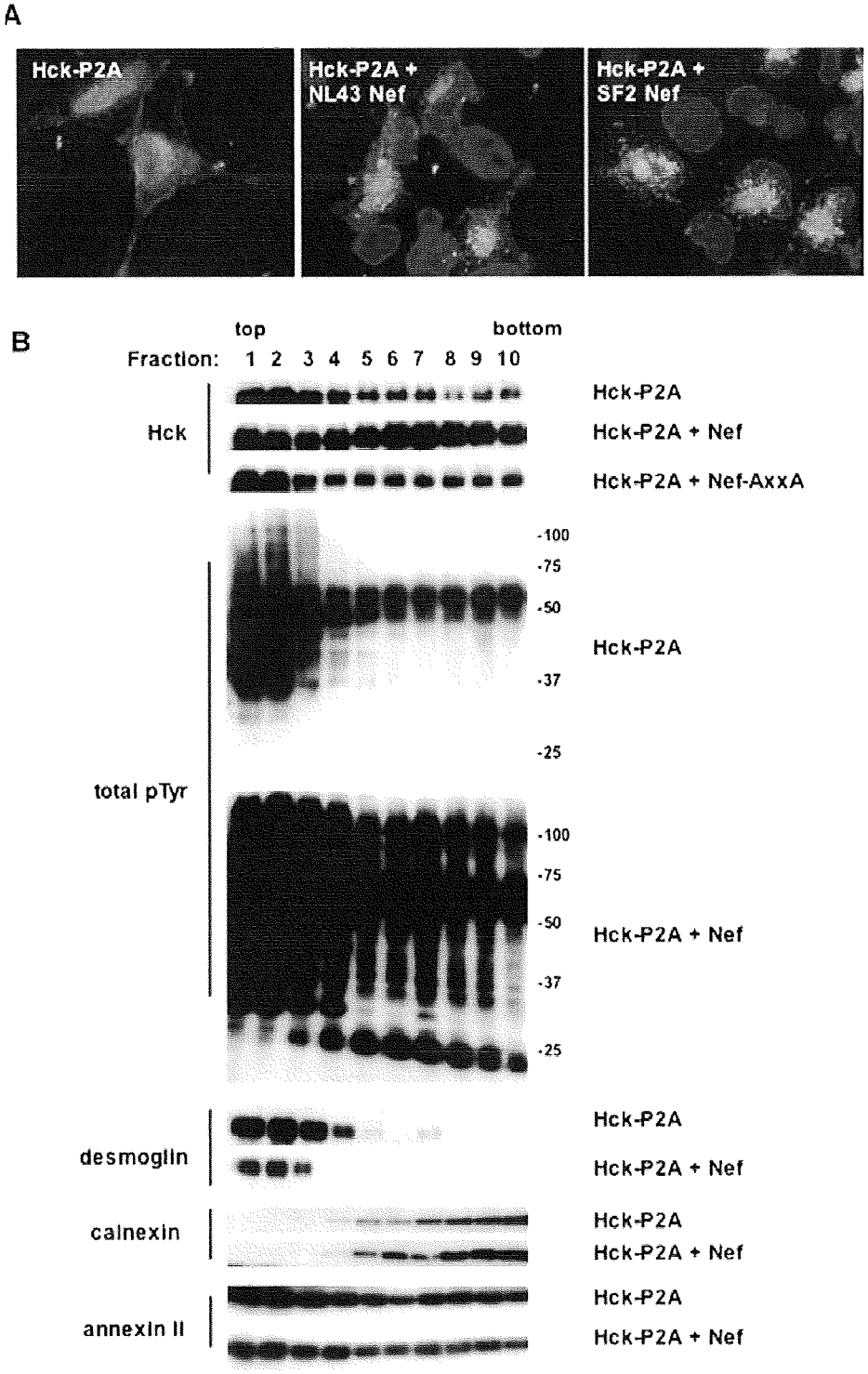

Fig. 2. Skewed Golgi-localization of Hck by Nef. A: HEK293 cells were transfected with Hck-P2A plasmid alone, or co-transfected with NL43 Nef or SF2 Nef plasmid. Cells were stained with antibody specific for active Hck (green) and DAPI (blue). B: Cells were transfected with Hck-P2A alone, or co-transfected 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

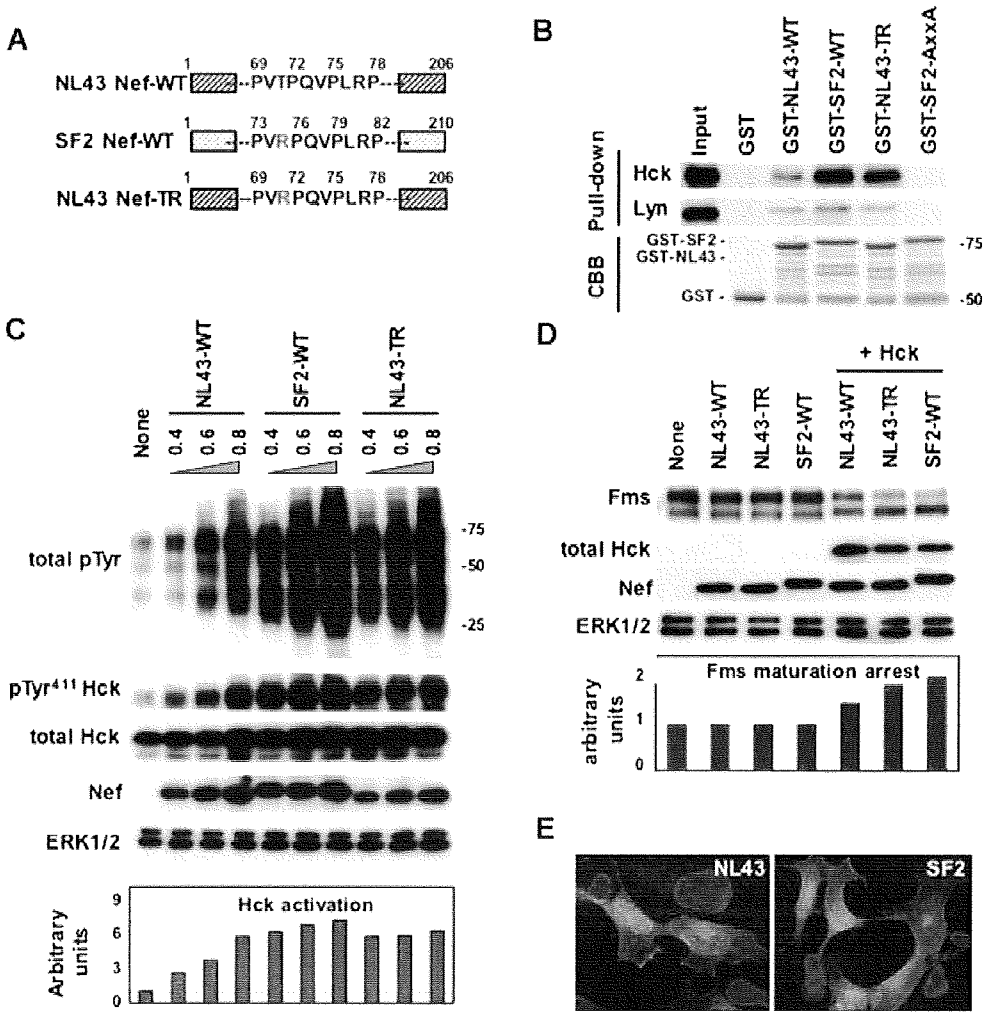

Fig. 3. Abilities of different Nef alleles to bind/activate Hck and to induce Fms maturation arrest. A: The NL43 Nef, SF2 Nef, and NL43 Nef-TR mutant used are schematically shown. B: The resins, to which the control GST or indicated GST-Nef proteins were bound, were incubated with the lysates of HEK293 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

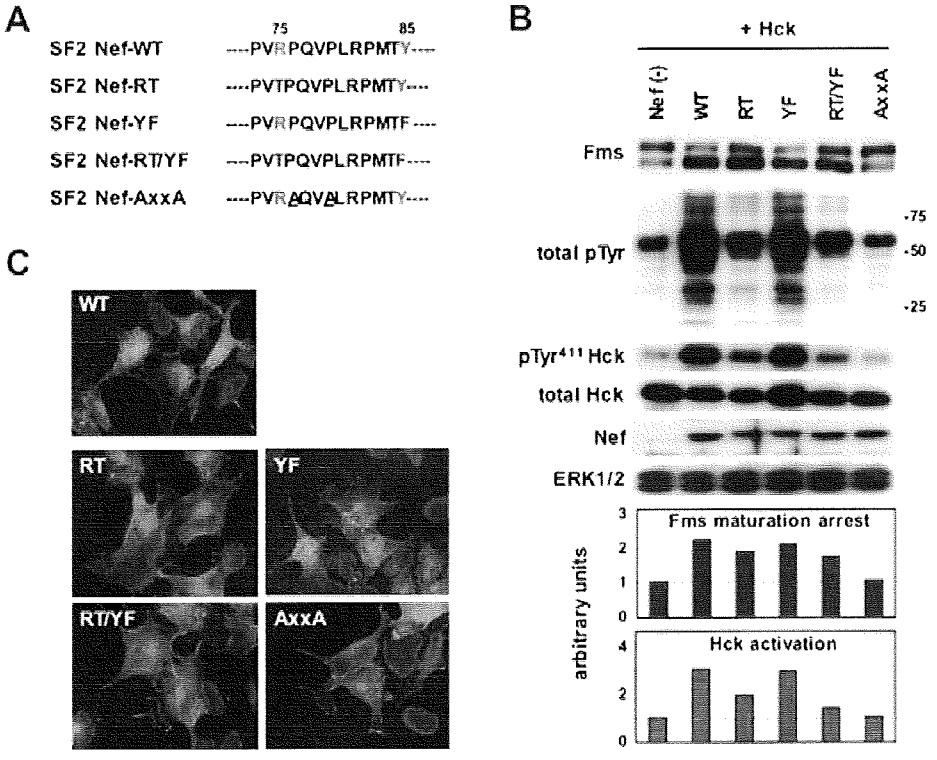

Fig. 4. Abilities of SF2 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The SF2 Nef mutants used (RT, YF, RT/YF, and AxxA) are schematically shown. B: HEK293 cells were transfected with Fms plasmid alone (None) or in combination with the plasmids for Nef and Hck, as indicated. These 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

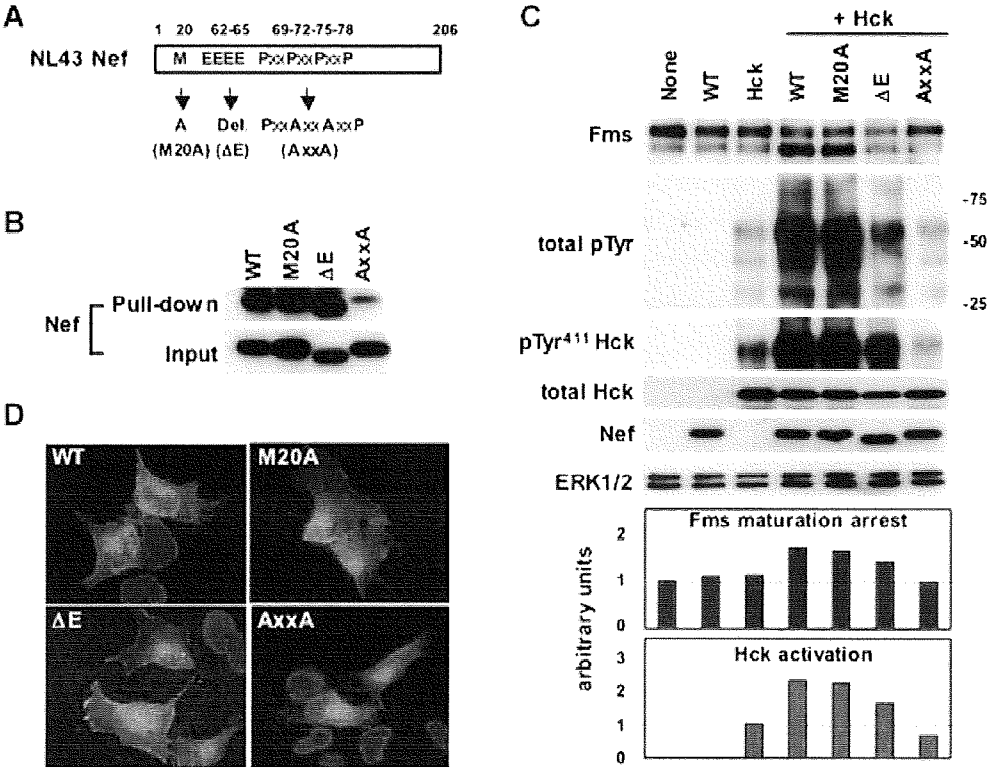

Fig. 5. Abilities of NL43 Nef mutants to activate Hck and to induce Fms maturation arrest. A: The NL43 Nef mutants used (M20A, ΔE, and AxxA) are schematically shown. B: The resin, to which GST-Hck SH3 fusion proteins were bound, were incubated with the lysates of HEK293 cells expressing the indicated 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.]

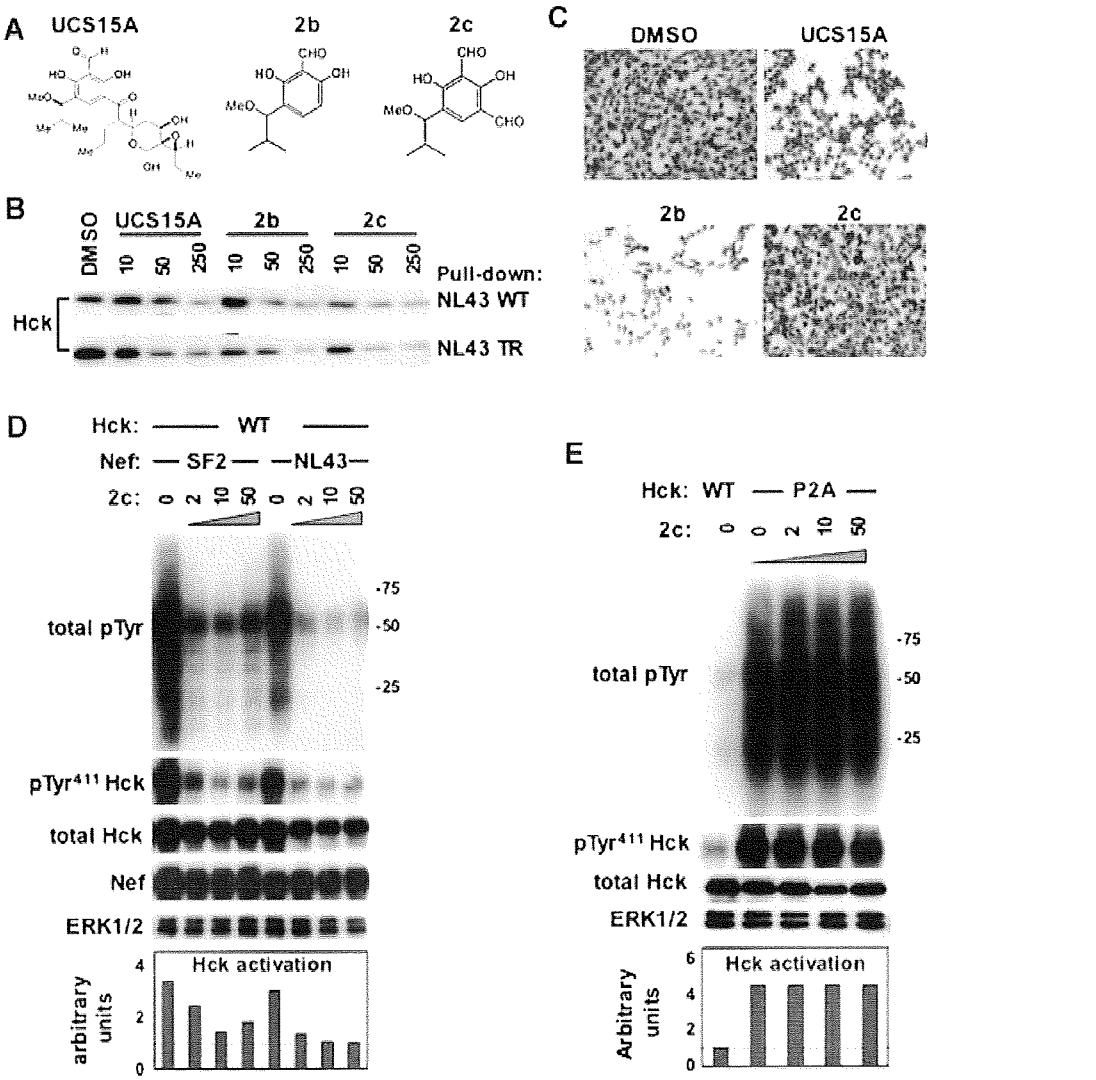

Fig. 6. Capability of 2c to block Nef-Hck binding and Nef-induced Hck activation. A: Chemical structures of UCS15A, 2b, and 2c are shown. B: The resins, to which GST-Nef (NL43 wild-type or TR mutant, see Fig. 2A) proteins were bound, were incubated with the lysates of Hck-expressing HEK293 cells in the 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

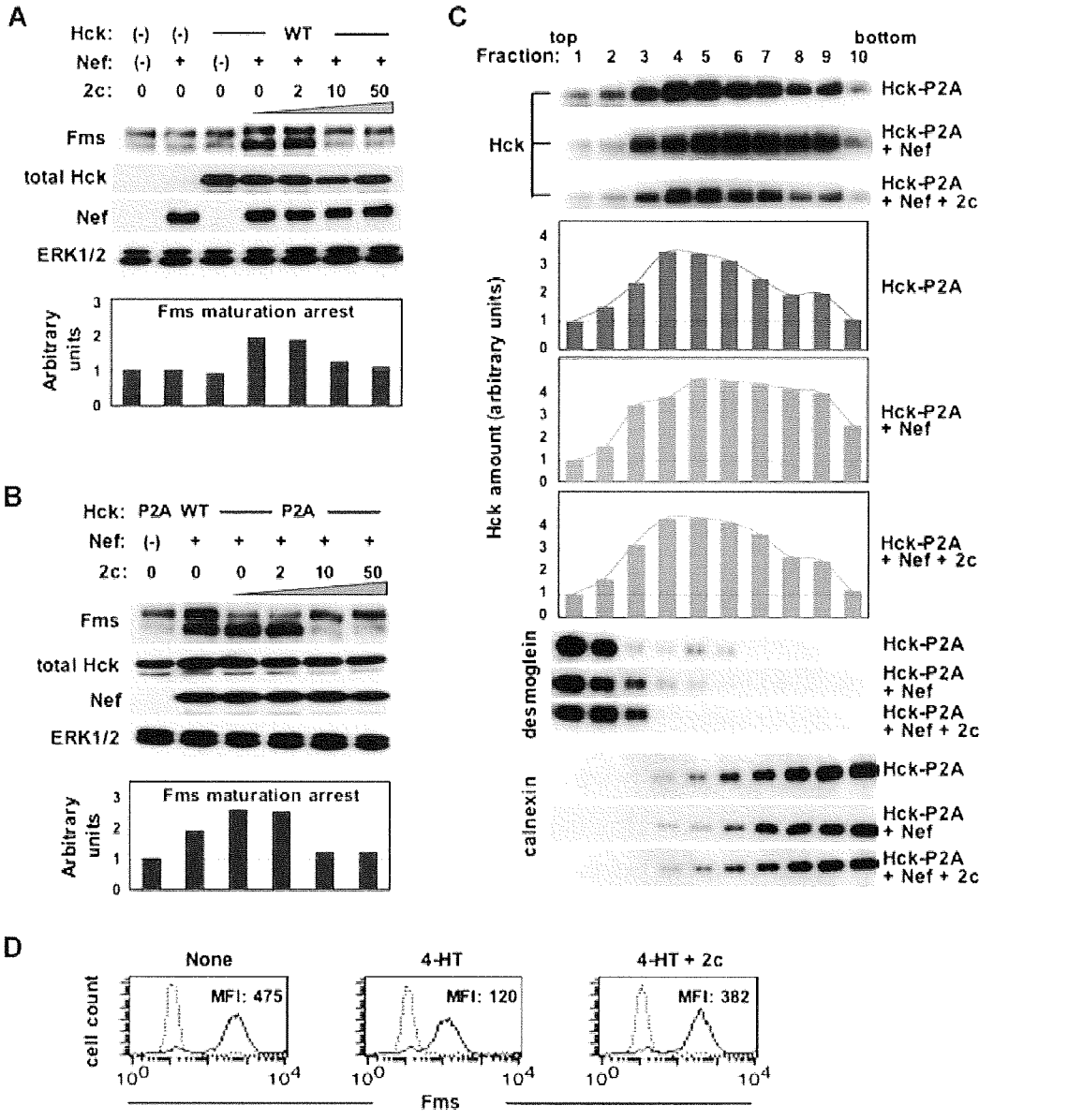

Fig. 7. Capability of 2c to block Fms maturation arrest and skewed Golgi-localization of Hck. A: HEK293 cells were transfected with the plasmids (Fms, NL43 Nef, and Hck-WT) in combination indicated, and cultured in the presence of increasing concentrations (μM) of 2c. These cells were then analyzed for 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

We thank Dr. G. Thomas (Vollum Institute) for critical reading of the manuscript. We thank Ms. Y. Endo and Ms. I. Suzu for experimental assistance.

Literature Cited

Akari H, Arold S, Fukumori T, Okazaki T, Strebel K, Adachi A. 2000. Nef-induced major histocompatibility complex class I down-regulation is functionally dissociated from its virion incorporation, enhancement of viral infectivity, and CD4 down-regulation. J Virol 74:2907–2912.

Arold S, O'Brien R, Franken P, Strub MP, Hoh F, Dumas C, Ladbury JE. 1998. RT loop flexibility enhances the specificity of Src family SH3 domains for HIV-1 Nef. Biochemistry 37:14683–14691.

Atkins KM, Thomas L, Youker RT, Harriff MJ, Pissani F, You H, Thomas G. 2008. HIV-1 Nef binds PACS-2 to assemble a multikinase cascade that triggers major histocompatibility complex class I (MHC-I) down-regulation: Analysis using short interfering RNA and knock-out mice. J Biol Chem 283:11772–11784.

Bard F, Mazelin L, Pechoux-Longin C, Malhotra V, Jurdic P. 2003. Src regulates Golgi structure and KDEL receptor-dependent retrograde transport to the endoplasmic reticulum. J Biol Chem 278:46601–46606.

Bijlmakers MJ, Isobe-Nakamura M, Ruddock LJ, Marsh M. 1997. Intrinsic signals in the unique domain target p56lck to the plasma membrane independently of CD4. J Cell Biol 137:1029–1040.

Brugge JS, Cotton PC, Queral AE, Barrett JN, Nonner D, Keane RW. 1985. Neurones express high lenels of a structurally modified, activated form of pp60c-src. Nature 316:554–557.

Carreno S, Gouze ME, Schaak S, Emorine LJ, Maridonneau-Parini I. 2000. Lack of palmitoylation redirects $p59^{Hck}$ from the plasma membrane to $p61^{Hck}$-positive lysosomes. J Biol Chem 275:36223–36229.

Chang AH, O'Shaughnessy MV, Jirik FR. 2001. Hck SH3 domain-dependent abrogation of Nef-induced class I MHC down-regulation. Eur J Immunol 31:2382–2387.

Chitu V, Stanley ER. 2006. Colony-stimulating factor-1 in immunity and inflammation. Curr Opin Immunol 18:39–48.

David-Pfeuty T, Nouvian-Dooghe Y. 1990. Immunolocalization of the cellular src protein in interphase and mitotic NIH c-src overexpresser cells. J Cell Biol 111:3097–3116.
Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont, Sullivan JS, Cunningham A, Dwyer D, Mills J. 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion and recipients. Science 270:988–991.
Drakesmith H, Chen N, Ledermann H, Screaton G, Townsend A, Xu XN. 2005. HIV-1 Nef down-regulates the hemochromatosis protein HFE, manipulating cellular iron homeostasis. Proc Natl Acad Sci USA 102:11017–11022.
Fackler OT, Baur AS. 2002. Live and let die: Nef functions beyond HIV replication. Immunity 16:493–497.
Fackler OT, Wolf D, Weber HO, Laffert B, D'Aloja P, Schuler-Thurner B, Geffin R, Saksela K, Geyer M, Peterlin BM, Schuler G, Baur AS. 2001. A natural variability in the proline-rich motif of Nef modulates HIV-1 replication in primary T cells. Curr Biol 11:1294–1299.
Greenberg ME, Iafrate AJ, Skowronski J. 1998. The SH3 domain-binding surface and an acidic motif in HIV-1 nef regulate trafficking of class I MHC complexes. EMBO J 17:2777–2789.
Haller C, Rauch S, Fackler OT. 2007. HIV-1 Nef employs two distinct mechanisms to modulate Lck subcellular localization and TCR induced actin remodeling. PLoS ONE 2:e1212.
Hamilton JA. 2008. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 8:533–544.
Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. 1998. Nef harbors a major determinant of pathogenicity for and AIDS-like disease induced by HIV-1 in transgenic mice. Cell 95:163–175.
Hanna Z, Weng X, Kay DG, Poudrier J, Lowell C, Jolicoeur P. 2001. The pathogenicity of human immunodeficiency virus (HIV) type 1 Nef in CD4C/HIV transgenic mice is fabolished by mutation of its SH3-binding domain, and disease development is delayed in the absence of Hck. J Virol 75:9378–9392.
Hashimoto S, Hirose M, Hashimoto A, Morishige M, Yamada A, Hosaka H, Akagi K, Ogawa E, Oneyama C, Agatsuma T, Okada M, Kobayashi H, Wada H, Nakano H, Ikegami T, Nakagawa A, Sabe H. 2006. Targeting AMAP1 and cortactin binding bearing an atypical src homology 3/proline interface for prevention of breast cancer invasion and metastasis. Proc Natl Acad Sci USA 103:7036–7041.
Hiyoshi M, Suzu S, Yoshidomi Y, Hassan R, Harada H, Sakashita N, Akari H, Motoyoshi K, Okada S. 2008. Interaction between Hck and HIV-1 Nef negatively regulates cell surface expression of M-CSF receptor. Blood 111:243–250.
Hung CH, Thomas L, Ruby CE, Atkins KM, Morris NP, Knight ZA, Scholz I, Barklis E, Weinberg AD, Shokat KM, Thomas G. 2007. HIV-1 Nef assembles a Src family kinase-ZAP-70/Syk-PI3K cascade to down-regulate cell surface MHC-I. Cell Host Microbe 1:121–133.
Kaplan KB, Swedlow JR, Varmus HE, Morgan DO. 1992. Association of $p60^{c\text{-}src}$ with endosomal membranes in mammalian fibroblasts. J Cell Biol 118:321–333.
Karkkainen S, Hiipakka M, Wang JH, Kleino I, Vaha-Jaakkola M, Renkema GH, Kiss M, Wagner R, Saksela K. 2006. Identification of preferred protein interactions by phage-display of the human Src homology-3 proteome. EMBO Rep 7:186–191.
Kasahara K, Nakayama Y, Ikeda K, Fukushima Y, Matsuda D, Horimoto S, Yamaguchi N. 2004. Trafficking of Lyn through the Golgi caveolin involves the charged residues on αE and αI helices in the kinase domain. J Cell Biol 165:641–652.
Kestler HW III, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC. 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65:651–662.
Kirchhoff F, Greenough TC, Brettler DB, Sullivan JL, Desrosiers RC. 1995. Brief report: Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. N Engl J Med 332:228–232.
Korade-Mirnics Z, Corey SJ. 2000. Src kinase-mediated signaling in leukocytes. J Leukocyte Biol 68:603–613.
Lerner EC, Smithgall TE. 2002. SH3-dependent stimulation of Src-family kinase autophosphorylation without tail release from the SH2 domain in vivo. Nat Struct Biol 9:365–369.
Ley SC, Marsh M, Bebbington CR, Proudfoot K, Jordan P. 1994. Distinct intracellular localization of Lck and Fyn protein tyrosine kinases in human T lymphocytes. J Cell Biol 125:639–649.
Lowell CA. 2004. Src-family kinases: Rheostats of immune cell signaling. Mol Immunol 41:631–643.
Mangasarian A, Piguet V, Wang JK, Chen YL, Trono D. 1999. Nef-induced CD4 and major histocompatibility complex class I (MHC-I) down-regulation are governed by distinct determinants: N-terminal alpha helix and proline repeat of Nef selectively regulate MHC-I trafficking. J Virol 73:1964–1973.
Matsuda D, Nakayama Y, Horimoto S, Kuga T, Ikeda K, Kasahara K, Yamaguchi N. 2006. Involvement of Golgi-associated Lyn tyrosine kinase in the translocation of annexin II to the endoplasmic reticulum under oxidative stress. Exp Cell Res 312:1205–1217.
Mitina O, Warmuth M, Krause G, Hallek M, Obermeier A. 2007. Src family tyrosine kinases phosphorylate Flt3 on juxtamembrane tyrosines and interfere with receptor maturation in a kinase-dependent manner. Ann Hematol 86:777–785.
Moarefi I, LaFevre-Bernt M, Sicheri F, Huse M, Lee CH, Kuriyan J, Miller WT. 1997. Activation of the Src-family tyrosine kianse Hck by SH3 domain displacement. Nature 385:650–653.
Oneyama C, Agatsuma T, Kanda Y, Nakano H, Sharma SV, Nakano S, Narazaki F, Tatsuta K. 2003. Synthetic inhibitors of proline-rich ligand-mediated protein-protein interaction: Potent analogs of UCS15A. Chem Biol 10:443–451.
Paliwal P, Radha V, Swarup G. 2007. Regulation of p73 by Hck through kinase-dependent and independent mechanisms. BMC Mol Biol 8:45.
Peterlin BM, Trono D. 2003. Hide, shield and strike back: How HIV-infected cells avoid immune eradication. Nat Rev Immunol 3:97–107.
Priceputu E, Hanna Z, Hu C, Simard MC, Vincent P, Wildum S, Schindler M, Kirchhoff F, Jolicoeur P. 2007. Primary human immunodeficiency virus type 1 Nef alleles show major differences in pathogenicity in transgenic mice. J Virol 81:4677–4693.
Pulvirenti T, Giannotta M, Capestrano M, Capitani M, Pisanu A, Polishchuk RS, San Pietro E, Beznoussenko GV, Mironov AA, Turacchio G, Hsu VW, Sallese M, Luini A. 2008. A traffic-activated Golgi-based signaling circuit coordinates the secretory pathway. Nat Cell Biol 10:912–922.
Roeth JF, Collins KL. 2006. Human immunodeficiency virus type 1 Nef: Adapting to intracellular trafficking pathways. Mol Biol Rev 70:548–563.
Saksela K, Cheng G, Baltimore D. 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef^+ viruses but not for down-regulation of CD4. EMBO J 14:484–491.
Schwartz O, Marechal V, Le Gall S, Lemonnier F, Heard JM. 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat Med 2:338–342.
Suzu S, Tanaka-Douzono M, Nomaguchi K, Yamada M, Hayasawa K, Kimura F, Motoyoshi K. 2000. $p56^{dok\text{-}2}$ as a cytokine-inducible inhibitor of cell proliferation and signal transduction. EMBO J 19:5114–5122.
Suzu S, Harada H, Matsumoto T, Okada S. 2005. HIV-1 Nef interferes with M-CSF receptor signaling through Hck activation and inhibits M-CSF bioactivities. Blood 105:3230–3237.
Trible RP, Emert-Sedlak L, Smithgall TE. 2006. HIV-1 Nef selecyively activates Src family kinases Hck, Lyn, and c-Src through SH3 domain interaction. J Biol Chem 281:27029–27038.
Ueno T, Motozono C, Dohki S, Mwimanzi P, Rauch S, Fackler OT, Oka S, Takiguchi M. 2008. CTL-mediated selective pressure influences dynamic evolution and pathogenetic functions of HIV-1 Nef. J Immunol 180:1107–1116.
van't Hof W, Resh MD. 1997. Rapid plasma membrane anchoring of newly synthesized p59fyn: Selective requirement for NH_2-terminal myristoylation and palmitoylation at cysteine-3. J Cell Biol 136:1023–1035.

Microbiol Immunol 2009; **53**: 53–57
doi:10.1111/j.1348-0421.2008.00087.x

MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

Hirofumi Akari, Yuki Iwasaki, Tomoyuki Yoshida and Sayuki Iijima

Laboratory of Disease Control, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

ABSTRACT

More than 170 million people worldwide are chronically infected by HCV, which is the causative agent of chronic hepatitis C, cirrhosis, and finally liver cancer. Although animal models of viral hepatitis are a prerequisite for the evaluation of antiviral and vaccine efficacy, the restricted host range of HCV has hampered the development of a suitable small animal model of HCV infection. Use of the chimpanzee, the only animal known to be susceptible to HCV infection, is limited by ethical and financial restrictions. In this regard GBV-B, being closely related to HCV, appears to be a promising non-human surrogate model for the study of HCV infection. This review describes the characteristic of GBV-B infection of New World monkeys, and discusses current issues concerning the GBV-B model and its future directions.

Key words GBV-B, HCV, hepatitis C, monkey.

INTRODUCTION

Since HCV was identified as a major causative agent for non-A, non-B hepatitis in 1989 by Choo *et al.* (1), it has become evident that HCV is disseminated worldwide and is carried by an estimated more than 170 million people (2). In most advanced nations, the prevalence of HCV infection is roughly 1–2% and further dissemination is suppressed. By contrast, among developing countries the number of HCV-infected patients is still increasing due to iatrogenic exposure, including blood transfusion from unscreened donors and reuse or inappropriate sterilization of contaminated medical equipment, and injecting drug use (3). After HCV exposure, about 70% of individuals who exhibit acute infection progress to chronic liver disease, and many of these patients develop hepatic cirrhosis and hepatocellular carcinoma (2). Currently, the only treatment available for patients with chronic HCV infections is combination therapy with pegylated interferon and ribavirin. As the standard therapy is effective in only approximately 50% of patients with chronic HCV hepatitis, the other half of affected patients are still threatened by poor prognosis (4). It is therefore urgent to develop more effective therapeutics for HCV infection. At the same time, prophylactic vaccines are indispensable for prevention of further spread of HCV in developing countries, including reduction of the risk to health care workers of occupational transmission.

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

Research in infectious diseases will never progress without animal models. Because conventional small animals are not susceptible to HCV infection due to its limited host range, development of an effective prophylactic vaccine, as well as unveiling of the molecular mechanism of viral pathogenesis, has been hampered. Nonetheless, decades

Correspondence
Hirofumi Akari, Laboratory of Disease Control, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan.
Tel: +81 29 837 2121; fax: +81 29 837 0218; email: akari@nibio.go.jp

Received 11 August 2008; revised 12 September 2008; accepted 17 September 2008

List of Abbreviations: ALT, alanine aminotransferase; CTL, cytotoxic T lymphocytes; GBV-B, GB virus-B; GE, genome equivalents; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency viruses; SIVmac, SIV derived from rhesus macaques.

of struggle have resulted in a number of animal models for HCV infection and hepatitis C.

Recently, a number of rodent models have been developed (for a review of this topic see reference 5). Rodent models, which permit HCV replication and involve transplantation of human hepatocytes, include immunotorelated fetal rat (transplantation of human hepatoma cell line Huh7 into newborn rats after prior injection of the same cells into pregnant females) (6) and immunodeficient mice such as trimera mice (7, 8) and uPA mice (9–11). These rodent models are highly useful for evaluating the efficacy of antiviral drugs and neutralizing monoclonal antibodies. In addition, a number of HCV transgenic mice have been developed. These enable direct characterization of the effects of expression of HCV genes on liver injury (5). These small animal models do not require costly facility for primates as mentioned later. While having a number of merits as mentioned above, these rodent models still have some limitations. For example the former models are not suitable for investigation of the pathogenesis of hepatitis C and the development of effective vaccine strategies, while in the latter models the proteins of interest are usually over-expressed as compared with natural HCV infection, and the integration site of the transgene may have an influence on the outcome of the study.

The chimpanzee model is the most straightforward since this animal can be experimentally infected with HCV. One third of HCV-inoculated chimpanzees develop chronic infection, while infection resolves in the remainder after an acute phase lasting 2–3 months, indicating that the chronicity rate in chimpanzees is somewhat lower than in humans (12). The chimpanzee model has been considered the primary choice for studying the relationship between the virus and host anti-viral immune responses, as well as for evaluating immunopathogenesis and the efficacy of prophylactic vaccination. However, irrespective of its benefits, many obstacles need to be overcome in order to use this model. For example in many countries it is illegal to employ the chimpanzee as an experimental animal, primarily due to ethical, (and secondly to financial), reasons. In fact, in 2004 the Dutch government decided to stop all research with chimpanzees at the biomedical primate research center in Rijswijk, Netherlands. As this was the only primate center in Europe where chimpanzees were used for biomedical research, this decision made chimpanzees unavailable as experimental animals in Europe. It is still possible to employ chimpanzees for biomedical research in some other countries, including the USA. However, the National Center for Research Resources of the National Institute of Health in the USA has recently decided not to continue to breed chimpanzees for research (13). It is estimated that the existing chimpanzees in the National Center for Research Resources will die within 30 years.

AIDS-related research has been one of the major purposes for using chimpanzees. However, due to the reasons mentioned above, as well as the endangered status of chimpanzees, nowadays AIDS scientists mainly make use of macaque monkeys infected with SIV as a non-human primate surrogate AIDS model (14, 15). The macaque/SIV model is useful since SIV is highly related to HIV-1, and induces AIDS-like diseases that are comparable to those of humans infected with HIV-1. Taking this into consideration, an alternative surrogate model which employs New World monkeys infected with GBV-B may be promising for future HCV/hepatitis C research.

GBV-B AS A NON-HUMAN PRIMATE SURROGATE MODEL OF HCV INFECTION

Among viruses so far known, GBV-B is the most closely related to HCV. However, due to a lack of epidemiological information as discussed below, GBV-B has been tentatively classified in the Hepacivirus genus of the Flavivirus family. Originally, Deinhardt *et al.* (16) found that some tamarins (genus *Saguinus*) developed hepatitis after inoculation with an inoculum obtained from a surgeon with the initials GB who had contracted hepatitis. After 11 passages in tamarins they obtained serum including GB agent(s), and were then able to achieve molecular cloning of GBV-A and GBV-B as flavivirus-like genomes (17) and to demonstrate GBV-B as an agent which could cause hepatitis in tamarins (18). Although it was unclear whether GBV-B originated from the GB inoculum or the tamarins themselves, later animal studies demonstrated that GBV-B is infectious for tamarins but not chimpanzees (19) and reciprocally that HCV is infectious for chimpanzees but not tamarins (20). These findings led to the retrospective conclusion that at least one of the tamarins employed for the *in vivo* passage study was persistently infected with GBV-B, and therefore GBV-B is probably a virus that originated in tamarins (20). However, GBV-B has not so far been isolated from additional tamarins, probably due to limited epidemiological analyses. Thus the natural host(s) and prevalence of GBV-B are yet to be determined.

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

Previous data have shown that a number of New World monkeys (parvorder *Platyrrhini*) including tamarins, the common marmoset (*Callithrix jacchus*) and the owl monkey (*Aotus trivirgatus*) are susceptible to GBV-B infection, as summarized in Table 1, although in tamarins peak concentrations of viruses in plasma are higher (10^7–10^{10} GE/ml) than in other monkeys (10^5–10^8 GE/ml) (21–29). In general, in any monkey species viremia

Table 1 Summary of characteristics of acute GBV-B infection in monkeys

Monkeys permissive of experimental infection	Tamarins (Genus *Saguinus*) Common marmoset (*Callithrix jacchus*) Owl monkey (*Aotus trivirgatus*)
Appearance of viremia	1–2 weeks post infection
Peak levels of viremia	Tamarins; 10^7–10^{10} GE/ml Marmoset and owl monkey; 10^5–10^8 GE/ml
Peak ALT levels	Approximately 200–500 IU/ml
Duration of viremia	2–3 months
Timing of seroconversion	A couple of weeks before clearance of viremia

persists for 2–3 months and is followed by clearance. GBV-B-infected monkeys with viremia usually develop self-resolving subacute hepatitis, as indicated by increases in the concentrations of serum enzymes such as ALT, gamma-glutamyltranspeptidase, and isocitrate dehydrogenase. Pathologically, degeneration and apoptosis of hepatocytes, as well as disruption and dilation of sinusoids, have been observed in the livers of GBV-B-infected tamarins with higher viremia and ALT activity (29). It is possible that GBV-B-specific CTL may cause the liver damage. However, a recent study reported that CTL are induced at a late stage of subacute GBV-B infection, and are inversely correlated with reduction in viremia (30). Since liver damage is usually found very early (1–2 weeks) after infection, when specific CTL are not observed, it is likely that viral replication in the hepatocytes leads directly to the early onset of cytopathic effects, while lower numbers of CTL may also contribute to cytotoxicity.

The clearance of viremia in the acute phase of GBV-B infection should require an effective antiviral immune response. In particular, in both GBV-B and HCV intrahepatic CTL appear to play a major role in viral clearance (30, 31). In addition, secondary GBV-B infection after clearance of the primary viremia induces a strong T cell response, leading to virtual absence of viremia, indicating that efficient memory is a key to protection from chronic viral infection (30, 32). In pre-immune chimpanzees antibody-mediated depletion of either CD4 or CD8 T lymphocytes affects their ability to control viral replication, resulting in prolonged viremia, demonstrating essential roles for both CD4 and CD8 memory in protection from viral persistence (33, 34).

On the other hand, the significance of humoral immunity in controlling GBV-B replication is still unclear. It is reasonable to assume that neutralizing antibodies also play important roles in the clearance of subacute viremia and protection from viral persistence. In the case of HCV, in one well characterized single-source outbreak of hepatitis C, viral clearance was associated with rapid induction of neutralizing antibodies in the early phase of infection, while chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in this phase. Patients with resolution of infection were shown to exhibit broader cross-neutralizing activity of antibodies in the early phase of infection (35). In one chronic HCV patient who was followed up for 30 years, it has also been shown that HCV continuously escaped the host's immune system by repeated mutational changes, resulting in loss of recognition of the HCV envelope glycoproteins by antibodies (36). The fact that the sequences of envelope glycoprotein and specificity of neutralizing antibody change over time suggests that neutralizing antibodies exert selective pressure on HCV evolution. Thus, although neutralizing antibodies (and/or CTL) are not necessarily capable of controlling chronic viral infection, frequent escape from the antibodies needs so called fitness cost, resulting in the partial suppression of viral loads. Indeed, HCV-infected patients with primary antibody deficiencies have accelerated rates of disease progression (37).

Although features of the subacute phase of GBV-B infection are similar to that of HCV, a major defect of GBV-B infection as a surrogate model for HCV is that it is difficult to chronically infect monkeys. While as many as 70% of humans with HCV infection become chronically infected, only approximately a third of chimpanzees do so (2, 12). By contrast, only a few cases regarding chronic GBV-B infection have been reported so far. The best example was a case of a tamarin persistently infected with GBV-B (24); the monkey exhibited acute mild hepatitis with viremia (peak level; $\sim 10^9$ GE/ml), which reduced to a set point level (less than 10^4 GE/ml) at 16 weeks post infection, followed by a gradual increase in viremia which reached $>10^7$ GE/ml at 112 weeks post infection, along with a significant ALT increase. However, the viremia suddenly declined thereafter and became undetectable, in association with a reduction in antibody titer, and subsequent *in vivo* passage of virus obtained from the tamarin failed to reproduce persistent infection in other tamarins (24). In addition, immunosuppression of a GBV-B-infected tamarin by FK506 treatment, or infection of GBV-B with deletion of poly(U) tract in the 3′ UTR, reportedly resulted in relatively long-term persistent infection of GBV-B for up to 46 and 90 weeks, respectively (23, 27). These results indicate that GBV-B may have the potential for establishing chronic infection.

Furthermore, our recent study has demonstrated that among four common marmosets infected with GBV-B derived from a molecular clone pGBB (21), two developed long-term chronic infection for up to three years, with recurrent viremia in which plasma viral RNA levels fluctuated between undetectable and 10^5 GE/ml, which is equivalent to the case of chimpanzees chronically infected with

HCV (Iwasaki *et al.*, manuscript in preparation). Notably, the induction of antiviral antibody response as measured by anti-Core and -NS3 antibodies was delayed in both cases, followed by a gradual increase, and then sustained high antibody titers. This was in contrast with an abrupt and transient increase at the end of periods of subacute viremia in marmosets and tamarins with viral clearance. Whether a delayed antibody response is associated with persistent GBV-B infection remains to be determined.

Taken together, these findings indicate the similarity between HCV and GBV-B in regard to their ability to induce chronic infection, and also shed light on the further potential of GBV-B as a surrogate model for HCV.

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

Although many questions are still to be addressed, accumulating evidence from extensive studies to date has greatly advanced the usefulness of the GBV-B as a surrogate model for HCV. The GBV-B model may be applicable for evaluating the feasibility and safety of anti-HCV vaccines employing novel viral vectors and gene therapy which creates RNA interference. For example, in a recent pilot study we showed that systemic administration of cationic liposome-encapsulated small interfering RNA to marmosets resulted in efficient regulation of GBV-B replication, indicating the usefulness of the surrogate model for proving the feasibility of RNA interference technology for future clinical application (38). This GBV-B model will also be helpful in identifying the virological and immunological factors which determine whether the outcome is acute resolving or chronic infection. While the GBV-B model appears to be valuable, development of an HCV/GBV-B chimeric virus would greatly expand the utility of the surrogate model, since it would enable us to directly evaluate antiviral vaccines and chemicals for HCV as a preclinical study. Rijnbrand *et al.* have reported that a chimeric GBV-B with 5′ untranslated region from HCV is infectious and causes hepatitis in tamarins (39). As recently demonstrated by Chevalier *et al.* (40), this will be a good model for evaluating the potential of small interfering RNA specific to HCV genome for future clinical application.

In regard to this, we may refer to an elegant precedent in the case of the macaques AIDS model. SIVmac is well known to efficiently infect, and result in the development of AIDS in macaques. Furthermore HIV-1, of which only 7% of the entire genome is derived from SIVmac, has been demonstrated to overcome the host range of authentic HIV-1, and to acquire the ability to productively infect macaque cells (41, 42). Instead of endangered chimpanzees, tamarins/marmosets which can be chronically infected with an HCV/GBV-B chimera (hopefully capable of inducing chronic hepatitis) should be the next generation of a promising non-human primate surrogate model for HCV infection, one which is similar to the macaques AIDS model. Whatever animals are used for pre-clinical study, it is important to keep in mind that results obtained from monkey models using either GBV-B or HCV/GBV-B chimera (as well as SIV or HIV/SIV chimera) may not necessarily be applicable to humans, because of potential differences in the molecular structure and/or mechanism by which antivirals and/or viral and host proteins function. Further characterization and understanding of the molecular biology and immunology of virus-host interactions will help in developing novel antiviral strategies.

REFERENCES

1. Choo Q.L., Kuo G., Weiner A.J., Overby L.R., Bradley D.W., Houghton M. (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244:** 359–62.
2. Chisari F.V. (2005) Unscrambling hepatitis C virus-host interactions. *Nature* **436:** 930–32.
3. Alter M.J. (2007) Epidemiology of hepatitis C virus infection. *World J Gastroenterol* **13:** 2436–441.
4. Feld J.J., Hoofnagle J.H. (2005) Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **436:** 967–72.
5. Kremsdorf D., Brezillon N. (2007) New animal models for hepatitis C viral infection and pathogenesis studies. *World J Gastroenterol* **13:** 2427–435.
6. Wu G.Y., Konishi M., Walton C.M., Olive D., Hayashi K., Wu C.H. (2005) A novel immunocompetent rat model of HCV infection and hepatitis. *Gastroenterology* **128:** 1416–423.
7. Ilan E., Arazi J., Nussbaum O., Nussbaum O., Zauberman A., Eren R., Lubin I., Neville L., Ben-Moshe O., Kischitzky A., Litchi A., Margalit I., Gopher J., Mounir S., Cai W., Daudi N., Eid A., Jurim O., Czerniak A., Galun E., Dagan S. (2002) The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. *J Infect Dis* **185:** 153–61.
8. Eren R., Landstein D., Terkieltaub D., Nussbaum O., Zauberman A., Ben-Porath J., Gopher J., Buchnick R., Kovjazin R., Rosenthal-Galili Z., Aviel S., Ilan E., Shoshany Y., Neville L., Waisman T., Ben-Moshe O., Kischitsky A., Foung S.K., Keck Z.Y., Pappo O., Eid A., Jurim O., Zamir G., Galun E., Dagan S. (2006) Preclinical evaluation of two neutralizing human monoclonal antibodies against hepatitis C virus (HCV): a potential treatment to prevent HCV reinfection in liver transplant patients. *J Virol* **80:** 2654–664.
9. Mercer D.F., Schiller D.E., Elliott J.F., Douglas D.N., Hao C., Rinfret A., Addison W.R., Fischer K.P., Churchill T.A., Lakey J.R., Tyrrell D.L., Kneteman N.M. (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7:** 927–33.
10. Meuleman P., Libbrecht L., De Vos R., de Hemptinne B., Gevaert K., Vandekerckhove J., Roskams T., Leroux-Roels G. (2005) Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* **41:** 847–56.
11. Kneteman N.M., Weiner A.J., O'Connell J., Collett M., Gao T., Aukerman L., Kovelsky R., Ni Z.J., Zhu Q., Hashash A., Kline J., Hsi B., Schiller D., Douglas D., Tyrrell D.L., Mercer D.F. (2006) Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* **43:** 1346–353.

12. Bassett S.E., Brasky K.M., Lanford R.E. (1998) Analysis of hepatitis C virus-inoculated chimpanzees reveals unexpected clinical profiles. *J Virol* **72:** 2589–599.
13. Cohen J. (2007) Animal studies: NIH to end chimp breeding for research. *Science* **316:** 1265.
14. Ambrose Z., KewalRamani V.N., Bieniasz P.D., Hatziioannou T. (2007) HIV/AIDS: in search of an animal model. *Trends Biotech* **25:** 333–37.
15. Lackner A.A., Veazey R.S. (2007) Current concepts in AIDS pathogenesis: insights from the SIV/macaque model. *Annu Rev Med* **58:** 461–76.
16. Deinhardt F., Holmes A.W., Capps R.B., Popper H. (1967) Studies on the transmission of human viral hepatitis to marmoset monkeys. I. Transmission of disease, serial passages, and description of liver lesions. *J Exp Med* **125:** 673–88.
17. Simons J.N., Pilot-Matias T.J., Leary T.P., Dawson G.J., Desai S.M., Schlauder G.G., Muerhoff A.S., Erker J.C., Buijk S.L., Chalmers M.L. (1995) Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc Natl Acad Sci USA* **92:** 3401–405.
18. Schaluder G.G., Dawson G.J., Simons J.N., Pilot-Matias T.J., Gutierrez R.A., Heynen C.A., Knigge M.F., Kurpiewski G.S., Buijk S.L., Leary T.P., Muerhoff A.S., Desai S.M., Mushahwar I.K. (1995) Molecular and serologic analysis in the transmission of the GB hepatitis agents. *J Med Virol* **46:** 81–90.
19. Bukh J., Apgar C.L., Govindarajan S., Purcell R.H. (2001) Host range studies of GB virus-B hepatitis agent, the closest relative of hepatitis C virus, in New World monkeys and chimpanzees. *J Med Virol* **65:** 694–97.
20. Beames B., Chavez D., Lanford R.E. (2001) GB virus B as a model for hepatitis C virus. *ILAR J* **42:** 152–60.
21. Bukh J., Apgar C.L., Yanagi M. (1999) Toward a surrogate model for hepatitis C virus: An infectious molecular clone of the GB virus-B hepatitis agent. *Virology* **262:** 470–78.
22. Sbardellati A., Scarselli E., Verschoor E., De Tomassi A., Lazzaro D., Traboni C. (2001) Generation of infectious and transmissible virions from a GB virus B full-length consensus clone in tamarins. *J Gen Virol* **82:** 2437–448.
23. Lanford R.E., Chavez D., Notvall L., Brasky K.M. (2003) Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. *Virology* **311:** 72–80.
24. Martin A., Bodola F., Sangar D.V., Goettge K., Popov V., Rijnbrand R., Lanford R.E., Lemon S.M. (2003) Chronic hepatitis associated with GB virus B persistence in a tamarin after intrahepatic inoculation of synthetic viral RNA. *Proc Natl Acad Sci USA* **100:** 9962–967.
25. Bright H., Carroll A.R., Watts P.A., Fenton R.J. (2004) Development of a GB virus B marmoset model and its validation with a novel series of hepatitis C virus NS3 protease inhibitors. *J Virol* **78:** 2062–071.
26. Jacob J.R., Lin K.C., Tennant B.C., Mansfield K.G. (2004) GB virus B infection of the common marmoset (*Callithrix jacchus*) and associated liver pathology. *J Gen Virol* **85:** 2525–533.
27. Nam J.H., Faulk K., Engle R.E., Govindarajan S., St Claire M., Bukh J. (2004) *In vivo* analysis of the 3′ untranslated region of GB virus B after *in vitro* mutagenesis of an infectious cDNA clone: persistent infection in a transfected tamarin. *J Virol* **78:** 9389–399.
28. Kyuregyan K.K., Poleschuk V.F., Zamyatina N.A., Isaeva O.V., Michailov M.I., Ross S., Bukh J., Roggendorf M., Viazov S. (2005) Acute GB virus B infection of marmosets is accompanied by mutations in the NS5A protein. *Virus Res* **114:** 154–57.
29. Ishii K., Iijima S., Kimura N., Lee Y.J., Ageyama N., Yagi S., Yamaguchi K., Maki N., Mori K., Yoshizaki S., Machida S., Suzuki T., Iwata N., Sata T., Terao K., Miyamura T., Akari H. (2007) GBV-B as a pleiotropic virus: distribution of GBV-B in extrahepatic tissues *in vivo*. *Microbes Infect* **9:** 515–21.
30. Woollard D.J., Haqshenas G., Dong X., Pratt B.F., Kent S.J., Gowans E.J. (2008) Virus-specific T-cell immunity correlates with control of GBV-B infection in marmosets. *J Virol* **82:** 3054–060.
31. Neumann-Haefelin C., Spangenberg H.C., Blum H.E., Thimme R. (2007) Host and viral factors contributing to CD8+ T cell failure in hepatitis C virus infection. *World J Gastroenterol* **13:** 4839–847.
32. Bukh J., Engle R.E., Govindarajan S., Purcell R.H. (2008) Immunity against the GBV-B hepatitis virus in tamarins can prevent productive infection following rechallenge and is long-lived. *J Med Virol* **80:** 87–94.
33. Shoukry N.H., Grakoui A., Houghton M., Chien D.Y., Ghrayeb J., Reimann K.A., Walker C.M. (2003) Memory CD8+ T cells are required for protection from persistent hepatitis C virus infection. *J Exp Med* **197:** 1645–655.
34. Grakoui A., Shoukry N.H., Woollard D.J., Han J.H., Hanson H.L., Ghrayeb J., Murthy K.K., Rice C.M., Walker C.M. (2003) HCV persistence and immune evasion in the absence of memory T cell help. *Science* **302:** 659–62.
35. Pestka J.M., Zeisel M.B., Bläser E., Schürmann P., Bartosch B., Cosset F.L., Patel A.H., Meisel H., Baumert J., Viazov S., Rispeter K., Blum H.E., Roggendorf M., Baumert T.F. (2007) Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci USA* **104:** 6025–030.
36. von Hahn T., Yoon J.C., Alter H., Rice C.M., Rehermann B., Balfe P., McKeating J.A. (2007) Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection *in vivo*. *Gastroenterology* **132:** 667–78.
37. Chapel H.M., Christie J.M., Peach V., Chapman R.W. (2001) Five-year follow-up of patients with primary antibody deficiencies following an outbreak of acute hepatitis C. *Clin Immunol* **99:** 320–24.
38. Yokota T., Iijima S., Kubodera T., Ishii K., Katakai Y., Ageyama N., Chen Y., Lee Y.J., Unno T., Nishina K., Iwasaki Y., Maki N., Mizusawa H., Akari H. (2007) Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C. *Biochem Biophys Res Commun* **361:** 294–300.
39. Rijnbrand R., Yang Y., Beales L., Bodola F., Goettge K., Cohen L., Lanford R.E., Lemon S.M., Martin A. (2005) A chimeric GB virus B with 5′ nontranslated RNA sequence from hepatitis C virus causes hepatitis in tamarins. *Hepatology* **41:** 986–94.
40. Chevalier C., Saulnier A., Benureau Y., Fléchet D., Delgrange D., Colbère-Garapin F., Wychowski C., Martin A. (2007) Inhibition of hepatitis C virus infection in cell culture by small interfering RNAs. *Mol Ther* **15:** 1452–462.
41. Kamada K., Igarashi T., Martin M.A., Khamsri B., Hatcho K., Yamashita T., Fujita M., Uchiyama T., Adachi A. (2006) Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. *Proc Natl Acad Sci USA* **103:** 16959–6964.
42. Hatziioannou T., Princiotta M., Piatak M. Jr, Yuan F., Zhang F., Lifson J.D., Bieniasz P.D. (2006) Generation of simian-tropic HIV-1 by restriction factor evasion. *Science* **314:** 95.

ORIGINAL

Amino acid alterations in Gag that confer the ability to grow in simian cells on HIV-1 are located at a narrow CA region

Tamiko Nagao*, Kazuki Hatcho*, Naoya Doi, Sachi Fujiwara, Akio Adachi, and Masako Nomaguchi

Department of Virology, Institute of Health Biosciences, the University of Tokushima Graduate School, Tokushima, Japan

Abstract : We previously generated a prototype monkey-tropic human immunodeficiency virus type 1 (HIV-1) designated NL-DT5R. This viral clone has a small region of simian immunodeficiency virus (SIV) within Gag capsid (CA) protein and also SIV Vif protein, but displays a poor growth phenotype in simian cells. To improve the growth potential of NL-DT5R, we have constructed a series of its *gag* variant viruses. Out of fourteen viral clones generated, five were infectious for simian HSC-F cells, and two of the infectious variants grew similarly with NL-DT5R. Taking their genome structures into consideration, our data here clearly show that a narrow CA region within the Gag protein, i.e., the domain around cyclophilin A (CypA)-binding loop, is critical for the growth ability of HIV-1 in simian cells. J. Med. Invest. 56 : 21-25, February, 2009

Keywords **:** *HIV-1, Gag, CA, CypA, TRIM5α*

INTRODUCTION

The narrow host range of human immunodeficiency virus type 1 (HIV-1) has been a major obstacle for establishing the animal model system for studies of viral replication and pathogenesis *in vivo* (1). HIV-1 infects and causes disease only in humans. To conquer this difficulty, we have recently generated a monkey-tropic HIV-1 designated NL-DT5R (1-3). Its genome contains a *gag* sequence encoding simian immunodeficiency virus from rhesus monkeys (SIVmac) capsid (CA) element, corresponding to the HIV-1 cyclophilin A (CypA)-binding loop, and the entire SIVmac *vif* gene (2). However, by subsequent studies, NL-DT5R was found to grow in simian cells more poorly both *in vitro* and *in vivo* than a standard SIVmac designated SIVmac 239, which induces the AIDS in monkeys and is widely used for model studies of HIV-1/AIDS. As a result of its biological property, NL-DT5R was unable to induce AIDS in the animals (3). Extensive attempts to improve the growth ability of NL-DT5R in simian cells through modification of Gag-CypA region and Vif by recombinant DNA techniques have been so far unsuccessful (4, 5). Another research group has also reported that a monkey cell-tropic HIV-1 designated stHIV-1 is successfully generated by genetic manipulation and virus adaptation in cells (6). It was suggested that three amino acid mutations in *gag* might be important for the ability of HIV-1 to grow in simian cells (6).

In this study, to potentiate growth capability in simian cells of a prototype monkey-tropic HIV-1 clone, various SIVmac sequences and mutations were introduced into the *gag* of NL-DT5R, and the resultant recombinants/mutants were monitored for

Received for publication November 17, 2008 ; accepted December 12, 2008.

*equal contribution

Address correspondence and reprint requests to Masako Nomaguchi, Department of Virology, Institute of Health Biosciences, the University of Tokushima Graduate School, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-88-633-7080.

their growth properties in simian HSC-F cells. We show here clearly that any viruses without the SIVmac CypA region do not grow in HSC-F cells, and demonstrate the importance of CypA region for the species tropism of HIV-1.

MATERIALS AND METHODS

Cells

A human monolayer cell line 293T (7) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum. A simian lymphocytic cell line HSC-F (8) was maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum.

Transfection

Sub-confluent 293T cells in 90 mm dishes were transfected with 20 μg of proviral clones by the calcium-phosphate co-precipitation method, and on day 2 post-transfection, cell-free virus samples for infection experiments were prepared as previously described (9).

Infection

HSC-F cells ($1\text{-}10\times10^6$) were infected with an equal amount of viruses [$1\text{-}10\times10^6$ reverse transcriptase (RT) units] prepared from transfected 293T cells, and monitored for RT production at intervals as previously described (9).

RT assay

Virus production in transfected 293T cells and viral growth property in infected HSC-F cells were determined by monitoring RT activity of the culture supernatants. RT assay using ^{32}P-dTTP has been previously described (10).

DNA constructs

Full-length infectious molecular clones of HIV-1 and SIVmac, designated pNL4-3 (9) and pMA239 (11), respectively, have been previously described. A monkey-tropic infectious DNA clone of HIV-1 designated NL-DT5R has been previously described (2). An infectious clone designated NL-DT5R/4-3 is a derivative of NL-DT5R carrying CA of NL4-3, and grows more poorly than NL-DT5R in simian HSC-F cells (4). Various *gag* recombinants and mutants of NL-DT5R were constructed by the QuikChange site-directed mutagenesisi kit (Stratagene, La Jolla, CA, USA) as previously described (12).

RESULTS AND DISCUSSION

Our previous results have indicated that modifications in *gag* (encoding CypA-binding loop) and *vif* genes of NL-DT5R do not improve its growth ability in simian cells (4, 5). We, therefore, constructed new *gag* recombinants between NL-DT5R and MA239 of SIVmac to obtain HIV-1 type viruses which would be more replication-competent than NL-DT5R in simian cells. We previously reported that recombinants between HIV-1 NL4-3 and SIVmac MA239 within five regions of CA-p2 are viable and infectious for human M8166 cells (12). Based on these results, recombinants in this study were carefully designed as shown in Fig. 1 and Table 1.

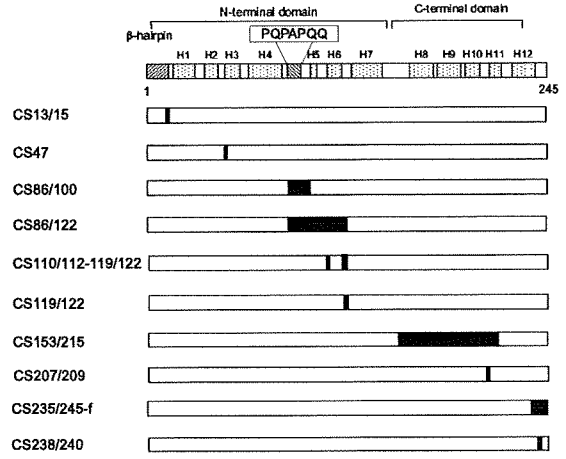

Figure 1 Structure of Gag CA-p2 region of various recombinant clones derived from a monkey-tropic HIV-1 NL-DT5R. Sequences of SIVmac239 are indicated by black areas (for details, see Table 1). Structural domains of HIV-1 Gag CA-p2 (19, 20) are shown at the top. Amino acid sequence PQPAPQQ represents the region of SIVmac239 corresponding to the CypA-binding loop of HIV-1 CA (PVHAGPIAP). H, α-helix.

To examine growth potentials in simian cells of new viral clones (10 clones in Fig. 1 and Table 1), they were transfected into 293T cells, and cell-free virus samples were prepared on day 2 post-transfection. Viruses obtained were then inoculated into HSC-F cells, and viral growth was monitored by RT assay. Representative results are shown in Fig. 2 and all the data are summarized in Table 1. As shown in Fig. 2, NL-DT5R readily established a spreading infection albeit grew more poorly than SIVmac239. The peak infection day for SIVmac239 and NL-DT 5R were 6 and 18, respectively. In contrast to NL-DT5R, eight of ten recombinants (Fig. 1 and Table 1) were not infectious at all for HSC-F cells (Fig. 2

Table 1. Amino acid sequences of SIVmac inserted into CA-p2 region of NL-DT5R, a derivative of HIV-1 NL4-3

Recombinants	Amino acid sequences of MA239 inserted[1]	Growth in HSC-F cells
CS13/15	(13) PLS (15)	(-)
CS47	(47) T (47)	(+)
CS86/100	(86) PQPAPQQGQLREPSGSD (100)	(+)
CS86/122	(86) PQPAPQQGQLREPSGSDIAGTTSSVDEQIQWMYRQQNPI (122)	(-)
CS110/112-119/122	(110) EQI (112) (119) YRQQN (122)	(-)
CS119/122	(119) YRQQN (122)	(-)
CS153/215	(153) QGPKEPFQSYVDRFYKSLRAEQTDAA VKNWMTQTLLIQNANPDCKLVLKGLGVNPTL EEMLTA (215)	(-)
CS207/209	(207) PTL (209)	(-)
CS235/245-f	(235) LKEALAPVPIPFAA (245)	(-)
CS238/240	(238) APV (240)	(-)

[1]The first and last amino acid numbers of HIV-1 NL4-3 CA-p2 replaced with sequences of SIVmac MA239 are indicated in parentheses. GenBank accession numbers for pNL4-3 and pMA239 are AF324493 and M33262, respectively.

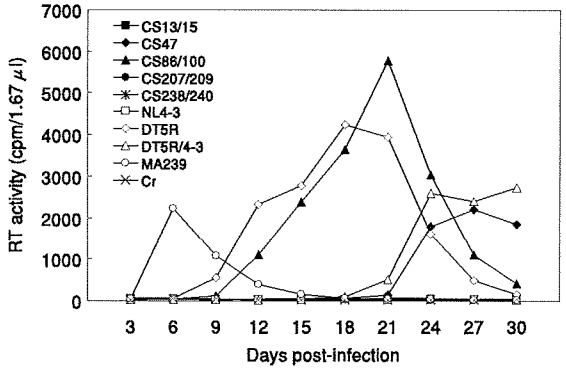

Figure 2 Growth kinetics in HSC-F cells of Gag CA-p2 recombinants. Input cell-free viral samples were prepared from 293T cells transfected with the clones indicated, and an equivalent RT units were inoculated into HSC-F cells. Viral growth was monitored at intervals by RT activity in the culture supernatants. As a negative control (Cr), pUC19 was used.

and Table 1). Even the two infectious recombinants (CS47 and CS86/100) did not grow better than NL-DT5R. In particular, CS47, which actually is a point mutant of NL-DT5R, grew much more poorly than NL-DT5R similarly with DT5R/4-3 carrying the HIV-1 type CA (4).

Recently, Hatziioannou, *et al.* reported that their monkey cell-tropic HIV-1 designated stHIV-1 differs from the parental HIV-1 clone in only minor ways other than the genetically engineered CA and Vif substitutions (6). They found three coding mutations in *gag* (K110I, A209V and P371L) and six silent mutations in *gag* and *pol* (6). Based on this report, we introduced the three nonsynonymous mutations into NL-DT5R to have viral clones that would grow better than NL-DT5R in simian cells (Fig. 3A).

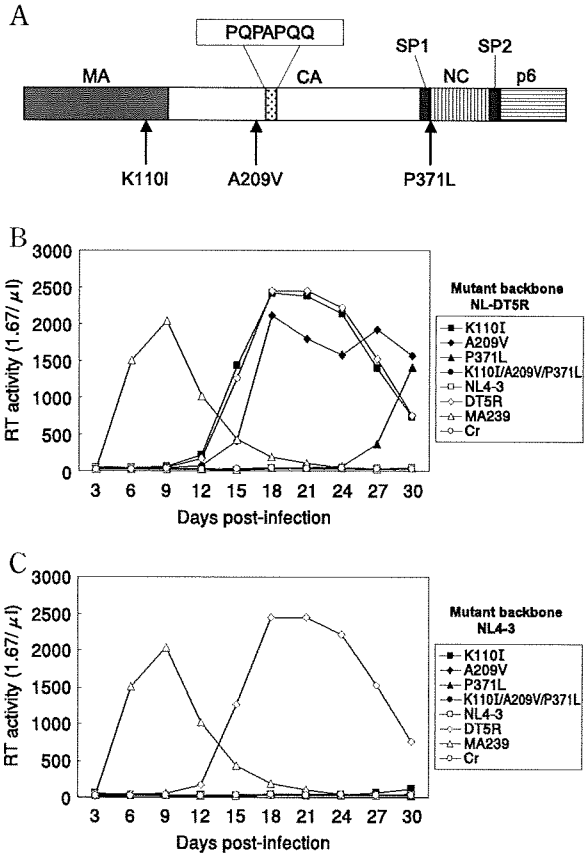

Figure 3 Growth kinetics in HSC-F cells of Gag mutants. (A) Locations of mutations in Gag. Mutations at the indicated positions are introduced into NL-DT5R and NL4-3 (mutants in panels B and C, respectively). Amino acid sequence PQPAPQQ represents the region of SIVmac239 corresponding to the CypA-binding loop of HIV-1 CA (PVHAGPIAP). MA, Gag matrix protein; NC, Gag nucleocapsid protein; SP1 and SP2, Gag spacer proteins 1 (p2) and 2 (p1), respectively. (B) and (C) Growth kinetics of various mutants clones in HSC-F cells were determined as described in the legend to Fig. 2. Results obtained in one experiment are shown separately in (B) and (C) for clarity.

Proviral clones containing each and all the three mutations were constructed for this purpose. Cell-free virus samples derived from these clones were prepared as described above, and inoculated into HSC-F cells. As shown in Fig. 3B, while control viruses SIVmac239 and NL-DT5R grew in a consistent manner, two (A209V and P371L) out of the four mutants displayed retarded growth phenotype. Although K 110I grew similarly well with NL-DT5R, the triple mutant K110I/A209V/P371L did not at all. To know whether the three mutations can confer the ability to grow in simian cells on HIV-1 type CA, four mutant clones in the context of NL4-3 were constructed as above and monitored for their growth potentials in HSC-F cells. As clearly seen Fig. 3C, the growth of the four mutants were undetectable or negligible during the observation period, indicating the reported coding mutations in *gag* (6) are biologically inactive or meaningless in simian cells.

In this study, we have constructed ten new recombinants between the NL-DT5R clone of monkey-tropic HIV-1 and the standard pathogenic clone SIVmac239, and four new *gag* mutants of NL-DT5R in anticipation of improved growth potentials in simian cells. In addition to the changes relative to HIV-1 (Gag-CypA region and Vif) which the prototype monkey-tropic virus NL-DT5R carries, the new viral clones contain alterations in scattered regions of Gag-CA and in the other parts of Gag. Our results here clearly showed that region(s) around the CypA-binding loop of HIV-1 is critical for viral growth ability in simian cells. Cellular TRIM5α is well known to interact with this region and restrict retroviral replication (13-18). In this regard, it is interesting to note that NL-DT5R does not overcome the barrier imposed by TRIM5α (4). Virological studies to evade the TRIM5α restriction are in progress in our laboratory.

ACKNOWLEDGMENTS

We thank Ms. Kazuko Yoshida for her excellent editorial assistance. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (19041051) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to A. A.), and by a Health Sciences Research Grant [Research on HIV/AIDS (2007-2009)] from the Ministry of Health, Labour and Welfare of Japan (to A. A.).

REFERENCES

1. Nomaguchi M, Doi N, Kamada K, Adachi A : Species barrier of HIV-1 and its jumping by virus engineering. Rev Med Virol 18 : 261-275, 2008
2. Kamada K, Igarashi T, Martin MA, Khamsri B, Hatcho K, Yamashita T, Fujita M, Uchiyama T, Adachi A : Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells. Proc Natl Acad Sci USA 103 : 16959-16964, 2006
3. Igarashi T, Iyengar R, Byrum RA, Buckler-White A, Dewar RL, Buckler CE, Lane HC, Kamada K, Adachi A, Martin MA : An HIV-1 derivative with 7% SIV genetic content is able to establish infections in pig-tailed macaques. J Virol 81 : 11549-11552, 2007
4. Kamada K, Yamashita T, Hatcho K, Adachi A, Nomaguchi M : Evasion from CypA- and APOBEC-mediated restrictions is insufficient for HIV-1 to efficienctly grow in simian cells. Microbes Infect, (in press)
5. Hatcho K, Kamada K, Yamashita T, Adachi A, Nomaguchi M : Replication potentials of vif variant viruses generated from monkey cell-tropic HIV-1 derivative clones NL-DT5/NL-DT5R. Microbes Infect 10 : 1218-1222, 2008
6. Hatziioannou T, Princiotta M, Piatak Jr M, Yuan F, Zhang F, Lifson JD, Bieniasz PD : Generation of simian-tropic HIV-1 by restriction factor evasion. Science 314 : 95, 2006
7. Lebkowski JS, Clancy S, Calos MP : Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression. Nature 12 : 169-171, 1985
8. Akari H, Fukumori T, Iida S, Adachi A : Induction of apoptosis in Herpesvirus saimiri-immortalized T lymphocytes by blocking interaction of CD28 with CD80/CD86. Biochem Biophys Res Commun 263 : 352-356, 1999
9. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, Rabson A, Martin MA : Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59 : 284-291, 1986
10. Willey RL, Smith DH, Lasky LA, Theodore TS, Earl PL, Moss B, Capon DJ, Martin MA : *in vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J Virol 62 :

139-147,1988
11. Shibata R, Kawamura M, Sakai H, Hayami M, Ishimoto A, Adachi A : Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J Virol 65 : 3514-3520, 1991
12. Kamada K, Yoshida A, Khamsri B, Piroozmand A, Yamashita T, Uchiyama T, Fujita M, Adachi A : Construction of *gag*-chimeric viruses between HIV-1 and SIVmac that are capable of productive multi-cycle infection. Microbes Infect 8 : 1075-1081, 2006
13. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J : The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427 : 848-853, 2004
14. Yap MW, Nisole S, Lynch C, Stoye JP : Trim5 alpha protein restricts both HIV-1 and murine leukemia virus. Proc Natl Acad Sci USA 101 : 10786-10792, 2004
15. Perron MJ, Stremlau M, Song B, Ulm W, Mulligan RC, Sodroski J : TRIM5alpha mediates the postentry block to N-tropic murine leukemia viruses in human cells. Proc Natl Acad Sci USA 101 : 11827-11832, 2004
16. Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J : Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. J Virol 79 : 3930-3937, 2005
17. Luban J : Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. J Virol 81 : 1054-1061, 2007
18. Takeuchi H, Matano T : Host factors involved in resistance to retroviral infection. Microbiol Immunol 52 : 318-325, 2008
19. Gamble TR, Vajdos FF, Yoo S, Worthylake DK, Houseweart M, Sundquist WI, Hill CP : Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell 87 : 1285-1294, 1996
20. Gamble TR, Yoo S, Vajdos FF, von Schwedler UK, Worthylake DK, Wang H, McCutcheon JP, Sundquist WI, Hill CP : Structure of the carboxyl^terminal dimerization domain of the HIV-1 capsid protein. Science 278 : 849-853, 1997

ELSEVIER

INSTITUT PASTEUR

Microbes and Infection 11 (2009) 164–171

www.elsevier.com/locate/micinf

Original article

Evasion from CypA- and APOBEC-mediated restrictions is insufficient for HIV-1 to efficiently grow in simian cells

Kazuya Kamada, Tomoki Yamashita, Kazuki Hatcho, Akio Adachi, Masako Nomaguchi*

Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan

Received 20 August 2008; accepted 5 November 2008
Available online 19 November 2008

Abstract

We have recently generated a monkey cell-tropic virus termed NL-DT5R from an HIV-1 NL4-3 clone and demonstrated that both cyclophilin A (CypA)-binding loop in Gag-capsid (CA) and Vif are responsible for the species-restriction of HIV-1. In this study, we constructed 16 CypA-binding loop mutants from the HIV-1-derivative NL-DT5R, and analyzed them biologically and biochemically. The mutants displayed various multi-cycle infection potencies in cynomolgus monkey (CyM) HSC-F cells, but none of them grew significantly better than NL-DT5R. Consistently, any of the HIV-1 variants examined here did not effectively counter CyM TRIM5α as judged by single-cycle infectivity assays. Assessment of their single-cycle infectivity in simian and CyM TRIM5α-expressing feline cells in the presence of cyclosporin A (CsA) showed that intervention of CypA–CA interaction did not restore full NL-DT5R infectivity, while CsA increased infectivity of DT5R/4-3 carrying the sequence of NL4-3 CypA-binding loop up to the NL-DT5R level. Almost similar data were obtained in the experiments utilizing CypA-targeting siRNA. Together with our previous results regarding NL-DT5R, these data suggested that evasion from CypA- and APOBEC-mediated restrictions is still insufficient for HIV-1 to completely overcome the species barrier.

Keywords: HIV-1; CypA; TRIM5α; APOBEC3; Species-tropism

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) possesses a tightly restricted species-tropism, and infects only humans and chimpanzees [1]. HIV-1 replication in simian cells is restricted at a post-entry step occurring before or during reverse transcription, but not the replication of simian immunodeficiency virus from rhesus monkeys (SIVmac). As cellular factors responsible for the growth-inhibition of HIV-1 in simian cells, APOBEC3 [2], cyclophilin A (CypA)[3–6], and TRIM5α [6,7] have been identified. Recently, based on these findings, we have successfully constructed a monkey cell-tropic (mt) HIV-1-derivative designated NL-ScaVR that grows considerably in simian cells by substituting the CypA-binding loop in Gag and the APOBEC-antagonizing Vif with homologues of SIVmac [8]. However, even its simian-cell-adapted version designated NL-DT5R still grew more poorly than a standard virus SIVmac239 pathogenic for macaque monkeys [8]. Of note, NL-ScaVR and NL-DT5R countered almost completely against anti-HIV-1 effects of APOBEC3G/F (reference [8] and our unpublished data). Therefore, it was reasonable to assume that the weak growth potential in simian cells of the prototype mt HIV-1s is ascribed to a viral protein(s) other than Vif. Together with the data obtained for another simian tropic HIV-1 (stHIV-1) recently reported [9], it was quite possible that some region(s) in Gag-capsid protein (CA) other than the CypA-binding loop contains sequence responsible for the poor growth phenotype of NL-ScaVR and NL-DT5R. The stHIV-1 having the entire Gag-CA of SIVmac grew similarly well with SIVmac239 in simian cells [9], while NL-ScaVR and NL-DT5R carrying a portion of SIVmac CA did inefficiently as described above.

CypA catalyzes cis-trans isomerization of peptidyl–prolyl bonds in target proteins [10,11]. It directly binds to HIV-1

* Corresponding author. Tel.: +81 88 633 9232; fax: +81 88 633 7080.
E-mail address: nomaguchi@basic.med.tokushima-u.ac.jp (M. Nomaguchi).

1286-4579/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2008.11.002

Gag-CA through proline 90 and adjacent residues on the surface loop between helices IV and V [12], and this association leads to CypA recruitment into nascent HIV-1 virions [13,14]. The interaction can be blocked by mutations around amino acid (aa) 90 and by an undecapeptide cyclosporin A (CsA), which competitively interferes with the interaction. The disruption of CypA–CA interaction decreases HIV-1 infectivity in human cells [15–18], but conversely enhances it in simian cells [3,4,8]. TRIM5α has been identified in cells of the rhesus macaque as a post-entry cellular restriction factor blocking HIV-1 infection [7]. Whereas human TRIM5α does not inhibit any primate lentiviruses, rhesus monkey TRIM5α suppresses HIV-1 replication but not SIVmac. TRIM5α forms trimer, interacts with hexametric CAs, and restricts HIV-1 in a sequence- or structure-dependent manner currently unidentified [7,19]. Proteasome inhibitors facilitate a reverse transcription (RT) of HIV-1 genome under monkey TRIM5α restriction but the late replication stage is still restricted, suggesting that proteasome-degradation machinery may be a part of important steps for TRIM5α-mediated restriction [20–22]. Nonetheless, the detailed mechanism of TRIM5α-mediated restriction is still uncertain. In total, although both CypA and TRIM5α target HIV-1 CA, the relationship between CypA and TRIM5α for species-specific HIV-1 restriction remains to be controversial [6,23].

In this study, to better understand the anti-viral activity of simian CypA and TRIM5α functionally associated with the HIV-1 Gag-CA, we have constructed a series of CypA-binding loop mutants in a genetic backbone of NL-DT5R, and analyzed their biological and biochemical properties in various cell lines. We demonstrate here that the HIV-1 replication in simian cells is also restricted via a viral sequence(s) in CA distinct from the CypA-binding region.

2. Materials and methods

2.1. Plasmids

An mt HIV-1 clone designated pNL-DT5R [8] was used as wild-type (WT) in this study. Mutations in CypA-binding loop in CA were introduced by the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described [8] using pNL-DT5R as template. For single-cycle infectivity assays, a GFP-encoding gene was inserted into the *nef* of proviral clones.

2.2. Cell culture

Monolayer cell lines such as 293T (human embryo kidney), LLC-MKII (rhesus monkey kidney), Vero (African green monkey (agm) kidney), and CRFK (feline kidney) cells were cultured in MEM medium supplemented with 10% heat-inactivated fetal bovine serum. A lymphocyte cell line of cynomolgus macaque monkey (CyM) designated HSC-F [8] was maintained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum.

2.3. Transfection, infection and RT assay

For determination of viral growth kinetics, test proviral clones were transfected into 293T cells by the calcium phosphate co-precipitation method [8], and on day 2 post-transfection, culture supernatants were collected and stored at −80 °C until use. Virion-associated RT activity was measured as described previously [24]. HSC-F cells (1×10^7) were infected with an equal amount of viruses (1×10^7 RT units). Viral growth kinetics was determined by RT production in the culture supernatants.

2.4. Monitoring CypA incorporation into virions

Culture supernatants from transfected 293T cells were passed through 0.25-μm filter, and then pelleted through 20% sucrose by ultra-centrifugation [25]. Pelleted virus fractions were lysed with CHAPS-based lysis buffer, and monitored for CypA by immunoblotting with an anti-CypA antibody (BIOMOL, Plymouth Meeting, PA).

2.5. Generation of expression vectors and cells for species-specific TRIM5α

Species-specific TRIM5α cDNA was isolated from HeLa (human) and HSC-F (CyM) cells. Total RNA obtained from cells was reverse-transcribed using oligo-dT_{12-18} as primer, and the resultant cDNA was subjected to PCR-amplification employing the sense (TRIM5αEco-5′: G*GAATTC*AGCTACTATGGCTT CTGGAATCCTG) and anti-sense (TRIM5αSacKp-3′: TTGGTACC*CCGCGG*TCACTTATCGTCATCATC) primers. The reactions were heated at: 95 °C for 5 min for 1 cycle; 95 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min for 10 cycles; 95 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min for 25 cycles, and 72 °C for 10 min for 1 cycle. The amplified fragments were cloned into pcDNA3.1-FLAG (Invitrogen, Carlsbad, CA) and termed pT5αHu-FLAG and pT5αCyM-FLAG for expression vectors of human and CyM TRIM5α, respectively. The sequences of human and CyM TRIM5α were deposited in the GenBank database (accession nos., AB362928 and AB362929 for TRIM5α from human and CyM, respectively). Expression level of TRIM5α in transfected 293T cells was monitored by immunoblotting using an anti-FLAG antibody (SIGMA–ALDRICH, St. Louis, MO). To obtain TRIM5α-expressing CRFK cells, linearlized plasmid DNAs were transfected into naïve CRFK cells, and TRIM5α-positive cells were cloned by G418 selection/limiting dilution method. Expression level of TRIM5α in each cell clone was checked by immunoblotting as above, and the best cell clones were used in this study.

2.6. Single-cycle infectivity assays

To determine single-cycle infectivity of test mutants, VSV-pseudotyped viruses were prepared from 293T cells co-transfected with an *env*-deficient GFP-reporter proviral clone and pCMV-G [26], which expresses VSV-G protein for pseudotyping, at a ratio of 9:1. Viruses released into the culture medium (normalized by RT activity) were added directly or as 3-fold serial dilutions to 293T,