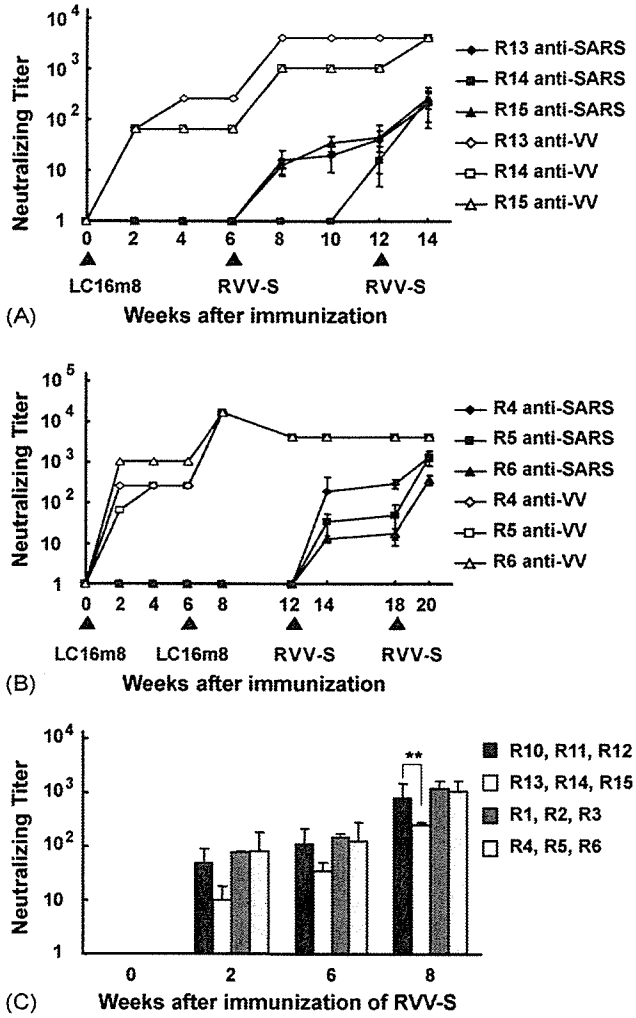

Fig. 4. Induction of NT antibodies against SARS-CoV in rabbits pre-immunized with LC16m8. (A) Three rabbits (R13–15) were immunized with 10^7 PFU of LC16m8 on day 0, and then immunized with 10^7 PFU of RVV-S at 6 and 12 weeks. (B) Three rabbits (R4–6) were immunized with 10^8 PFU of LC16m8 at 0 and 6 weeks, and then immunized with 10^8 PFU of RVV-S at 12 and 18 weeks. Immunized rabbit sera were analyzed by *in vitro* NT assay against SARS-CoV (closed symbols) or vaccinia virus (open symbols). Each type of symbol indicates one and the same individual, and the schedule of immunization with RVV-S or LC16m8 is indicated by arrowheads. (C) Comparison of NT antibodies against SARS-CoV induced by RVV-S in VV-immunized or naïve rabbits. RVV-S (10^7 or 10^8 PFU) was injected into rabbits at 0 and 6 weeks in the presence (10^7 PFU, R13–15; 10^8 PFU, R4–6) or absence (10^7 PFU, R10–12; 10^8 PFU, R1–3) of pre-immunization with an equal titer of LC16m8. Immunized rabbit sera were analyzed by *in vitro* NT assay against SARS-CoV. $^{**}p < 0.01$.

of LC16m8. Since the NT titer against VV induced by 10^7 PFU of LC16m8 was comparable to that in people vaccinated with the smallpox vaccine [32], RVV-S may induce NT antibodies against SARS-CoV in such people. On the other hand, 10^8 PFU of RVV-S also induced NT antibodies against SARS-CoV in rabbits that had an extremely high titer of NT antibodies against VV due to two pre-immunizations with 10^8 PFU of LC16m8. Furthermore, there was no difference in the NT titer against SARS-CoV induced by RVV-S between naïve rabbits and LC16m8 pre-immunized rabbits. The immune response against a protein encoded by a transduced gene may be influenced by the amount of antigen expression, the antigenicity of the protein encoded by a transduced gene, the route of immunization and viral proliferation in the host, and thus further analysis is required to resolve the precise mechanism involved. Furthermore, the vaccine effect of RVV-S still needs to be confirmed in humans pre-immunized with the smallpox vaccine.

In the present study, immunization with RVV-S manifested a vaccine effect against SARS-CoV, in spite of the pre-existing NT antibodies against VV. This finding indicates that an RVV vaccine derived from LC16m8 can be used for people previously immunized with the smallpox vaccine. Furthermore, this RVV vaccine could be repeatedly used against various microbes, such as influenza virus, by alteration of the protein encoded by the transduced gene. Therefore, the use of an RVV vaccine generated from LC16m8 is a promising vaccine strategy against various infectious diseases.

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References

[1] Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 2003;348(20):1967–76.
[2] Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 2003;348(20):1953–66.
[3] Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, et al. Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 2003;361(9366):1319–25.
[4] Gallagher TM, Buchmeier MJ. Coronavirus spike proteins in viral entry and pathogenesis. Virology 2001;279(2):371–4.
[5] Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, et al. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 2003;426(6965):450–4.
[6] Spaan W, Cavanagh D, Horzinek MC. Coronaviruses: structure and genome expression. J Gen Virol 1988;69(Pt. 12):2939–52.
[7] Nie Y, Wang G, Shi X, Zhang H, Qiu Y, He Z, et al. Neutralizing antibodies in patients with severe acute respiratory syndrome-associated coronavirus infection. J Infect Dis 2004;190(6):1119–26.

[8] Wang YD, Sin WY, Xu GB, Yang HH, Wong TY, Pang XW, et al. T-cell epitopes in severe acute respiratory syndrome (SARS) coronavirus spike protein elicit a specific T-cell immune response in patients who recover from SARS. J Virol 2004;78(11):5612–8.
[9] Normile D, Enserink M. SARS in China. Tracking the roots of a killer. Science 2003;301(5631):297–9.
[10] Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science 2005;310(5748):676–9.
[11] Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci USA 2005;102(39):14040–5.
[12] Haagmans BL, Kuiken T, Martina BE, Fouchier RA, Rimmelzwaan GF, van Amerongen G, et al. Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. Nat Med 2004;10(3):290–3.
[13] Tang L, Zhu Q, Qin E, Yu M, Ding Z, Shi H, et al. Inactivated SARS-CoV vaccine prepared from whole virus induces a high level of neutralizing antibodies in BALB/c mice. DNA Cell Biol 2004;23(6):391–4.
[14] Takasuka N, Fujii H, Takahashi Y, Kasai M, Morikawa S, Itamura S, et al. A subcutaneously injected UV-inactivated SARS coronavirus vaccine elicits systemic humoral immunity in mice. Int Immunol 2004;16(10):1423–30.
[15] Yang ZY, Kong WP, Huang Y, Roberts A, Murphy BR, Subbarao K, et al. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature 2004;428(6982):561–4.
[16] Kim TW, Lee JH, Hung CF, Peng S, Roden R, Wang MC, et al. Generation and characterization of DNA vaccines targeting the nucleocapsid protein of severe acute respiratory syndrome coronavirus. J Virol 2004;78(9):4638–45.
[17] Gao W, Tamin A, Soloff A, D'Aiuto L, Nwanegbo E, Robbins PD, et al. Effects of a SARS-associated coronavirus vaccine in monkeys. Lancet 2003;362(9399):1895–6.
[18] Bisht H, Roberts A, Vogel L, Bukreyev A, Collins PL, Murphy BR, et al. Severe acute respiratory syndrome coronavirus spike protein expressed by attenuated vaccinia virus protectively immunizes mice. Proc Natl Acad Sci USA 2004;101(17):6641–6.
[19] Buchholz UJ, Bukreyev A, Yang L, Lamirande EW, Murphy BR, Subbarao K, et al. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. Proc Natl Acad Sci USA 2004;101(26):9804–9.
[20] Moss B. Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proc Natl Acad Sci USA 1996;93(21):11341–8.
[21] Sugimoto M, Yasuda A, Miki K, Morita M, Suzuki K, Uchida N, et al. Gene structures of low-neurovirulent vaccinia virus LC16m0, LC16m8, and their Lister original (LO) strains. Microbiol Immunol 1985;29(5):421–8.
[22] Yamaguchi M, Kimura M, Hirayama M. Report of the National Smallpox Vaccination Research Committee: study of side effects, complications and their treatments. Clin Virol 1975;3:269–78.
[23] Jin NY, Funahashi S, Shida H. Constructions of vaccinia virus A-type inclusion body protein, tandemly repeated mutant 7.5 kDa protein, and hemagglutinin gene promoters support high levels of expression. Arch Virol 1994;138(3–4):315–30.
[24] Funahashi S, Itamura S, Iinuma H, Nerome K, Sugimoto M, Shida H. Increased expression in vivo and in vitro of foreign genes directed by A-type inclusion body hybrid promoters in recombinant vaccinia viruses. J Virol 1991;65(10):5584–8.
[25] Hong TC, Mai QL, Cuong DV, Parida M, Minekawa H, Notomi T, et al. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol 2004;42(5):1956–61.
[26] Shida H, Tochikura T, Sato T, Konno T, Hirayoshi K, Seki M, et al. Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection. Embo J 1987;6(11):3379–84.
[27] Keng CT, Zhang A, Shen S, Lip KM, Fielding BC, Tan TH, et al. Amino acids 1055 to 1192 in the S2 region of severe acute respiratory syndrome coronavirus S protein induce neutralizing antibodies: implications for the development of vaccines and antiviral agents. J Virol 2005;79(6):3289–96.
[28] Chen Z, Zhang L, Qin C, Ba L, Yi CE, Zhang F, et al. Recombinant modified vaccinia virus Ankara expressing the spike glycoprotein of severe acute respiratory syndrome coronavirus induces protective neutralizing antibodies primarily targeting the receptor binding region. J Virol 2005;79(5):2678–88.
[29] Subbarao K, McAuliffe J, Vogel L, Fahle G, Fischer S, Tatti K, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J Virol 2004;78(7):3572–7.
[30] Czub M, Weingartl H, Czub S, He R, Cao J. Evaluation of modified vaccinia virus Ankara based recombinant SARS vaccine in ferrets. Vaccine 2005;23(17–18):2273–9.
[31] Olsen CW, Corapi WV, Ngichabe CK, Baines JD, Scott FW. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J Virol 1992;66(2):956–65.
[32] Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, et al. Duration of antiviral immunity after smallpox vaccination. Nat Med 2003;9(9):1131–7.

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Original article

CRM1, an RNA transporter, is a major species-specific restriction factor of human T cell leukemia virus type 1 (HTLV-1) in rat cells

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Abstract

Rat ortholog of human CRM1 has been found to be responsible for the poor activity of viral Rex protein, which is essential for RNA export of human T cell leukemia virus type 1 (HTLV-1). Here, we examined the species-specific barrier of HTLV-1 by establishing rat cell lines, including both adherent and $CD4^+$ T cells, which express human CRM1 at physiological levels. We demonstrated that expression of human CRM1 in rat cells is not harmful to cell growth and is sufficient to restore the synthesis of the viral structural proteins, Gag and Env, at levels similar to those in human cells. Gag precursor proteins were efficiently processed to the mature forms in rat cells and released into the culture medium as sedimentable viral particles. An HTLV-1 pseudovirus infection system suggested that the released virus particles are fully infectious. Our newly developed reporter cell system revealed that Env proteins produced in rat cells are fully fusogenic, which is the basis for cell–cell HTLV-1 infection. Moreover, we show that the early steps in infection, from post-entry uncoating to integration into the host chromosomes, occur efficiently in rat cells. These results, in conjunction with reports describing efficient entry of HTLV-1 into rat cells, may indicate that HTLV-1 is unique in that its major species-specific barrier is determined by CRM1 at a viral RNA export step. These observations will enable us to construct a transgenic rat model expressing human CRM1 that is sensitive to HTLV-1 infection.

Keywords: Human T cell leukemia virus type 1; CRM1; Species barrier

1. Introduction

The human T lymphotropic virus type 1 (HTLV-1) is a type C retrovirus, whose etiological role in adult T cell leukemia (ATL) and tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) has been well established [1,2]. In its small genome, the virus encodes not only viral structural and enzymatic proteins, but also several regulatory proteins, using alternative splicing and alternate codon usage. The actions of these regulatory proteins are critical for the virus life cycle. Tax, identified as a viral onco-protein, activates viral and cellular transcription to promote T-cell growth and ultimately, malignant transformation [3,4]. Rex, which is a nucleocytoplasmic shuttling protein, mediates nuclear export of unspliced or incompletely spliced viral mRNAs, which encode the viral structural and enzymatic proteins, Gag, Pol and Env [5–7]. In the nucleus, Rex interacts with the Rex response element (RxRE), which is located in the 3′ long terminal repeat (LTR) of the viral mRNA [8,9]. To form an export complex, Rex binds to human CRM1 (hCRM1), a member of the karyopherin family of nuclear transport receptors, in cooperation with a GTP-bound form of the small G protein Ran (Ran-GTP) and RanBP3 [10–12]. Moreover, multimerization of Rex on the viral RNA is critical for its full biological activity [13], since the Rex multimer may shield the viral RNA from being spliced or down-regulated [14]. Previously, we reported that hCRM1 is dually involved in both the export of the target

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viral mRNA complex and the multimerization of Rex on the cognate RNA [11]. This suggests that hCRM1 is the most critical cofactor guiding Rex function.

Several animal models to investigate the mechanisms underlying the onset of HTLV-1 related diseases have been developed over the past years. Monkeys and rabbits have been used to examine HTLV-1 infection, replication, disease manifestation, immune response, and vaccine development [15–20]. However, rats and mice are more attractive models for HTLV-1 study because of the ease with which they can be genetically manipulated. HTLV-1 transmission to newborn mice has been reported and the HTLV-1 provirus in mouse spleen has been detected [21]. Nevertheless, no viral expression or antibody production was detected in these mice and, furthermore, mouse cells seem to be less susceptible to HTLV-1 envelope fusion [22,23], even though some conflicting results have been reported [24]. In contrast, HTLV-1 infection in rats establishes a persistent infection and elicits specific antibody responses [25,26]. Moreover, HAM/TSP-like diseases develop in HTLV-1-infected WKA/H rats [27,28]. However, until now, efficient replication of HTLV-1 in rat cells has not been reported.

To develop better rat models, it is essential to identify the step at which viral replication is blocked and the host factor(s) responsible. HTLV-1 has been reported to infect several types of rat cells, which indicates that the rat cells possess receptors for viral attachment and penetration [29–31]. Recent identification of a highly conserved molecule, Glut-1, a glucose transporter, as a receptor [32] is consistent with these observations. Previously, we found the inability of the host factor rat CRM1 to support Rex function and thus that viral RNA export from nucleus was a possible block in the viral life cycle. Rat CRM1 induces minimal amount of Rex multimerization on cognate RNA, although it efficiently exports Rex protein to the cytoplasm. This may cause the defect in viral RNA transport [33]. Two residues (amino acids 411 and 414) in the central region of human CRM1 are crucial for multimerization [34]. These results suggest that a transgenic (Tg) rat, which expresses human CRM1, may be a model animal to support replication of HTLV-1. Prior to constructing the Tg rat, we examined the effects of hCRM1, expressed at physiological levels in rat cells, because the above results were obtained by overexpression of human and rat CRM1 and toxic effects from overexpressed CRM1 and a dominant-negative influence of rCRM1 over hCRM1 have been reported [33,35]. Moreover, an understanding of the entire viral life cycle is needed. In the case of the human immunodeficiency virus (HIV), nonhuman cells have been reported to contain inhibitors such as Trim5α and Apobec3G, which act at uncoating and reverse-transcription steps, respectively [36,37].

In this study, we constructed rat cells expressing hCRM1 at physiological levels and examined the effects on HTLV-1 replication. We investigated the early steps of the HTLV-1 life-cycle, between entry and transcription, and the late steps, including formation of infectious virus and cell to cell infection. To investigate these steps quantitatively, we used a pseudovirus system [38] and our newly devised cell fusion assay, because the extremely poor infectivity of free HTLV-1 virions [38,39] makes it impractical to evaluate them by conventional virological methods. Here, we show that expression of hCRM1 in rat cells may be sufficient to enhance production of HTLV-1 proteins and infectious viruses at levels similar to those in human cells.

2. Materials and methods

2.1. Retro-vector preparation

To construct an hCRM1 expressing retro-vector, the 3 kb fragment, which encodes the 3′ part of the hCRM1 coding frame, was isolated from pSRαhCRM1 plasmid [11] by digestion with AatII and XhoI, and the left 5′ part was amplified by PCR using the primer pair: hCrm15′F: CCG AAT TCT CTC TGG TAA TCT ATG CCA GCA A; hCrm15′R: CAA GTT GGG TCA GAT GAC GTC TT on pSRαhCRM1 as a template. The PCR was performed by a single step of 94 °C for 90 s and 10 cycles of a three-temperature PCR (94 °C for 30 s, 56 °C for 60 s, and 72 °C for 30 s) followed by one step of 72 °C for 5 min. The amplified fragment was then digested with AatII and EcoRI. The two fragments of hCRM1 cDNA were then ligated to retrovector pMX-neo digested with EcoRI and XhoI [40]. The resultant expression plasmid, named pMXneohCRM1, was transfected to packaging PLAT-E cells, the supernatants were collected, and stored at −80 °C.

2.2. Construction of stable cell lines

Rat mammary adenocarcinoma ER-1 cells, maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FCS), were seeded into 6-well plates at a density of 1×10^5 cells/well 1 day before infection. To construct stable rat cell lines which express hCRM1, ER-1 cells were incubated with 0.5 ml of MXneohCRM1 virus solution for 4 h in the presence of 10 μg/ml polybrene, and then fresh medium was added. The hCRM1 expressing clones were selected in the presence of 300 μg/ml neomycin. We picked two neomycin resistant clones and designated them ER-1/hCRM1-1 and ER-1/hCRM1-2, respectively. The control cells, designated as ER-1neo1, were infected with virus carrying the MX-neo plasmid.

To construct an hCRM1 expressing rat T cell line, 1×10^5 FPM1 cells [44], an HTLV-1 transformed rat $CD4^+$ T cell line, were infected with MXneohCRM1 and selected with 100 μg/ml neomycin. The resistant cells were then divided into 96-well plates at 0.1 cells per well to clone the FPM1hCRM1-14 line.

To establish reporter cells for detection of HTLV-1-induced cell fusion, 5×10^5 293T cells in 10 cm petri dishes were transfected with 2.5 μg of pLTR-GL3 [41] and 0.5 μg of pTK-Hyg (Clontech, Palo Alto, CA) using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Forty-eight hours after transfection, the medium was replaced with fresh DMEM supplemented with

10% FCS and 300 μg/ml hygromycin B. Antibiotic resistant colonies were picked with a cloning cylinder. A cell clone, designated as 293T/LTR-Luc8, was used in this study.

2.3. Measurement of Rex activity

The cells (1×10^5) were transfected with 0.4 μg of pDM128RxRE [33], 0.05 μg of pSRαRex, and 0.1 μg of pCDMβ-Gal. Twenty-four hours after transfection the cells were lysed and the amount of CAT was quantified using a CAT ELISA kit (Roche). The β-galactosidase (β-gal) activity was measured by standard colorimetric methods, to normalize the transfection efficiency. The Rex activity was represented by a ratio of CAT/β-gal for each sample as described previously [33].

2.4. Measurement of HTLV-1 Gag production

The human and rat cell lines (1×10^5) were transfected with 0.5 μg of HTLV-1 infectious clone K30 and 0.1 μg of pCDMβ-Gal. Forty-eight hours after transfection, the medium of the cell culture was harvested and centrifuged at low speed to remove the cell debris. The transfected cells were washed twice with PBS then scraped off and transferred to microcentrifuge tubes; the cells were suspended in 50 μl of lysis buffer (10 mM Tris–HCl, 140 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40, 1 μg/ml of aprotinin, leupeptin and pepstain). Gag was quantified by a RETRO-TEK HTLV-1 p19 Gag ELISA kit according to the manufacturer's protocol.

2.5. Immunoblot analysis

Immunoblot analysis was performed for detection of CRM1 using affinity purified chicken anti-hCRM1 and rabbit anti-rCRM1 antibodies [33]. To detect HTLV-1 proteins, we used rabbit anti-Rex antisera, mouse anti-Tax MAb Lt-4, and mouse anti-p24 Gag MAb NOR-1 [42]. To detect the HTLV-1 Env protein, we concentrated the Env proteins using Concanavalin A (Con-A) Sepharose and then detected with rat anti-gp46 MAb LAT-27 [43]. For detection of Gag in released virions, the culture medium was ultracentrifuged at 40,000 rpm for 1 h in a Beckman TLA100.3 rotor at 4 °C. The pellets were suspended in the sample buffer and processed for immunoblotting. To detect the HTLV-1 Env protein in the cell lysate, 5×10^5 rat or human cells in 10 cm petri dishes were transfected with 2.5 μg of HTLV-1 K30. The cells were lysed 48 h after infection and applied to Con-A Sepharose. The concentrated glycoproteins were eluted using sample loading buffer.

2.6. Quantification of fusion activity of HTLV-1 infected cells

The infectious clone HTLV-1 K30 (0.5 μg) was used to transfect various rat and human cells, and 24 h later the cells were trypsinized and suspended in fresh medium. The cells were mixed with an equal number (1×10^5) of 293T/LTR-Luc8 cells, and cultured for an additional 48 h. The cells were lysed and luciferase expression was measured using the Steady-Glo luciferase assay system (Promega, Madison, WI) to evaluate fusion activity.

2.7. Cell free infection and gene transduction analysis

The preparation of pseudotyped HTLV-1 virus and virus infection were performed, as described previously, except that the reporter plasmid, pHTC-GFP-Luc was used instead of pHTC-Luc-tsa [38]. pHTC-GFP-Luc, a newly developed reporter vector by David Derse's group, encodes a GFP-luciferase fusion protein, otherwise identical to pHTC-Luc-tsa. Briefly, pCMVHT-Δenv and pHTC-GFP-Luc, which were kindly provided by Dr David Derse, and pMD-VSV-G were transfected into 1×10^6 cells. The culture supernatant, which contains resultant viruses, was harvested 28 h after transfection and used to infect various cell types. Seventy-two hours later luciferase activity in the infected cells was measured. For AZT inhibition, 100 nM of 3′-azido-3′-deoxythymidine (AZT) (Sigma) was used to treat the infected cells as described [38].

3. Results

3.1. Effects of hCRM1 expressed in rat adenocarcinoma cell lines

We first established the stable rat adenocarcinoma cell lines, ER-1/hCRM1-1 and -2, which express hCRM1, by transduction with MxneohCRM1, a retro-vector encoding the hCRM1 cDNA. A control cell line named ER-1neo1, which carries only the neomycin resistance gene, was also generated. ER-1/hCRM1-1 and ER-1/hCRM1-2 expressed hCRM1 at levels similar to human HeLa cells and CD4-HeLa cells, as judged by immunoblotting. hCRM1 was not detected in the parental ER-1 or control ER-1neo1 cell samples (Fig. 1A). Both cell lines expressing hCRM1 propagated similarly or a little faster than parental ER-1 (Fig. 1B). It is conceivable that double the amount of CRM1 in the hCRM1 expressing rat cells, which also express rat CRM1, might facilitate rat mRNA/protein export, leading to better growth. However, it is less likely, because ER-1/neo1 grew equally well as the hCRM1 expressing ER-1 cells. In any case our results suggest that expression of physiological levels of hCRM1 does not negatively affect the replication dynamics of cells.

Next, we examined whether the hCRM1 transgene can restore Rex activity in rat cell lines. We co-transfected a CAT expressing reporter, the pDM128RxRE, pSRαRex, and pCDMβ-gal plasmids, and quantified Rex activity based on the amount of CAT protein production. In the parental rat ER-1 and control ER-1neo1 cells, Rex activity was undetectable, while in the hCRM1-expressing cells Rex activity was significantly augmented to levels found in HeLa and CD4-HeLa cells (Fig. 1C). As predicted, CAT expression in cells transfected only with pCDM128RxRE was very low. These results clearly demonstrate that expression of hCRM1 in rat cells

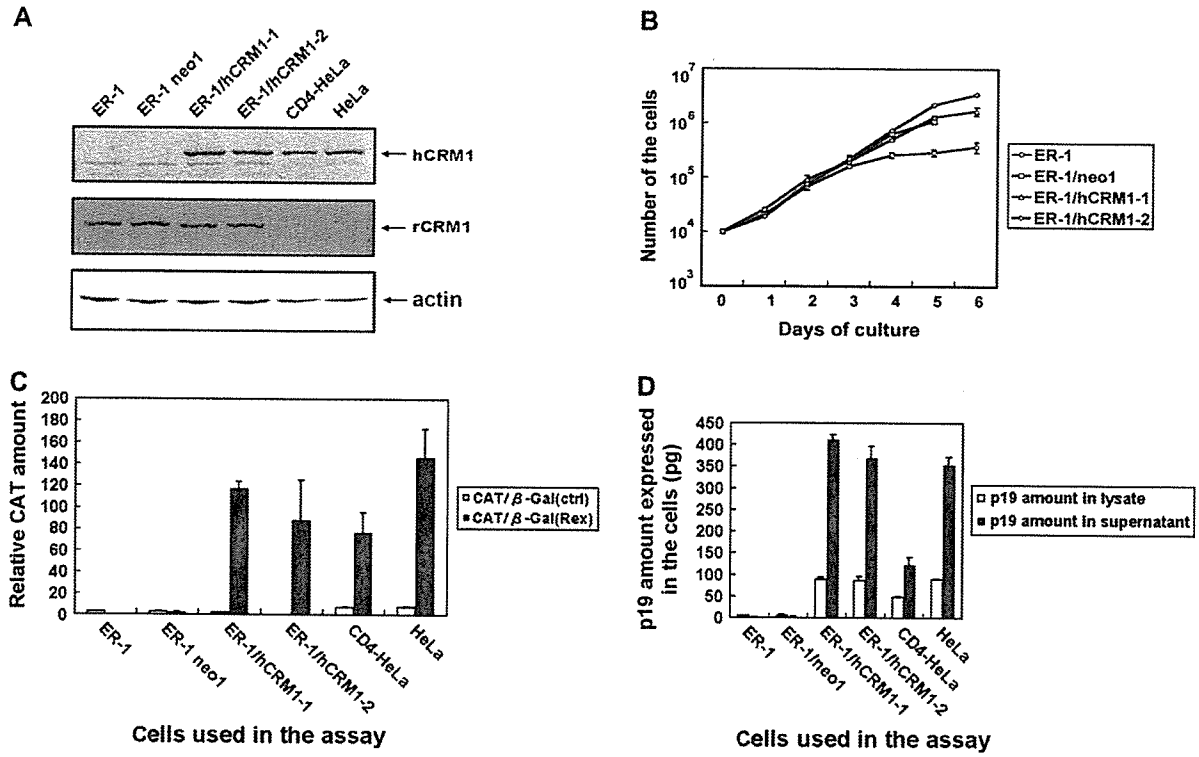

Fig. 1. Properties of hCRM1 expressing adherent rat cell lines. (A) The rat mammary tumor cell line, ER-1, was transduced with the retrovector Mx-neo-hCRM1 or Mx-neo. The expression of hCRM1 and rCRM1 in the selected clones as well as the parental rat cell and control human cell lines was examined by immunoblotting. The same amounts of the cell lysates were loaded on the SDS-PAGE. The expression of β-actin was also examined as a control to monitor the amounts of samples applied in the assay. (B) The cell growth of hCRM1-transduced rat cell clones was compared with parental ER-1 The cells (1×10^4/ml) were seeded into a 6-well plate, and counted every 24 h. (C) hCRM1 expression enhances Rex activity in rat cells. The results are shown as the means of three independent experiments. (D) hCRM1 augments HTLV-1 Gag production in the rat cells. The rat and human cell lines were transfected with the HTLV-1 K30 and Gag products in the cell lysate and culture medium were quantified by HTLV-1 p19 ELISA. The results are shown as the mean of three independent experiments.

is sufficient to augment Rex activity to levels similar to those found endogenously in human cells, regardless of the presence of endogenous rCRM1.

The restoration of Rex activity may directly result in enhanced expression of the HTLV-1 viral structural protein. To test this possibility, we transfected rat and human cells with the HTLV-1 molecular clone K30. Gag production was first quantified using an HTLV-1 p19 antigen ELISA (Fig. 1D). The p19 antigens were produced at similar levels in hCRM1 expressing rat and human cells, whereas very low levels of p19 were detected in the parental ER-1 and control ER-1neo1 samples. The ratio of p19 in the medium to that in the cell lysate was 3–4 in both human and rat cells expressing hCRM1. Approximately 30% of the secreted Gag protein, which was produced in all types of hCRM1 expressing cells, could be pelleted by ultracentrifugation (Table 1). Taken together, these results suggest that viral particles budded from rat cells as efficiently as human cells.

To further examine the expression of the HTLV-1 structural proteins in rat cells expressing hCRM1, we performed a Western blot analysis (Fig. 2). Gag proteins including p24 and its precursor p55 were expressed equally well in human and rat cells expressing hCRM1, but were not expressed in control ER-1neo1 rat cells. The efficiency of processing p55 to p24 was similar in human and rat cells and the p38 intermediate was detected in both cell lines. Similar amounts of the HTLV-1 gp46 Env protein and its precursor gp61 were detected in both human and rat cells expressing hCRM1, but not in control ER-1neo1 rat cells. In contrast, the two trans-regulatory proteins, Tax and Rex, were expressed at similar levels in all rat and human cells upon HTLV-1 K30 transfection.

Since it is difficult to measure the infectivity of HTLV-1 by conventional methods, we applied a reporter virus assay [38], in which the HTLV-1 pseudovirus harboring a luciferase gene is coated with G proteins of vesicular stomatitis virus (VSV), which shows a broad tropism. Luciferase is driven by the

Table 1
Amount of HTLV-1 p19 Gag protein in K30-transfected rat and human cells[a]

Cells	P19 amount (pg) in		
	Culture medium	Concentrated pellet	Lysate
ER-1	UD[b]	UD	UD
ER-1/neo1	UD	UD	UD
ER-1/hCRM1-1	412 ± 12	116 ± 5	89 ± 6
ER-1/hCRM1-2	369 ± 27	122 ± 9	87 ± 13
CD4-HeLa	122 ± 18	41 ± 1	48 ± 5
HeLa	355 ± 17	96 ± 2	90 ± 5

[a] Cells (1×10^5) were transfected with 0.5 μg of the HTLV-1 K30. Forty-eight hours after transfection, the cells and medium of the culture were harvested and applied to HTLV-1 p19 ELISA. The total amount of p19 was calculated. The results are shown as the mean of three independent experiments.
[b] UD, under detection limit.

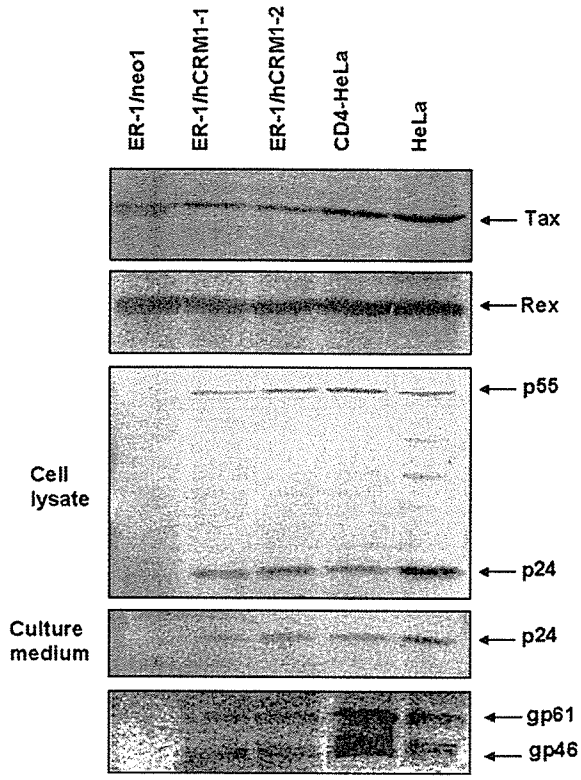

Fig. 2. hCRM1 enhances expression of HTLV-1 structural proteins, but not regulatory proteins, in rat cell lines. HTLV-1 K30-transfected cells and their culture medium were harvested and immunoblot assays were performed to detect the expression of viral proteins. The amount of samples was normalized by transfection efficiency based on β-galactosidase activity before applying to SDS-PAGE. The samples for detection of Gag in released virions and Env protein in the cell lysate were prepared as described in Section 2.

strong CMV promoter, and used as a sensitive marker of the gene expression from viral genomes, which have integrated into their host cells. We produced the pseudotyped viruses in ER-1/neo1, ER-1/hCRM1-2, HeLa and CD4-HeLa cell lines, and compared their luciferase inducing capacity after infection of 293 cells (Table 2). The pseudovirus produced in ER-1/hCRM1-2 or CD4-HeLa induced similar levels of luciferase activity, which was greater than that produced by HeLa cell derived pseudoviruses. The luciferase activity was positively correlated to the amount of HTLV-1 p19 in the medium. The luciferase activity from ER-1neo1 samples represents the background. The luciferase activity was reduced to background levels by AZT, an inhibitor of the viral reverse transcriptase, indicating that the infection occurred through the normal retrovirus infection route. These results suggest that HTLV-1 virions produced from the hCRM1 expressing rat cells are fully infectious.

3.2. hCRM1 expression converts rat CD4$^+$ T cells into high efficiency HTLV-1 producers

To examine the effect of hCRM1 expression in rat CD4$^+$ T cells, we transduced MXhCRM1 into FPM1, an HTLV-1-transformed rat CD4$^+$ T cell line (Fig. 3). The FPM1-derived

Table 2
Infectivity of pseudotyped HTLV-1 produced in rat and human cells[a]

Cells	Luciferase activity in infected 293 cells		p19 ELISA titer (pg/ml)
	Without AZT	With AZT	
ER-1/neo1	239[b]	261	UD
ER-1/hCRM1-2	1654	265	638
HeLa	444	132	347
CD4-HeLa	1729	370	1219

[a] The pseudotyped viruses produced in various cells were quantified by p19 ELISA and infected to 293 cells in the absence or presence of AZT. Seventy-two hours later luciferase activity in the infected cells was measured. Representative results of two independent experiments are shown.
[b] RLU, relative light units.

hCRM1 expressing cells, FPM1-hCRM1-14, produced hCRM1 levels comparable to the Jurkat human T cell line and to MT-4, an HTLV-1 producing human T cell line. FPM1-hCRM1-14 grew as well as the parental FPM1 cells (Fig. 3A). FPM1 has been reported to selectively express viral regulatory proteins, such as Tax, but not structural proteins [44]. Our ELISA data consistently showed very low levels of p19 expression (approximately 25 pg/ml) in the culture medium of FPM1, whereas FPM1-hCRM1-14 produced very high levels of secreted Gag antigen (approximately 7400 pg/ml), comparable to MT-4 cells (data not shown).

Western blotting showed that expression of hCRM1 in FPM1 did not affect the amount of Tax and Rex proteins, which are encoded by mRNAs that are exported independently of CRM1. In contrast, hCRM1 augmented the production of Gag and Env proteins. The Gag precursor p55 was processed to mature p24 as efficiently as the human T cells (Fig. 3B).

3.3. Fusion ability of HTLV-1-infected rat cells

Efficient spread of HTLV-1 requires cell contact [45,46]. Lymphocytes naturally infected with HTLV-1 produce very few cell-free HTLV-1 virions, and one in 10^5 to 10^6 virions is estimated to be infectious [38,39]. The cell-to-cell spread of HTLV-1 is mediated through fusion of the two cell membranes caused by Env proteins [23,47,48]. Certain integrins, including the intercellular and vascular cell adhesion molecules ICAM-1, ICAM-3, and VCAM, act as cofactors for HTLV-1-induced cell fusion [49,50]. To quantify the fusion efficiency of HTLV-1-infected cells, we established a 293T derived reporter cell, 293/LTR-luc8, which harbors an HTLV-1 LTR promoter-driven luciferase reporter gene. When cell fusion occurs following co-culture of this reporter cell with HTLV-1 producing cells, Tax protein is transferred from the donor cells and activates the LTR promoter. An alternative route to activate the luciferase gene is through newly synthesized Tax protein from an HTLV-1 genome, which has been transferred from the donor cells, reverse-transcribed, and integrated into a reporter cell chromosome. In either case, the fusion ability of the HTLV-1 infected cells can be evaluated by luciferase expression. The reporter cells express very low levels of luciferase under normal culture conditions,

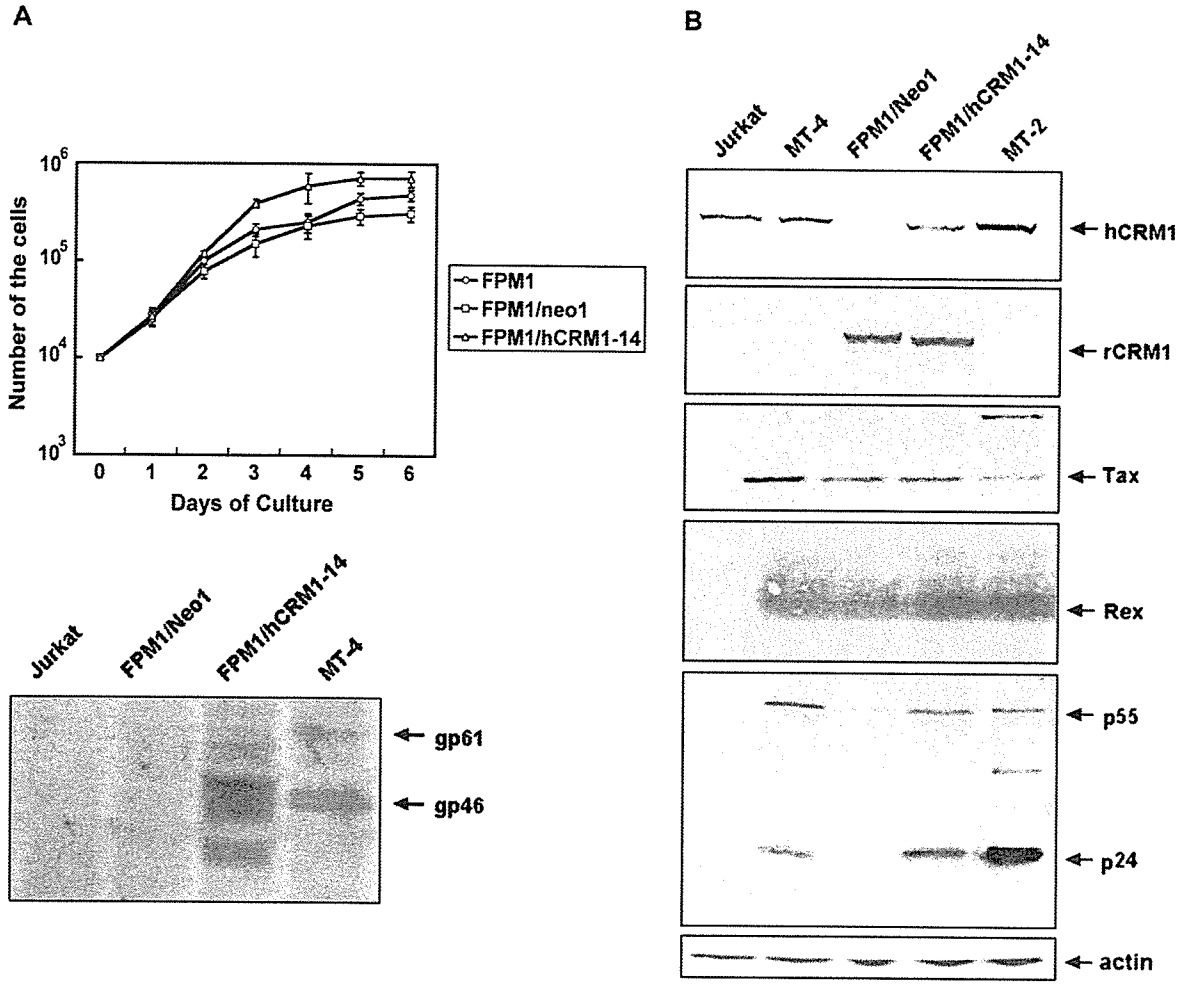

Fig. 3. hCRM1 enhanced viral structural protein expression in HTLV-1-infected rat T-cell lines. (A) The cell growth of hCRM1-transduced rat T cell clones was compared with parental FPM1 cells. The cells (1×10^4/ml) were seeded into a 6-well plate, and counted every 24 h. (B) HTLV-1-infected and uninfected rat and human T cells (1×10^6 cells) were harvested in their log growth phase. Immunoblotting was performed as in Fig. 1A and Fig. 2. The expression of β-actin was also examined as a control to monitor the amounts of samples applied in the assay.

but have a very sensitive LTR response upon Tax stimulation, as demonstrated by transient expression of a Tax encoding plasmid (data not shown), or co-culture with as few as 1×10^3 HTLV-1 producing MT-2 cells. Luciferase activity increased linearly with the number of MT-2 cells, up to 5×10^4 cells. In contrast, co-culture with 5×10^4 Jurkat cells did not induce luciferase activity (Fig. 4A).

To compare the fusion capability of virus-producing cells, we first transfected various rat and human cells with HTLV-1 K30, incubated the cells for 24 h after transfection, and then co-cultured them with 293T/LTR-luc8 cells for a further 72 h. As shown in Fig. 4B, the luciferase activity induced by co-culture with ER-1/hCRM1-1 and ER-1/hCRM1-2 was as high as that resulting from co-culture with HeLa or CD4-HeLa cells, as well as the HTLV-1 high producing line C77, suggesting that K30-infected rat cells could be highly infectious. In contrast, the luciferase expression in reporter cells co-cultured with ER-1neo1 was close to the basal level. As a control, rat and human cells transfected with a Tax expressing plasmid pSRαTax in the absence of Env expression were also unable to increase luciferase expression when co-cultured with 293T/LTR-luc8 cells (data not shown), indicating that luciferase expression depends on cell fusion mediated by Env proteins.

The fusion ability of HTLV-1-infected rat T cells was also investigated. The luciferase level in reporter cells co-cultured with both human T cells (MT-4) and FPM1-mxhCRM1-14 were unexpectedly lower than the adherent cells described above. Nevertheless, the hCRM1 expressing rat T cells stimulated luciferase activity more efficiently than MT-4 cells (Fig. 4C). Luciferase activity was proportional to the amount of Env gp46 expressed (see Figs. 3 and 4). These results clearly demonstrate that the Env protein induced by hCRM1 in the rat cells is fully fusogenic, supporting HTLV-1 infectivity in rat cells.

To discriminate whether a Tax protein transferred from the donor cells or Tax protein produced from the HTLV-1 genome which had infected the reporter cells was the primary inducer of luciferase, we co-cultured the infected and reporter cells in the presence of AZT. The former alternative would be insensitive to AZT, while the latter would be sensitive to AZT. AZT at 100 nM had a negligible effect on luciferase activity (data not

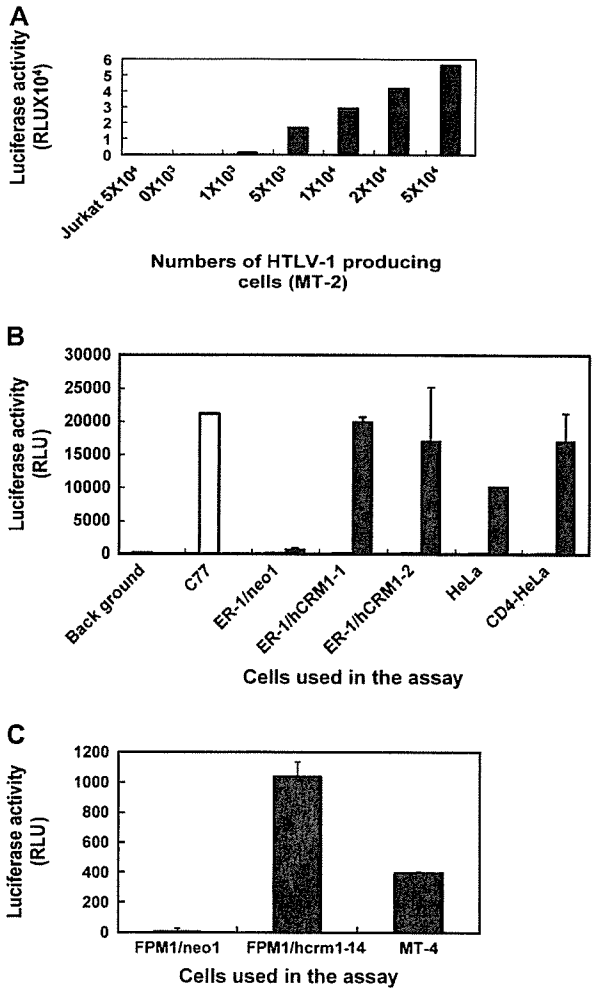

Fig. 4. Env proteins expressed in the rat cells are fusogenic. (A) Various numbers of MT-2 cells were co-cultured with 1×10^5 of the reporter cell 293T/LTR-Luc8. One-fourth of the cell lysate was for the luciferase assay. RLU, relative light units. (B) Luciferase activity induced in reporter cells co-cultured with the HTLV-1 K30-transfected rat and human cells. The results are shown as the mean of three independent experiments. (C) Luciferase activity in the reporter cells co-cultured with HTLV-1 producing rat and human T cells. The results are shown as the mean of two independent experiments.

shown), consistent with poor formation of HTLV-1 infectious virus.

3.4. Efficient early events of HTLV-1 replication in rat cells

To determine the efficiency of early replication events, from entry to the genome integration step, human and rat cells were infected with VSV G-pseudotyped HTLV-1 virus containing the GFP-luciferase gene. ER-1/neo1 and ER1/hCRM1-2 cells produced luciferase signals similar to 293 and 293T cells (Table 3, Experiment 1). Unexpectedly, HeLa and CD4-HeLa cells produced much weaker signals. Luciferase activity was inhibited by the reverse transcriptase inhibitor AZT, indicating that luciferase expression occurred as a result of retrovirus replication, implying reverse-transcription and integration. We next compared the efficiency of the early events

Table 3
Transduction efficiencies of pseudotyped HTLV-1 to various cells

Cells	Luciferase activity (RLU)	
	Without AZT	With AZT
Experiment 1[a]		
ER-1/neo1	3320	228
ER-1/hCRM1-2	2798	126
HeLa	98	103
CD4-HeLa	175	308
293	1946	197
293T	4485	293
Experiment 2[b]		
FPM1	995	275
FPM1/hCRM1-14	465	132
Nb2	860	156
MT-4	2908	416
Jurkat	758	307
Molt-4	884	300

[a] Filtered supernatant of 293T cotransfected with pCMVHT-Δenv, pHTC-GFP-Luc, and pMD-VSV-G were applied to infect 1×10^5 adherent cells. The p19 in the supernatant was 19 ng/ml. Representative results of two independent experiments are shown.

[b] The filtered supernatant was ultra-centrifuged at 14,000 rpm for 90 min. The pellet resultant was suspended in 100 μl of fresh medium, and used to infect the rat and human cells.

in rat T cells with those in human T cells. As shown in Table 3 Experiment 2, both HTLV-1-uninfected (Nb2) and infected rat T cells (FPM1) induced luciferase as much as the human T cells. These results suggest that the early events of HTLV-1 infection indeed occurred in the rat cells at the same or slightly higher levels than in human cells. Free HTLV-1 viruses prepared from feline HTLV-1 producing C77 cells were used to infect activated primary T cells prepared from human PBMC or rat spleen. PCR was used to detect the pX region of HTLV-1 in genomic DNA, extracted from the cells 3 days after infection. We found more intensive signals in the rat T cell samples than the human samples (data not shown). This result supports the above notion, although it is not a quantitative method.

4. Discussion

Previously, we have shown that rCRM1, even though it is able to export Rex protein from the nucleus, does not support Rex multimerization, resulting in poor export of HTLV-1 RNAs in rat cells [33,34]. In this study, we demonstrated that the expression of hCRM1 at physiological levels in rat cells, including epithelial and $CD4^+$ T cells, is not harmful to cell growth, but augments the synthesis of HTLV-1 Gag and Env proteins to levels similar to those seen in human cells. The endogenous rCRM1 does not inhibit the function of Rex, although rCRM1 overexpressed by transfection has been reported to act as a dominant negative inhibitor of hCRM1 function [33]. The Gag precursor synthesized in hCRM1 expressing rat cells is normally processed to the mature p24 and possibly other Gag proteins and then released into the culture medium as sedimentable viral particles, with

an efficiency comparable to human cell lines. The fact that only 25–30% of the Gag protein detected in the medium of both human and rat cells was sedimentable suggests that only a fraction of the Gag proteins may be incorporated into virions, or that the non-sedimentable Gag proteins may reflect the fragility of the virus. Finally, the results using the HTLV-1 pseudovirus [38] suggest that HTLV-1 virions released from the rat cells may have the same infectivity as those released from human cells.

A major route for the spread of HTLV-1 is cell to cell infection mediated by cell fusion caused by the Env proteins in cooperation with the cell adhesion molecules ICAM-1, ICAM-3, and VCAM [48,49]. Some reports suggest that the main function of the Env protein is to mediate cell fusion. These data led us to develop a reporter cell line to quantify Env-mediated cell fusion, based on activation of an HTLV-1 LTR-driven luciferase gene. This system can detect fusion events between as little as approximately 1×10^3 MT-2 cells and 1×10^4 K30-infected cells. Given that this fusion has been shown to be dependent on the Env protein, quantitation of functional Env proteins by this system should be much more sensitive than Western blotting, which requires Env protein concentrated by Con A Sepharose from at least 5×10^5 K30-infected cells. Using this system, we demonstrated that hCRM1 expressing rat cells infected with HTLV-1 mediate cell fusion as efficiently as human cell lines. The results suggest that the fusion capacity of human and rat cells is proportional to the amount of the Env gp46 protein detected by Western blotting (compare Figs. 2, 3 and 4), and indicates that the Env expressed on rat cells is fully fusogenic, which is the basis for HTLV-1 cell to cell transmission.

The poor replication of HTLV-1 in rat cells is unlikely to be related to efficiency of viral entry, since HTLV-1 has a broad host range of infection using Env-coated virus systems [29–31]. The results of our cell fusion-dependent reporter assay (Fig. 4) are consistent with these observations and with the data suggesting that the ubiquitously expressed Glut-1 acts as a HTLV-1 receptor [32]. The post entry steps, including reverse-transcription, nuclear entry, and integration of the HTLV-1 genome, should not be severely inhibited in rat cells since our results using the HTLV-1 pseudovirus system [9] indicate that the infected rat cells induced luciferase, a quantitative marker for successful integration of the viral genome, at levels similar to human cells. Moreover, the early viral proteins, such as Tax and Rex, which are expressed independently of Rex function, are efficiently synthesized in rat cells (Figs. 2 and 3) [44]. Our results suggest that rat cells do not have serious blockages in viral replication other than rCRM1 in the late stage.

In conclusion, rCRM1 can be considered a major species-specific barrier for HTLV-1 replication in rat cells. This barrier is unique to HTLV-1, since, for many viruses, this restriction is determined by species-specific receptor interactions. The fact that expression of hCRM1 allows rat cells to produce high amounts of fully functional Gag and Env proteins and assemble infectious HTLV-1 suggests the feasibility of constructing a transgenic rat expressing hCRM1, which could present a better animal model to study HTLV-1 infection and develop preventive and therapeutic intervention strategies.

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References

[1] Y. Hinuma, K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K.I. Kinoshita, S. Shirakawa, I. Miyoshi, Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 6476–6480.
[2] M. Osame, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, M. Tara, HTLV-I associated myelopathy, a new clinical entity, Lancet 1 (1986) 1031–1032.
[3] G. Franchini, Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection, Blood 86 (1995) 3619–3639.
[4] M. Yoshida, Multiple viral strategies of HTLV-1 for dysregulation of cell growth control, Annu. Rev. Immunol. 19 (2001) 475–496.
[5] M. Hidaka, J. Inoue, M. Yoshida, M. Seiki, Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins, EMBO J. 7 (1988) 519–523.
[6] B.R. Cullen, Regulation of human immunodeficiency virus replication, Annu. Rev. Microbiol. 45 (1991) 219–250.
[7] J. Inoue, M. Yoshida, M. Seiki, Transcriptional (p40x) and post-transcriptional (p27x-III) regulators are required for the expression and replication of human T-cell leukemia virus type I genes, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 3653–3657.
[8] C. Ballaun, G.K. Farrington, M. Dobrovnik, J. Rusche, J. Hauber, E. Bohslein, Functional analysis of human T-cell leukemia virus type I rex-response element: direct RNA binding of Rex protein correlates with in vivo activity, J. Virol. 65 (1991) 4408–4413.
[9] H.P. Bogerd, G.L. Huckaby, Y.F. Ahmed, S.M. Hanly, W.C. Greene, The type I human T-cell leukemia virus (HTLV-I) Rex trans-activator binds directly to the HTLV-I Rex and the type 1 human immunodeficiency virus Rev RNA response elements, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 5704–5708.
[10] M. Fornerod, M. Ohno, M. Yoshida, I.W. Mattaj, CRM1 is an export receptor for leucine-rich nuclear export signals, Cell 90 (1997) 1051–1060.
[11] Y. Hakata, T. Umemoto, S. Matsushita, H. Shida, Involvement of human CRM1 (exportin 1) in the export and multimerization of the Rex protein of human T-cell leukemia virus type 1, J. Virol. 72 (1998) 6602–6607.
[12] M.E. Nemergut, M.E. Lindsay, A.M. Brownawell, I.G. Macara, Ran-binding protein 3 links Crm1 to the Ran guanine nucleotide exchange factor, J. Biol. Chem. 277 (2002) 17385–17388.
[13] H. Bogerd, W.C. Greene, Dominant negative mutants of human T-cell leukemia virus type I Rex and human immunodeficiency virus type 1 Rev fail to multimerize in vivo, J. Virol. 67 (1993) 2496–2502.
[14] H. Shida, Y. Hakata, Multiple roles of cellular export machinery in HTLV-1 Rex functioning, Gann monogr. 50 (2003) 61–72.
[15] H. Shida, T. Tochikura, T. Sato, T. Konno, K. Hirayoshi, M. Seki, Y. Ito, M. Hatanaka, Y. Hinuma, M. Sugimoto, et al., Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection, EMBO J. 6 (1987) 3379–3384.
[16] K. Ogawa, S. Matsuda, A. Seto, Induction of leukemic infiltration by allogeneic transfer of HTLV-I-transformed T cells in rabbits, Leuk. Res. 13 (1989) 399–406.
[17] Y. Tanaka, R. Tanaka, E. Terada, Y. Koyanagi, N. Miyano-Kurosaki, N. Yamamoto, E. Baba, M. Nakamura, H. Shida, Induction of antibody responses that neutralize human T-cell leukemia virus type I infection

in vitro and in vivo by peptide immunization, J. Virol. 68 (1994) 6323–6331.
[18] M. Kazanji, A. Ureta-Vidal, S. Ozden, F. Tangy, B. de Thoisy, L. Fiette, A. Talarmin, A. Gessain, G. de, The Lymphoid organs as a major reservoir for human T-cell leukemia virus type 1 in experimentally infected squirrel monkeys (*Saimiri sciureus*): provirus expression, persistence, and humoral and cellular immune responses, J. Virol. 74 (2000) 4860–4867.
[19] T.M. McGinn, Q. Wei, J. Stallworth, P.N. Fultz, Immune responses to HTLV-I(ACH) during acute infection of pig-tailed macaques, AIDS Res. Hum. Retroviruses 20 (2004) 443–456.
[20] K. Ibuki, S.I. Funahashi, H. Yamamoto, M. Nakamura, T. Igarashi, T. Miura, E. Ido, M. Hayami, H. Shida, Long-term persistence of protective immunity in cynomolgus monkeys immunized with a recombinant vaccinia virus expressing the human T cell leukaemia virus type I envelope gene, J. Gen. Virol. 78 (Pt 1) (1997) 147–152.
[21] R. Feng, A. Kabayama, K. Uchida, H. Hoshino, M. Miwa, Cell-free entry of human T-cell leukemia virus type 1 to mouse cells, Jpn. J. Cancer Res. 92 (2001) 410–416.
[22] C. Denesvre, P. Sonigo, A. Corbin, H. Ellerbrok, M. Sitbon, Influence of transmembrane domains on the fusogenic abilities of human and murine leukemia retrovirus envelopes, J. Virol. 69 (1995) 4149–4157.
[23] K. Nagy, P. Clapham, R. Cheingsong-Popov, R.A. Weiss, Human T-cell leukemia virus type I: induction of syncytia and inhibition by patients' sera, Int. J. Cancer 32 (1983) 321–328.
[24] B. Sun, T. Nitta, M. Shoda, M. Tanaka, S. Hanai, H. Hoshino, M. Miwa, Cell-free human T-cell leukemia virus type 1 binds to, and efficiently enters mouse cells, Jpn. J. Cancer Res. 93 (2002) 760–766.
[25] F. Ibrahim, L. Fiette, A. Gessain, N. Buisson, G. de-The, R. Bomford, Infection of rats with human T-cell leukemia virus type-I: susceptibility of inbred strains, antibody response and provirus location, Int. J. Cancer 58 (1994) 446–451.
[26] T. Suga, T. Kameyama, T. Kinoshita, K. Shimotohno, M. Matsumura, H. Tanaka, S. Kushida, Y. Ami, M. Uchida, K. Uchida, et al., Infection of rats with HTLV-1: a small-animal model for HTLV-1 carriers, Int. J. Cancer 49 (1991) 764–769.
[27] N. Ishiguro, M. Abe, K. Seto, H. Sakurai, H. Ikeda, A. Wakisaka, T. Togashi, M. Tateno, T. Yoshiki, A rat model of human T lymphocyte virus type I (HTLV-I) infection. 1. Humoral antibody response, provirus integration, and HTLV-I-associated myelopathy/tropical spastic paraparesis-like myelopathy in seronegative HTLV-I carrier rats, J. Exp. Med. 176 (1992) 981–989.
[28] T. Kasai, H. Ikeda, U. Tomaru, I. Yamashita, O. Ohya, K. Morita, A. Wakisaka, E. Matsuoka, T. Moritoyo, K. Hashimoto, I. Higuchi, S. Izumo, M. Osame, T. Yoshiki, A rat model of human T lymphocyte virus type I (HTLV-I) infection: in situ detection of HTLV-I provirus DNA in microglia/macrophages in affected spinal cords of rats with HTLV-I-induced chronic progressive myeloneuropathy, Acta. Neuropathol. (Berl) 97 (1999) 107–112.
[29] Q.X. Li, D. Camerini, Y. Xie, M. Greenwald, D.R. Kuritzkes, I.S. Chen, Syncytium formation by recombinant HTLV-II envelope glycoprotein, Virology 218 (1996) 279–284.
[30] R.E. Sutton, D.R. Littman, Broad host range of human T-cell leukemia virus type I demonstrated with an improved pseudotyping system, J. Virol. 70 (1996) 7322–7326.
[31] K. Okuma, M. Nakamura, S. Nakano, Y. Niho, Y. Matsuura, Host range of human T-cell leukemia virus type I analyzed by a cell fusion-dependent reporter gene activation assay, Virology 254 (1999) 235–244.
[32] N. Manel, F.J. Kim, S. Kinet, N. Taylor, M. Sitbon, J.L. Battini, The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV, Cell 115 (2003) 449–459.
[33] Y. Hakata, M. Yamada, H. Shida, Rat CRM1 is responsible for the poor activity of human T-cell leukemia virus type I Rex protein in rat cells, J. Virol. 75 (2001) 11515–11525.
[34] Y. Hakata, M. Yamada, H. Shida, A multifunctional domain in human CRM1 (exportin 1) mediates RanBP3 binding and multimerization of human T-cell leukemia virus type 1 Rex protein, Mol. Cell. Biol. 2 (2003) 8751–8761.
[35] M. Callanan, N. Kudo, S. Gout, M. Brocard, M. Yoshida, S. Dimitrov, S. Khochbin, Developmentally regulated activity of CRM1/XPO1 during early Xenopus embryogenesis, J. Cell Sci. 113 (Pt 3) (2000) 451–459.
[36] M. Stremlau, C.M. Owens, M.J. Perron, M. Kiessling, P. Autissier, J. Sodroski, The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys, Nature 427 (2004) 848–853.
[37] A.M. Sheehy, N.C. Gaddis, J.D. Choi, M.H. Malim, Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein, Nature 418 (2002) 646–650.
[38] D. Derse, S.A. Hill, P.A. Lloyd, H. Chung, B.A. Morse, Examining human T-lymphotropic virus type 1 infection and replication by cell-free infection with recombinant virus vectors, J. Virol. 75 (2001) 8461–8468.
[39] N. Fan, J. Gavalchin, B. Paul, K.H. Wells, M.J. Lane, B.J. Poiesz, Infection of peripheral blood mononuclear cells and cell lines by cell-free human T-cell lymphoma/leukemia virus type I, J. Clin. Microbiol. 30 (1992) 905–910.
[40] M. Onishi, S. Kinoshita, Y. Morikawa, A. Shibuya, J. Phillips, L.L. .Lanier, D.M. Gorman, G.P. Nolan, A. Miyajima, T. Kitamura, Application of retrovirus-mediated expression cloning, Exp. Hematol. 24 (1996) 324–329.
[41] J. Fujisawa, M. Seiki, T. Kiyokawa, M. Yoshida, Functional activation of the long terminal repeat of human T-cell leukemia virus type I by a trans-acting factor, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 2277–2281.
[42] Y. Tanaka, M. Yasumoto, H. Nyunoya, T. Ogura, M. Kikuchi, K. Shimotohno, H. Shiraki, N. Kuroda, H. Shida, H. Tozawa, Generation and characterization of monoclonal antibodies against multiple epitopes on the C-terminal half of envelope gp46 of human T-cell leukemia virus type-I (HTLV-I), Int. J. Cancer 46 (1990) 675–681.
[43] Y. Tanaka, L. Zeng, H. Shiraki, H. Shida, H. Tozawa, Identification of a neutralization epitope on the envelope gp46 antigen of human T cell leukemia virus type I and induction of neutralizing antibody by peptide immunization, J. Immunol. 147 (1991) 354–360.
[44] Y. Koya, T. Ohashi, H. Kato, S. Hanabuchi, T. Tsukahara, F. Takemura, K. Etoh, M. Matsuoka, M. Fujii, M. Kannagi, Establishment of a seronegative human T-cell leukemia virus type 1 (HTLV-1) carrier state in rats inoculated with a syngeneic HTLV-1-immortalized T-cell line preferentially expressing Tax, J. Virol. 73 (1999) 6436–6443.
[45] N. Yamamoto, M. Okada, Y. Koyanagi, M. Kannagi, Y. Hinuma, Transformation of human leukocytes by cocultivation with an adult T cell leukemia Virus producer cell line, Science 217 (1982) 737–739.
[46] M. Popovic, P.S. Sarin, M. Robert-Gurroff, V.S. Kalyanaraman, D. Mann, J. Minowada, R.C. Gallo, Isolation and transmission of human retrovirus (human t-cell leukemia virus), Science 219 (1983) 856–859.
[47] L. Delamarre, A.R. Rosenberg, C. Pique, D. Pham, M.C. Dokhelar, A novel human T-leukemia virus type 1 cell-to-cell transmission assay permits definition of SU glycoprotein amino acids important for infectivity, J. Virol. 71 (1997) 259–266.
[48] S.R. Jassal, M.D. Lairmore, A.J. Leigh-Brown, D.W. Brighty, Soluble recombinant HTLV-1 surface glycoprotein competitively inhibits syncytia formation and viral infection of cells, Virus. Res. 78 (2001) 17–34.
[49] J.E. Hildreth, A. Subramanium, R.A. Hampton, Human T-cell lymphotropic virus type 1 (HTLV-1)-induced syncytium formation mediated by vascular cell adhesion molecule-1: evidence for involvement of cell adhesion molecules in HTLV-1 biology, J. Virol. 71 (1997) 1173–1180.
[50] S. Daenke, S.A. McCracken, S. Booth, Human T-cell leukaemia/lymphoma virus type 1 syncytium formation is regulated in a cell-specific manner by ICAM-1, ICAM-3 and VCAM-1 and can be inhibited by antibodies to integrin beta2 or beta7, J. Gen. Virol. 80 (Pt 6) (1991) 429–436.

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Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8

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A highly attenuated LC16m8 (m8) smallpox vaccine has been licensed in Japan because of its extremely low neurovirulence profile, which is comparable to that of replication incompetent strains of vaccinia virus. From 1973 to 1975, m8 was administrated to >100,000 infants where it induced levels of immunity similar to that of the originating Lister strain, without any serious side effects. Recently, we observed that m8 reverts spontaneously to large plaque forming clones that possess virulence equivalent to that of LC16mO, a parental virus strain of m8. Here, we report that the *B5R* gene is responsible for the reversion, and that we could construct a more genetically stable virus by deleting *B5R* from m8. The protective immunogenicity of the vaccine candidate proved to be equivalent to that of the U.S.-licensed product Dryvax, and much superior to modified vaccinia Ankara in a mouse model. Furthermore, the vaccine strain never elicited any symptoms in severe combined immunodeficiency disease mice, even at a dose 1,000-fold greater than that used in the immune protection experiments, which is in contrast to the lethal pathogenicity induced by Dryvax inoculation of severe combined immunodeficiency disease mice. Our results suggest that this vaccine strain is a good candidate as a suitable smallpox vaccine and a vector virus, and that B5R is not essential for protective immunity against smallpox.

B5R gene | reversion | Lister strain | extracellular enveloped virion

Although smallpox was eradicated >20 years ago (1), the necessity of a smallpox vaccine has been reawakened by concerns of bioterrorism using the smallpox virus (2) and outbreaks of monkeypox (3). However, the current vaccine in the United States, Dryvax, occasionally elicits serious adverse effects, including postvaccinal encephalitis (4). Accordingly, a safer smallpox vaccine is much needed.

In Japan, a highly attenuated form of vaccine referred to as LC16m8 (m8) was administrated to >100,000 infants without any serious adverse events and proved to be as immunogenic as the Lister (LO) strain (5, 6), a once widely used vaccine. m8 was indirectly isolated from LO through intermediate strains, such as LC16mO (mO) and LC16. m8, a variant that forms small-sized pocks, is a direct descendant of mO, which itself is a clone that forms medium-sized pocks, isolated from the LC16 strain (5). LC16 was selected from LO based on its temperature sensitivity (5, 7, 8). In rabbit and monkey models, the neurovirulence of m8 was markedly reduced in comparison with other vaccine strains (5, 7–9), including LO and Dryvax (10, 11), and comparable to the replication-defective mutant DIs (Dairen I-derived small-sized pock variant) (12). Moreover, m8 exhibited a markedly diminished dermal reaction in both rabbits and humans and a lower fever ratio compared with mO in clinical trials (5, 6). Therefore, m8 was finally adopted as a vaccine strain instead of mO (6).

Takahashi-Nishimaki *et al.* (13) first identified the vaccinia virus (VV) gene *B5R* as responsible for large plaque formation and proliferating ability in Vero cells. m8 has lost the *B5R* function as the result of a frameshift mutation brought about by a single base deletion in the ORF. *B5R* encodes a 42-kDa glycoprotein that is involved in packaging the intracellular matured virion with trans-Golgi membrane or endosomal cisternae to form an intracellular enveloped virion (IEV) (14–16). IEV is transported along microtubules to the cell periphery (17, 18) where it adheres to the cell surface as a cell-associated enveloped virion (CEV). B5R, in cooperation with the A36R and A33R proteins, also participates in the Src kinase-dependent process of forming of actin-containing microvilli and releasing CEV from the cell surface to form an extracellular enveloped virion (EEV) (19, 20). Despite its relative paucity of whole progeny virions, EEV plays an important role in dissemination within the host (21). Because anti-B5R antibodies can neutralize EEV, expression of *B5R* has been proposed as an effective smallpox vaccine (14, 22–25). In contrast, the results of the field trial in Japan showed that neutralizing (NT) antibody titers induced by m8 were similar to a conventional LO vaccine (5, 6).

Recently, we found that m8 reverted spontaneously to large plaque-forming clones (LPCs).[§] The content of LPCs seemed to increase rapidly in proportion to passage number of the virus. Because LPCs emerged from plaque-purified m8, their generation appears to be an intrinsic property of m8. We were concerned that LPC contamination might ruin the safety of the m8 vaccine. Therefore, to improve the m8 strain, we tested whether *B5R* was the gene responsible for the reversion, because this gene has been correlated with large plaque formation. We then constructed genetically more stable virus by deleting *B5R*. Moreover, by using this virus, we were able to evaluate the contribution of *B5R* to protective immunity against smallpox.

Methods

Virus Preparations. m8 was obtained from Chiba Serum Institute (Chiba, Japan). m8rc (plaque-purified m8 to minimize contamination by revertants) and the revertant viruses (LPCs) were isolated from the m8 stock by three serial plaque purifications in RK13 cells. The modified VV Ankara (MVA) (26, 27) and Western Reserve (WR) viruses were obtained from S. Morikawa (National Institute of Infectious Diseases, Tokyo). MVA was propagated and titrated in chicken embryo fibroblasts. Other viruses were propagated and titrated in RK13 cells, and purified by sedimentation through a 36% sucrose cushion. A vial of Dryvax vaccine, obtained from I. K. Damon and J. Becher (Centers for Disease Control and Prevention, Atlanta), was

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Abbreviations: m8, LC16m8; LO, Lister; mO; LC16mO; VV, vaccinia virus; EEV, extracellular enveloped virion; NT, neutralizing; LPC, large plaque-forming clone; MVA, modified VV Ankara; WR, Western Reserve; PRK, primary rabbit kidney; SCID, severe combined immunodeficiency disease; ErD_{50}, 50% erythema dose; RED_{50}, 50% rash expression dose; pfu, plaque-forming units.

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dissolved in the enclosed solvent, aliquoted, and stored at −80°C. Construction of m8B5R, which harbors the intact *B5R* gene, m8Δ, which lacks the entire *B5R* gene, and m8dTM, which expresses only the ectodomain of the B5R protein, and characterization of their properties, including the structures of *B5R* in the viruses used, are described in more detail (*Supporting Text*, Table 1, and Fig. 5, which are published as supporting information on the PNAS web site).

Western Blotting. We performed immunoblotting by using an antiserum from rabbits that were immunized with baculovirus-expressed recombinant B5R protein. The anti-B5R sera were used at a dilution of 1:200, and detected with a horseradish peroxidase-labeled secondary antibody and an ECL Plus kit (Amersham Pharmacia Biosciences, Piscataway, NJ).

Evaluation of Genetic Stabilities of VVs. We passaged the VVs in primary rabbit kidney (PRK) cells that are used for vaccine production 7 times at 30°C or 34°C, then in Vero cells 2 times at 34°C to amplify LPCs, or 10 times in PRK at 30°C or 34°C. We estimated the fraction of LPCs as the ratio of plaque counts on Vero cells to those on RK13 cells.

Animals. Severe combined immunodeficiency disease (SCID) mice (female, 6 weeks old) and BALB/c mice (female, 6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Female Japan white rabbits (16 weeks old) were obtained from Kitayama Labes (Nagano, Japan). All animal experiments were approved by the National Institute of Infectious Diseases Animal Experiment Committee and were performed in accordance with guidelines for animal experiments performed at the National Institute of Infectious Diseases.

Skin Reaction Test in Rabbits. We conducted a skin reaction test as described (10). Briefly, after inoculating tenfold serial dilutions of VVs intradermally on rabbit backs, the diameters of erythema were measured daily for 1 week. Two animals were used for each viral strain, and each rabbit received two injections of the serial dilution series of a virus. Erythemas >10 mm in diameter was scored as positive. The time at which erythemas reached their peak was determined for each animal, and the 50% erythema dose (ErD_{50}) was calculated by the Beherns and Karber method (28).

SCID Mice Infection Test. To establish an index for pathogenicity of VV against SCID mice, we defined a 50% rash expression dose (RED_{50}), which indicates the virus dose needed to induce a rash in 50% of the animals. After inoculating 10-fold serial dilutions

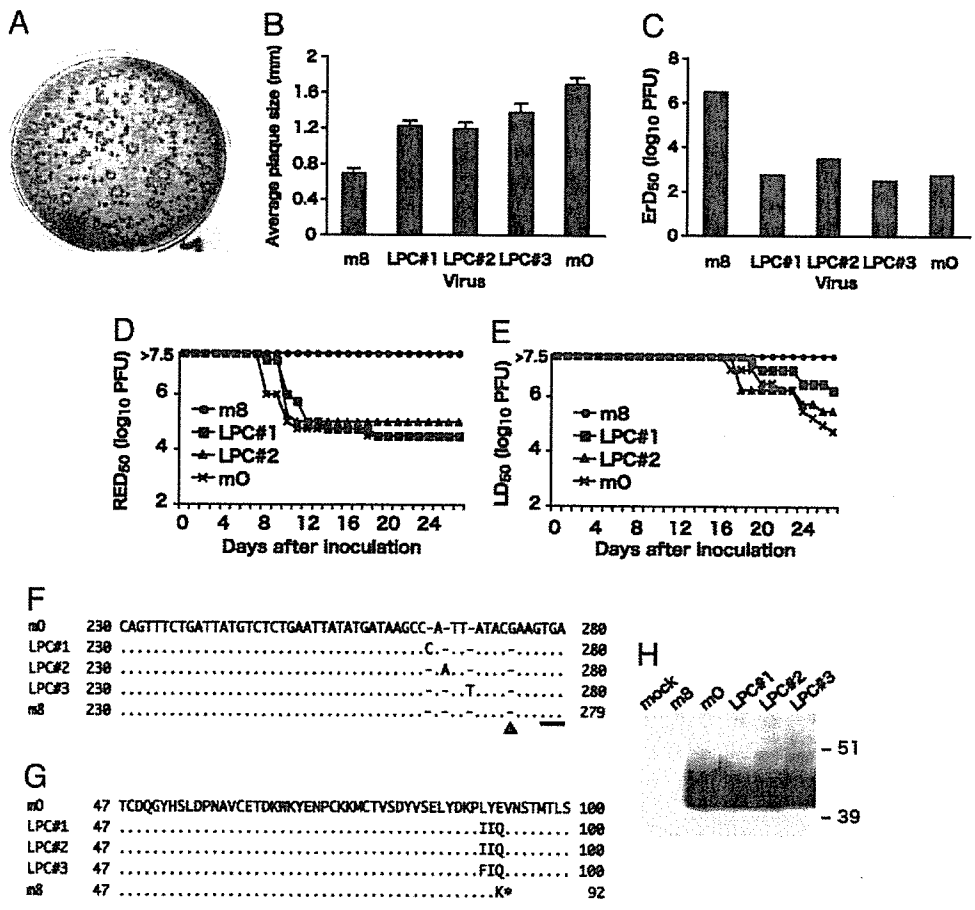

Fig. 1. Biological properties of LPC viruses. (*A*) The plaque configurations of LPCs contaminating an m8 virus stock. LPC viruses make considerably larger plaques than m8. (*B*) The mean plaque sizes of m8, mO, and plaque-purified LPCs. LPCs were isolated from an m8 stock solution. The data are presented as mean ± SD ($P < 0.05$). (*C*) The dermal reaction scores (ErD_{50}) of the LPCs intradermally inoculated in rabbits. (*D* and *E*) Pathogenicity of LPCs against SCID mice. The graphs show temporal changes of RED_{50} (*D*) and LD_{50} (*E*) for a 4-week period after inoculation. The m8 strain was asymptomatic even at the highest viral doses in this experiment (10^7 pfu). If all mice are killed by inoculation of 10^8 pfu of m8, its LD_{50} is $10^{7.5}$ pfu. Therefore, pathogenicity of asymptomatic group ought to be $>10^{7.5}$ pfu. (*F* and *G*) Alignment of the *B5R* nucleotide sequences (*F*) and amino acid sequences (*G*) of mO, m8, and three LPC viruses. Numbers at both ends of the alignments indicate residue numbers. Dots, hyphens, and black triangles in the alignments show identical sequences, gaps, and the single-nucleotide deletion of m8, respectively. The bar and asterisk in the alignments indicate the termination codon. (*H*) Western blots of B5R in VV-infected RK13 cell-lysates. Duplex bands of B5R may be the result of differential glycosylation. Molecular weight markers are shown in kDa.

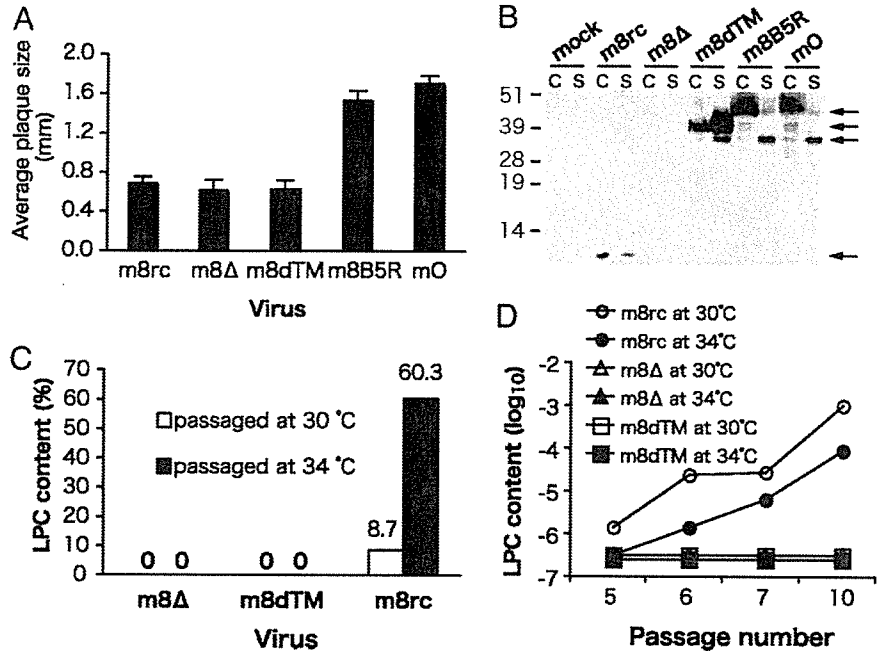

Fig. 2. Characterization of *B5R*-defective viruses. (*A*) The average plaque sizes of m8rc-, m8Δ-, m8dTM-, and *B5R*-positive viruses (m8B5R and mO). The data are presented as mean ± SD. (*B*) Western blots of VV-infected RK13 cell lysates (lane C) and supernatants (lane S). m8rc expresses a short peptide (10 kDa) in the cell and supernatant lanes. The soluble ectodomain of B5R (38 kDa) is expressed from m8dTM. The smaller molecule (35 kDa) in the supernatant lanes of m8dTM, m8B5R, and mO is proteolytically cleaved B5R by cellular proteases. (*C* and *D*) Evaluation of genetic stability by serial passages in PRK and Vero cells under different temperatures (at 30°C or 34°C). The revertant contents of viruses that were passaged seven times in PRK cells and two times in Vero cells are shown in *C*, and those contents that were passaged in PRK cells are shown in *D*.

[10^3 to 10^7 plaque-forming units (pfu)] of VVs i.p. into a series of SCID mice, we calculated the viral doses required for inducing rash (RED_{50}) or killing (LD_{50}) in 50% of the animals by the Reed–Muench method, and followed both values for 4 weeks and 8 weeks.

BALB/c Protection Study. BALB/c mice (eight animals per group) were injected intramuscularly with a single dose of 10^4 to 10^6 pfu of VVs, bled at the tail artery 3 weeks later, and then challenged intranasally with 10^6 pfu of the WR strain 4 weeks after vaccination. Individual body weight was measured daily for 3 weeks, and animals with a weight loss of >30% were killed.

Neutralization Assays. Serial 4-fold dilutions (from 2^{-1} to 2^{-7}) of heat-inactivated mouse serum were mixed with solution containing ≈200 pfu of the WR strain, incubated for 16 h at 37°C, and inoculated on RK13 cells cultured in 48-well plates. Antibody titers were defined as the reciprocal of serum dilution that reduces viral plaques by 50%. All assays were performed in triplicate. The antibody titers of sera from a mock-immunized group were <2 in our assay system.

Statistical Methods. We used Microsoft EXCEL and ORIGIN (OriginLab, Northampton, MA) for statistical analysis. The differences in the mean plaque sizes and in body weight changes measured 5 days after viral challenge in the mouse model were determined by Student's *t* test, with $P < 0.05$ as the criterion for statistical significance. The results are summarized in Table 2, which is published as supporting information on the PNAS web site.

Results

We isolated three LPC clones from an m8 stock and compared several biomarkers with m8 and mO (Fig. 1). All of the clones exhibited phenotypical characteristics similar to mO, such as plaque size (Fig. 1*B*), dermal reactions in rabbits (ErD_{50}) (Fig. 1*C*), and pathogenicity to SCID mice (Fig. 1 *D* and *E*). Specifically, i.p. injection of 10^7 pfu of m8 elicited no overt symptoms over a 4-week period, whereas mO and two LPC clones induced a severe rash and then killed mice, even when administered at a dose (10^5 pfu) 100-fold lower than that of m8 (Fig. 1 *D* and *E*). The accelerated viral replication of LPCs in Vero cells (data not shown) also supported the similarity of the mO and LPC clones. Because the growth ability of mO has been linked to the *B5R* gene product, we hypothesized that the *B5R* gene might be involved in the reversion. Sequencing the LPC genomes revealed that the *B5R* ORF was restored in all of the LPCs, by a one-base insertion at sites just upstream of the deletion site in the m8 *B5R* (Fig. 1 *F* and *G*). Western blotting confirmed the expression of B5R proteins from these LPCs (Fig. 1*H*).

To prevent the reversion of the m8 *B5R* gene, we constructed *B5R*-knockout viruses (see *Supporting Text*). First, we constructed a $B5R^+$ virus (named m8B5R) from m8 by introducing the complete *B5R* cloned from mO (*Supporting Text*, Fig. 5, and Table 1). We then deleted the entire *B5R* sequence from m8B5R to construct m8Δ (*Supporting Text*, Fig. 5, and Table 1). The resultant knockout virus formed plaques as small as the m8rc plaques that were then plaque-purified from m8 stock to minimize LPC contamination (Fig. 2*A*), and did not express the B5R protein in infected RK13 cells, whereas m8B5R and mO did (Fig. 2*B*).

One method by which to augment the immunogenicity of VV without increasing its pathogenicity may be the construction of VV that overexpresses a B5R derivative, which is fully immunogenic but loses its original function in the formation of EEV. The ectodomain of B5R has been reported to possess all epitopes necessary for induction of NT antibody production (22, 29), whereas B5R must be anchored in the membrane for EEV formation (30). We constructed a VV named m8dTM (*Supporting Text*, Fig. 5, and Table 1) that expresses only the ectodomain

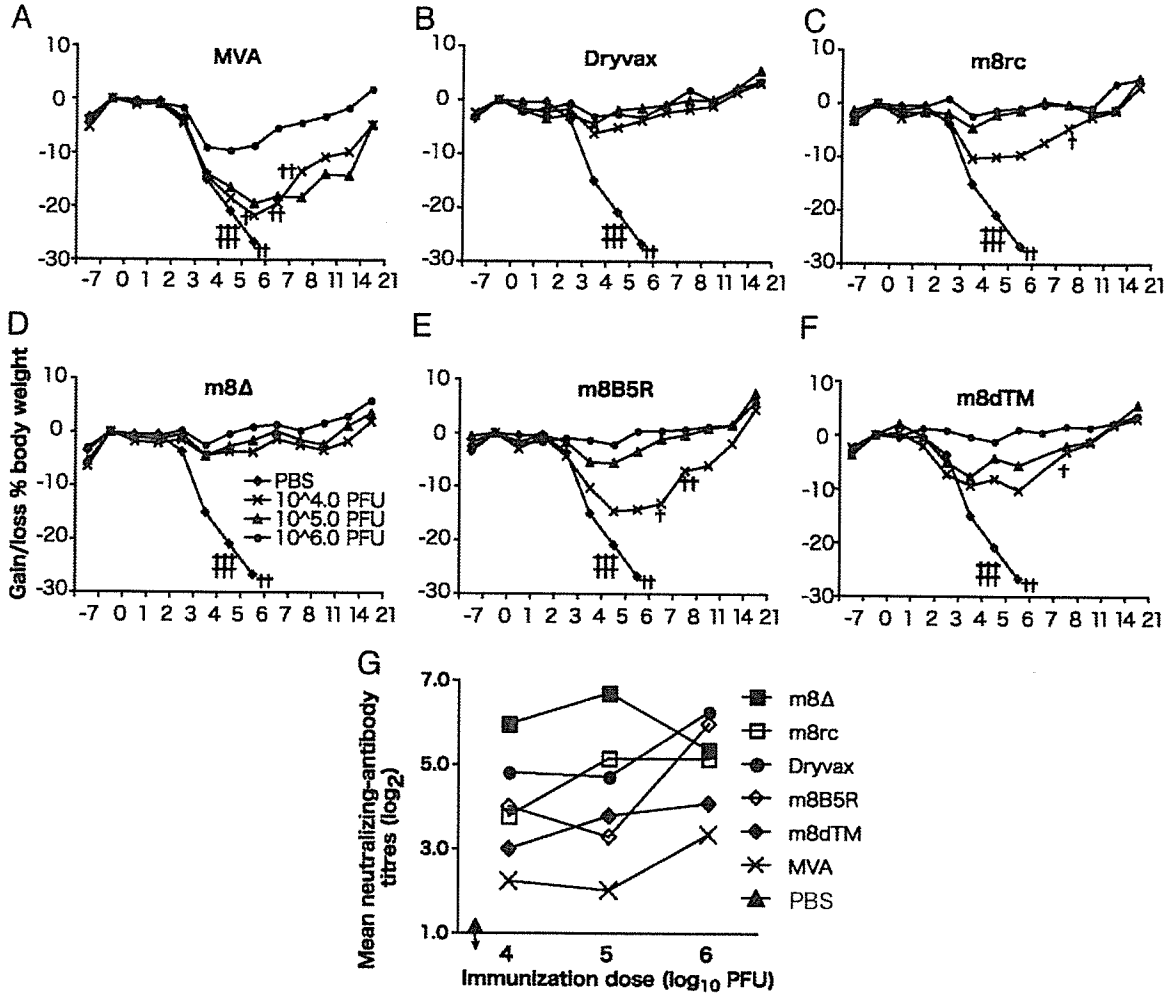

Fig. 3. Protective immunogenicity of vaccine candidate virus in mice. (*A–F*) Average body weight of mice immunized with $10^{4.0}$ to $10^{6.0}$ pfu of VVs intramuscularly and challenged intranasally with the WR strain. Cross marks indicate the mice that died or were killed because of a 30% weight loss. (*G*) Average NT antibody titers in mouse sera collected 3 weeks after immunization with VVs. The titers in sera from a Sham-immunized group were below the limit of detection.

of the B5R protein by replacing the whole *B5R* region of m8B5R with the *B5R* ectodomain sequence placed downstream of the strong promoter PSFJ1–10 (31). m8dTM also formed as small plaques as m8Δ, suggesting that this truncated B5R was not functional for EEV formation. As expected, m8dTM expresses a large quantity of a 38-kDa truncated protein in the culture medium of infected cells (Fig. 2*B*).

To evaluate the genetic stability of the viruses and m8rc, we serially passaged the viruses in PRK cells and Vero cells. Under all conditions tested, including that for vaccine production (passage in PRK cells at 30°C), detectable levels (i.e., levels of $>10^{-6}$) of LPCs failed to emerge from either m8Δ or m8dTM, which is in contrast to the LPC generation from m8rc (Fig. 2 *C* and *D*). Each of the three viruses propagated at similar levels in the cultured cells. It should be noted that once LPCs appeared in the cultures, the fraction of LPCs derived from m8rc rapidly increased with the number of passages (Fig. 2*D*), suggesting it is of vital importance to prevent the emergence of LPCs for optimum quality control of the vaccine.

The protective immunogenicities of smallpox vaccine candidates were compared with other vaccine strains by using a mouse model challenged with a highly pathogenic VV, the WR strain (32) (Fig. 3 and Table 2). All mice immunized with doses of m8Δ or Dryvax survived, whereas all Sham-immunized mice, and 5/8, 3/8, 1/8, and 1/8 mice immunized with 10^4 pfu of MVA, m8B5R, m8rc, or m8dTM, respectively, died or were killed because of a 30% weight loss (Fig. 3 *A–F*). At the lower doses, the mice immunized with m8Δ or Dryvax did not exhibit any significant differences in weight in a challenge after 5 days (*t* test, $P < 0.05$, Table 2). Moreover, the m8Δ-immunized group lost less weight than the Dryvax-immunized group at the highest dose (Table 2). In contrast, the groups immunized with 10^4 pfu of m8rc, m8B5R, and all mice immunized with MVA, experienced a significant weight loss in comparison to m8Δ ($P < 0.05$, Table 2). The m8dTM-immunized group also showed significant weight loss by days 4 and 6 ($P = 0.012$ and 0.038, respectively, data not shown). Measurement of the NT antibody titers elicited in the mice at 3 weeks after immunization (Fig. 3*G*) showed that m8Δ induced the highest titers among the viral strains at lower doses than the other immunizations. The next group, including Dryvax, m8rc, m8B5R, and m8dTM, induced NT antibodies with an efficiency intermediate between m8Δ and MVA. MVA was the least immunogenic virus: 10^6 pfu of MVA was required to induce significant NT antibodies.

The pathogenicity of the *B5R*-defective viruses was examined by ErD_{50} in rabbits (Fig. 4*A*) and by RED_{50} and LD_{50} in SCID mice (Fig. 4 *B* and *C*). m8Δ and m8dTM exhibited an ErD_{50} in rabbits similar to that of m8rc, whereas m8B5R induced the most

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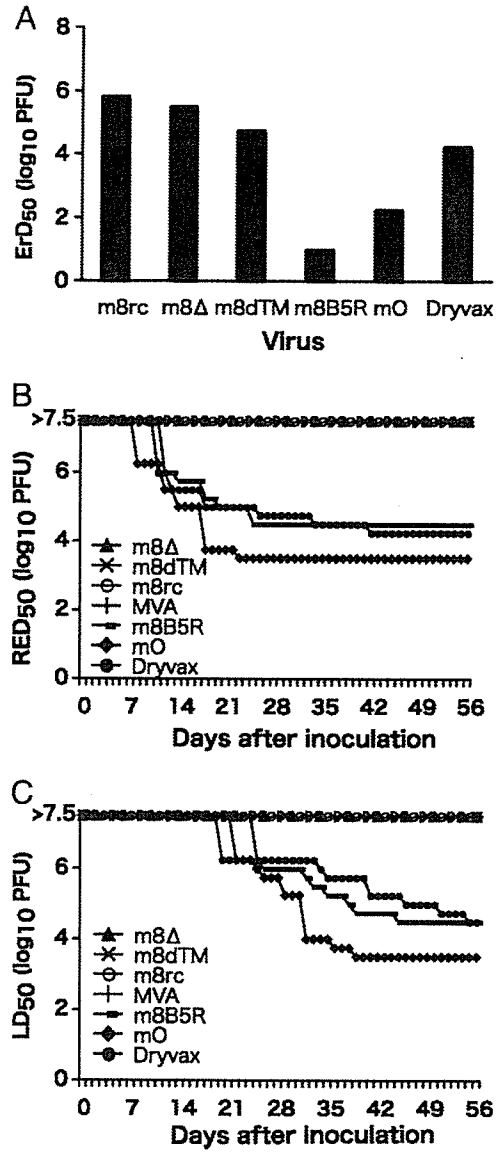

Fig. 4. Pathogenicity of vaccine candidate virus in animals. (*A*) The dermal reaction scores (ErD_{50}) of the *B5R*-defective viruses in rabbits. (*B* and *C*) Virulence of the *B5R*-defective viruses in SCID mice. RED_{50} and LD_{50} are shown in *B* and *C*, respectively.

severe dermal reaction among the strains examined (Fig. 4*A*). The pathogenicity of m8Δ and m8dTM to SCID mice was particularly weak, as demonstrated by the fact that 10^7 pfu of m8Δ or m8dTM did not elicit any symptoms in SCID mice over a period of 56 days (Fig. 4 *B* and *C*). It should be noted that this dose is 1,000-fold higher than that conferring protective immunity (see Fig. 3*D*). MVA and m8rc were also safe in SCID mice, whereas mO, m8B5R, and Dryvax exhibited lethal pathogenicities at lower doses ($10^{3.5}$, $10^{4.5}$, and $10^{4.5}$ pfu, respectively) (Fig. 4 *B* and *C*).

Discussion

One of our goals is to develop a safe and effective smallpox vaccine and vector virus. The m8 strain could be used as a prototype because it has been proven to induce an effective immune response without serious complications in humans (5, 6). It is important to note that the neurovirulence of these strains was separable from their dermal replicability, as suggested by previous experiments with LO-derived strains expressing the envelope protein of human T cell Leukemia virus type 1 (10).

A major safety drawback of m8 is its spontaneous reversion to the mO-like viruses. We identified the *B5R* gene as being responsible for the reversion, and constructed the *B5R*-defective viruses, m8Δ and m8dTM. These viruses are genetically stable and evidently retain the properties of the highly attenuated m8. Moreover, m8Δ shows a level of immunogenicity similar to that of Dryvax.

A previous study (33) reported that the m8-derived recombinant virus, which expresses the hepatitis B surface antigen, maintained its plaque size during 10 passages in the cell culture. The discrepancy between these data and our results in this study may be due to differences in the cell types used to measure plaque sizes. In the earlier study, plaque assays were performed on PRK cell monolayers on which m8 and mO form plaques that are indistinguishable in size, in contrast to the RK13 cell line used in our study. This may be one reason why the reversion of m8 has been previously undocumented.

Another purpose of this study was to evaluate the importance of *B5R* in generating an immune response that confers protection against smallpox infection. B5R protein and a DNA vaccine expressing *B5R* have been reported to induce production of NT antibodies and achieve partial protection against the virus (22–25). Therefore, we assessed the ability of *B5R*, which is expressed during viral replication in mice, to induce protective immunity. The *B5R*-defective virus (m8Δ) was able to elicit NT antibodies, leading to protection comparable to that of the wild-type *B5R*-harboring vaccine, Dryvax. Moreover, the protective efficacies of m8dTM and m8B5R were, unexpectedly, never superior to the *B5R*-defective virus (Fig. 3 and Table 2), and m8B5R was statistically inferior to m8Δ. Western blotting confirmed that B5R proteins were expressed by m8dTM, m8B5R, and Dryvax, and that these proteins were immunogenic and could induce anti-B5R antibody production in mice (data not shown). The subtle difference in protection between m8rc and the *B5R*-knockout virus may be due to the 10-kDa truncated B5R protein synthesized by m8rc (Fig. 2*B*). These data indicate that B5R does not play a major role in inducing protective immunity in response to live vaccinia inoculation in mice. The clinical trial data on m8 in Japan (5, 6) also support our conclusion.

However, the NT antibody titers induced in mice were correlated with body weight changes to some extent, but not completely. Quantitation of antibody titers by ELISAs against the outer membrane proteins of intracellular matured virion (34, 35), which includes L1R, a major target of NT antibodies, showed a similar tendency with NT antibody titers (data not shown). Moreover, the levels of A33R EEV-specific antibodies in mice, which had been suggested to be important for protective immunity (22, 23), did not correlate with the protection level (data not shown). These results may suggest that there may be a contribution of cell-mediated immunities to the protection (36, 37).

Recently, several groups have reevaluated the available vaccinia strains, including the replication-defective MVA, in a search for safer smallpox vaccines (37–40). Although 10^9 pfu of MVA was shown to be safe in monkeys (41), a large quantity of virus, 10^8 pfu, an amount that is 1,000-fold more than a conventional vaccination dosage, was necessary to induce protective immunity (40). Because m8Δ can replicate in the host, it can induce protective immunity comparable to that of the Dryvax strain at a 100-fold lower dose of the virus, making it clearly more effective than MVA. Moreover, m8Δ was not pathogenic in SCID mice at a dose 1,000-fold greater than the lethal dose of Dryvax, a dose that was also 1,000-fold greater than the dose required for its effective protective immunity. m8Δ replicated at the injection site in rabbit skin and caused temporary viremia in SCID mice (data not shown). The preliminary experiments suggested that the viral loads of VV correlate with their pathogenicity to SCID mouse. The virus seems to be eliminated rapidly thereafter and seldom replicates in the CNS (5);

therefore, the magnitude and the region of replication should be restricted, which may explain its safety and efficacy. Therefore, m8Δ should be eminently suitable as a safe and effective vaccine virus and viral vector.

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1. World Health Organization. (1980) *Wkly. Epidemiol. Rec.* **55**, 148.
2. Henderson, D. A., Inglesby, T. V., Bartlett, J. G., Ascher, M. S., Eitzen, E., Jahrling, P. B., Hauer, J., Layton, M., McDade, J., Osterholm, M. T., *et al.* (1999) *J. Am. Med. Assoc.* **281**, 2127–2137.
3. Reed, K. D., Melski, J. W., Graham, M. B., Regnery, R. L., Sotir, M. J., Wegner, M. V., Kazmierczak, J. J., Stratman, E. J., Li, Y., Fairley, J. A., *et al.* (2004) *N. Engl. J. Med.* **350**, 342–350.
4. Centers for Disease Control and Prevention (CDC). (2004) *Morbid. Mortal. Wkly. Rep.* **53**, 106–107.
5. Hashizume, S., Yoshizawa, H., Morita, M. & Suzuki, K. (1985) in *Vaccinia viruses as Vectors for Vaccine Antigens*, ed. Ouinnan, G. V. (Elsevier, Amsterdam), pp. 421–428.
6. Yamaguchi, M., Kimura, M. & Hirayama, M. (1975) *Clin. Virol.* **3**, 269–278.
7. Morita, M., Arita, M., Komatsu, T., Amano, H. & Hashizume, S. (1977) *Microbiol. Immunol.* **21**, 417–418.
8. Morita, M., Aoyama, Y., Arita, M., Amano, H., Yoshizawa, H., Hashizume, S., Komatsu, T. & Tagaya, I. (1977) *Arch. Virol.* **53**, 197–208.
9. Kempe, C. H., Fuliginiti, V., Minamitani, M. & Shinefield, H. (1968) *Pediatrics* **42**, 980–985.
10. Shida, H. Hinuma, Y., Hatanaka, M., Morita, M., Kidokoro, M., Suzuki, K., Maruyama, T., Takahashi-Nishimaki, F., Sugimoto, M., Kitamura, R., *et al.* (1988) *J. Virol.* **62**, 4474–4480.
11. Lee, M. S., Roos, J. M., McGuigan, L. C., Smith, K. A., Cormier, N., Cohen, L. K., Roberts, B. E. & Payne, L. G. (1992) *J. Virol.* **66**, 2617–2630.
12. Kitamura, T., Kitamura, Y. & Tagaya, I. (1967) *Nature* **215**, 1187–1188.
13. Takahashi-Nishimaki, F., Funahashi, S., Miki, K., Hashizume, S. & Sugimoto, M. (1991) *Virology* **181**, 158–164.
14. Smith, G. L., Vanderplasschenb, A. & Law, M. (2002) *J. Gen. Virol.* **83**, 2915–2931.
15. Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E. J., Shida, H., Hiller, G. & Griffiths, G. (1994) *J. Virol.* **68**, 130–147.
16. Hollinshead, M., Rodger, G., Van Eijl, H., Law, M., Hollinshead, R., Vaux, D. J. & Smith, G. L. (2001) *J. Cell Biol.* **154**, 389–402.
17. Rietdorf, J., Ploubidou, A., Reckmann, I., Holmström, A., Frischknecht, F., Zettl, M., Zimmerman, T. & Way, M. (2001) *Nat. Cell Biol.* **3**, 992–1000.
18. Ward, B. M. & Moss, B. (2001) *J. Virol.* **75**, 4802–4813.
19. Katz, E., Ward, B. M., Weisberg, A. S. & Moss, B. (2003) *J. Virol.* **77**, 12266–12275.
20. Newsome, T. P., Scaplehorn, N. & Way, M. (2004) *Science* **306**, 124–129.
21. Payne, L. G. & Kristensson, K. (1985) *J. Gen. Virol.* **66**, 643–646.
22. Galmiche, M. C., Goenaga, J., Wittek, R. & Rindisbacher, L. (1999) *Virology* **254**, 71–80.
23. Hooper, J. W., Custer, D. M. & Thompson, E. (2003) *Virology* **306**, 181–195.
24. Pulford, D. J., Gates, A., Bridge, S. H., Robinson, J. H. & Ulaeto, D. (2004) *Vaccine* **22**, 3358–3366.
25. Hooper, J. W., Thompson, E., Wilhelmsen, C., Zimmerman, M., Ichou, M, A., Steffen, S. E., Schmaljohn, C. S., Schmaljohn, A. L. & Jahrling, P. B. (2004) *J. Virol.* **78**, 4433–4443.
26. Stickl, H., Hochstein-Mintzel, V., Mayr, A., Huber, H. C., Schafer H. & Holzner, A. (1974) *Dtsch. Med. Wochenschr.* **99**, 2386–2392.
27. Mayr, A., Hochstein-Mintzel, V. & Stickl, H. (1974) *Infection* **3**, 6–14.
28. Finney, D. J. (1959) *Acta Microbiol.* **6**, 341–368.
29. Law, M. & Smith, G. L. (2001) *Virology* **280**, 132–142.
30. Herrera, E., Lorenzo, M. M., Blasco, R. & Isaacs, S. N. (1998) *J. Virol.* **72**, 294–302.
31. Jin, N. Y., Funahashi, S. & Shida, H. (1994) *Arch. Virol.* **138**, 315–330.
32. Williamson, J. D., Reith, R. W., Jeffrey, L. J., Arrand, J. R. & Mackett, M. (1990) *J. Gen. Virol.* **71**, 2761–2767.
33. Watanabe, K., Morita, M. & Kojima, A. (1989) *Vaccine* **7**, 499–502.
34. Rodriguez, J. F., Janeczko, R. & Esteban, M. (1985) *J. Virol.* **56**, 482–488.
35. Wolffe, E. J., Vijaya, S. & Moss, B. (1995) *Virology* **211**, 53–63.
36. Xu, R., Johnson, A. J., Liggitt, D. & Bevan, M. J. (2004) *J. Immunol.* **172**, 6265–6271.
37. Wyatt, L. S., Earl, P. L., Eller, L. A. & Moss B. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 4590–4595.
38. Ober, B. T., Bruhl, P., Schmidt, M., Wieser, V., Gritschenberger, W., Coulibaly, S., Savidis-Dacho, H., Gerencer, M. & Falkner, F. G. (2002) *J. Virol.* **76**, 7713–7723.
39. Drexler, I., Staib, C., Kastenmuller, W., Stevanovic, S., Schmidt, B., Lemonnier, F. A., Rammensee, H. G., Busch, D. H., Bernhard, H., Erfle, V., & Sutter, G. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 217–222.
40. Earl, P. L., Americo, J. L., Wyatt, L. S., Eller, L. A., Whitbeck, J. C., Cohen, G. H., Eisenberg, R. J., Hartmann, C. J., Jackson, D. L., Kulesh, D. A., *et al.* (2004) *Nature* **428**, 182–185.
41. Stittelaar, K. J., Kuiken, T., de Swart, R. L., van Amerongen, G., Vos, H. W., Niesters, H. G., van Schalkwijk, P., van der Kwast, T., Wyatt, L. S., Moss, B. & Osterhaus, A. D. (2001) *Vaccine* **19**, 3700–3709.

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Original article

Regulation of human T-cell leukemia virus type 1 (HTLV-1) budding by ubiquitin ligase Nedd4

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Abstract

The Gag protein of human T-cell leukemia virus type 1 (HTLV-1) contains the conserved sequences PPxY and PTAP, which are putative viral motifs required for budding (L-domain motifs). We show here that the PPxY motif, but not the PTAP motif, is essential for HTLV-1 virion budding from the plasma membrane. In addition, we show that overexpression of Nedd4 enhances HTLV-1 budding and that Nedd4 interacts with Gag via its WW domain. The HECT domain of Nedd4 is also required for budding. These results indicate that Nedd4 or a Nedd4-related ubiquitin ligase plays a critical role in HTLV-1 budding.

Keywords: HTLV-1; Budding; Nedd4; Ubiquitin

1. Introduction

Retroviral assembly is driven by the Gag precursor proteins. For most of the retroviruses, including human T-cell leukemia virus type 1 (HTLV-1), Gag polyproteins assemble at the plasma membrane, grow a spherical bud form, and are released from the membrane. The HTLV-1 Gag precursor is cleaved by a viral protease into three peptides, termed 19-kDa matrix (MA), 24-kDa capsid (CA) and 15-kDa nucleocapsid (NC). Three functional domains in the Gag protein that are critical for the assembly and budding processes have been identified. The membrane-binding domain (M domain) is required for the myristylation of the Gag N-terminal region and the subsequent targeting of the protein to the plasma membrane. The interaction domain (I domain) appears to be a major region for Gag multimerization. The late assembly domain (L domain) plays a critical role in the pinching off of virus particles from the plasma membrane of infected cells [1–5].

In the Mason–Pfizer monkey virus (M-PMV), Rous sarcoma virus and murine leukemia virus (MuLV), a highly conserved PPxY sequence functions as the core of the L domain [6–8]. In contrast, the human immunodeficiency viruses type 1 (HIV-1) and type 2 (HIV-2) have another crucial motif, PTAP, that acts as the core sequence of the L domain [9,10]. However, the PTAP motif of HIV can be interchanged with other retroviral PPxY motifs without these viruses losing their ability to bud [11,12]. Interestingly, the Ebola virus has overlapping PPxY and PTAP motifs (PTAP-PxY) in the VP40 matrix protein [13], both of which seem to be essential for Ebola viral budding [13,14].

PPxY motifs have been reported to bind to Nedd4-like E3 ligases [7,15,16], suggesting that ubiquitin E3 ligases play an important role in retroviral budding. Supporting this notion is our study showing that expression of a novel Nedd4-like ubiquitin ligase, BUL1, enhances the budding of M-PMV and that when BUL1 is mutated, the ability to bud is lost [17]. The PTAP motif in HIV-1, HIV-2 and the Ebola virus has also been found to interact with a ubiquitin E2 ligase variant (UEV), namely, Tsg101 [4,9,10,18,19]. Recent studies have reported that RNA interference (RNAi) of Tsg101 and the expression of a dominant negative form of Tsg101 block the budding of these viruses [20,21]. Thus, Tsg101 appears to be crucially involved in the budding of some viruses.

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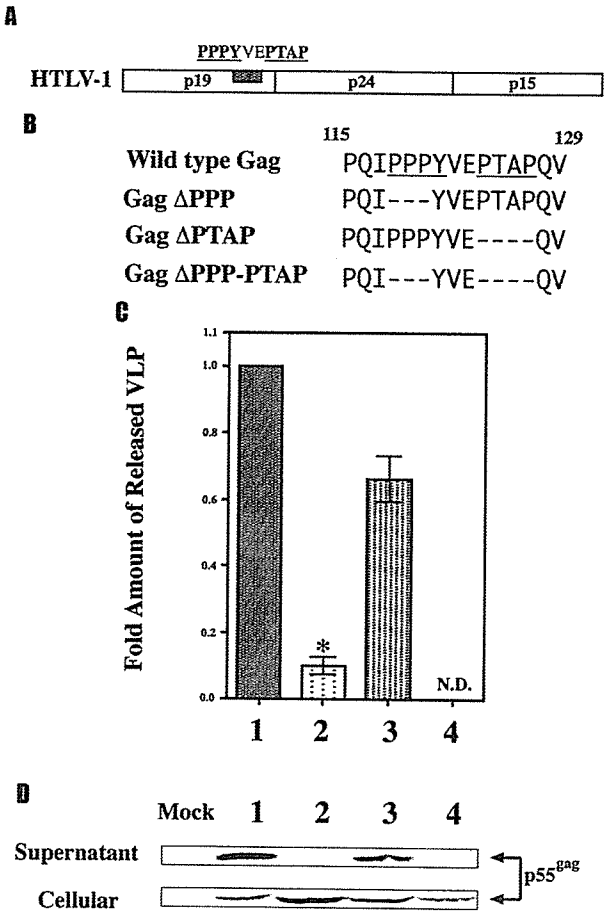

Fig. 1. (A) Location of the PPxY and PT/SAP late domain sequences in HTLV-1. Both the PPxY and PTAP sequences are located in the C-terminal region of the HTLV-1 p19 matrix protein. (B) Schematic representation of the Gag mutants analyzed in this study. (C, D) The PPxY sequence is required for VLP budding. Gag protein in the VLPs and cell lysates was detected by western blotting with the α-p24 antibody (CA). The intensities of the bands for p24 in the VLPs and the cell lysates were quantitated by FLA-3000. The graphical data are presented as the ratio of VLP p24/cellular p24. The extent of VLP budding in wild type was set to 1.0. The data represent averages and S.D. of three independent experiments. Lane 1, wild-type Gag; lane 2, ΔPPPY; lane 3, ΔPTAP; lane 4, ΔPPP–PTAP. The asterisk indicates P value <0.05. N.D., Not detected.

HTLV-1 has both PPxY and PTAP sequences in the MA protein (Fig. 1A), unlike HTLV-2 or the bovine leukemia virus (BLV), which bear only PPxY sequences [22,23]. Therefore, HTLV-1 could bud by using one of three possible mechanisms. First, it could employ the PPxY/E3-mediated pathway like that used by M-PMV. Second, it could bud with the PTAP/UEV-mediated pathway like that in HIV-1. Third, it could use both pathways, as does the Ebola virus. Recently, Le Blanc et al. [26] reported that the PPPY sequence of HTLV-1 was required for the viral budding. However, they did not examine the effect of the PTAP sequence. In this study, we examined which of the HTLV-1 L domain sequences are critical for HTLV-1 budding. We also determined the cellular ligase that is involved in HTLV-1 budding.

2. Materials and methods

2.1. Plasmid

Plasmids that express Nedd4, Nedd4WW, Nedd4C894A, BUL1, KIAA1301, or Tsg101 have been described previously [8,17,24]. To express wild-type and mutant Gag proteins, we used a pJW322 expression vector, which is a pBR322-based plasmid from pJW4303 that includes a CMV promoter. The HTLV-1 *gag* gene was amplified by PCR from HTLV-1 K30, an infectious molecular clone, using a forward primer containing an *Eco*RI site (CGGAATTCCGTCCCTAGGCAATGGGCCAAA) and a reverse primer containing a *Bam*HI site (CGCGGATCCGCGTGTGGGGGGGGAGGTTAAACCT). The amplified product was digested with *Eco*RI and *Bam*HI and cloned into pJW322 to construct the wild-type pGag. Mutated Gag expression vectors were constructed by using QuickChange™ (STRATAGENE) and the wild-type Gag expression vector. The mutated Gag expression vectors, pGag ΔPPP and ΔPTAP, lack the PPP and PTAP sequences of L domain, respectively (Fig. 1B). The pGag ΔPPP–PTAP lacks both the PPP and PTAP sequences (Fig. 1B). The resulting plasmids were verified by sequencing.

2.2. Cell culture and transfection

HeLa cells were cultured in Dulbecco modified Eagle medium, which was supplemented with 10% fetal bovine serum, in a 5% CO_2 atmosphere at 37 °C. Transfection experiments were carried out using LipofectAmine™ 2000 (Invitrogen). The cells were harvested 48 h posttransfection.

2.3. Western blotting

Forty-eight hours after the transfection, the virus-like particles (VLPs) or virions in the culture medium were collected and prepared as previously described [8]. VLPs, virion and cell lysates were resolved by SDS-PAGE, and the proteins were then transferred to nitrocellulose filters. The anti-p24 mouse monoclonal antibody (MAb) NOR-1 [25] or anti-c-myc MAb 9E10 (SIGMA) was used as primary antibody. Horseradish peroxidase- or alkaline phosphatase-conjugated anti-immunoglobulin G (IgG) antibodies (Promega) were used as secondary antibodies. Immunoreactive bands were visualized using ECL+ (Amersham Pharmacia Biotech), followed by the LAS-1000 plus system (Fujifilm) or 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium solution. For quantitation, the signal intensity on the western blot membrane was evaluated by Image Gauge version 3.4 software (Fuji Film) using the LAS-1000 plus system.

2.4. ELISA

The amount of p19 (MA) in the virion and the cell lysates was quantified using the HTLV-I/II p19Antigen ELISA (ZeptoMetrix).