through the degradation of viral proteins. Recently, MDM2 was also reported to ubiquitinate HIV-1 Tat protein and activate its transcriptional activity in a non-proteolytic manner [26]. Our experiment using MDM2 knockdown macrophages showed that HIV-1 replication in these macrophages was more efficient than in control siRNA-transfected macrophages. These data are consistent with MDM2 negatively regulateing HIV-1 replication through Vif ubiquitination (Fig. 5C). However, the replication efficiency of HIV-1 in MDM2 knockdown macrophages was only 2-fold higher and was slower than in control siRNA-transfected macrophages. This suggests the possibilities that the ubiquitination of Tat might work as a positive regulatory factor at an earlier phase of infection and that MDM2 might be involved in both positive and negative regulation of HIV-1 replication at different stages. Further studies on the detailed effect of MDM2 on HIV-1 replication are needed.

We also demonstrated that Vif can bind MDM2 directly. We also mapped the interaction domain of MDM2 with Vif to amino acids 168–320 which is located in its central acidic and Zn finger domains. This central domain is different from the primary p53-binding site of MDM2 which is located in its N-terminal region; however, this central deomain was recently reported as a second p53-binding site and was shown to be important for the regulation of p53 stability [27-30] (Fig. 2B &2C). Interestingly, several proteins including p300, $p14^{ARF}$, and pRB bind to the central domain of MDM2 and regulate the stability and function of p53 via MDM2 [28,31]. Thus, it is possible that Vif might affect the stability and function of p53. Indeed, we confirmed that Vif can stabilize p53 (*Izumi et al., unpublished data*), which could explain why the effect of MDM2 on p53 degradation was weaker than that on Vif as shown in Fig. 1A. A further study is under way to elucidate this new function of Vif (*Izumi et al., HIV-1 Vif induces G2 cell cycle arrest via the p53 pathway, unpublished*).

Finally, expanding evidence suggests that the ubiquitination system plays important roles in many aspects of HIV-1 replication including the degradation of A3G by Vif [9-11], the degradation of CD4 by Vpu [32], HIV-1 viral budding [33], Tat-mediated transactivation [26], and Vpr-induced G2 cell cycle arrest [34,35]. The functional linkage between Vif and MDM2 also suggests that ubiquitin processes such as the A3G/Vif interplay is highly complex. It is obvious that HIV-1 replication in target CD4+ T cells is strongly affected by the interplay of these proteins. From the viral point of view, this interplay might give an advantage to HIV-1 replication. One possibility is that MDM2 regulates cellular Vif levels appropriately, such as not to affect viral replication [36] but just enough to antagonize A3G. Recent studies suggest that the G-to-A mutations induced by A3G may not be the mechanism by which A3G restricts or controls viral replication [37] and that a partially effective Vif inhibitor may actually accelerate the evolution of drug resistance and immune escape [38]. The inhibitory activity of MDM2 toward Vif could be partially effective and therefore could lead to viral evolution of drug resistance and immune escape. More recently, Nathans et al. have reported a small molecule that specifically antagonizes Vif function and inhibits viral replication by targeting the A3G/Vif axis. This compound enhances Vif degradation only in the presence of A3G, but does not induce A3G degradation and rather stabilizes A3G. They suggested the possibility of a new proteolytic enzyme for Vif degradation and that their new compound interferes with Vif interaction with a host protein in a Vif-A3G-host protein complex, thereby making Vif less stable. The precise biological significance of this Vif-A3G-host protein complex requires future elucidation. Nevertheless, modification or intervention of such Vif-A3G-host protein interplay could lead to the development of new therapeutic strategies for HIV-1 infection.

Conclusion

MDM2 is a novel E3 ligase for Vif which induces the polyubiquitination and degradation of Vif to negatively regulate HIV-1 replication.

Methods

Plasmid constructs

Expression vectors for hemagglutinin (HA)- or FLAG-tagged MDM2, pCMV4/HA-MDM2 or pCMV4/FLAG-MDM2, and their mutants were constructed as previously described [19]. An expression vector for HA-tagged human APOBEC3G, pcDNA3/HA-hA3G [39], and HIV-1 reporter plasmids, pNL43/Δenv-Luc (WT) and pNL43/ΔenvΔvif-Luc (ΔVif) [8], were constructed as previously described. Expression vectors for FLAG-tagged Parkin and Cul5 (pcDNA3/FLAG-Parkin and pcDNA3/FLAG-Cul5, respectively) were constructed by the PCR method. Complementary DNA for HIV-1 Vif was also cloned into pDON-AI (TAKARA BIO INC.) and pDON/EGFP for expression of Vif and EGFP-fused Vif (EGFP-Vif). The subgenomic expression vector pNL-A1, which expresses all HIV-1 proteins except for *gag* and *pol* products, and its mutants expressing Vif deletion mutants were kind gifts from Dr. K. Strebel [22].

Co-immunoprecipitation assays

We performed an immunoprecipitation assay for protein-protein interaction *in vivo*, as described previously [8]. HEK293T cells were cotransfected with pCMV4/HA-MDM2 and pNL-A1 by the calcium phosphate method. Two days after transfection, cells were lysed in lysis buffer (25 mM HEPES pH7.4/150 mM NaCl/1 mM $MgCl_2$/0.5% TritonX-100/10% Glycerol) and complexes were immunoprecipitated with anti-MDM2 monoclonal antibody

(mAb) (SMP-14, Santa Cruz Biotechnology, Inc., Santa Cruz, CA and Ab-1, Calbiochem, EMD Biosciences, Inc, Darmstadt, Germany) and Protein A-Sepharose beads (Amersham Biosciences Corp.) at 4°C. The beads were washed with RIPA buffer (50 mM Tris-HCl pH8.0/150 mM NaCl/1% Triton-X 100/0.1% SDS/0.1% DOC) and analyzed by immunoblotting with anti-Vif mAb (#319) (A kind gift from Dr. M. Malim through the AIDS Research and Reference Reagent Program) [40] or anti-HA mAb (12CA5). To map the regions of MDM2 necessary for binding to Vif, HEK293T cells were cotransfected with expression vectors for a series of MDM2 deletion mutants together with pNL-A1. Complexes were immunoprecipitated with anti-HA mAb and analyzed by immunoblotting with anti-Vif mAb. To map the regions of Vif necessary for binding to MDM2, HEK293T cells were cotransfected with expression vectors for a series of Vif deletion mutants together with pCMV4/HA-MDM2. Complexes were immunoprecipitated with anti-Vif mAb and analyzed by immunoblotting with anti-MDM2 mAb. In all these experiments, transfected cells were treated with MG132 for 6 hrs prior to harvesting in order to stabilize both Vif and MDM2; otherwise we could not detect the expression of MDM2 because of its rapid degradation, as seen in Fig. 1A.

In vitro and in vivo ubiquitination assays

In vitro ubiquitination assays were carried out in ubiquitin reaction buffer (50 mM Tris-HCl/2 mM ATP/5 mM $MgCl_2$/2 μM DTT) with E1(200 ng), E2(Ubc5c)(150 ng), and GST-tagged ubiquitin (GST-Ub) (10 μg) as described previously [13]. MDM2 and Vif were expressed as GST-fusion proteins in Escherichia coli strain DH5α and BL21, respectively. The reactions were incubated at 30°C for 90 min. The samples were subjected to immunoblotting with anti-Vif mAb to detect GST-ubiquitin conjugated Vif.

For *in vivo* ubiquitination assays, HEK 293T cells were cotransfected with plasmids expressing Vif, FLAG-MDM2 or its mutants, and His-tagged ubiquitin (His-Ub) as indicated. Cells were treated with 10 μM MG132 for 6 hrs prior to harvesting. Forty-eight hours post transfection, cell lysates were affinity-purified with Ni-NTA-agarose beads (Invitrogen corporation, Carlsbad, CA) and analyzed by immunoblotting with anti-Vif mAb.

For production of RNAi within the cells, we used the pSuper vector as described previously [19]. pSuper-MDM2-1 contained the 19 nt derived from the *mdm2* cDNA (nt 404–422) as the target sequence. Double-stranded RNA containing scrambled 19 nt was used as a control. HEK293T cells were transfected with pSuper plasmids together with plasmids expressing Vif and HA-Ub. Cell lysates were immunoprecipitated with anti-Vif mAb followed by immunoblottimg with anti-HA mAb.

Single round infection assays with HIV-1 luciferase reporter virus

Luciferase reporter viruses with or without Vif were prepared by cotransfection of pNL43/Δenv-Luc (Wt) or pNL43/ΔenvΔvif-Luc (ΔVif) plus pVSV-G together with a mock vector or an expression vector for MDM2 or a mutant in the presence or absence of pcDNA3/hA3G by calcium phosphate as previously described [8]. The reporter viruses were adjusted according to p24 values and used to infect M8166 target cells. Productive infection was measured by luciferase activity and values were presented as percent infectivity relative to the value of each virus without the expression of hA3G.

Knockdown of MDM2 in macrophages and replication assays

Monocyte-derived macrophages (MDM) were cultured for 7 days from CD14+ monocytes isolated from the peripheral blood of an HIV-1-negative healthy individual. Electroporation with Stealth Select RNAi for MDM2 or Control (Invitrogen Corporation) was performed using the Nucleofector machine (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's instructions. Twenty four hours after transfection, MDM were challenged with R5 HIV-1_{JR-FL} at multiplicity of infection of 0.1 at 37°C for 3 hrs. The cells were cultured from day 4 to 21 after infection, and the concentration of p24 antigen in the supernatant was measured with an HIV-1 p24 antigen enzyme-linked immunosorbent assay [ELISA] kit (ZeptMetrix, Buffalo, NY).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TI. designed research, performed research, contributed vital new reagents, analyzed data, and wrote the paper. ATK designed research, analyzed data, wrote the paper, and organized the research. KS, KIo, and MM prepared the materials and performed a part of the research. KIwai, HK, TS, MT, SI., and HA contributed vital new reagents. YK contributed vital new reagents, performed a part of the research, and analyzed the data. HH, KItoh, and JF designed the research, contributed vital new reagents, and analyzed the data. TU analyzed the data, drafted the paper, and organized the research.

Additional material

Additional file 1

Supplementary figure 1 – the stability of Vif protein in p53-/- MEF and p53-/-MDM2-/- MEF cells. MEF cells were transfected with pDON/Vif or pcDNA3/HA-A3G. Twenty-two hours after transfection, the cells were treated with cycloheximide (CHX) for the indicated times, and cell lysates were subjected to immunoblotting with the indicated Abs.

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Additional file 2

Supplementary figure 2 – immunopurified MDM2 induced the polyubiquitination of Vif in vitro. *(A) MDM2 as well as Cul5 induced the polyubiquitination of Vif. HEK293T cells were transfected with expression vectors for His-MDM2 and His-Cul5. His-tagged proteins were purified using Ni-NTA agarose and subjected to* in vitro *ubiquitination assays as described in a legend to Fig. 4A. Reactions were subjected to immunoblotting with anti-Vif Ab. Arrows indicate GST-Ub-conjugated Vif. Asterisks indicate non-specific bands associated with GST-Vif protein recognized by anti-Vif Ab, as they are seen in lanes 1 and 3. (B) MDM2 induced the polyubiquitination of Vif Wt but not that of Δ22 that was defective for binding MDM2. Filled asterisks indicate non-specific bands associated with GST-Vif protein, while white asterisks indicate those associated with GST-Vif Δ22.*

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Additional file 3

Supplementary figure 3 – the overexpression of MDM2 inhibited HIV-1 replication in the presence of A3F. Single round infection assays were performed in the presence or absence of A3F as described in a legend to Fig. 5A. Values are presented as averages of more than 3 independent experiments.

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Acknowledgements

We thank Drs. K. Strebel for the pNL-A1 plasmid and its derivative mutants, D. P. Lane for p53$^{-/-}$MDM2$^{-/-}$ DKO-MEF, and M. Malim for the anti-Vif mAb (#319) through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This study was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, from the Ministry of Health, Labour and Welfare, Japan, from the Naito Foundation, and from Mitsubishi Pharma Research Foundation.

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

MINI-REVIEW

Non-human primate surrogate model of hepatitis C virus infection

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ABSTRACT

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

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

INTRODUCTION

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

ANIMAL MODELS OF HCV INFECTION: RODENTS AND CHIMPANZEES

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

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

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

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

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

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

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

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

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

CHARACTERISTICS OF GBV-B INFECTION OF NEW WORLD MONKEYS

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

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

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

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

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

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

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

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

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

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

FUTURE PROSPECT OF GBV-B SURROGATE MODEL

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

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

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Gene 432 (2009) 60–66

Contents lists available at ScienceDirect

Gene

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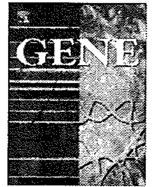

Molecular cloning and characterization of the common marmoset huntingtin gene

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ARTICLE INFO

Article history:
Received 24 July 2008
Received in revised form 4 November 2008
Accepted 5 November 2008
Available online 24 November 2008

Received by M. Di Giulio

Keywords:
Common marmoset
Huntingtin
Gene silencing
Immortalized cell line

ABSTRACT

We report here for the first time the isolation and identification of the common marmoset (*Callithrix jacchus*) *huntingtin* (*Htt*) gene, whose ortholog in humans is known to be related to Huntington's disease (HD). A 9396 nucleotide complementary DNA (cDNA) carrying the putative full-length open reading frame of the marmoset *Htt* gene was identified, and highly conserved nucleotide and amino acid sequences among primates were observed. Based on this data and using tools evaluated for the detection of the marmoset *Htt* gene, we have demonstrated gene silencing against the expression of endogenous *Htt* gene in immortalized common marmoset mononuclear cells by means of RNA interference (RNAi). Taken together, the data presented here may assist us in realizing a non-human primate HD model with the common marmoset.
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1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease characterized by progressive and selective neural cell death associated with choreic movement and dementia (Walker, 2007). The responsible gene for HD, the huntingtin (Htt) gene, has been identified on chromosome 4q16.3 (Gusella et al., 1983; Gilliam et al., 1987), and an aberrant length of a CAG triplet repeat in exon 1, followed by expanded tracts of polyglutamine in the Htt polypeptide, is greatly involved in the onset of HD (Huntington's-Disease, 1993). Although the molecular mechanisms of either normal or aberrant Htt protein are still poorly understood, HD model animals (Mangiarini et al., 1996; Kazemi-Esfarjani and Benzer, 2000; von Horsten et al., 2003) and cells (Lunkes and Mandel, 1998) for understanding the pathogenesis of HD and developing therapies have been established by means of genetic engineering based on the genetic information of *Htt*. The use of an animal model that is closely related to humans may be particularly promising.

The common marmoset (*Callithrix jacchus*) is classified into the Callitrichidae family of Platyrrini (New World monkeys) and has been used as a non-human primate experimental animal in various research fields including gene therapy, autoimmune disease, organ transplantation, and pharmacology (Kendall et al., 1998; Doods et al., 2000; Deisboeck et al., 2003; t'Hart et al., 2003). Accordingly, it is worth promoting studies with the common marmoset aimed at overcoming neurodegenerative diseases such as HD, as the animal's close relationship to humans makes it well suited to this kind of study. Indeed, a recent study has generated a non-human primate HD model with the rhesus monkey (*Macaca mulatta*) (Palfi et al., 2007; Yang et al., 2008).

In this report, we describe for the first time the isolation and characterization of a cDNA encoding the putative full-length open reading frame of the common marmoset *Htt* gene, and present experimental data based on the isolated cDNA. The data presented here may provide us with useful information for establishing non-human primate HD models with the common marmoset.

2. Materials and methods

2.1. Preparation of total RNA

Common marmoset total RNA was isolated from the brain tissue of a stillborn marmoset fetus and immortalized monocytes (described below) using Trizol (Invitrogen). The experiments with the common marmoset complied with protocols approved by the ethical committee for primate research of the National Center of Neurology and Psychiatry and adhered to the legal requirements of Japan.

Abbreviations: HD, Huntington's disease; Htt, huntingtn; RNAi, RNA interference; cDNA, complementary DNA; PBMC, peripheral blood mononuclear cell; RT, reverse transcription; PCR, polymerase chain reaction; ORF, open reading frame; APP, amyloid precursor protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescence protein; CMV, Cytomegalovirus.

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doi:10.1016/j.gene.2008.11.012

2.2. Established common marmoset cell lines

Adult common marmosets being reared at the Primate Research Institute of Kyoto University or Tsukuba Primate Research Center were anesthetized by ketamine, which was approved by the Animal Welfare and Animal Care Committees of both institutes, and peripheral blood was collected. From the collected blood samples, peripheral blood mononuclear cells (PBMCs) were purified and immortalized by infection of a 488-77 strain of *Herpesvirus saimiri* (kindly provided by Dr. R. C. Desrosiers) as previously described (Akari et al., 1996). The established marmoset cell lines, designated HSCj-110, HSCj-009, and HSCj-002, were phenotypically activated CD3+T lymphocytic cells and grown in RPMI-1640 medium (Sigma) supplemented with 10% FCS, 50 mM 2-mercaptoethanol, and antibiotics.

2.3. Reverse transcription – (real time) polymerase chain reaction [RT-(real time) PCR]

The common marmoset total RNAs were subjected to complementary DNA (cDNA) synthesis using oligo(dT) primers and a Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions, and polymerase chain reaction (PCR) using the cDNAs as templates was carried out by means of the ABI GeneAmp PCR system 9700 (Applied Biosystems). In the case of real time PCR, the cDNAs were examined by the AB 7300 Real Time PCR System (Applied Biosystems) with a TaqMan Universal PCR Master Mix together with Assays-on-Demand Gene Expression products (Applied Biosystems) or a SYBR Green PCR Master Mix together with Perfect Real Time Primers (Takara Bio) or designed PCR primers, according to the manufacturers' instructions. Synthesized oligonucleotide primers and purchased primer and probe were as follows:

Synthesized oligonucleotide primers:

HD1-F: 5′-TATAGAATTCGGGAGACCGCCATGGCGAC-3′
HD1-ORF-R: 5′-TCAAGCGGCCGCTCAGCAGGTGGTGACCTTG-3′
HD1-1900R2: 5′-TAAAGGATCCCCGTCTAACACAATTTCAG-3′
cjHtt(1139)-F: 5′-TTATAGCTGGAGGCGGTTCC-3′
cjHtt(1254)-R: 5′-GACGTCCGACCTCGATTCAG-3′

Purchased primer and probe:

Assays-on-Demand Gene Expression product for the human *Htt* gene (Assay ID: Hs00169273_m1) (Applied Biosystems).

Perfect Real Time Primers for the human *GAPDH* gene (Primer-Set ID: HA067812) (Takara Bio).

2.4. Cloning and sequence analysis of the full-length ORF of the marmoset Htt gene

Complementary DNA derived from the common marmoset total RNA was subjected to PCR amplification using *TaKaRa LA Taq* polymerase (TAKARA BIO) with the HD1-F and HD1-ORF-R primers under the following thermal cycling conditions: heat denaturation at 94 °C for 1 min, 30 cycles of amplification including denaturation at 94 °C for 20 s and extension at 68 °C for 12 min, and a final extension at 72 °C for 10 min. The PCR product was examined by agarose gel electrophoresis followed by ethidium bromide staining, and an approximately 9.4 kb PCR band (Fig. 1) was purified from the gels using a TOPO XL gel purification kit (Invitrogen). The resultant PCR product was inserted into the pCR-XL-TOPO plasmid with a TOPO XL PCR cloning kit (Invitrogen) and then sequence determination of the insert was carried out. To clarify uncertain nucleotide sequences, additional RT-PCR targeting of uncertain regions followed by sequence determination was performed and the precise nucleotide sequence was confirmed. The determined nucleotide sequence encoding a putative full-length ORF of the common marmoset *Htt* gene has been registered in the GenBank database: accession number, AB443866.

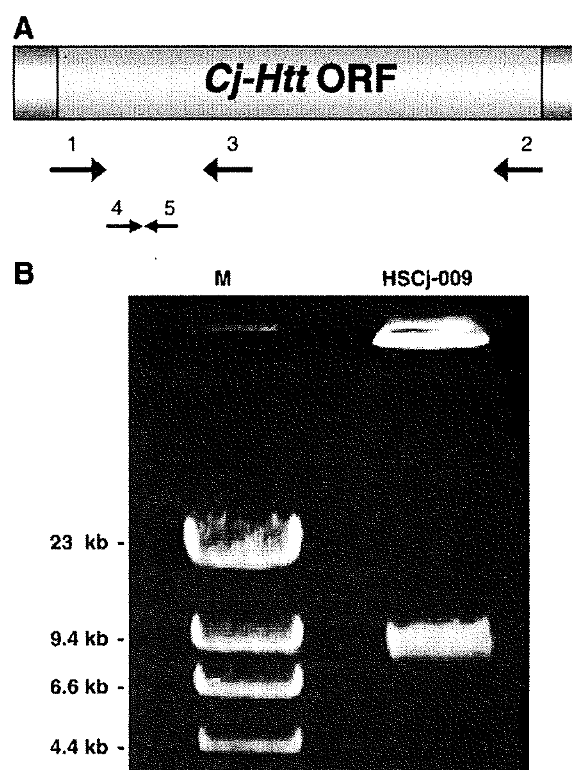

Fig. 1. RT-PCR amplification. (A) Schematic drawing of putative *Htt* cDNA. Open reading frame (ORF) is indicated by a yellow box. Arrows indicate synthesized PCR primers, which are designed in possibly conserved nucleotide sequences: 1, HD1-F; 2, HD1-ORF-R; 3, HD1-1900R2; 4, cjHtt(1139)-F; 5, cjHtt(1254)-R (detailed in Materials and methods). (B) RT-PCR. The first strand cDNA was synthesized by RT using RNA isolated from immortalized common marmoset mononuclear cells (HSCj-009) as a template and oligo(dT) as a primer. The following PCR was carried out using HD1-F and HD1-ORF-R primers. The resultant PCR products were analyzed by gel electrophoresis with 0.6 % agarose gel followed by ethidium bromide staining. Hind III-digested λDNA was used as a DNA size marker (M).

2.5. Western blotting

Equal amounts (~35 μg) of protein extracts from the common marmoset and mouse brain tissues and established PBMC lines (described above) were separated by SDS-PAGE with 5% polyacrylamide gels and electrophoretically blotted onto PVDF membranes (Millipore). Membranes were blocked for 1 h in blocking solution [5% non-fat milk in TBST buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween-20)] and incubated with 1/1000 dilution of mouse anti-huntingtin protein monoclonal antibodies [MAB2166 and MAB2170 (Chemicon); ab7666 (Abcam)] followed by washing in TBST buffer and further incubation with sheep anti-mouse Ig, HRP-linked whole Ab (GE Healthcare). Antigen-antibody complexes were visualized using ECL plus Western Blotting Detection Reagent (GE Healthcare). After detection of signals, the membranes were subjected to antibody removal in Re-Blot Plus strong antibody stripping solution (Chemicon) followed by washing in TBST buffer, and then incubated with 1/1000 dilution of mouse anti-APP [MAB348 (Chemicon)] monoclonal antibody. Subsequent processes were the same as described above.

2.6. Gene silencing of marmoset Htt by RNA interference

To monitor gene silencing against the common marmoset *Htt* gene, we constructed a reporter plasmid carrying the 5′-terminal region of the marmoset *Htt* linked with the *GFP* reporter gene: the PCR product obtained from RT-PCR with the HD1-F and HD1-1900R2 primers was

Table 1
Sequence homologies (%) among various species' *Htt* genes

Homo sapiens	*Callithrix jacchus*	*Canis lupus familiaris*	*Bos taurus*	*Sus scrofa*	*Mus musclus*	*Rattus norvegicus*
Homo sapiens	**95.1** **97.0**	87.0 92.0	84.0 89.5	84.1 88.6	86.1 91.2	85.8 91.2
Canis lupus familiaris		86.6 91.4	84.0 88.4	83.9 87.9	85.6 90.8	85.1 90.9
Callithrix jacchus			84.5 89.4	84.4 89.7	84.0 89.2	83.8 89.3
Bos taurus				86.8 89.3	81.2 87.1	81.3 87.4
Sus scrofa					80.9 86.9	80.8 87.2
Mus musclus						95.9 97.6
Rattus norvegicus						

Figures in upper and lower stands represent nucleotide and amino acid sequence homologies, respectively, between two species.

trimmed with EcoRI and BamHI, and inserted into the pd2EGFP-N1 plasmid (Clontech) treated with the same restriction enzymes. The resultant reporter (5′*Cj-Htt-GFP*) plasmid and synthetic siRNA duplex targeting the marmoset *Htt* (cjHtt-1 siRNA duplex) were cotransfected into mouse neuroblastoma Neuro2a cells by Lipofectamine 2000 transfection reagent (Invitrogen) as described previously (Sakai and Hohjoh, 2006). Two days after transfection, the cells were examined by a fluorescent microscope. When the endogenous marmoset *Htt* gene was inhibited by RNAi, the cjHtt-1 siRNA duplex (0.4 nmol/transfection) was introduced into HSCj-009 cells (1×10^6 cells/transfection) by means of a Nucleofector system (Amaxa Biosystems) according to the manufacturer's instructions. Two days after transfection, total RNA and cell lysate were prepared from the cells and examined by RT-real time PCR and Western blotting, respectively.

The nucleotide sequences of synthesized cjHtt-1 siRNA were as follows:

Sense: 5′-GCCUUUGAGUCCCUCAAGUUU-3′
Antisense: 5′-ACUUGAGGGACUCAAAGGCUU-3′

2.7. Sequence data and computational analyses

The *Htt* sequnece data derived from various species were as follows [GenBank accession number]: human (*Homo sapiens*) [NM_002111]; chimp (*Pan troglodytes*) [XM_517080]; rhesus macaque (*Macaca mulatta*) [XM_001086119]; canine (*Canis lupus familiaris*) [XM_536221]; bovine (*Bos taurus*) [XM_866758]; wild boar (*Sus scrofa*) [NM_213964]; mouse (*Mus musclus*) [NM_010414]; rat (*Rattus norvegicus*) [XM_573634]; chicken (*Gallus gallus*) [XM_420822]. Although the rhesus macaque Htt sequence [XM_001086119] contains 20 undetermined nuclotides at positons 4932–4951 followed by 6 suspensive amino acid sequences, the sequence was used and examined together with the other sequneces in this study.

Sequence homology analysis of either nucleotide or amino acid sequences was carried out by means of the GENETYX software (Software Development Co., Ltd., Tokyo, Japan), where all the parameters were set at default. For identification of the HEAT repeats in the Cj-Htt protein sequences, the REP program (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html) developed by Andrade et al. was used.

3. Results and discussion

3.1. Isolation and characterization of the common marmoset Htt gene

To isolate and identify the common marmoset *Htt* (*Cj-Htt*) gene and/or gene products, we focused on conserved regions in the *Htt* gene and isolate cDNA clone of the *Cj-Htt* transcript. Highly homologous regions (sequences) between the human and mouse *Htt* genes, whose corresponding regions in the *Cj-Htt* gene were also expected to remain conserved, were selected, and PCR primers were designed for such regions. We add that such conserved regions are also detectable by BLAST search with the human *Htt* as a query on the Trace archive of the Cj-database in NCBI. RT-PCR with the designed primers and total RNA extracted from common marmoset brain tissue and established cell lines was carried out, and an approximately 9.4 kb long PCR product, which was expected to contain the full-length open reading frame (ORF) of *Cj-Htt*, was obtained (Fig. 1). The PCR product was subjected to sequence determination and then compared with various species' *Htt* genes. From the results, it was clear that the PCR product, which is 9396 nucleotides in length, was derived from the common marmoset *Htt* gene which encodes a predicted 3131 amino acid long Cj-Htt polypeptide (the sequence accession number in GenBank is AB443866). Sequence homologies in the *Htt* gene among various species are indicated in Table 1. From the data, it appears that both the nucleotide and predicted amino acid sequences of the *Cj-Htt*

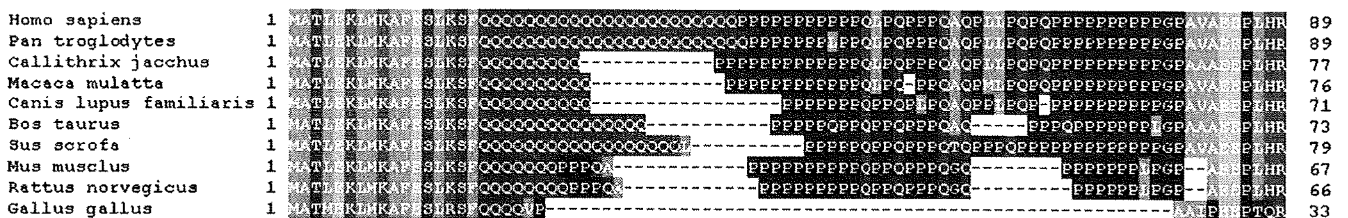

Fig. 2. Alignment of amino acid sequences in the Htt exon 1 and its corresponding regions. Sequence data were aligned based on the human Htt exon 1 (top line). Amino acid residues are color-coded based on the biochemical properties of the residues: hydrophobic amino acids in orange, polar amino acids with uncharged R groups in green, acidic amino acids in pink, basic amino acids in light blue, and special amino acids in dark blue.

Table 2
Alignment of HEAT repeats

Repeat*	Species**	AA position From	AA position To	Fragment†	Score	E-value
HEAT_AAA	Hs - Htt	124	162	QKLLGIAMELFLLCSDDAESDVRMVADECLNKVIKALMD	1510	1.26E-06
	Cj - Htt	112	150	QKLLGIAMELLLLCSDDAESDVRMVADECLNKVIKALMD	1590	5.48E-07
HEAT_AAA	Hs - Htt	205	243	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	1.03E-04
	Cj - Htt	193	231	RPYLVNLLPCLTRTSKRPEESVQETLAAAVPKIMASFGN	1990	2.37E-08
HEAT_AAA	Hs - Htt	247	285	DNEIKVLLKAFIANLKSSSPTIRRTAAGSAVSICQHSRR	1590	5.48E-07
	Cj - Htt	235	273	DNEIKILLKAFIANLKSSSPTIRRTAAGSAVSICQHSRR	1590	1.97E-06
HEAT_AAA	Hs - Htt	317	355	LLTLRYLVPLLQQQVKDTSLKGSFGVTRKEMEVSPSAEQ	1620	1.11E-06
	Cj - Htt	305	343	LLTLRYLVPLLHQQVKDTSLKGSFGVTRKEMEVSPSAEQ	1570	1.46E-07
HEAT_ADB	Hs - Htt					
	Cj - Htt	734	771	YPEEQYVSDILNYIDHGDPQVRGATAILCGTLVCSILS	1450	3.29E-07
HEAT_AAA	Hs - Htt	803	841	TFSLADCIPLLRKTLKDESSVTCKLACTAVRNCVMSLCS	1500	5.78E-07
	Cj - Htt	791	829	TFSLADCVPLLRKTLKDESSVTCKLACTAVRHCVMSLCS	1449	2.90E-06
HEAT_AAA	Hs - Htt	904	942	KLQERVLNNVVIHLLGDEDPRVRHVAAASLIRLVPKLFY	1930	6.69E-08
	Cj - Htt	892	930	TLQERVLTSVVIHLLGDEDPRVRHVAAASLIRLVPKLFY	2150	2.51E-05
HEAT_AAA	Hs - Htt	984	1025	RIYRGYNLLPSITDVTMENNLSRVIAAVSHELITSTTRALTF	1370	9.05E-06
	Cj - Htt	972	1013	RIYRGYNLLPSIIDVTMENNLSRVIAAVSHELITSTTRALTF	1410	2.71E-06
HEAT_AAA	Hs - Htt	1425	1463	RLFEPLVIKALKQYTTTTCVQLQKQVLDLLAQLVQLRVN	1370	5.62E-06
	Cj - Htt	1413	1451	RLFEPLVIKALKQYTTTTSVQLQKQVLDLLAQLVQLRVN	1580	3.20E-07
HEAT_AAA	Hs - Htt	2798	2836	DDTAKQLIPVISDYLLSNLKGIAHCVNIHSQQHVLVMCA	1430	3.51E-06
	Cj - Htt	2785	2823	DDTAKQLIPVISDYLLSSLKGLAHCVNTHSQQHVLVMCA	1430	3.29E-06

* HEAT_AAA and HEAT_ADB indicate subsets of HEAT repeats representing PP2A and adaptin families, respectively.
** Hs-Htt and Cj-Htt indicate the human and common marmoset Htt proteins, respectively.
†Amino acids which are different from the sequence of Hs-Htt are indicated in red.

cDNA have significant sequence homology to that of other species' *Htt* genes. In addition, it should be noted that *Htt* sequences between the human and common marmoset (colored in yellow in Table 1) appear to be particularly conserved as compared with sequence conservation within non-primate *Htt* genes, suggesting that the *Htt* gene is highly conserved in primates.

Fig. 2 shows the alignment of amino acid sequences encoded by *Htt* exon 1 and its corresponding region in various species. From the alignment, *Cj-Htt* appears to possess a short polyglutamine tract of nine glutamines compared with that of the human and chimpanzee *Htt* genes; but other than the polyglutamine tract, the exon 1 corresponding region in *Cj-Htt* exhibits high sequence homology to the human *Htt* exon 1. It is also interesting that polyproline region adjacent to the polyglutamine tract has differences between primates and non-primates: amino acid substitutions and deletions are observed, and the lack of the polyproline region in the *Gallus gallus Htt* exon 1 is particularly remarkable. These differences may influence folding and aggregation of the Htt protein, and might represent adaptive evolution of *Htt* to each species. The difference in the exon 1 among various species may provide us with a hint for understanding the expansion of the polyglutamine tract in Huntington's disease in human.

Other than the exon 1 region, we also investigated the HEAT repeats possessing tandem arrayed bihelical structure, which appear to wrap around target substrates (Andrade and Bork, 1995; Neuwald and Hirano, 2000), and found that the HEAT repeats are also conserved in the Cj-Htt protein (Table 2). In addition, it may be interesting that HEAT_ADB, a subset of HEAT repeats representing adaptin family, is present in Cj-Htt, but not in Hs-Htt.

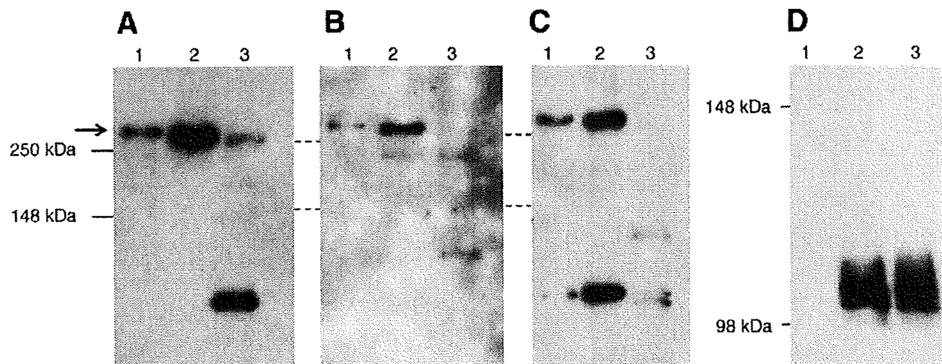

Fig. 3. Assessment of anti-human Htt antibodies against the common marmoset Htt polypeptide. Cell lysate derived from the common marmoset cell line (HSCj-110) (lane 1), brain tissue (lane 2), and mouse brain tissue as a control (lane 3) was examined by Western blotting with anti-human Htt antibodies. Tested antibodies were as follows: (A) MAB2166 (Chemicon), (B) MAB2170 (Chemicon), and (C) ab7666 (Abcam). Arrow indicates the signals of Htt proteins. The same results as those of HSCj-110 were also obtained when HSCj-002 and-009 were used (data not shown). After detection of signals, blotted membranes were subjected to antibody removal and then incubated with anti-APP antibody [MAB348 (Chemicon)] (D) followed by the same procedure as in the anti-human Htt antibodies described above.

3.2. Detection of *Cj-Htt* gene products

It is important to be able to properly detect the *Cj-Htt* gene and its gene products. Since the nucleotide and predicted amino acid sequences of the *Cj-Htt* cDNA showed significantly high sequence homologies to those of the human *Htt* gene, we tested whether commercially-available TaqMan probe and antibodies against human *Htt* gene products (mRNA and protein) were also able to detect *Cj-Htt* gene products. As a result, the TaqMan probe (Fig. 5A) appears to be able to detect *Cj-Htt* mRNA. In addition, newly designed PCR primers, which are perfectly matched to *Cj-Htt*, also appear to be able to detect *Cj-Htt* mRNA (Fig. 5B).

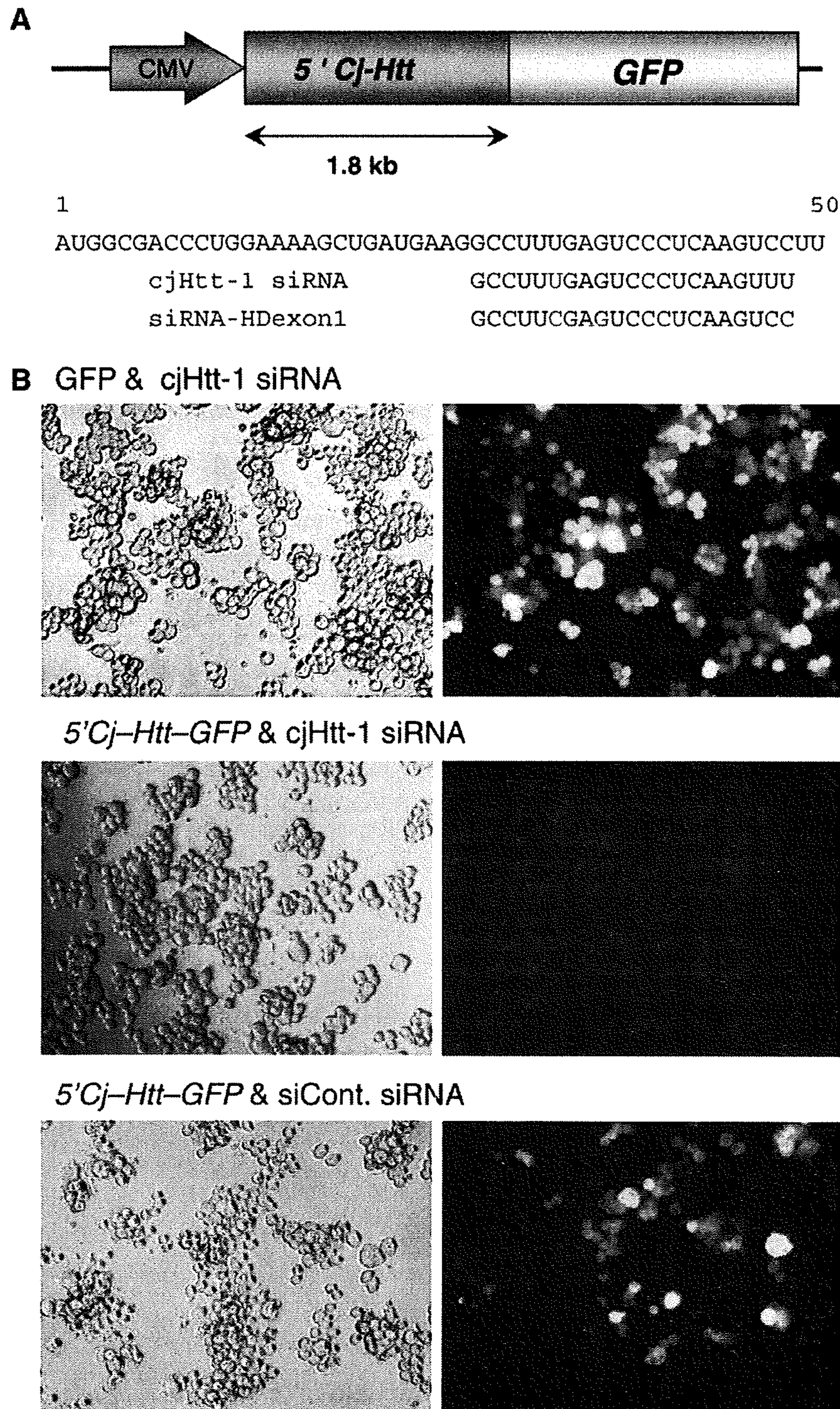

Fig. 4. Gene silencing against the 5′*Cj-Htt-GFP* fusion gene. (A) Schematic drawing of the 5′*Cj-Htt-GFP* fusion gene and designed cjHtt-1 siRNA. The fusion gene is composed of the 5′ terminal region of the Cj-Htt ORF and GFP, and driven by the Human cytomegalovirus promoter. The *Cj-Htt* sequence from the first ATG to position 50 is shown together with sequences of cjHtt-1 siRNA and siRNA-HDexon1 targeting human *Htt*. A variant nucleotide between the siRNAs is indicated in red. (B) Effect of cjHtt-1 siRNA on gene silencing. Reporter genes [5′*Cj-Htt-GFP* or *GFP* (empty reporter)] and siRNAs [cjHtt-1 or siCont (non-silencing siRNA)] were introduced into mouse neuroblastoma Neuro2a (N2a) cells. Two days after transfection, the cells were examined by a fluorescent microscope. Left (differential interference contrast) and right (fluorescence image) panels are identical in the visual field.

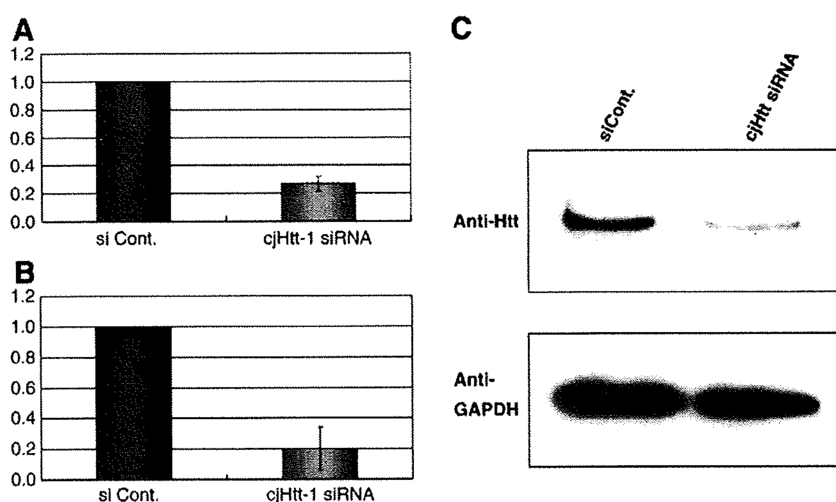

Fig. 5. Inhibition of expression of endogenous *Cj-Htt* by RNAi. The cjHtt-1 siRNA was introduced into HSCj-009 cells by means of electroporation. Two days after transfection, total RNA and cell lysate were prepared and examined by RT-real time PCR and Western blotting, respectively. Total RNA was subjected to cDNA synthesis as in Fig. 1. The resultant cDNA was examined by real time PCR with a TaqMan probe for the human *Htt* gene (A) and newly designed PCR primers [cjHtt(1139)-F and cjHtt(1254)-R] (B). The expression of *Gapdh* as a control was also examined using Perfect Real Time Primers for the human *GAPDH* gene (TAKARA BIO). The expression level of *Cj-Htt* was normalized against that of *Gapdh*, and the ratios of *Cj-Htt* expression level in the presence of cjHtt-1 siRNA were normalized against the ratio obtained in the presence of the siControl siRNA (siCont.). Data are means of at least three independent determinations. Error bars represent standard deviations. (C) Western blot. Cell lysate was examined by Western blotting with anti-human Htt antibody (MAB2166; Chemicon) as in Fig. 3. After detection of signals, the expression of GAPDH as a control was also examined by anti-GAPDH antibody (AM4300; Ambion).

Western blot analyses indicate that a polypeptide of approximately 350 kDa, which is almost the same as the molecular weight estimated from the amino acid sequence (346 kDa) in the *Cj-Htt* cDNA, can be detected in the common marmoset specimens by the antibodies tested, suggesting that the Cj-Htt protein is detectable with the antibodies (Figs. 3A–C). The 350 kDa mouse Htt protein was detected by the MAB2166 antibody (Fig. 3A), but hardly with the other antibodies (Figs. 3B and C). This may be caused by possibly low expression level of mouse Htt in the brain tissue, and/or by difference in the epitope sequences between the common marmoset and mouse Htt proteins. Other than the 350 kDa band, a few bands migrated faster than the 350 kDa band were also observed. They may be degradation products of the Htt protein, and different cells and/or species might have different degradation of the protein. To clarify these, further studies need to be carried out.

In addition to the Htt protein, we also examined the expression of amyloid precursor protein (App) with the 22C11 antibody, which can recognize the same amino acid sequence at positions 66–81 of either the human or mouse App. As a result, the App signal was able to be detected in either the common marmoset or mouse brain tissue, but not in the common marmoset immortalized peripheral blood mononuclear cells (PBMCs) (Fig. 3D), suggesting little or no expression of App in PBMCs and availability of the antibody for detection of the Cj-App protein.

3.3. Gene silencing against the Cj-Htt gene

To verify the data presented here and tools for the detection of *Cj-Htt*, we carried out gene silencing against the expression of endogenous *Cj-Htt* by means of RNA interference (RNAi), and assessed the knockdown potency of designed siRNA targeting *Cj-Htt* using the tools evaluated above. Based on a previous study where a competent siRNA duplex, siRNA-HDexon 1, conferring strong inhibition against the expression of the human *Htt* gene was used (Liu et al., 2003), we chemically synthesized an siRNA duplex, cjHtt-1 siRNA, corresponding to the siRNA-HDexon 1 duplex; note that there is one nucleotide change between cjHtt-1 siRNA and siRNA-HDexon 1 (Fig. 4A).

To examine the effect of the siRNA duplex on gene silencing, we constructed a reporter plasmid carrying the 5′ terminal region of *Cj-Htt* cDNA linked with the *GFP* reporter gene (the 5′*Cj-Htt-GFP* fusion gene). The reporter plasmid and the siRNA were cotransfected into mouse Neuro2a cells, and the expression of the 5′*Cj-Htt-GFP* fusion gene was examined by a fluorescent microscope. As shown in Fig. 4B, the data indicated that the cjHtt-1 siRNA duplex was able to induce strong RNAi activity against the fusion gene expression.

Next, we introduced the cjHtt-1 siRNA duplex into immortalized common marmoset mononuclear cells by means of electroporation, and two days after transfection, the expression levels of the endogenous *Cj-Htt* mRNA and protein were examined by RT-real time PCR and Western blotting, respectively. As shown in Fig. 5, the results consistently indicated that *Cj-Htt* mRNA (A and B) and protein (C) levels markedly decreased in the presence of the cjHtt-1 siRNA duplex, i.e., potent RNAi knockdown against the endogenous *Cj-Htt* gene was induced by the siRNA duplex. Finally, the data presented here also indicate that proper detection of the newly identified *Cj-Htt* gene and its products can be performed by means of the methods and tools assessed in this study.

In conclusion, we described for the first time the common marmoset *Htt* gene, and also detection methods and tools for the gene and its gene products. The data presented here may assist us in promoting a non-human primate HD model with the common marmoset.

Acknowledgments

We would like to thank Dr. R.C. Desrosiers for kindly providing HS viruses. We also thank Drs. K. Nakamura, T. Kabuta, M. Suzuki, and C. Konya for their helpful advice and discussion. Finally, we would like to thank Dr I. Kanazawa for his encouragement and helpful advice. This work was supported by research grants from the Ministry of Health, Labour and Welfare of Japan.

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Transworld Research Network
37/661 (2), Fort P.O., Trivandrum-695 023, Kerala, India

Virus Expression Vectors, 2007: 123-146 ISBN: 81-7895-273-4
Editor: Kathleen L. Hefferon

5 Sendai virus engineering: From reverse genetics to vector development

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Abstract

We established Sendai virus (SeV) reverse genetics that allows engineering of the viral genome and assessment of its outcome not only in terms of virus proliferation at the cellular level but also in the context of viral pathogenesis in the entire host organism. This technological innovation has played a prominent role in settling outstanding issues and resolving enigmas in virology, which had been impenetrable using conventional

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methodology. For instance, it was established that the viral accessory genes are not mere "accessories" but rather are the central players in the realm of in vivo pathogenesis. At the same time, the technology is about to bear the impressive fruit of multipurpose cytoplasmic RNA vectors with extremely high potential in transgene expression capacity and target range breadth. Diverse applications of the SeV vector to gene therapy, cancer treatment, AIDS prophylaxis, and other therapies are now in the pipeline and poised for testing in clinical trials.

1. Introduction

Sendai virus (SeV) was first isolated in 1952 in Japan as a possible causative agent of neonatal pneumonia (1), but this causal relationship has not yet been established. In fact, no report thus far has identified its involvement in candidate human diseases, including neonatal pneumonia, although it contaminates laboratory animal houses and can pose a serious threat to the rodents. Therefore, SeV may be a virus that eventually disappears from the human virological scene. Nevertheless, it remains a major player in virology as research on SeV has continued to elucidate basic biological or virological concepts. Establishment of SeV engineering (reverse genetics) is one of the major results of these efforts. The idea was hatched out of pure academic interest and evolved into a business development using SeV as a novel class of vector. In this review article, we first describe how much academia anticipated this technology and struggled for its establishment and how successfully the technology has been applied to settle a series of long-held questions in virology. We then illustrate our joint efforts among academia, industry, and government in developing the SeV vector, including conceptual and laboratory work necessary to demonstrate its feasibility; generation of safer versions; and diverse applications now in the pipeline and awaiting clinical trials, as well as safety issues.

2. SeV structure and biology

SeV, also known as the murine parainfluenza virus type 1 or hemagglutinating virus of Japan, is an enveloped virus with a negative-strand RNA genome; it is spherical, with a diameter of approximately 200 nm. SeV is a member of the family *Paramyxoviridae* in the *Mononegavirales* superfamily comprising a wide range of "classic" viruses such as rabies, measles, mumps, and Newcastle disease, pathogens of medical or veterinary significance, and "new" deadly emergent viruses such as Nipah, Ebola, and Marburg viruses.

Inside SeV is a ribonucleoprotein (RNP) complex in which the 15,384-base RNA genome is encapsidated with the nucleocapsid (N) proteins (Fig. 1). The RNP complex has a left-handed helical structure 1.1 μm in length and 18 nm

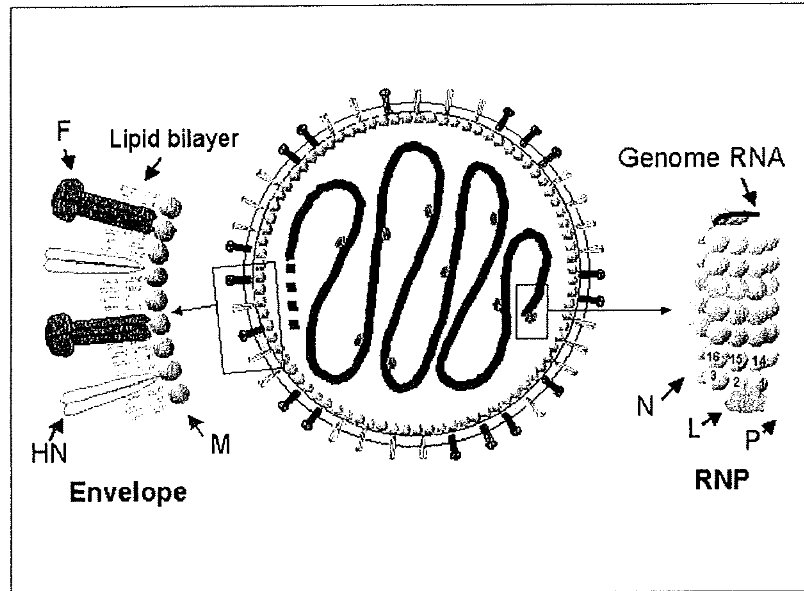

Figure 1. Structure of SeV.

in diameter, with a pitch of 5 nm. It has 13 N proteins per turn. Because one N protein binds to every 6 RNA bases, the entire RNP complex is calculated to contain 2,564 (= 15,384/6) units of N protein. The large (L) and phospho- (P) proteins, subunits of the RNA polymerase complex involved in genome transcription and replication, attach to the 3'-terminal site as well as to occasional sites in the RNP complex (Fig. 1). The outermost structure, the envelope, contains two types of glycoproteins protruding in the form of spikes, anchored within the lipid bilayer derived from the plasma membrane of host cells (Fig. 1). One of the spike glycoproteins has hemagglutinin (HA) and neuraminidase (NA) activities and is named HN. The HN protein recognizes terminal sialic acids in the sugar chains as the receptor on the target cell surface. It also contributes to the release of progeny virus from the cell surface. The other spike glycoprotein is called F protein because it induces fusion of the virus envelope with the host cell plasma membrane, thereby introducing viral RNP into the cytoplasm. The F protein is produced as an inactive precursor molecule F_0, which is cleaved by host proteases to F_1 and F_2 to be biologically active. The matrix (M) protein is located between the envelope and RNP (Fig. 1). The M protein serves as an anchor to stabilize the spike glycoprotein molecules floating in the lipid bilayer by binding to their cytoplasmic

tails; it also binds to RNP, crosslinking the internal and external structures. This M protein is indispensable for structural proteins to assemble into virus particles as well as for viral budding from the plasma membrane (reviewed in ref. 2).

The genes coding for the above six structural proteins lie in the order of 3'-*N*-*P*-*M*-*F*-*HN*-*L*-5'. The *P* gene further produces two accessory proteins, V and C, which are the focus of active investigation by reverse genetics (see Fig. 3 and Section 4).

3. Establishment of SeV reverse genetics. A historical review

Reverse genetics in the post-genome era

The nucleotide sequencing of the entire paramyxovirus genome was first achieved for SeV in the mid 1980s (3). This achievement proved to be a milestone in genome-wide explorations of paramyxovirus gene expression and genome replication mechanisms. It paved the way for the popularization of forward genetics, which involves determining the gene or the genotype that specifies a particular virus phenotype. It also stimulated studies on cloning and expressing individual virus genes, allowing cellular-level evaluation of functions of the respective genes.

However, mere summation of the functions of individual genes does not guarantee an accurate overall picture of the virus, a viable organism in which various gene functions are intertwined with one another in a complex and systematic manner. Forward genetics fell short of providing methods of reproducing (and thus confirming) phenotypes identified as corresponding to certain genotypes. Furthermore, there were a number of unsolved issues in paramyxovirus biology that had been unapproachable using conventional virology, including gene expression studies. Typical of such problems are the so-called accessory genes; in case of SeV and many other paramyxoviruses, these are the genes encoding the V and C proteins. The virological definition of an accessory gene is that the gene can be deleted (knocked out) without destroying infectivity at the cellular level. The actual roles of the accessory gene can be defined by comparing the phenotypes of the knockout virus with those of the parental virus at the cellular level and in the susceptible host organism.

Under these circumstances, post-genome research looked forward to the establishment of reverse genetics, which would allow gene manipulation at will and assessment of its outcome not only in terms of cellular-level proliferation but also in the context of viral pathogenesis at the level of the entire organism. Hence, the intense competition for virus recovery (rescue) from cDNA started in the field of *Paramyxoviridae* as