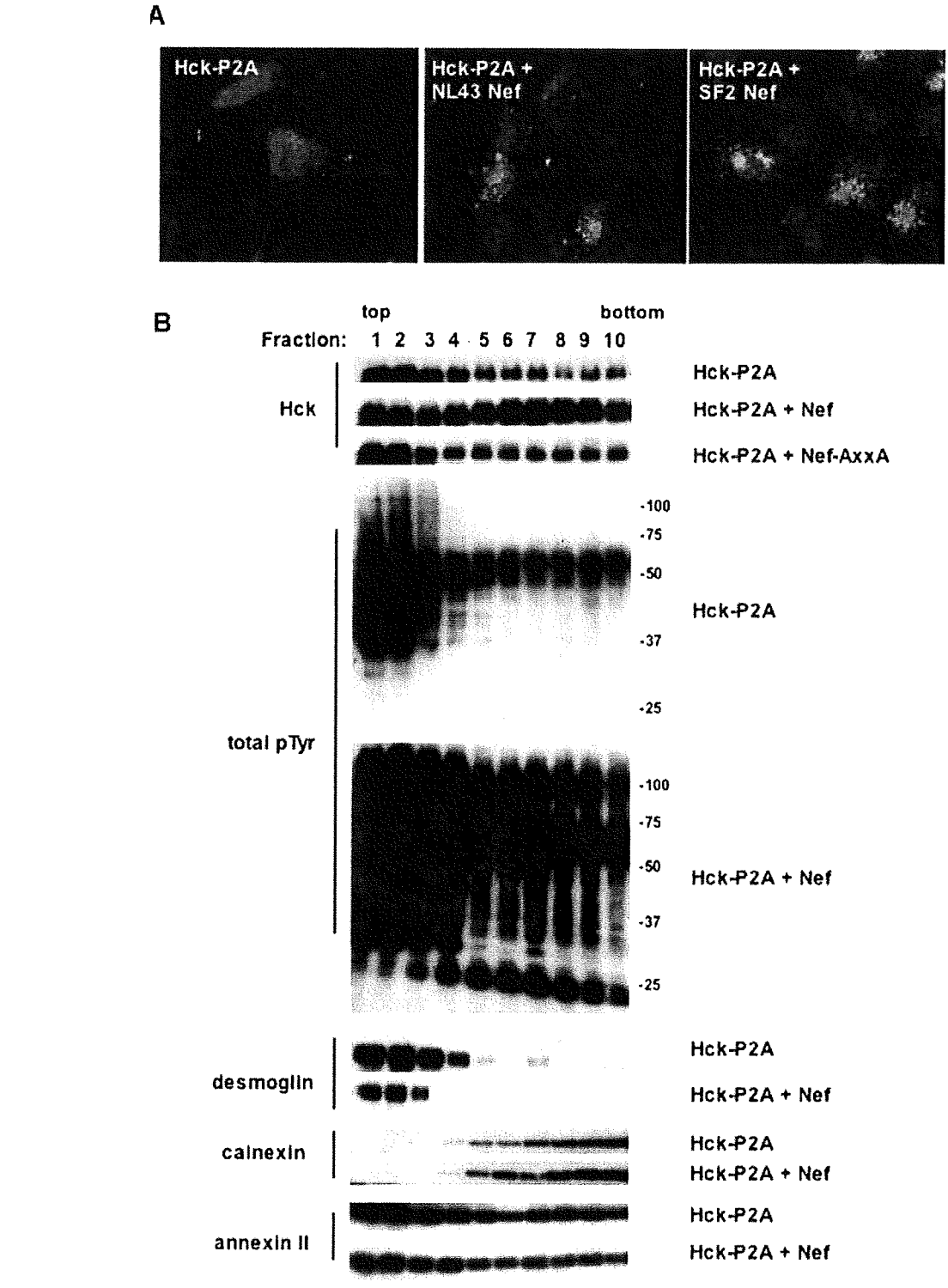

Fig. 2. Skewed Golgi-localization of Hck by Nef. A: HEK293 cells were transfected with Hck-P2A plasmid alone, or co-transfected with NL43 Nef or SF2 Nef plasmid. Cells were stained with antibody specific for active Hck (green) and DAPI (blue). B: Cells were transfected with Hck-P2A alone, or co-transfected with NL43 Nef. Then, cells were subjected to subcellular fractionation on sucrose gradients and Western blotting with antibodies against Hck, phosphotyrosine (pTyr), desmoglein, calnexin, or annexin II. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

swollen in hypotonic buffer containing protease inhibitors followed by homogenization. Then, the post-nuclear supernatants were fractionated by ultracentrifugation on discontinuous sucrose gradients. All steps were carried out on ice. The fractions obtained were subjected to Western blotting with antibodies to Hck (clone 18; Transduction Laboratories), desmoglein (clone 62; Transduction Laboratories), annexin II (C-10; Santa Cruz), or calnexin (H-70; Santa Cruz).

Flow cytometry

Human myeloid TF-1-fms cells expressing Nef-ER fusion protein were maintained as described previously (Suzu et al., 2005; Hiyoshi et al., 2008). To activate the Nef-ER fusion protein, we used the estrogen analog 4-HT (Sigma) at a final concentration of 0.1 μM. The cells were stained with PE-labeled anti-Fms antibodies (Santa Cruz), and the level of cell surface Fms was analyzed by flow cytometry on a FACS Calibur using Cell Quest software (Becton Dickinson, Mountain View, CA).

Results

Analyses with Src kinase inhibitor and Hck mutant

As reported, Nef induces Fms maturation arrest when co-expressed with Hck in HEK293 cells (Fig. 1A). HEK293 cells do not express Hck endogenously, and the upper and lower band was the fully *N*-glycosylated and under-*N*-glycosylated Fms, respectively (Hiyoshi et al., 2008). The low molecular weight Fms was sensitive to Endo-H (Endo-β-*N*-acetylglucosaminindase H), which selectively cleaves high-mannose type oligosaccharide, and their increase was clearly associated with the intense staining of Fms mainly at

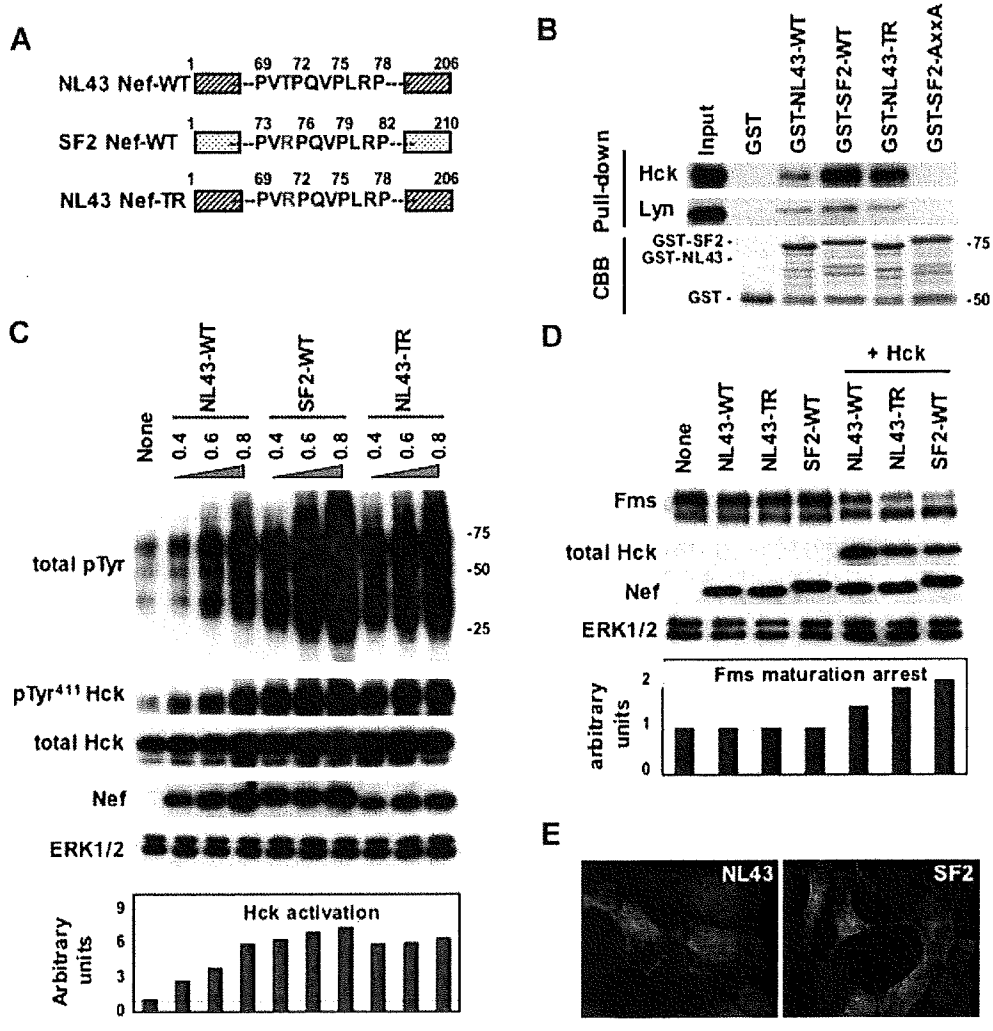

Fig. 3. Abilities of different Nef alleles to bind/activate Hck and to induce Fms maturation arrest. A: The NL43 Nef, SF2 Nef, and NL43 Nef-TR mutant used are schematically shown. B: The resins, to which the control GST or indicated GST-Nef proteins were bound, were incubated with the lysates of HEK293 cells expressing Hck or Lyn. The amount of Hck or Lyn bound to the resins was determined by Western blotting (Pull-down). The amount of GST and GST-Nef fusion proteins bound to the resins was verified by the elution from the resins followed by SDS–PAGE/Coomassie brilliant blue (CBB) staining. C: HEK293 cells were co-transfected with the wild-type Hck and indicated Nef alleles. The amounts of Nef plasmids used are shown (0.4, 0.6, or 0.8 μg/well). Total cell lysates were subjected to Western blotting with antibodies against phosphotyrosine (total pTyr), active-Hck (pTyr^{411} Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Hck activation is shown in the bar graph. D: Cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. Western blotting was done as in (C). E: Cells were transfected with indicated GFP-Nef plasmid (green). Nuclei were stained with DAPI (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the perinuclear region, which overlapped well with the signal of GM130 or Vti1a, the markers for the Golgi (Hiyoshi et al., 2008). These results strongly suggested that the low molecular weight Fms was the immature under-*N*-glycosylated form. The increase of the lower molecular weight species was obvious in the cells co-expressing Nef and Hck (Fig. 1A, left blot), and this Fms maturation arrest was blocked by a Src kinase inhibitor PP2 (Fig. 1A, right blot). However, the expression of a constitutive-active Hck mutant (Hck-P2A; Lerner and Smithgall, 2002) was not sufficient to induce Fms maturation arrest when expressed alone (Fig. 1B, Fms blot), despite its strong kinase activity (total pTyr and $pTyr^{411}$ Hck blots). In this study, we monitored kinase activity of Hck by overall protein tyrosine-phosphorylation (total pTyr) and auto-phosphorylation of Hck ($pTyr^{411}$ Hck) (reviewed in Korade-Mirnics and Corey, 2000). Nonetheless, Hck-P2A/Nef co-expression induced more severe Fms maturation arrest than wild-type Hck/Nef co-expression (Fig. 1B), and Nef did not enhance the kinase activity of Hck-P2A (Fig. 1C), confirming our previous finding that Hck activation was necessary but not sufficient for Nef-induced Fms maturation arrest.

Analyses with different Nef alleles and their mutants

In this study, we first found that Nef derived from SF2 strain of HIV-1 induced more severe Golgi-localization of Hck-P2A than Nef derived from NL43 strain. Hck-P2A signal at the plasma membrane was still observed in some NL43 Nef-transfected cells, whereas such signal was not observed in SF2 Nef-transfected cells (Fig. 2A). The Nef-induced skewed Golgi-localization of Hck-2PA was confirmed by a quantitative analysis, that is, subcellular fractionation on sucrose gradients. Based on a previous report (Matsuda et al., 2006), we used desmoglein, annexin II and calnexin as marker proteins for the plasma membrane, both the plasma membrane and the Golgi, and the endoplasmic reticulum, respectively. As shown (Fig. 2B), the plasma membrane was recovered in light fractions whereas the Golgi and the endoplasmic reticulum were recovered in heavy fractions, and the peak of Hck-P2A shifted to heavy fractions by the co-expression with NL43 Nef but not a Nef-AxxA mutant defective in the binding to Hck (Saksela et al., 1995). The peak shift was also associated with the appearance of many tyrosine-phosphorylated proteins in these fractions (Fig. 2B).

Both NL43 Nef and SF2 Nef had intact PxxP motif (Fig. 3A), but SF2 Nef showed much higher affinity to Hck than NL43 Nef (Fig. 3B). In the control experiments, we confirmed that the binding of both Nef to Lyn remained low and the PxxP motif-disrupted SF2 Nef mutant (AxxA) bound neither Hck nor Lyn. Reflecting the higher affinity to Hck, SF2 Nef induced stronger Hck activation (Fig. 3C) and more severe Fms maturation arrest (Fig. 3D). As expected, even SF2 Nef failed to induce Fms maturation arrest when co-expressed with Lyn (data not shown). However, SF2 Nef and NL43 Nef showed no obvious change in the pattern of predominant Golgi-localization (Fig. 3E). It was therefore likely that SF2 Nef bound Hck at

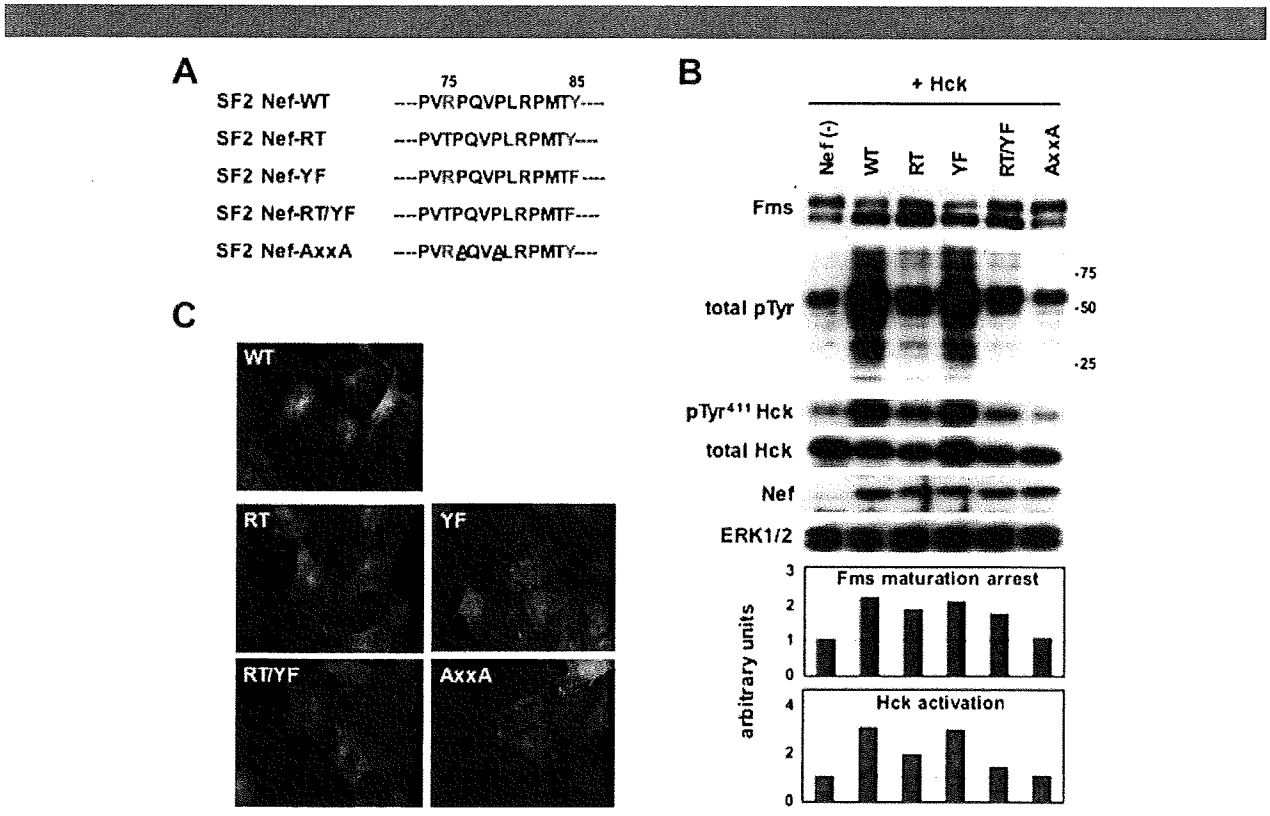

Fig. 4. Abilities of SF2 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The SF2 Nef mutants used (RT, YF, RT/YF, and AxxA) are schematically shown. B: HEK293 cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. These cells were then analyzed for the expression of Fms, phosphotyrosine (total pTyr), active-Hck ($pTyr^{411}$ Hck), total Hck, GFP-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest and Hck activation are shown in the bar graphs. C: Cells were transfected with indicated GFP-Nef plasmid (green). Nuclei were stained with DAPI (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the Golgi with higher affinity and thereby induced stronger Hck activation and more severe Fms maturation arrest.

There was a single amino acid difference within the PxxP motif, Thr^{71} in NL43 Nef and Arg^{75} in SF2 Nef (Fig. 3A). We found that an NL43 Nef with $Thr^{71}Arg$ substitution (NL43 Nef-TR) showed higher affinity to Hck than wild-type NL43 Nef (Fig. 3B), and induced stronger Hck activation (Fig. 3C) and more severe Fms maturation arrest (Fig. 3D) than wild-type NL43 Nef. We also performed a complementary experiment with SF2 Nef mutants (Fig. 4A; Ueno et al., 2008). As a result, we found that mutants with $Arg^{75}Thr$ substitution (SF2 Nef-RT and SF2-RT/YF) induced moderate Hck activation/Fms maturation arrest (Fig. 4B). However, both showed no obvious change in the pattern of predominant Golgi-localization (Fig. 4C). These results indicated that the single amino acid difference (Thr to Arg) within the PxxP motif governed the higher ability of SF2 Nef to induce Golgi-localization and activation of Hck, and Fms maturation arrest.

Although PxxP motif is essential for Nef to bind Hck, a recent study showed that an acidic region of Nef facilitated Nef-Hck binding at the Golgi (Hung et al., 2007). Although an NL43 Nef mutant lacking this region (ΔE; Fig. 5A) bound GST-Hck SH3 fusion proteins as with wild-type NL43 Nef (Fig. 5B), ΔE mutant was indeed less active than wild-type in transfected HEK293 cells, that is, in both Hck and activation Fms maturation arrest (Fig. 5C). Another mutant (M20; Fig. 5A), which was defective in the down-regulation of MHC I, another hallmark function of Nef (Akari et al., 2000), retained the ability to induce Hck activation and Fms maturation arrest (Fig. 5C). Both ΔE and M20A mutants showed no obvious change in the pattern of predominant Golgi-localization (Fig. 5C). The result further supported the idea that stronger Hck activation, which took place at the Golgi, induced severe Fms maturation arrest.

Analyses with a newly discovered Nef-Hck binding blocker

To directly show that the Golgi-localization of active Hck determines Nef-induced Fms maturation arrest, we sought to discover Nef-Hck binding blockers. In this study, we focused on UCS15A and its analogs 2b and 2c (Fig. 6A), because these small-molecule compounds were shown to block several proline-rich motif-SH3 domain binding such as Sam68-Fyn binding (Oneyama et al., 2003) and AMAP1-cortactin binding (Hashimoto et al., 2006). As they have not been used before for HIV-1 studies, we tested their capability to block Nef-Hck binding by the GST pull-down assay. As shown (Fig. 6B), all compounds blocked the binding of Hck to NL43 Nef or NL43 Nef-TR mutant (more potent than the wild-type, see Fig. 3), in a dose-dependent manner. Like the case of Sam68-Fyn binding (Oneyama et al., 2003), 2c was the most effective in blocking Nef-Hck binding (Fig. 6B), and showed no obvious toxicity to HEK293 cells (Fig. 6C). As shown (Fig. 6D), 2c indeed inhibited

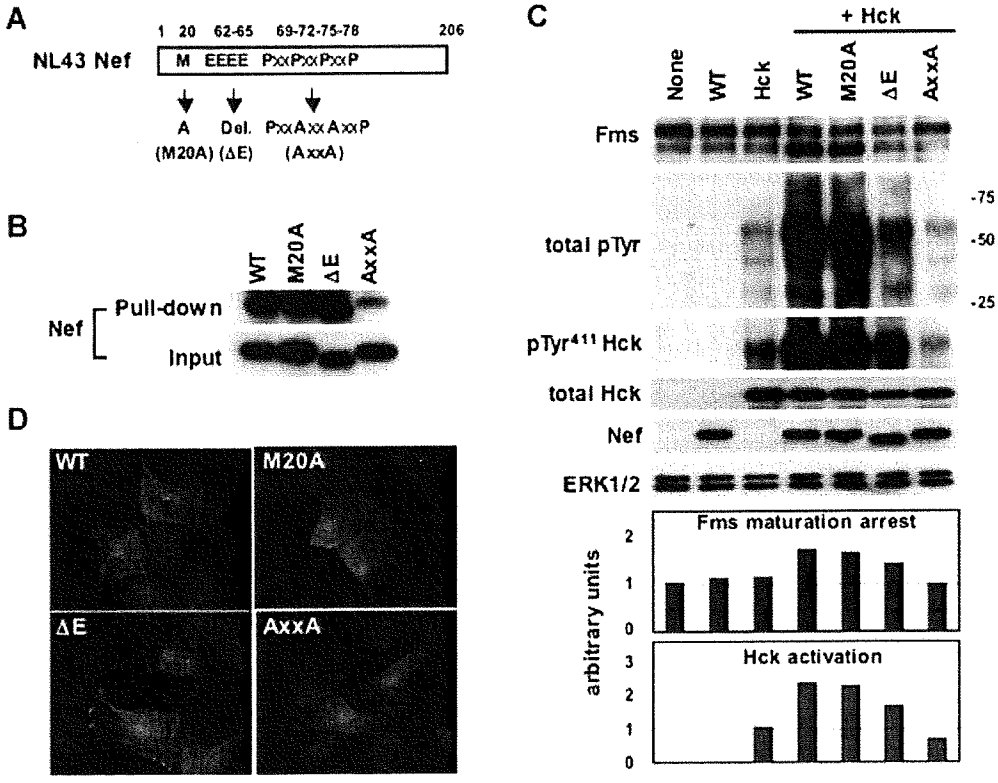

Fig. 5. Abilities of NL43 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The NL43 Nef mutants used (M20A, ΔE, and AxxA) are schematically shown. B: The resin, to which GST-Hck SH3 fusion proteins were bound, were incubated with the lysates of HEK293 cells expressing the indicated Nef proteins. The amount of Nef proteins in the lysates (Input) or bound to the resins (Pull-down) was verified by Western blotting. C: HEK293 cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. These cells were then analyzed for the expression of Fms, phosphotyrosine (total pTyr), active-Hck ($pTyr^{411}$Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest and Hck activation are shown in the bar graphs. D: Cells were transfected with indicated GFP-Nef (green). Nuclei were stained with DAPI (blue). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

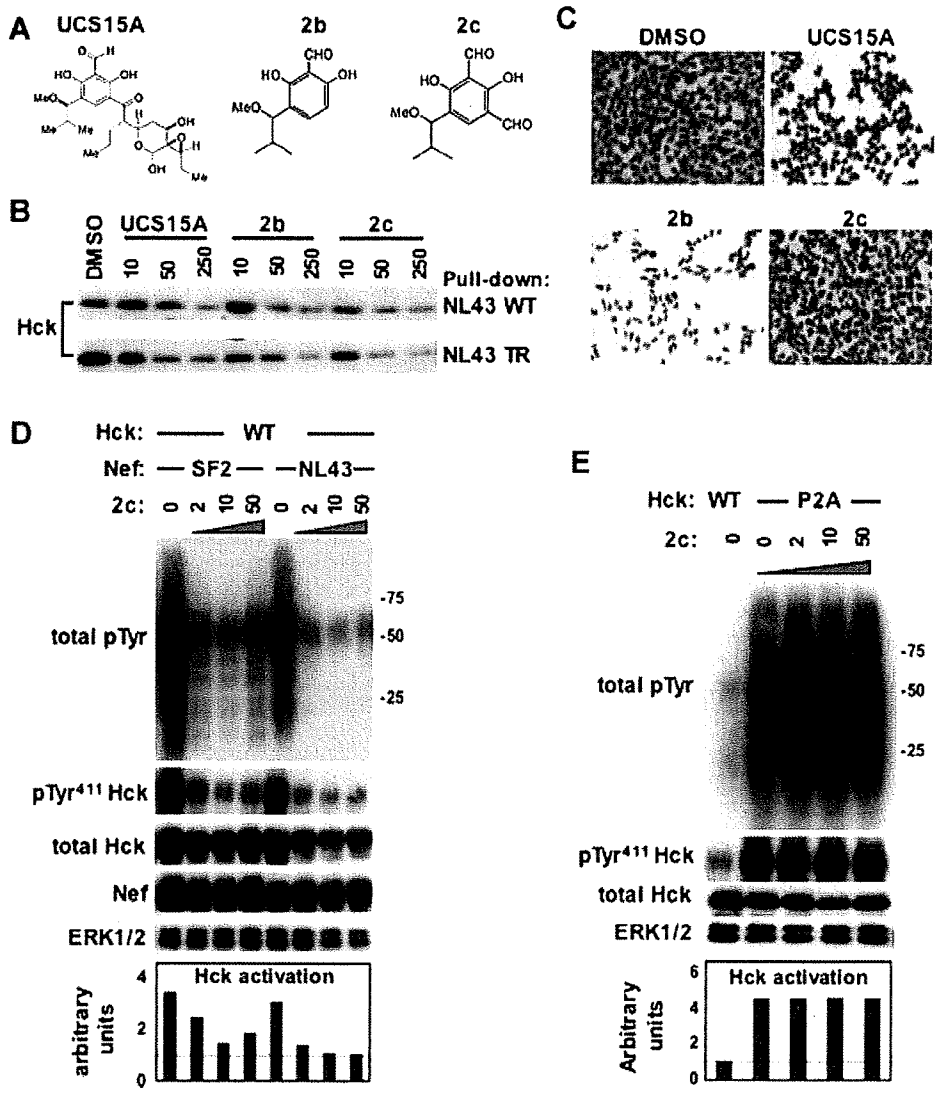

Fig. 6. Capability of 2c to block Nef-Hck binding and Nef-induced Hck activation. A: Chemical structures of UCS15A, 2b, and 2c are shown. B: The resins, to which GST-Nef (NL43 wild-type or TR mutant, see Fig. 2A) proteins were bound, were incubated with the lysates of Hck-expressing HEK293 cells in the absence (DMSO) or presence of the indicated concentration (0, 10, 50, or 250 μM) of UCS15A, 2b, or 2c. The amount of Hck proteins bound to the resins was determined by Western blotting. C: HEK293 cells were cultured in the absence (DMSO) or presence of 50 μM of UCS15A, 2b, or 2c for 2 days, and subjected to Wright-Giemsa staining. D: Cells were co-transfected with Hck-WT and indicated Nef alleles (SF2 or NL43), and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed for the expression of tyrosine-phosphorylated proteins (total pTyr), active-Hck (pTyr^{411}Hck), total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Hck activation is shown in the bar graphs. E: Cells were transfected with Hck-WT or Hck-P2A, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were analyzed as in (D). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Hck activation by NL43 Nef and more potent SF2 Nef (see Fig. 3). Importantly, 2c had little inhibitory effect on kinase activity of the constitutive-active Hck P2A mutant, even when used at a concentration as high as 50 μM (Fig. 6E). These results indicated that 2c was not a kinase inhibitor but inhibited Nef-induced Hck activation by blocking Nef-Hck binding.

This unique feature of 2c prompted us to examine whether 2c blocks Nef/Hck-induced Fms maturation arrest and Nef-induced skewed Golgi-localization of Hck. As shown (Fig. 7A), 2c completely blocked Fms maturation arrest induced by Nef and wild-type Hck as expected. However, of particular importance was that 2c also completely blocked severe Fms maturation arrest induced by Nef and the constitutive-active Hck P2A (Fig. 7B). Because 2c had little inhibitory effect on kinase activity of Hck-P2A (see Fig. 6E), these results strongly supported that the presence of Hck-P2A at the Golgi caused by its binding with Nef (see Fig. 2) was a direct cause of severe Fms maturation arrest. We therefore sought to verify that 2c indeed blocked Nef-induced skewed Golgi-localization of Hck-P2A. To this end, we employed the quantitative analysis, that is, subcellular fractionation on sucrose gradients (see Fig. 2B). The peak of Hck-P2A shifted to heavier fractions by the co-expression with Nef, and such change in the intracellular localization of Hck-P2A was restored to normal by the addition of 2c (Fig. 7C). We also tested whether 2c blocked Nef-induced Fms abnormality in another culture system. We previously showed that the cell surface expression of Fms was impaired in human myeloid TF-1-fms cells expressing a conditionally active

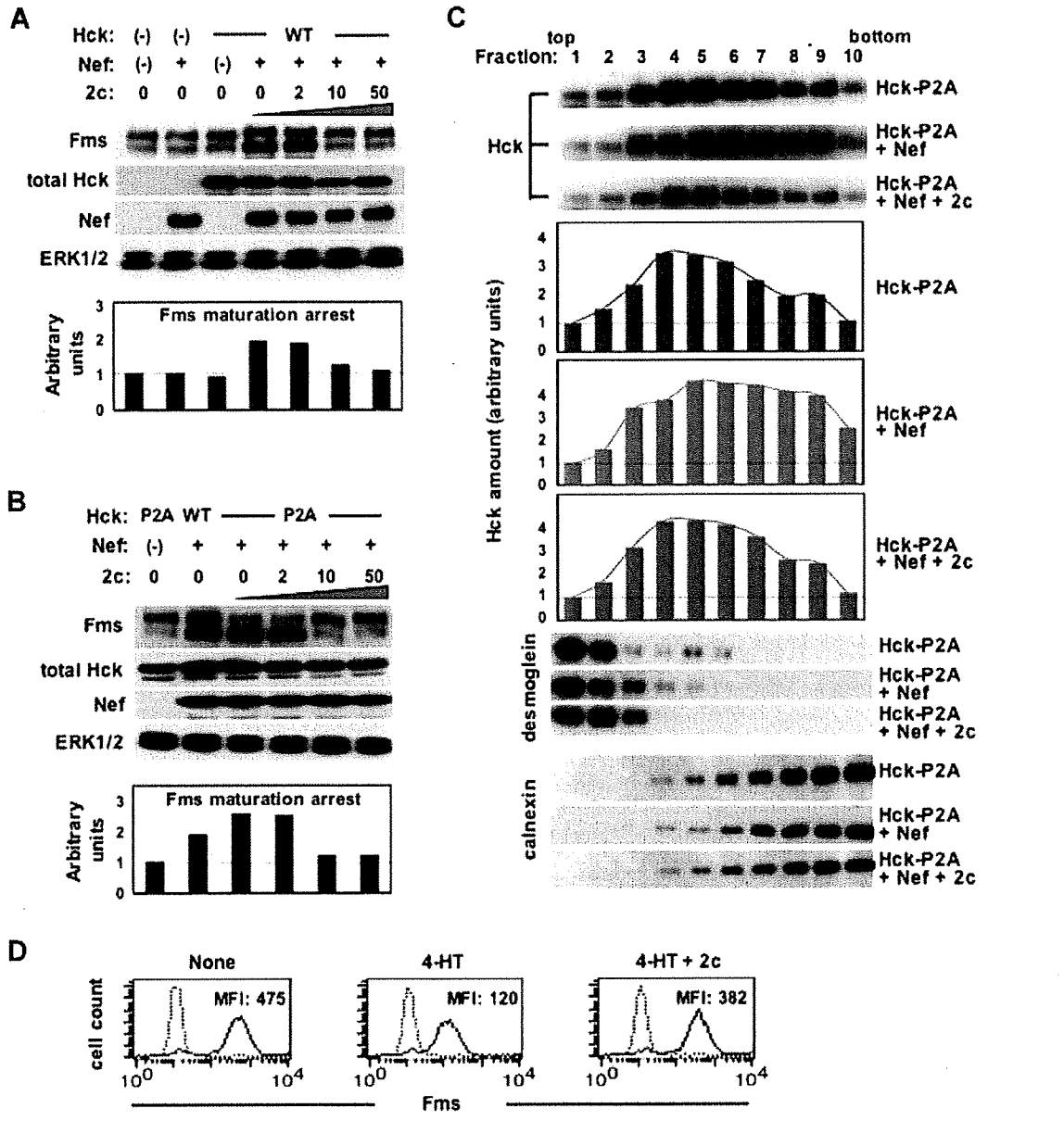

Fig. 7. Capability of 2c to block Fms maturation arrest and skewed Golgi-localization of Hck. A: HEK293 cells were transfected with the plasmids (Fms, NL43 Nef, and Hck-WT) in combination indicated, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed for the expression of Fms, total Hck, CD8-Nef (Nef), or ERK by Western blotting. The quantified Fms maturation arrest is shown in the bar graphs. B: Cells were transfected with the plasmids (Fms, NL43 Nef, Hck-WT, and Hck-P2A) in combination indicated, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed as in A. C: Cells were transfected with Hck-P2A alone (top), or co-transfected with NL43 Nef (middle). 2c was added to a final concentration of 50 μM to selected wells (bottom). Then, cells were subjected to subcellular fractionation on sucrose gradients and Hck Western blotting. The quantified Hck amounts are shown in the bar graph. The fractions were also analyzed for the amount of desmoglein and calnexin. D: TF-1-fms-Nef-ER cells cultured with M-CSF-free media in the absence (left) or presence of 0.1 μM 4-HT (middle), or the co-presence of 0.1 μM 4-HT and 50 μM 2c (right) for 12 h. The expression of Fms on the surface of treated cells was analyzed by flow cytometry with PE-labeled anti-Fms. The mean fluorescence intensity (MFI) of Fms expression is indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Nef-ER fusion protein when the Nef-ER in the cells was activated by the estrogen analog 4-HT (Hiyoshi et al., 2008). This impaired cell surface Fms expression was highly likely due to intracellular Fms maturation arrest (Hiyoshi et al., 2008). Finally, we found that the Fms down-regulation in Nef-active TF-1-fms-Nef-ER cells was also restored to normal by the addition of 2c (Fig. 7D). All taken together, our present study clearly demonstrated that skewed Golgi-localization of active Hck induced by Nef was indeed the direct cause of Fms maturation arrest.

Discussion

M-CSF is a cytokine essential not only for the survival of macrophages but also for the maintenance of macrophages at an

anti-inflammatory state (reviewed in Chitu and Stanley, 2006; Hamilton, 2008). Thus, the inhibitory effect of Nef on M-CSF signal through Fms maturation arrest at the Golgi is a possible trigger to worsen uncontrolled immune systems in patients (Suzu et al., 2005; Hiyoshi et al., 2008). In this study, we therefore sought to define molecular basis of this important function of Nef, by using different Nef alleles, various Nef mutants, constitutive-active Hck mutant, and Nef-Hck binding blocker 2c. The study with various Nef proteins supported the idea that high affinity Nef-Hck binding and subsequent stronger Hck activation, both of which took place mainly at the Golgi, determined Fms maturation arrest at the Golgi (Figs. 2–5). Moreover, the study with 2c enabled us to conclude that skewed Golgi-localization of active Hck by Nef was indeed the direct cause of Fms maturation arrest (Figs. 6 and 7). By analogy with the Sam68-Fyn binding inhibition (Oneyama et al., 2003), the inhibitory effect of 2c on Nef-Hck binding was supposed to be mediated by its interaction with Nef PxxP motif.

As mentioned earlier, it has been known for a long time that most members of Src kinases including Hck localize to the Golgi as well as to the plasma membrane. For example, it was shown that newly synthesized Lyn initially localized and accumulated to the Golgi, and then moved toward the plasma membrane (Kasahara et al., 2004). Importantly, Pulvirenti et al. (2008) recently revealed that coordinated regulation of activity of the Golgi-localized Src kinases is crucial to maintain intra-Golgi trafficking of proteins. Our present finding that skewed Golgi-localization of active Hck leads to Fms maturation arrest at the Golgi is in line with the new concept. It appears that long-lasting and dys-regulated activation of the Golgi-localized Src kinases disturbs glycosylation and/or trafficking of proteins, exemplified by Fms maturation arrest. Indeed, N-Src, a c-Src isoform with a higher basal tyrosine kinase activity (Brugge et al., 1985), showed more obvious perinuclear localization than the constitutive-active Hck-P2A and induced Fms maturation arrest even in the absence of Nef (unpublished result). Moreover, Mitina et al. (2007) reported that over-expression of an active form of Hck disturbed *N*-glycosylation of another cytokine receptor Flt3 even in the absence of Nef. These results may further support the idea that long-lasting and dys-regulated activation of the Golgi-localized Src kinases per se affects protein glycosylation and/or trafficking at the Golgi. Anyhow, our system with Nef provides a useful model to elucidate how Src kinases regulate the Golgi structure/function. It will be important to identify which Golgi proteins are phosphorylated directly or indirectly by Hck activated at the Golgi and to clarify how such phosphorylation cascade leads to Nef-induced Fms maturation arrest.

Nef has been shown to affect protein trafficking and a well-characterized target is major histocompatibility complex class I (MHC I). Nef reduced the cell surface expression of MHC I, which diminishes the recognition of infected cells by cytotoxic T cells (reviewed in Fackler and Baur, 2002; Peterlin and Trono, 2003). However, it is still in intense debate whether Nef requires SH3 domain-containing proteins such as Hck to reduce the cell surface level of MHC I (Schwartz et al., 1996; Greenberg et al., 1998; Mangasarian et al., 1999; Akari et al., 2000; Chang et al., 2001; Roeth and Collins, 2006; Hung et al., 2007; Atkins et al., 2008). In this regard, Fms is not the direct target of Nef. However, as shown, Nef disturbed the cell surface expression of Fms, which is triggered by skewed Golgi-localization of active Hck. Moreover, it was shown that Nef disturbed the cell surface expression of another macrophage-specific protein HFE, an iron homeostasis regulator, which was blocked by a dominant-negative Hck (Drakesmith et al., 2005). Although whether the reduced cell surface level of HFE by Nef relates to skewed Golgi-localization of active Hck is unclear, it is conceivable that Nef acquires an additional machinery to manipulate protein trafficking in macrophages by exploiting the Golgi-localized Hck. Of interest, the *N*-glycosylation of Flt3, which is structurally related to Fms, was also impaired in Nef/Hck-expressing HEK293 cells, but the degree of maturation arrest of Flt3 was quite weak when compared to that of Fms (data not shown). The finding may imply that Fms maturation arrest is not necessarily due to the general disruption in the Golgi structure or function. Future studies, in which we determine to what extent overall protein *N*-glycosylation and trafficking are affected by Nef-Hck binding, will further clarify pathological significance of the molecular binding in macrophages. The newly discovered Nef-Hck binding blocker 2c will be useful in such studies and may provide a strategy to complement current anti-HIV-1 therapy for better treatment outcomes.

In this study, we showed that SF2 Nef had much higher affinity to Hck than NL43 Nef and thereby induced stronger Hck activation/severe Golgi-localization of Hck (Figs. 2 and 3) and that the single amino acid difference (Thr^{71} in NL43 Nef and Arg^{75} in SF2 Nef) within PxxP motif largely governs the higher ability of SF2 Nef (Figs. 3 and 4). This difference might reflect that the $Thr^{71}Arg$ substitution in NL43 Nef (NL43 Nef-TR, see Fig. 3) altered the flexibility of a loop containing the PxxP motif (Fackler et al., 2001). Importantly, for reasons not clearly understood, NL43 Nef-TR was more pathogenic in HIV-1 Tg mice than wild-type NL43 Nef and the pathogenicity of SF2 Nef in Tg mice was evident despite very low levels of expression (Priceputu et al., 2007). It is therefore possible that more severe Golgi-localization of active Hck followed by perturbed *N*-glycosylation and trafficking of proteins including Fms account for the high pathogenicity of SF2 Nef in Tg mice.

In summary, our present study clearly demonstrated that skewed Golgi-localization of active Hck was the direct cause of Fms maturation arrest by Nef. Our findings establishes an intriguing link between the pathogenesis of HIV-1 Nef and the newly emerging concept that the Golgi-localized Src kinases regulate the Golgi function. The identification of Golgi proteins phosphorylated by the Golgi-localized active Hck will provide novel insights into molecular mechanisms by which Nef functions as an HIV-1 pathogenetic factor through Hck and the Gogi-localized Src kinases regulate the Golgi function.

Acknowledgments

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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.

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 [3].

2.2. Plasmids

The SEC14L1a CTD1 was amplified from MT-4 polyA RNA by reverse transcriptase PCR (RT-PCR) using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACCCTGCGTGCGCCGCCAGCAGC-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'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACTGCGAAGTGCCAGAGGGTGGAC-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-3'. Full length (FL) SEC14L1a was amplified by PCR from a plasmid containing the SEC14L1a open reading frame (ORF, CS0DL004YN18, Invitrogen), using the primers 5'-GCACCGGTCTCGAGCCACCATGGACTACAAAGACGATGACGACGTGCAGAAATACCAGTCCCCAG-3' and 5'-CCAATTGCTACCTGGAGATCATGGAGCTG-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′-GGATCCTCAGTCGTCATCGTCTTTGTAGTCTTCCTTCGGGCCTGTCGGGTC-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'-TGCTGGGATTACAGGCGTGAG-3'; and for the second Alu-LTR PCR reaction, 5'-AACTAGGGAACCCACTGCTTAAG-3' and 5'-CTGCTAGAGATTTTCCACACTGAC-3'. For amplification of HIV-1 mRNA, 5'-ATGGAGCCAGTAGATCCTAGAC-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'-TATTGGTCTCCTTAAACCTGTCTTG-3' and 5'-CTGACACAACTGTGTTCACTAGC-3'; and cyclophilin A, 5'- CACCGCCACCATGGTCAACCCCACCGTGTTCTTCGAC-3' and 5'- CCCGGGCCTCGAGCTTTCGAGTTGTCCACAGTCAGCAATGG-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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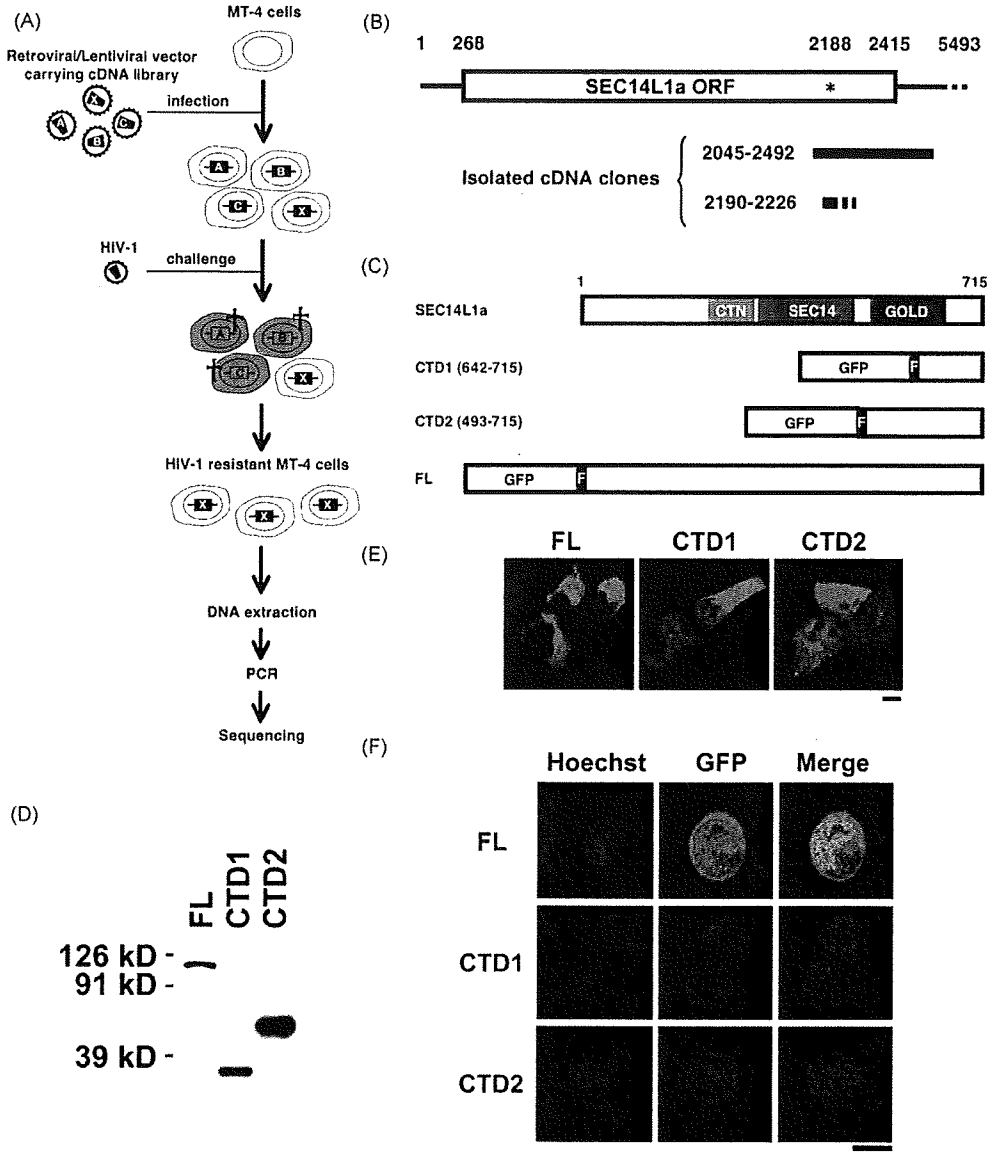

Fig. 1. Identification of SEC14L1a CTD as a potential regulator of HIV-1 replication. (A) The experimental strategy used to screen a cDNA library for genes rendering cells resistant to HIV-1. MT-4 cells were infected with a retroviral or lentiviral vector carrying cDNA libraries and were challenged with 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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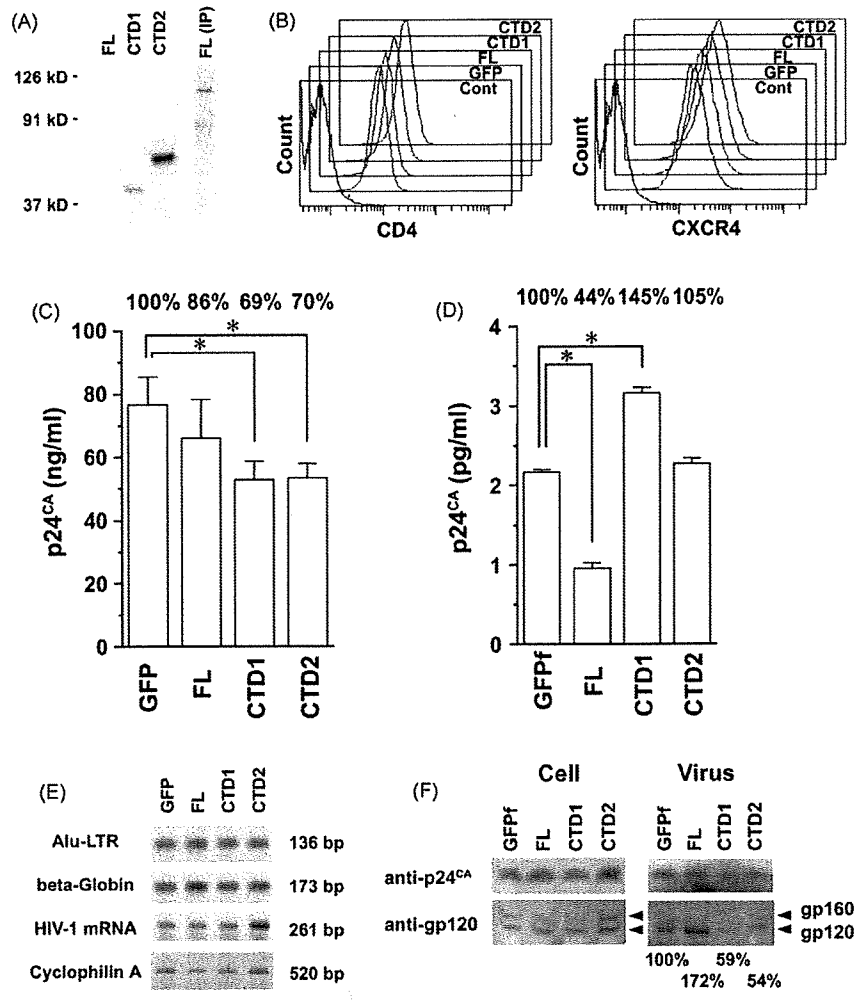

Fig. 2. Functional characterization of the SEC14L1a derivatives. (A) Detection of stable expression of FL, CTD1, and CTD2 in MT-4 cells by Western blotting using anti-FLAG antibody. FL was detected by the immunoprecipitation (IP) assay using agarose beads conjugated with anti-FLAG antibody. The flow 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.

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Conflict of interest: None.

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Inhibition of HIV replication by a CD4-reactive Fab of an IgM clone isolated from a healthy HIV-seronegative individual

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HIV replication is restricted by some anti-CD4 mouse mAb *in vitro* and *in vivo*. However, a human monoclonal anti-CD4 Ab has not been isolated. We screened EBV-transformed peripheral B cells from 12 adult donors for CD4-reactive Ab production followed by functional reconstitution of Fab genes. Three independent IgM Fab clones reactive specifically to CD4 were isolated from a healthy HIV-seronegative adult (~0.0013% of the peripheral B cells). The germ line combinations for the VH and VL genes were VH3-33/L6, VH3-33/L12, and VH4-4/L12, respectively, accompanied by somatic hypermutations. Genetic analysis revealed a preference for V-gene usage to develop CD4-reactive Ab. Notably, one of the CD4-reactive clones, HO538-213, bearing 1×10^{-8} M dissociation constant (Kd) to recombinant human CD4, limited the replication of R5-tropic and X4-tropic HIV-1 strains at 1–2.5 μg/mL in primary mononuclear cells. This is the first clonal genetic analysis of human monoclonal CD4-reactive Ab. A mAb against CD4 isolated from a healthy individual could be useful in the intervention of HIV/AIDS.

Key words: Autoimmunity · CD4-reactive Ab · IgM · Inhibition of HIV replication

Supporting Information available online

Introduction

CD4 is a T-cell marker that serves as a principal receptor for HIV. CD4-reactive Ab are detected in HIV-infected individuals (~13%) [1, 2] and HIV-exposed seronegative individuals (34%) [3]. In addition, some healthy individuals are positive for anti-CD4 Ab (~0.6%) [4]. Replication of multiple HIV clades is blocked by mouse mAb against CD4 *in vitro* and *in vivo* [5–12]. Thus, it is possible that anti-CD4 Ab play a role in protecting individuals from HIV infection and delaying AIDS disease progression. Similar arguments have been made for Ab against CCR5, a coreceptor for HIV [3, 10, 13]. Furthermore, some clinical studies suggest that CD4-reactive Ab, including a humanized mAb, has therapeutic potential against HIV infection and AIDS progression [5, 8, 10, 12]. However, the development and pathophysiological roles of self-recognizing Ab in healthy individuals are still largely unknown, and a human mAb against CD4 has not yet been isolated.

To gain insights into the genesis of auto-reactive Ab and to characterize the nature of CD4-reactive auto-Ab, we conducted experiments to isolate human monoclonal anti-CD4 Ab from PBMC of 12 HIV-seronegative adult donors. We succeeded in isolating three independent IgM clones recognizing CD4 from a healthy donor. Analysis of the V-region sequences of CD4-reactive Ab

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revealed a preference for V gene usage to give rise to CD4-reactive Ab. This is the first report describing CD4-reactive human mAb.

Results and discussion

Isolation of CD4-reactive IgM clones from a healthy individual

PBMC were collected from 12 HIV-seronegative adult volunteers, including two healthy and ten with autoimmune disorders, and B-lymphoblastoid cell lines (B-LCL) were established by infecting the cells with EBV (for experimental procedure, see Supporting Information Fig. 1). B-LCL were propagated in oligoclonal pools. In 790 cultures from one healthy donor, we identified two cultures positive for recombinant human CD4 (rhCD4) reactivity, HO538 and HO702, using ELISA (Fig. 1A). This donor may have a unique Ab repertoire, as auto-reactive B-LCL cultures were identified significantly more frequently in this donor than in the others (Fig. 1A). The rhCD4 reactivity was specific, as no binding was observed to 72 other viral, bacterial, and auto-Ag screened in parallel (Supporting Information Fig. 2). We amplified the Ig genes encoding Fab regions by RT-PCR and cloned them into the bacterial expression vector pFabI-His2 that produces Fab fragments of an inserted set of VH and VL genes. We expected that some clones should reconstitute the CD4-reactive Fab present in the original B-LCL cultures. After screening by ELISA, one CD4-reactive Fab clone, HO538-213, was isolated from the HO538 culture, and two independent clones, HO702-001 and HO702-016, were isolated from the HO702 culture. These Fab clones originated from IgM, as determined by the sequence analysis. The estimated efficiency of peripheral B cells producing CD4-reactive Ab was ~0.0013% (three clones/2.4×10^5 estimated screened B cells × 100 (%), given that the B cells compose 10% of PBMC and that EBV immortalization is 30% efficient on average) [14]. According to the ELISA data, the Fab concentrations that yielded 50% maximal binding were ~8 μg/mL for HO538-213, and ~1 μg/mL for HO702-001 and HO702-016 (Fig. 1B). Consistent with these data, the BIACORE assay revealed that the dissociation constant (Kd) of HO538-213, HO702-001, and HO702-016 to rhCD4 was 6.5×10^{-8}, 7.7×10^{-9}, and 2.7×10^{-9} M, respectively (Fig. 1C), which is relatively weak compared with average Ab–Ag interactions (e.g. the Kd of mouse mAb Leu-3a to rhCD4 is 2.2×10^{-10} M).

Genetic analysis of CD4-reactive IgM clones

The Fab sequences were analyzed by the Kabat database (http://www.ncbi.nlm.nih.gov/igblast/) in GenBank, as previously described [15, 16]. The Ig gene family of each gene and the most homologous germ line are indicated (Fig. 2A). All the three clones were of the IgM class and had a κ-chain for VL. Comparison of the

A

Donor	Number of tested cultures	CD4	Nuclear antigen	Blood type i-glycolipid
Healthy 1	790	2 (0.3%)*	21 (2.6%)*	28 (3.5%)*
Healthy 2	160	0	0	0
Autoimmune (10 individuals)	435	0	3 (0.7%)	0

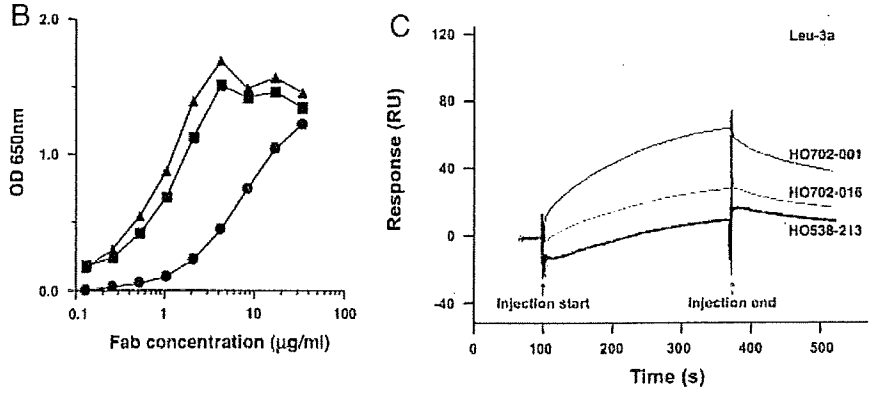

Figure 1. Isolation and characterization of healthy human-derived CD4-reactive Ab. (A) Summary of the frequency of B-LCL cultures that reacted to representative auto-Ag. The number of cultures positive for rhCD4 reactivity, HeLa cell nuclear staining, and blood type i-glycolipid are shown. *$p<0.05$, compared with other donor groups, Fisher's exact test. (B) CD4-binding kinetics of CD4-reactive IgM Fab. Serial dilutions of HO538-213 (circles), HO702-001 (triangles), and HO702-016 (squares) were incubated in microtiter plates pre-coated with rhCD4. (C) Surface plasmon resonance analysis of CD4-reactive IgM FAb HO702-001 (black), HO702-016 (dark gray), HO538-213 (bold), and mAb Leu-3a (gray) binding to immorbilized rhCD4. The concentration of Ab was 0.3 μg/mL, flow rate 20 μL/min, and reaction time 270 s. RU, resonance units.

heavy chain with the germ lines revealed that the μ-chains of HO538-213 and HO702-001 were 95 and 97% homologous to germ line VH 3-33, respectively, while HO702-016 was 96% homologous to germ line VH 4-4 [17]. For the light chains, the κ-chain Vkappa3 of HO538-213 was 97% homologous to germ line L6 [6, 18, 19], and κ-chain Vkappa1 of both HO702-001 and HO702-016 was 97% homologous to the germ line L12 [6, 18, 19]. These data suggest that there is a preferential use of VH and VL genes to develop CD4-reactive Ab, considering the number of VH and VL genes present before the Ig gene rearrangement. According to the sequence analysis, the VH amino acid sequences of HO538-213 and HO702-001 carried distinct mutations, although both were derived from the same germ line VH3-33. The mutations were more frequent in the CDR regions (Fig. 2B and C, Supporting Information Fig. 3), which is characteristic of somatic hypermutation (sHM) associated with affinity maturation. Unlike most sHM, however, mutations involving G/C were not dominant.

A

Clone	Ig class	VH family	Closest germline	% identity	VL family	Closest germline	% identity
HO538-213	IgM	VH 3	VH3-33	95%	Vk3	L6	97%
HO702-001	IgM	VH 3	VH3-33	97%	Vk-1	L12	97%
HO702-016	IgM	VH 4	VH4-4	98%	Vk-1	L12	97%

B

Clone			Number of mutations associated with: A	G	C	T	Ins[a]	del[a]	GC involvement (%)[b]	Total	aa changes[a]	(%)
HO702-001	heavy chain	FR	3	2	1	0	0	0	50.0	6	2	33.3
		CDR	0	0	0	0	5	0	0.0	5	0	0.0
	light chain	FR	0	0	2	2	0	0	0.0	2	1	50.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	4	2	1	2	11	0	30.3	20	3	15.0
HO702-016	heavy chain	FR	1	0	0	0	0	0	0.0	1	0	0.0
		CDR	0	2	1	0	11	0	100.0	9	0	0.0
	light chain	FR	0	0	0	2	0	0	0.0	3	1	33.0
		CDR	1	0	0	0	6	0	0.0	7	0	0.0
		Total	2	2	1	2	17	0	42.9	21	1	4.2
HO538-213	heavy chain	FR	2	1	1	4	0	0	25.0	8	3	37.5
		CDR	2	2	3	1	10	0	70.0	23	5	22.0
	light chain	FR	1	2	1	0	0	0	75.0	4	3	75.0
		CDR	0	1	0	0	2	0	25.0	6	3	50.0
		Total	8	6	7	5	17	0	60.9	43	19	55.2

C

Light chain

	FR1	CDR1	FR2	CDR2
HO538-213	ETVMTQSPATLSLSPGERATLSC	RASQSVNNYLA	WYQQRPGQAPSLLIY	DASNRAT
HO702-001/016	D-QL----S---A-V-D-V-IT-	-----TSS-----	----K--K--K----	X--SLES

	FR3	CDR3	FR4
HO538-213	GIPARFSGSGSGTDFTLTISSLEPEDFAVYYC	QQ.RG.NWFHT	FGQGTKLEIKRT
HO702-001/016	-V-S---------E---------Q-D---T---	--YNSYSWEY-	----------

Heavy chain

	FR1	CDR1	FR2	CDR2
HO538-213	QVQLVQSGGGVVQPGRSLRLSCA.ASG.FTFS	SYGMH	WVRQAPGKGLEWVA	VVCYDGRTTYYADSVKG
HO702-001	----QEW----------	-----	----------	-IN---SNK--------
HO702-016	---QE--P L-K-SQT-S-T-TVSG-SISSG	--YWS	-I- PA------IG	RI TSGS-N NP-R-S

	FR3	CDR3	FR4
HO538-213	RFTISRDISENTLYLQMNSLRAEDTAVYYCAK	DLNKGYCNNGGCYG.VDC	WGQGTLVTVSS
HO702-001	-------N-K----------	--.RD.-SG-S--SWF-P	----------
HO702-016	-V--SV-T-K-QFS-KLS-VT-A--------	T.-PTL.GR-L.F...-Y	----------

Figure 2. Genetic analysis of CD4-reactive Ab. (A) Summary of the Ig class, V-gene family, closest germ line, and percentage identity of the closest germ line of CD4-reactive Ab. (B) The mutation profiles of the CD4-reactive IgM Fab fragments. (C) The deduced protein sequences of the VH and VL genes of the CD4-reactive Fab fragments are aligned. FR, framework region. Dashes and dots indicate identical residues and deletions, respectively. See Supporting Information Fig. 2 for further detail.

Inhibition of HIV replication by a Fab fragment of a CD4-reactive IgM

We next examined the potential impact of these CD4-reactive Fab Ab on HIV replication. Viral replication was monitored in PBMC by measuring $p24^{CA}$ viral Ag levels in the culture supernatant. Among the three IgM Fab clones, HO538-213 suppressed R5-tropic virus HIV-$1_{JR\text{-}FL}$ replication by 3.5±1.5-fold at 1–2.5 μg/mL (average±SD from four independent experiments, Fig. 3A). There was a modest but consistent suppression of X4-tropic virus HIV-1_{HXB2} replication (1.4±0.2-fold, average±SD from three independent experiments). A BIACORE and ELISA revealed that HO538-213 did not compete with the anti-CD4 mAb Leu-3a [20, 21] for CD4 binding. Leu-3a restricts HIV-1 replication by physically blocking *Env*-CD4 interaction (data not shown), suggesting that the epitope recognized by HO538-213 is distinct from the *Env*-interacting domain of CD4 [7, 22, 23]. The monoclonal anti-CD4 Ab OKT4a does not block *Env*-CD4 interaction, but restricts HIV-1 infection, although decreasing CD4 lateral diffusion on the cell surface [24–26]. We hypothesized that HO538-213 may have a similar mechanism of action. CD4 localizes to lipid rafts, and CD4-crosslinking activates signal transduction involving tyrosine kinases [27–29]. Thus, we treated MOLT-4 cells with HO538-213, and the lipid raft fraction was isolated by a membrane floatation assay as verified by the raft markers glycosphyngomyelin 1 and sphingomyelin (Fig. 3B, left panel). Tyrosine kinase activitiy was examined by immunoblotting, the lipid raft fractions using a PY20 anti-phosphotyrosine mAb (Fig. 3B, right panel, arrowhead). We detected a significant amount of tyrosine phosphorylation in the lipid raft fraction after HO538-213 treatment, indicating that HO538-213 can assemble cell surface CD4. This is consistent with our hypothesis that HO538-213 inhibits HIV-1 infection by decreasing the lateral movement of cell surface CD4.

Does CD4-reactive IgM function as a natural HIV resistance factor?

We then further characterized the donor from which the CD4-reactive Ab was isolated. The donor serum did not show a strong reactivity to rhCD4 at 1:10 dilution, where the non-specific effect was no longer detected. We analyzed the HIV-inhibition titer of the donor plasma. In a TZM-bl cell assay, the plasma did not block HIV replication at 1:50 dilution (data not shown). These data suggest that the CD4-reactive IgM circulates at very low titers in the donor and may not be sufficient to block HIV infection *in vitro*. However, it is possible that the CD4-reactive IgM may be able to limit HIV-1 propagation under *in vivo* conditions.

We next investigated the immunological status of the donor. IgG and IgM levels were within the normal range, and the plasma was negative for rheumatoid factor, anti-DNA, and anti-ribonucleoprotein Ab. However, the donor serum reacted to nuclear Ag at a titer of 1:160 (1:40 or less is considered normal), and the staining patterns were nucleolar (1:160) and speckled (1:80). Consistent with these data, the frequency of auto-reactive

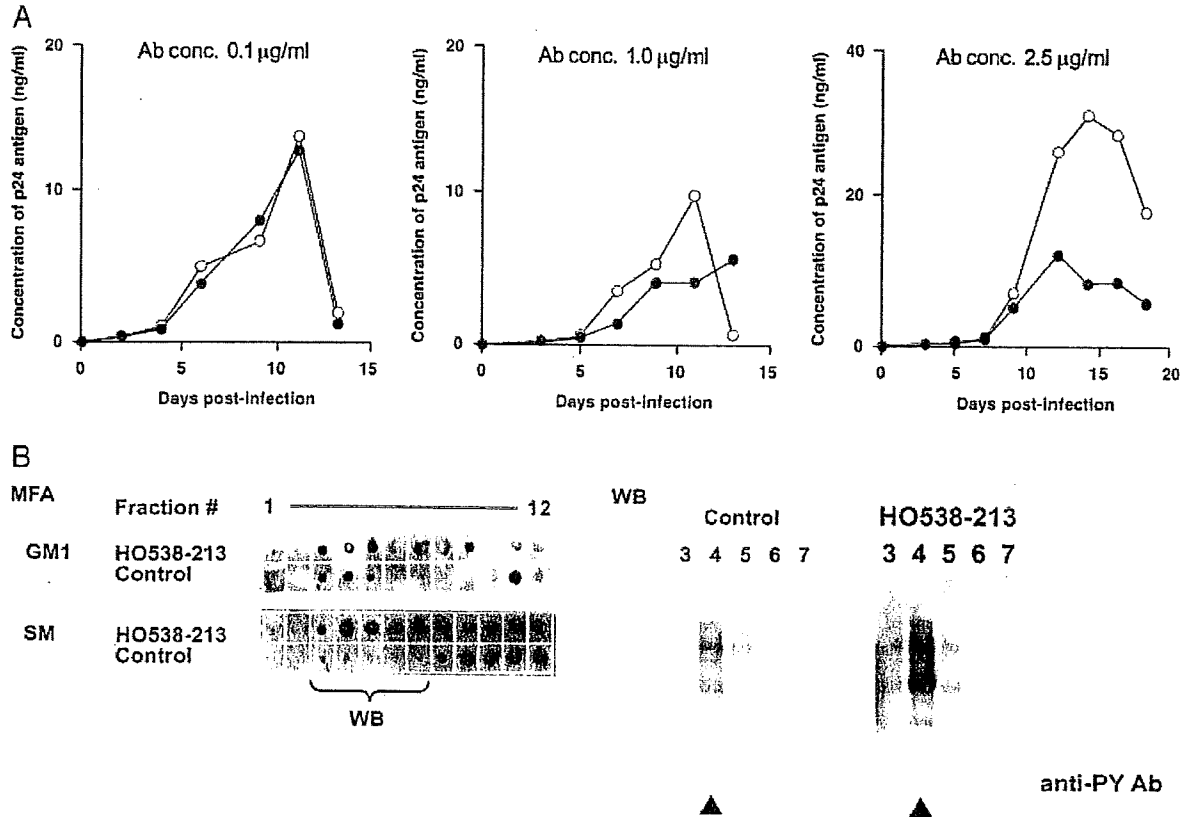

Figure 3. The effect of CD4-reactive Fab clone HO538-213 on HIV-1 replication. (A) The Fab clone HO538-213 (filled circles) was tested for its ability to inhibit HIV-$1_{JR\text{-}FL}$ replication at a concentration of 0.1 (left), 1.0 (middle), and 2.5 (right) μg/mL. The CD4 non-reactive Fab clone 13-3 (open circles) was used as a negative control. Representative data from four independent experiments are shown. (B) Activation of the tyrosine kinase signaling cascade by HO538-213 in MOLT-4 cells. The detergent-resistant membrane fraction (arrowhead) was isolated by a membrane floatation assay (MFA) from MOLT-4 cells treated with HO538-213, and phosphotyrosine levels were examined by immunoblotting.

Ab-producing cells from the same donor, namely against nuclear Ag and blood group i-glycolipid, was significantly higher than the other donors (Fig. 1A). In addition, we isolated anti-TNF-α IgG and IgM clones from this donor [16]. Although clinical manifestations of autoimmune disorders were lacking, it is likely that the donor may have an immunological background that generates auto-reactive Ab and tolerates them. Moreover, the donor has been healthy for 29 years, as the CD4-reactive Ab was first isolated, suggesting that such CD4-reactive Ab may not disturb host immunity.

Considering that the IgM-producing B cells we isolated went through positive/negative selection, their original target should not be CD4. It is thus likely that the IgM genes accumulated sHM that resulted in cross-reactivity to CD4 in periphery after B-cell maturation. To better understand the unique immunological features of individuals with CD4-reactive Ab and their auto-reactive Ab repertoire, more human monoclonal self-reactive Ab are needed to analyze both their V-region sequences and cross-reactivities. Our experimental approach might be useful for addressing these issues. Unfortunately, however, we were unable to characterize the CD4-reactive Ab-producing cells, as the oligoclonal cultures of B-LCL were terminated after RNA extraction for our Ig gene cloning strategy. We speculate that B-1 cells could be the source of the CD4-reactive Ab, because B-1 cells produce IgM that often cross-reacts with auto-Ag.

Our genetic data indicated that only a fraction of the CD4-reactive Ab could have some HIV-inhibitory function. It is an open question whether such CD4-reactive HIV-inhibitory Ab may be present in the other healthy individuals, as well as in HIV-seropositive long-term non-progressors.

HIV-inhibitory CD4-reactive Ab are effective against multiple HIV clades, as CD4 is the major HIV receptor for all the viral clades [11]. A clinical trial is being conducted to examine the therapeutic efficacy of a humanized CD4-reactive mAb in patients with HIV infection [8, 12]. Although CD4-reactive Ab can be detected in healthy individuals, safety is always a concern when using self-recognizing Ab as therapeutic drugs. Given that HO538-213 was isolated from a healthy individual and that it recognized a different epitope than Leu-3a, HO538-213 might effectively inhibit HIV without disturbing $CD4^+$ T-cell functions. As noted above, the donor from which the three CD4-reactive IgM Fab were isolated has been healthy for more than 29 years since PBMC collection, suggesting that these Ab may not seriously inhibit $CD4^+$ T-cell functions *in vivo* and thus may be useful in treating HIV infection and other disorders [4].

Concluding remarks

This report provides the first clonal genetic analyses of human monoclonal anti-CD4 Ab. IgM is considered to function in "natural humoral immunity", as it has a relatively low affinity for pathogens and confers natural resistance to infectious agents. However, the pathogen-specific immunity function of IgM has not been demonstrated at a clonal level. Our data suggest that CD4-reactive IgM is present in healthy individuals and can contribute to natural resistance to HIV infection and AIDS progression. This is the first clear demonstration of a natural humoral immunity function of IgM against HIV.

Materials and methods

Functional cloning of heavy and light chain Ab genes

The establishment of Ab-producing cells, cloning of Ig genes encoding V regions, ELISA, and the purification of Fab fragments from *Escherichia coli* have been described previously [16]. The experimental procedure is schematically shown in the Supporting Information Fig. 1. In brief, PBMC from 12 donors, including two healthy individuals and ten individuals with autoimmune disorders, were infected with the B95-8 strain of EBV, and 1×10^4 cells were propagated in 96-well plates. The supernatant was analyzed by ELISA using rhCD4 derived from a baculovirus system (50 ng/well; INTRACELL) as an Ag. Other Ag tested, including viral, bacterial, and auto-Ag, are listed in the Supporting Information Fig. 2. Total cellular RNA was isolated from oligoclonal cell populations positive for anti-CD4 Ab production (RNeasy mini kit, Qiagen). cDNAs were synthesized and amplified by PCR with specific primers for human Ig μ-, γ-, λ-, and κ-chains. Only the μ- and κ-chains were amplified from HO538 and HO702 cultures and cloned into the pFab1-His2 vector, generating bacterial Fab-expression libraries [30]. The pFab libraries were screened for the production of CD4-reactive Fab by ELISA. The Fab fragments were purified using an anti-Fab Ab affinity column. The eluted Fab was dialyzed against PBS and concentrated by centrifugation (VIVASPIN concentrator, Vivascience AG). The purity of the Fab Ab was greater than 95% as determined by SDS-PAGE analysis (data not shown).

Surface plasmon resonance biosensor analysis

Surface plasmon resonance analyses were performed using a BIACORE 3000 (GE Healthcare). The hrCD4 was immobilized onto CM5 sensor chips using standard amine-coupling chemistry. The purified Fab was diluted in a running buffer (10 mM HEPES, 0.15 M NaCL, 3 mM EDTA, surfactant P 20, pH 7.4) to 0.3–20 μg/mL and injected at a rate of 20–30 μL/min. The Fab was allowed to associate and dissociate for 120–270 s.

Cells

B-LCL and 293 T cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Sigma) supplemented with 10% fetal bovine serum (Japan Bioserum), penicillin, and streptomycin (Invitrogen). The primary mononuclear cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin, 5 μg/mL plasmocin (InvivoGen), 10 mM HEPES, 5 μg/mL anti-CD3 mAb (OKT3, Janssen Pharmaceutical), 70 U/mL recombinant human IL-2 (Shionogi Pharmaceutical), GlutaMax-I (Invitrogen), insulin–transferrin–selenium-A (Invitrogen), and 10 mM HEPES (Invitrogen). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Other experimental procedures

Procedures for monitoring HIV-1 replication [31] and membrane floatation assays [32] were described previously. Standard auto-Ab was tested by the clinical laboratory testing service SRL (Tokyo, Japan).

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