGACGA CGACGACAAGAGACCAGAGCCAACAGCCCCAC-3′. Addition of the HA epitope tag sequence to the C-terminus of the *pol* gene was similarly carried out using a reverse primer 5′-GCTATGTCGACCATATGGCT AAGCGTAATCTGGAACATCGTATGGGTATCTAGAAT CCTCATCCTGTCTACTTGCCAC-3′. For expression of Gag without GagPol protein, two termination codons were introduced into the *pol* frame immediately downstream of the gag termination codon using 5′-CCCCTCGTCACAA<u>TAA</u>AG<u>TAA</u>GGGGGG<u>TAA</u>T<u>TAA</u>A GGAAGCTCTAT-3′ (termination codons in the *gag* and *pol* frames are underlined). For expression of GagPol protein alone, the *gag* and *pol* genes were placed into the same reading frame by deleting the 5T nucleotides at the frameshifting site (nucleotide positions 2086 to 2090). For FRET imaging, unique *XbaI* and *NotI* sites were initially introduced at the junction of PR/RT and the sequences encoding EGFP and Strawberry protein were inserted in-frame between the *XbaI* and *NotI* sites. The sequences encoding EGFP and Strawberry fluorescent protein were similarly added to the C-termini of Gag (for positive controls).

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Cell culture and DNA transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10% FBS. DNA transfection was carried out using Lipofectamine 2000 (Invitrogen, Tokyo, Japan).

Western blotting

Following transfection, culture media were subjected to centrifugation through 20% (wt/vol) sucrose cushions at 120,000 × *g* for 2 hr at 4°C. Cells and virus particles were analyzed by Western blotting using anti-FLAG rabbit (Sigma), anti-HA mouse (Sigma), anti-HIV-1 RT mouse (ICN Pharmaceuticals, Cleveland, OH, USA), anti-HIV-1 p24/CA mouse (19), and anti-HIV-1 p17/MA mouse (Advanced Bio-technologies, Suwanee, GA, USA) antibodies. In some experiments, virus particles were further subjected to equilibrium centrifugation in 20–60% (wt/vol) sucrose gradients at 120,000 × *g* for 16 hr at 4°C and subjected to fractionation.

Confocal microscopy

HeLa cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature, then permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Following blocking, the cells were incubated with anti-FLAG mouse or rabbit (Sigma), anti-HA mouse or rabbit (Santa Cruz, Santa Crz, CA, USA), anti-HIV-1 RT mouse, and anti-HIV-1 p17/MA mouse antibodies, and subsequently with Alexa Fluor 488 or 568-conjugated anti-mouse and anti-rabbit antibodies (Molecular Probes, Eugene, OR, USA). Nuclear staining was carried out with TOPRO-3 (Molecular Probes) and the cells observed with a laser scanning confocal microscope (Leica, Heidelberg, Germany).

Electron microscopy

HeLa cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hr at 4°C prior to treatment with 2% osmium tetroxide for 1 hr at 4°C. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (H-7500, Hitachi, Tokyo, Japan) at 80 kV.

Fluorescence resonance energy transfer imaging

HeLa cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at room temperature and subjected to laser scanning confocal microscopy (Yokogawa, Sugar Land, TX, USA). Images were acquired using three filter combinations: EGFP excitation/EGFP emission (donor channel), Strawberry excitation/Strawberry emission (acceptor channel), and GFP excitation/Strawberry emission (FRET channel). FRET was confirmed by two changes in fluorescence signals upon photobleaching of the acceptor: (i) a decrease in donor fluorescence, and (ii) a proportional increase in acceptor fluorescence. Pixel by pixel analysis of FRET was carried out using the PixFRET plug-in of ImageJ software (20).

RESULTS

Addition of epitope tags had no detrimental effect on viral particle production

HIV-1 molecular clone pNL43 (17) is referred as WT in this study. To discriminate between Gag and GagPol proteins, we added FLAG and HA epitope tag sequences to the C-terminal regions of HIV-1 Gag and GagPol proteins, respectively (Fig. 1a). The Gag/GagPol constructs were introduced into pNL43. The C-terminal p6 domain of Gag has three functional motifs as follows. The first two, namely the PTAP and YPXnL sequences, are binding sites of tumor susceptibility gene 101 and ALG-2-interacting protein X/ALG-2-interacting protein 1, respectively, and both are required for particle budding (21–25). The third, the LXXLFG sequence, is a binding site of viral accessory protein Vpr (26, 27). The FLAG-tag sequence (DYKDDDDK) was inserted upstream of the PTAP sequence, in-frame in the p6 domain, which generated insertion of the RLQGRRRQE sequence in the *pol* frame. The HA-tag sequence was added to the C-terminus of the GagPol protein. The construct expressing both Gag-FLAG and GagPol-HA is referred to here as Gag-FLAG/Pol-HA. We also created a pNL43 derivative expressing GagPol-HA alone by deletion of the frameshift signal (referred to as GagPol-HA).

To assess the effect of the addition of the epitope tags, HeLa cells were transfected with the Gag-FLAG/Pol-HA construct, GagPol-HA construct, and its parental clone without the epitope tags, all of which are pNL43 derivatives containing inactive PR. Then, intracellular expression of Gag/GagPol proteins and particle production were examined by Western blotting using anti-HIV-1 p24/CA and anti-FLAG (for Gag protein) and anti-HIV-1 RT and anti-HA (for GagPol protein) antibodies (Fig. 1b). Gag-FLAG and GagPol-HA proteins were specifically detected by anti-FLAG and anti-HA antibodies and the levels of expression were comparable to those of the parental Gag and GagPol proteins when probed with anti-HIV-1 p24/CA and anti-HIV-1 RT antibodies. When viral particles were purified through sucrose cushions and analyzed by Western blotting, in the context of Gag-FLAG/Pol-HA, the amounts of Gag-FLAG and GagPol-HA were also comparable to those of Gag and GagPol in the parental virus

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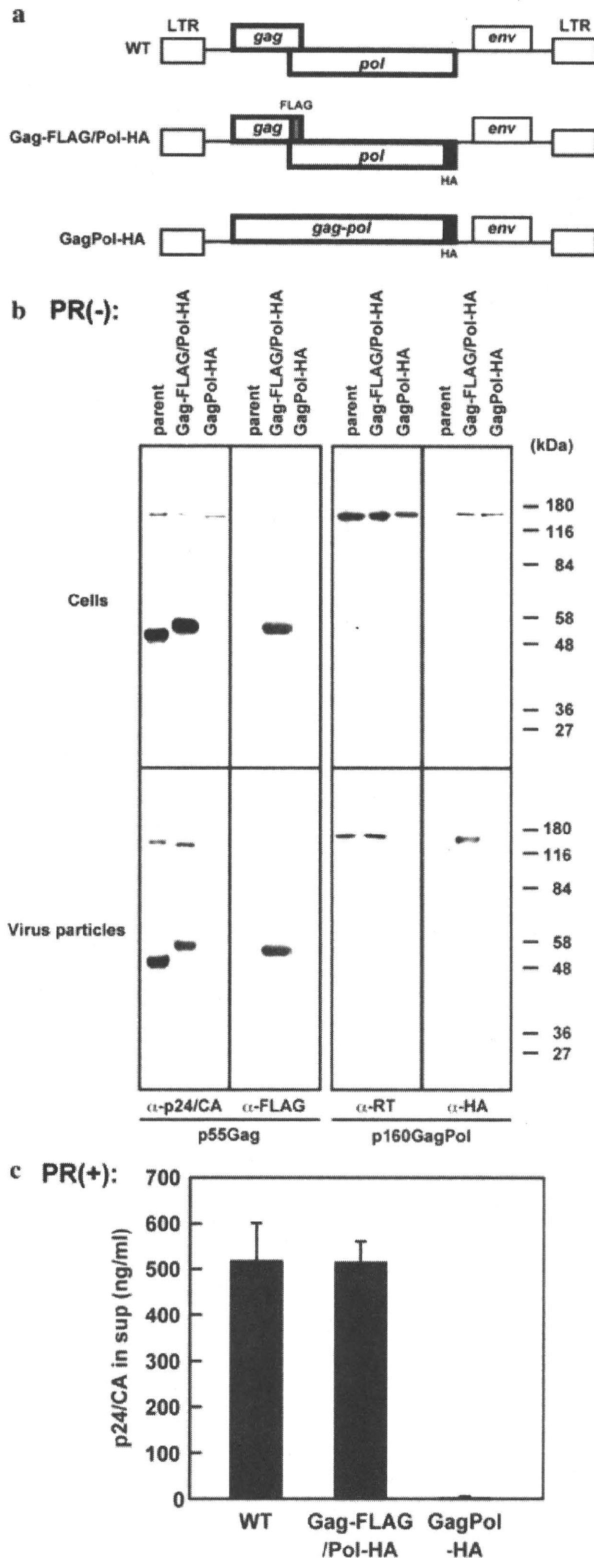

Fig. 1. Effects of epitope tagging on viral particle production. (a) Schematic representation of HIV-1 cDNA molecular clones tagged with the FLAG and HA sequences. HIV-1 molecular clone pNL43 is referred to as WT. The FLAG and HA tag sequences were inserted in-frame in the C-terminal p6 domain of the Gag protein and the C-terminus of the GagPol protein, respectively (referred to as Gag-FLAG/Pol-HA). For expression of GagPol-HA alone, the HA tag sequence was added to the C-terminus of GagPol, and the 5T sequence at the frameshift site was deleted. The same set of HIV-1 molecular clones except for an inactive form (D25N mutation) of the PR domain was similarly made. LTR, long terminal repeat. (b) Intracellular expression of Gag/GagPol and production of viral particles. HeLa cells were transfected with Gag-FLAG/Pol-HA, GagPol-HA and their parental molecular clones, all of which contain an inactive form of PR. After 48 hr, cells and viral particles were subjected to Western blotting using anti-HIV-1 p24/CA, anti-HIV-1 RT, anti-FLAG, and anti-HA antibodies. (c) Production of viral particles. HeLa cells were transfected with the WT, Gag-FLAG/Pol-HA and GagPol-HA constructs, all of which had an active form of PR. Culture media were clarified and virus particles yields (from three independent experiments) were measured by p24 antigen capture ELISA.

particles, respectively, indicating that both Gag-FLAG and GagPol-HA were incorporated into the viral particles to the same degree as occurs with authentic Gag and GagPol. Thus, the Gag-FLAG/Pol-HA construct produced a particle yield largely equivalent to that of the parental molecular clone. Particle production of the Gag-FLAG/Pol-HA construct was further examined using its molecular clone containing an active PR by HIV-1 p24/CA antigen capture ELISA, the results indicating that the Gag-FLAG/Pol-HA construct produced virus particles at a level nearly equivalent to that of WT (Fig. 1c). In contrast, expression of GagPol-HA alone did not yield virus particles, a finding which is consistent with previous studies (4, 5). These results indicate that the addition of epitope tags exerts no detrimental effects on particle production.

Gag and GagPol proteins were relocated from the cytoplasm to the plasma membrane

To gain a better understanding of the intracellular trafficking of Gag/GagPol proteins, their intracellular localization was observed by confocal microscopy at various time points (6, 12, and 24 hr post-transfection) (Fig. 2b). HeLa cells were transfected with the Gag-FLAG/Pol-HA construct, which had inactive PR, and stained with anti-FLAG and anti-HA antibodies. As shown in Figure 2a, three patterns of antigen distribution, consistent with previous studies (28, 29), were observed, namely, diffuse cytosolic distribution alone, accumulation at the plasma membrane, and punctate staining in the cytoplasm. We observed approximately 100 cells at each time point and sorted them into the three categories (Fig. 2c). As expected, Gag was diffusely distributed at 6 hr post-transfection (83.3% of Gag-positive cells) and had accumulated at the

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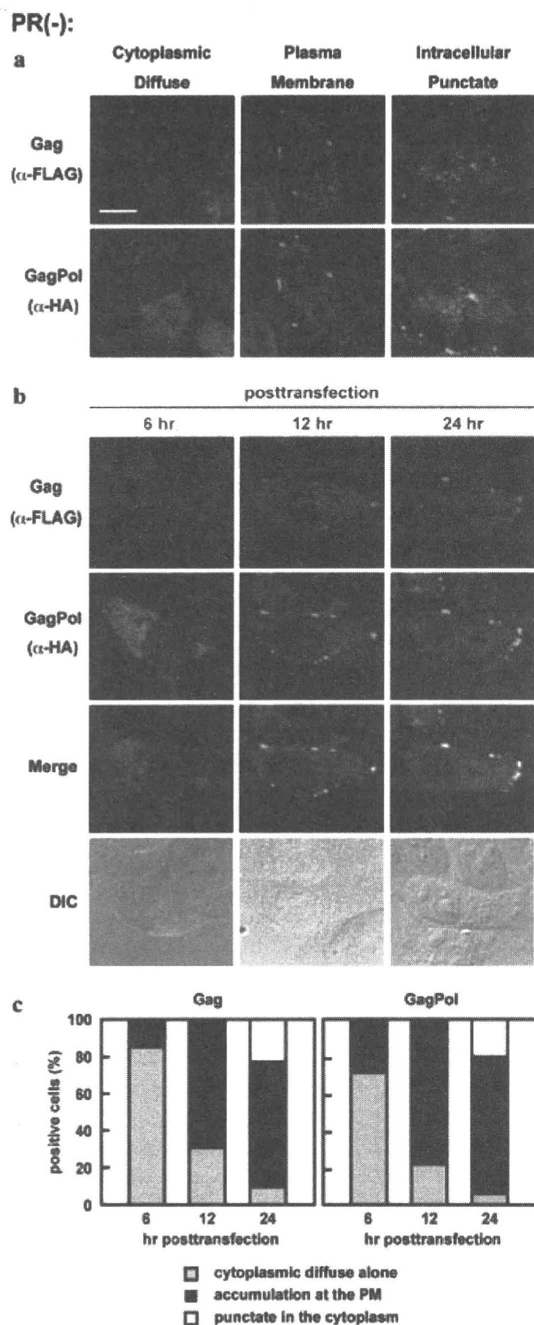

Fig. 2. Temporal study of Gag and GagPol localization. HeLa cells were transfected with the Gag-FLAG/Pol-HA construct containing an inactive form of PR. At 6, 12, and 24 hr post-transfection, cells were immunostained with anti-FLAG (for Gag) and anti-HA (for GagPol) antibodies. Nuclei were stained with TOPRO-3. All micrographs are shown at the same magnification. Bar, 10 μm. (a) Representation patterns of intracellular localization. (b) Temporal analysis of intracellular localization patterns. DIC, differential interference contrast. (c) Semiquantification of Gag and GagPol localization. Approximately 100 Gag and GagPol-positive cells were observed at each time point and the number of cells with each pattern of Gag and GagPol distribution was counted. PM, plasma membrane.

plasma membrane at 12 and 24 hr post-transfection (69.0 and 66.7% of Gag-positive cells, respectively). Punctate staining of Gag in the cytoplasm was observed frequently (24.0% of Gag-positive cells) at 24 hr post-transfection. When we observed GagPol, we found a very similar pattern of relocation: GagPol was diffusely distributed at 6 hr post-transfection (70.8% of GagPol-positive cells) and had accumulated at the plasma membrane at 12 and 24 hr post-transfection (78.9 and 74.9% of GagPol-positive cells, respectively). Overall, these data indicate that Gag and GagPol relocated from the cytoplasm to the plasma membrane. Similar observations were made when a molecular clone containing a reverse combination of tagging, Gag-HA/Pol-FLAG (data not shown) was used. Previous confocal studies have indicated that the intracellular puncta observed at late time points correspond to Gag internalized to endosomes (28).

Gag/GagPol processing occurs predominantly at the plasma membrane where Gag and GagPol co-assemble

The Pol region of GagPol contains PR that cleaves Gag and GagPol proteins and generates mature functional domains such as p17/MA, p24/CA, and RT. The profiles of Gag and GagPol processing in the Gag-FLAG/Pol-HA construct containing an active PR were examined by Western blotting using anti-RT (for p66 and p51 subunits), anti-p24/CA (for p55 precursor and mature p24/CA domain), and anti-p17/MA antibodies (for mature p17/MA domain alone) (Fig. 3a). The anti-p17/MA antibody specifically recognized the mature form of p17/MA, but not the unprocessed p55 precursor, consistent with previous studies (16). Expression of Gag/GagPol proteins was faintly detected at 6 hr post-transfection and had increased by 24 hr. Efficient processing of Gag/GagPol was observed at 24 hr post-transfection but not before that. When HeLa cells were co-stained with anti-FLAG and anti-HA antibodies and observed by confocal microscopy, Gag and GagPol were diffusely distributed at 6 hr post-transfection and had accumulated at the plasma membrane at later time points (12 and 24 hr post-transfection) (Fig. 3b), profiles which are essentially similar to those of the Gag/GagPol construct containing inactive PR (Fig. 2b). To examine intracellular sites of Gag/GagPol processing, we co-stained HeLa cells with anti-p17/MA and anti-HA antibodies. No p17/MA signals were seen at 6 hr post-transfection, by which time Gag and GagPol were diffusely distributed in the cytoplasm. At 12 and 24 hr post-transfection, punctate p17/MA signals were observed at the plasma membrane, where both Gag and GagPol had accumulated (Fig. 3b). These results suggest that Gag/GagPol processing by PR

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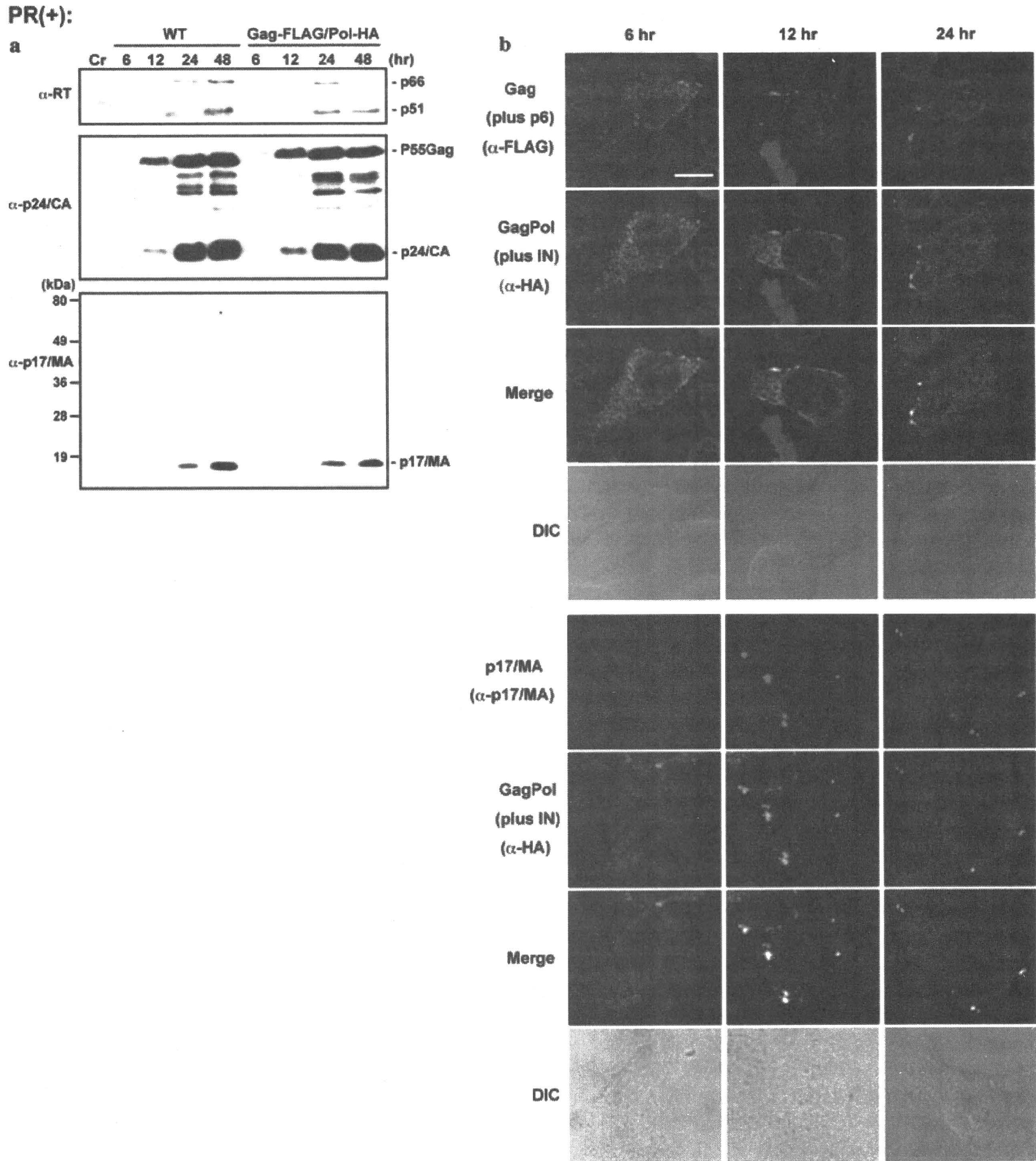

Fig. 3. Intracellular sites of Gag/GagPol processing. (a) Intracellular processing of Gag and GagPol proteins. HeLa cells were transfected with the WT and Gag-FLAG/Pol-HA constructs, both of which have an active form of PR. At 6, 12, and 24 hr post-transfection, cells were subjected to Western blotting using anti-HIV-1 p24/CA and anti-RT antibodies. Western blotting was also probed with anti-HIV-1 p17/MA antibody that specifically recognizes the mature form of p17/MA alone. (b) Intracellular localization of the mature form of p17/MA and Gag/GagPol products. HeLa cells were transfected with the Gag-FLAG/Pol-HA constructs, which have an active form of PR and were immunostained with a combination of anti-FLAG (for Gag and p6) and anti-HA (for GagPol and IN), and with a combination of anti-p17/MA (for mature MA) and anti-HA antibodies. Nuclei were stained with TOPRO-3. All micrographs are shown at the same magnification. Bar, 10 μm. DIC, differential interference contrast.

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