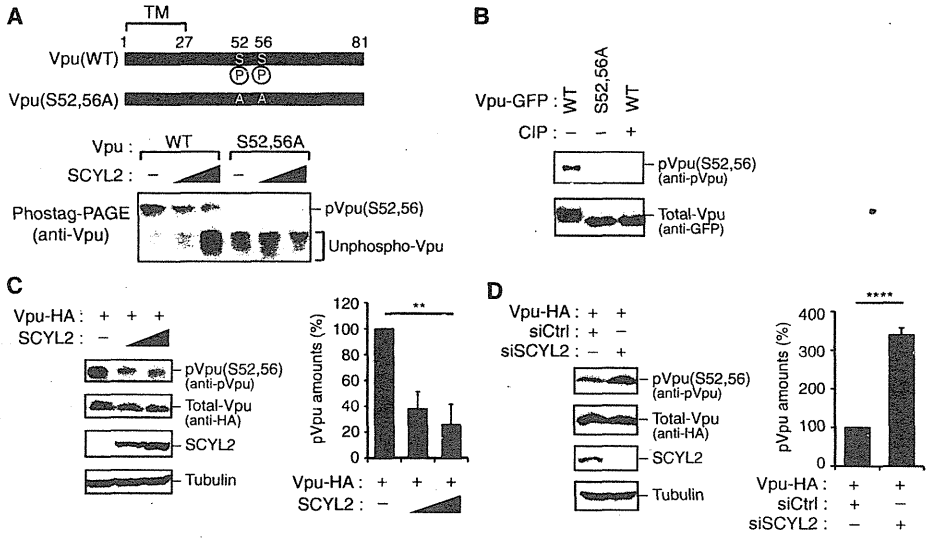

Fig. 4. SCYL2 inhibits the phosphorylation of Vpu on Ser^{52} and Ser^{56}. (A) Schematic representation of Vpu phosphorylated at Ser^{52} and Ser^{56} and of alanine substitution mutants at these sites. HEK 293T cells were cotransfected with plasmids encoding either WT Vpu or the Vpu(S52,56A) mutant (100 ng) together with the SCYL2 expression plasmid (at 0, 500, or 1000 ng). Forty-eight hours after transfection, cell lysates were subjected to Phos-tag PAGE analysis. Blots were incubated with anti-Vpu antibody. (B) Specific recognition of pVpu by an anti-pVpu($Ser^{52,56}$) antibody. HEK 293T cells were transfected with expression plasmids encoding GFP-tagged WT Vpu or the Vpu(S52,56A) mutant. Twenty-four hours after transfection, cell lysates were incubated with buffer alone or with calf intestinal alkaline phosphatase for 30 min before Western blotting analysis was performed with the indicated antibodies. (C) SCYL2 facilitates the dephosphorylation of Vpu on Ser^{52} and Ser^{56}. HEK 293T cells were transfected with plasmids encoding Vpu-HA and SCYL2 at a molar ratio of 1:5 or 1:10. Twenty-four hours after transfection, cell lysates were processed for Western blotting analysis with antibodies against the indicated proteins. The bar chart indicates the amounts of pVpu as determined by densitometric analysis of Western blots. $^{**}P = 0.0012$. (D) HEK 293T cells were treated with either control (siCtrl) or SCYL2-specific siRNA (siSCYL2) for 24 hours before being transfected with 100 ng of Vpu expression plasmids. Cell lysates were subjected to Western blotting analysis with antibodies against the indicated proteins. The bar chart indicates the amounts of pVpu as determined by densitometric analysis of Western blots. $^{****}P < 0.0001$. All data are from single experiments and are representative of three experiments.

data suggested that the interaction between SCYL2 and Vpu might have developed during the evolution of primate lentiviruses.

SCYL2 promotes PP2A-mediated dephosphorylation of Vpu

Our earlier results suggested that SCYL2 interacted with Vpu and inhibited its function by promoting the dephosphorylation of Vpu at residues Ser^{52} and Ser^{56}. To investigate the molecular mechanism underlying this process, we next addressed whether SCYL2 had phosphatase activity toward Ser^{52} and Ser^{56} on Vpu. To this end, we incubated SCYL2 immunoprecipitates with either phosphorylated or nonphosphorylated Vpu peptide containing Ser^{52} and Ser^{56} (AEDpS^{52}GNEpS^{56}EGE) and then measured the amounts of free phosphate released. We found that SCYL2 immunoprecipitates had phosphatase activity toward Ser^{52} and Ser^{56} on Vpu, which was blocked by a phosphatase inhibitor, okadaic acid (OA) (Fig. 6A). Furthermore, treatment of cells with OA blocked SCYL2-mediated dephosphorylation of Vpu and inhibition of viral release (Fig. 6, B and C). Because OA specifically inhibits the activity of PP2A in vivo, we next asked whether PP2A physically associated with SCYL2. The core structure of PP2A consists of a scaffold A subunit (PP2A/A) and a catalytic C subunit ($PP2A_C$) (*53*). Immunoprecipitation analysis revealed that SCYL2 specifically interacted with PP2A/A, but not with $PP2A_C$ (Fig. 6D), suggesting the possibility that SCYL2 recruits the scaffold subunit of PP2A to Vpu. As expected, in the presence of SCYL2, the interaction between Vpu and PP2A/A was enhanced (Fig. 6E). Moreover, Vpu interacted with endogenous SCYL2 and PP2A in HEK 293T cells (Fig. 6F). Depletion of PP2A/A by specific siRNA abrogated the SCYL2-mediated dephosphorylation of Vpu (Fig. 6G). Furthermore, immunofluorescence analysis revealed that SCYL2 induced the colocalization of PP2A/A with both Vpu and SCYL2 in the perinuclear region (Fig. 6H). These observations suggested that SCYL2 promoted Vpu dephosphorylation by recruiting PP2A to pVpu.

SCYL2 affects Vpu function through a phosphorylation-dependent mechanism

Several studies have suggested that the mechanism by which BST2 antagonizes Vpu function is partly independent of β-TrCP and BST2 degradation (and therefore partly independent of the phosphorylation of Ser^{52} and Ser^{56}) (*54*, *55*). We thus explored the possibility that SCYL2 might affect Vpu function through a phosphorylation-independent mechanism. To this end, we first investigated whether SCYL2 associated with the nonphosphorylated form of Vpu. Immunoprecipitation analysis demonstrated that both wild-type Vpu and the Vpu(S52,56A) mutant interacted with endogenous SCYL2 (fig. S4A). We next addressed whether SCYL2 interfered with the direct interaction between Vpu and BST2 and thereby inhibited viral release. Bimolecular fluorescence complementation analysis (*56*, *57*) revealed that SCYL2 did not interfere with the association of BST2 with either wild-type Vpu or the Vpu(S52,56A) mutant in live cells (fig. S4C). In contrast, viral release assays demonstrated that overexpression of SCYL2 inhibited the release of wild-type HIV-1 but not of its derivative encoding the Vpu(S52,56A) mutant (fig. S4, D and E). Together, these data suggested that SCYL2 interfered with the anti-BST2 activity of Vpu through a phosphorylation-dependent mechanism.

SCYL2 affects the type I IFN–mediated antiviral response

BST2 acts as a key effector of the type I IFN–inducible antiviral response (*58*). We thus investigated whether SCYL2 might also affect this response. We treated epithelial (HeLa) and $CD4^+$ T lymphocyte (H9) cell lines with type I IFN (that is, IFN-α and IFN-β) for 6 hours and then analyzed the expression of SCYL2 by reverse transcription polymerase chain reaction (RT-PCR) or Western blotting analysis. Type I IFN increased the abundance of SCYL2 mRNA and protein in both cell lines (Fig. 7A). Moreover, analysis of reporter gene expression revealed that the 5′ untranslated region of the gene encoding

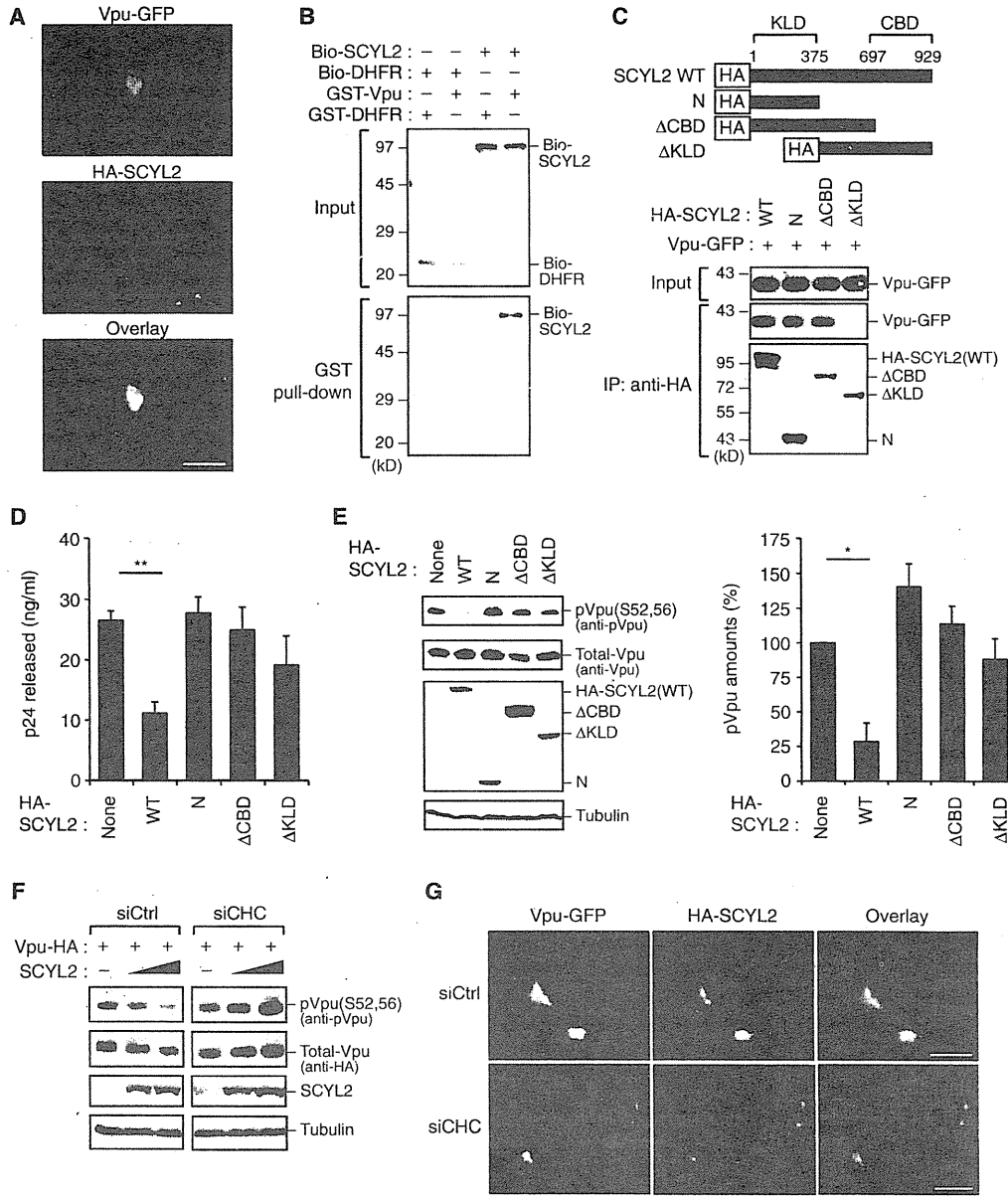

Fig. 5. Both the KLD and CBD of SCYL2 are required for its ability to antagonize Vpu activity. (**A**) Colocalization of Vpu and SCYL2 in cells. HeLa cells were transfected with plasmids encoding Vpu-GFP and HA-SCYL2. After 24 hours, the cells were fixed, permeabilized, and stained with anti-HA antibody (red), followed by confocal microscopic analysis. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and are shown in blue. Scale bar, 10 μm. (**B**) SCYL2 interacts with Vpu in vitro. Recombinant biotinylated SCYL2 (Bio-SCYL2) and biotinylated dihydrofolate reductase (Bio-DHFR) were processed for GST pull-down analysis with either GST-Vpu or GST-DHFR. DHFR was used as a negative control. Captured proteins were analyzed by Western blotting with streptavidin-HRP. (**C**) The N-terminal KLD of SCYL2 interacts with Vpu. HEK 293T cells were cotransfected with plasmids encoding Vpu-GFP together with plasmid encoding WT SCYL2 or one of its truncation mutants. Cell lysates were immunoprecipitated (IP) with anti-HA antibody and then analyzed by Western blotting with anti-GFP or anti-HA antibodies. (**D** and **E**) SCYL2 mutants lacking the CBD have no observable effect on (D) viral release or (E) Vpu dephosphorylation. Cells were cotransfected with pNL4-3 (100 ng) together with the indicated SCYL2 expression plasmids (100 ng). Forty-eight hours after transfection, culture supernatants and cell lysates were subjected to (D) p24 ELISA or (E) Western blotting analysis with anti-pVpu, anti-Vpu, anti-HA, and anti-tubulin antibodies. The bar chart in (E) indicates the amounts of pVpu as determined by densitometric analysis of Western blots. $^{**}P$ = 0.0045; $^{*}P$ = 0.0172; n = 3 experiments. (**F**) CHC depletion abrogates SCYL2-mediated dephosphorylation of Vpu. HEK 293T cells were treated with either control (siCtrl) or CHC-targeted siRNA (siCHC) for 24 hours before being transfected with plasmids expressing Vpu-HA and SCYL2 at a molar ratio of 1:5 or 1:10. Forty-eight hours after transfection, cell lysates were subjected to Western blotting with the indicated antibodies. (**G**) CHC depletion interferes with the colocalization of SCYL2 and Vpu. HeLa cells were treated with either control or CHC-specific siRNA for 24 hours before being transfected with plasmids encoding Vpu-GFP and HA-SCYL2. After 24 hours, the cells were fixed, permeabilized, stained with anti-HA antibody (red), and analyzed by confocal microscopy. Nuclei were stained with DAPI (blue). Scale bars, 10 μm. Data shown in (A) to (C) and (E) to (G) are representative of three experiments.

SCYL2 was transactivated by IFN-β (Fig. 7B). We next examined whether type I IFN promoted the SCYL2-mediated dephosphorylation of Vpu. To this end, we infected HeLa and H9 cells with vesicular stomatitis virus glycoprotein (VSVG)–pseudotyped HIV-1 and then treated the cells with IFN-β. As expected, the addition of IFN-β induced the dephosphorylation of Vpu (Fig. 7C). Single-cycle virus release analysis revealed that IFN-β decreased the amounts of virus released (Fig. 7D), as reported previously (*41*). However, the targeted depletion of SCYL2 by siRNA partially blocked IFN-β–mediated viral restriction (Fig. 7D). This was not the case in parallel experiments with Vpu-deficient viruses or BST2-deficient cells (Fig. 7, E and F). Together,

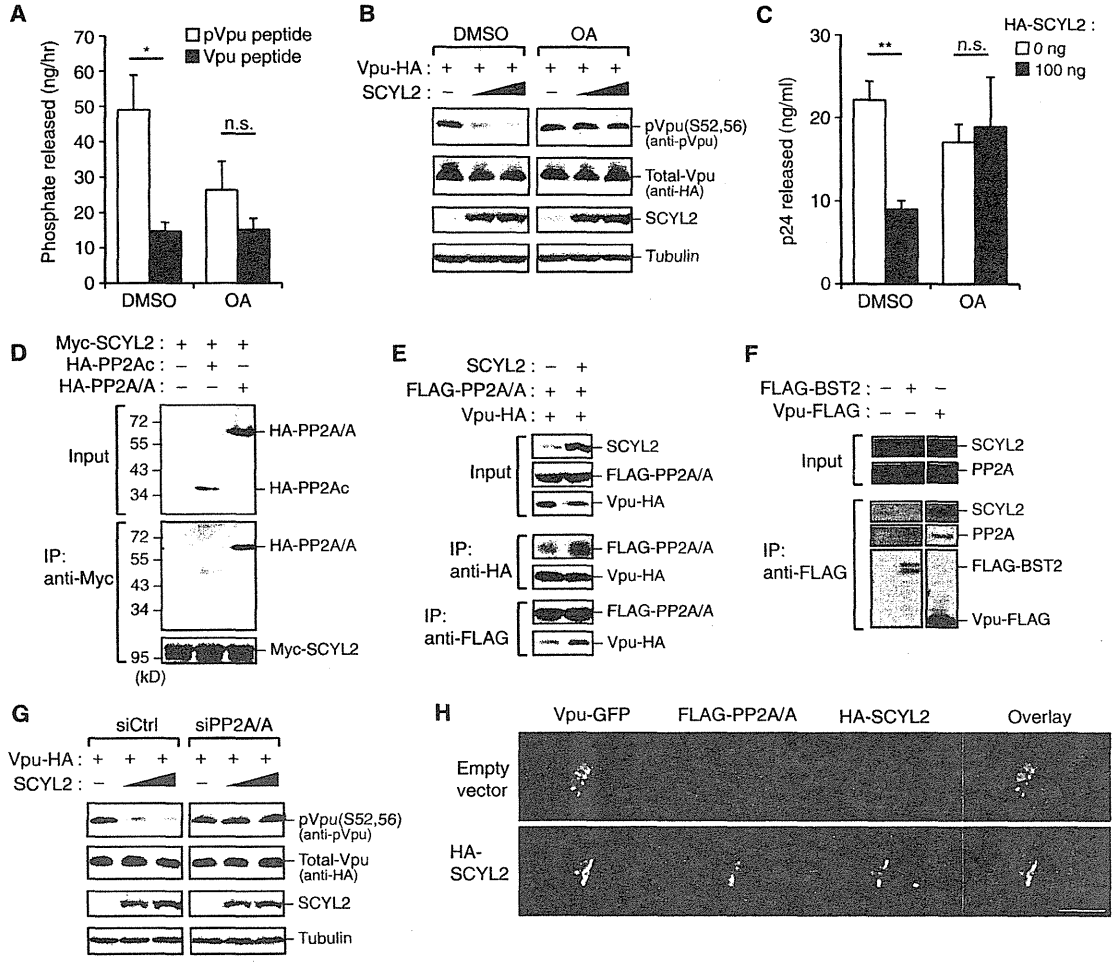

Fig. 6. SCYL2 promotes PP2A-mediated dephosphorylation of Vpu. (A) SCYL2 immunoprecipitates have phosphatase activity toward Ser^{52} and Ser^{56} on Vpu. HEK 293T cells expressing HA-SCYL2 were lysed and subjected to immunoprecipitation with an anti-HA antibody in buffer containing dimethyl sulfoxide (DMSO) or 10 nM OA. The immunoprecipitates were incubated with either pVpu peptide (white bar) or the nonphosphorylated equivalent (black bar). After 8 hours, the amounts of released phosphates in the reaction mixture were measured. n.s., not significant; $^*P = 0.0410$; $n = 3$ experiments. (B) OA inhibits the SCYL2-mediated dephosphorylation of Vpu. HEK 293T cells were transfected with plasmids expressing Vpu-HA and SCYL2 at a molar ratio of 1:5 or 1:10. Cells were treated with DMSO or 10 nM OA for 18 hours before being harvested. Forty-eight hours after transfection, cell lysates were subjected to Western blotting with the indicated antibodies. (C) OA blocks SCYL2-mediated restriction of viral release. HeLa cells were cotransfected with pNL4-3 (100 ng) with or without plasmid encoding SCYL2. Cells were then treated with DMSO control or 10 nM OA for 18 hours before being harvested. Forty-eight hours after transfection, culture supernatants were subjected to p24 ELISA. n.s., not significant; $^{**}P = 0.0039$; $n = 3$ experiments. (D) SCYL2 interacts with the scaffold A subunit of PP2A (PP2A/A). HEK 293T cells were transfected with expression plasmids for the indicated proteins. Cell lysates were immunoprecipitated with an anti-Myc antibody, and bound proteins were visualized by Western blotting. (E) SCYL2 enhances the interaction between Vpu and PP2A/A. HEK 293T cells were transfected with vectors encoding the indicated proteins. Cell lysates were immunoprecipitated with anti-HA or anti-FLAG antibodies, and bound proteins were visualized by Western blotting analysis. (F) Vpu interacts with endogenous SCYL2 and PP2A. HEK 293T cells were transfected with vectors encoding the indicated proteins. Cell lysates were immunoprecipitated with anti-FLAG antibody, and bound proteins were visualized by Western blotting analysis. (G) Depletion of PP2A/A inhibits SCYL2-mediated dephosphorylation of Vpu. HEK 293T cells were treated with either control (siCtrl) or PP2A/A-specific siRNA (siPP2A/A) for 24 hours before being transfected with plasmids expressing Vpu-HA and SCYL2 at molar ratios of 1:5 and 1:10. Forty-eight hours after transfection, cell lysates were subjected to Western blotting with antibodies against the indicated proteins. (H) Immunofluorescence analysis of HeLa cells transiently coexpressing Vpu-GFP (green), FLAG-PP2A/A (red), and either HA-SCYL2 (blue) or empty vector. Scale bar, 10 μm. Data shown in (B) and (D) to (H) are representative of three experiments.

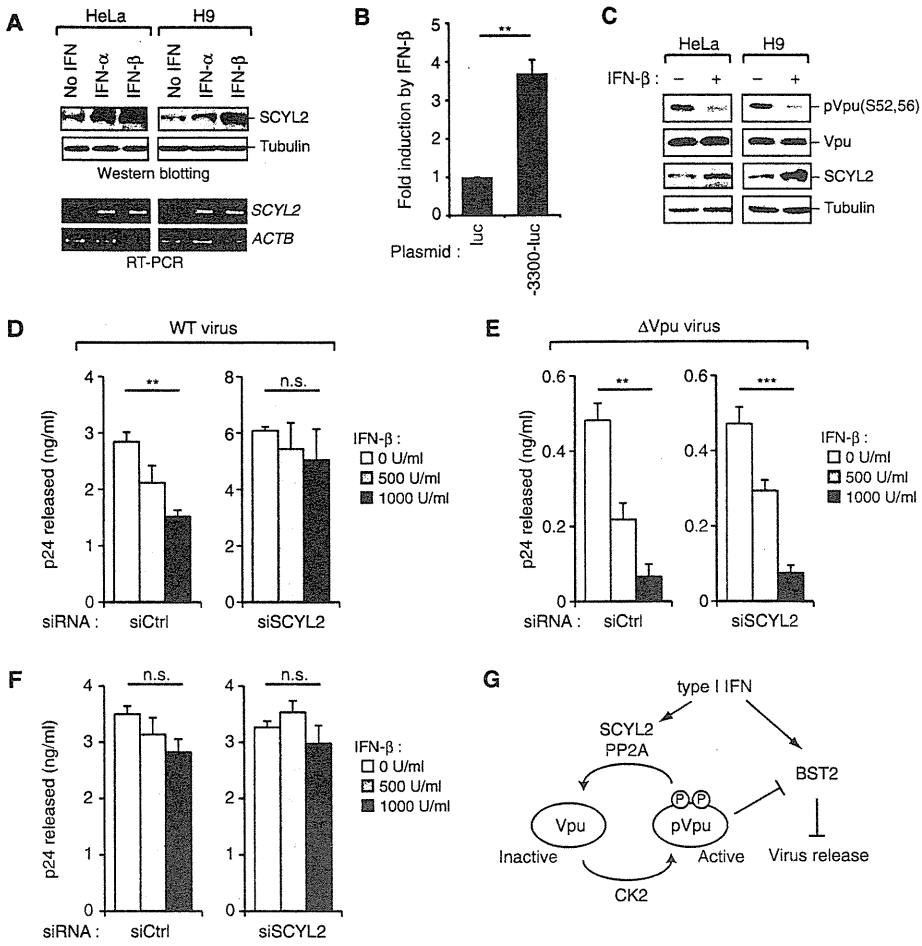

Fig. 7. SCYL2 mediates the type I IFN–induced antiviral response. (A) Type I IFN increases the abundance of SCYL2. Western blotting analysis (top) and RT-PCR analysis (bottom) of the indicated cells treated with IFN-α or IFN-β (1000 U/ml) for 6 hours before harvesting. Representative data from three experiments are shown. (B) The SCYL2 promoter is transactivated by IFN-β. HeLa cells were cotransfected with the pGL4-luc vector encoding SCYL2 promoter (-3300-luc) together with pGL4-TK-Rluc as a transfection control. Forty-eight hours after transfection, cells were treated with IFN-β (1000 U/ml) for 6 hours and cell lysates were subjected to a luciferase reporter assay. The fold induction in IFN-treated cells was determined. $^{**}P$ = 0.0036; n = 3 experiments. (C) IFN-β induces Vpu dephosphorylation in infected T cells. The indicated cells were infected with VSVG-pseudotyped HIV-1_{NL4-3} [at a multiplicity of infection (MOI) of 0.2] and treated with IFN-β (1000 U/ml) for 8 hours before being harvested. Cell lysates were subjected to Western blotting with antibodies against the indicated proteins. Data are representative of three experiments. (D to F) SCYL2 depletion partially blocks the antiviral effect of IFN-β. (D and E) HeLa cells or (F) BST2-knockdown HeLa cells were treated with either control or SCYL2-specific siRNA for 24 hours before being infected with (D and F) VSVG-pseudotyped HIV-1_{NL4-3} or (E) its Vpu-deficient derivative at an MOI of 0.01. Forty-eight hours after infection, the cells were washed and treated with IFN-β (0, 500, or 1000 U/ml) for 8 hours. Culture supernatants and cell lysates were subjected to p24 ELISA. n.s., not significant; $^{**}P$ = 0.0079 for (D); $^{**}P$ = 0.0012 and $^{***}P$ = 0.0007 for (E); n = 3 experiments. (G) A model depicting the proposed signaling cascade in type I IFN–mediated viral release restriction. Type I IFN induces the production of both BST2 and SCYL2 in infected cells. SCYL2 binds to Vpu, recruits PP2A, and inhibits the anti-BST2 activity of Vpu, thereby facilitating the BST2-mediated restriction of viral release.

these observations indicated that SCYL2 is itself IFN-inducible, and that by antagonizing Vpu function and consequently facilitating BST2-mediated restriction, SCYL2 stimulates the anti-HIV activity of type I IFN (Fig. 7G).

DISCUSSION

Here, we demonstrated that SCYL2 is an IFN-inducible gene that regulates the phosphorylation of HIV-1 Vpu by recruiting PP2A to the viral protein, thereby inhibiting Vpu function and enhancing the antiviral activity of BST2 in HIV-1–infected cells. SCYL2 was originally identified as a poly-L-lysine–stimulated kinase that phosphorylates the β2 subunit of the adaptor protein AP-2 (*50*). Although the idea of whether SCYL2 has kinase activity is still controversial (*52*), a subsequent study suggested that SCYL2 is a multifunctional protein involved in membrane trafficking between the *trans*-Golgi and endosomes (*51*). Other studies have reported a role for SCYL2 in the clathrin-mediated sorting of t-SNARE (target-soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor) proteins (*52*) and in the Wnt signaling pathway (*59*). Here, we reveal an additional role for SCYL2 as an inhibitor of viral release in HIV-1–infected cells through its inhibition of the phosphorylation of Vpu.

A study predicted the existence of at least 4000 as yet unidentified phosphorylation sites on hundreds of viral proteins (*60*). Indeed, the phosphorylation of viral proteins is one of the most important processes for efficient viral replication and pathogenesis (*61*). Previous biochemical analyses have reported that the phosphorylation of Vpu on key serine residues (Ser^{52} and Ser^{56}) is catalyzed by CKII (*29–31*). Because CKII is a ubiquitously expressed serine and threonine (Ser/Thr) kinase (*62*), the Ser^{52} and Ser^{56} phospho-acceptor sites are likely to be constitutively phosphorylated in infected cells, suggesting that dephosphorylation could be a key step for exerting control over Vpu function. However, the sequence requirements of major cellular phosphatases are less specific than those of protein kinases (*63*), and additional cofactors are likely needed for phosphatases to target a specific substrate. Indeed, many transporter or adaptor proteins interact with subunits of phosphatases to promote substrate recognition by these dephosphorylating enzymes (*64*).

Although the phosphorylation of Vpu is required for optimal anti-BST2 activity, the precise molecular mechanism by which the phosphorylation status of Vpu is controlled has been elusive. The association of SCYL2 with both Vpu and PP2A suggests a previously unrecognized mechanism through which the activity of Vpu is regulated by SCYL2-mediated recruitment of PP2A and dephosphorylation. Our data suggest that SCYL2 induces the dephosphorylation

of Vpu, abrogating Vpu function and consequently inhibiting viral particle release through BST2. Our model of the SCYL2-PP2A-Vpu virus-host interaction is reminiscent of previously described interactions among cellular proteins; for example, cyclin G recruits PP2A to Mdm2 in a similar manner to facilitate the dephosphorylation and functional modification of Mdm2 (*65*).

Our model, however, does not directly address the original role of SCYL2 as a component of clathrin-coated vesicles. Although the role of clathrin during Vpu-induced BST2 inactivation is still uncertain, substantial data suggest that a clathrin-dependent pathway contributes to the antagonism of BST2 by Vpu (*19*, *24*, *66*, *67*). On the basis of our present findings that clathrin is required for SCYL2 function with respect to Vpu, SCYL2 could conceivably inhibit Vpu by modulating clathrin-mediated membrane trafficking. Of note in this regard, several studies have proposed that Vpu counteracts BST2 at least partly through a β-TrCP–independent (phosphorylation-independent) pathway (*54*, *55*). Indeed, we observed that SCYL2 interacted with a nonphosphorylated mutant of Vpu, as well as with wild-type Vpu, implying that SCYL2 might interfere with the anti-BST2 activity of Vpu irrespective of the phosphorylation status of Vpu. However, the phosphatase inhibitor OA almost completely abrogated the activity of SCYL2. Moreover, SCYL2 did not affect the virion release of an HIV-1 derivative encoding a nonphosphorylatable form of Vpu. Together, these results suggest that the function of SCYL2 as a Vpu inhibitor is likely dependent on the phosphorylation of the Vpu residues Ser^{52} and Ser^{56}.

Our data indicate that human SCYL2 has no obvious efficacy against ancestral Vpu, which suggests that the SCYL2-Vpu interaction might have appeared during the evolution of primate lentiviruses. However, whether SCYL2 from GSN monkeys might interfere with SIV_{GSN} Vpu and whether primate SCYL2 has evolved to gain an antagonistic interaction with HIV-1 Vpu are currently unresolved questions. How can the acquisition of SCYL2-binding activity by HIV-1 Vpu be reconciled with the seemingly inhibitory function of SCYL2 on HIV-1 release? One possibility is that the SCYL2-Vpu interaction is associated with an unrecognized benefit in some aspect of the viral life cycle. For example, SCYL2 might increase the stability of Vpu through dephosphorylation. Indeed, a previous report suggested that nonphosphorylated Vpu is more stable than wild-type Vpu (*68*). Another possibility is that the cycle of phosphorylation and dephosphorylation is important for Vpu activity. Nonetheless, our observation that overexpression of SCYL2 inhibits the antagonism of BST2 by Vpu, whereas depletion of endogenous SCYL2 has the opposite effect, suggests that, at least with regard to this function, SCYL2 behaves as an inhibitor rather than a cofactor of Vpu.

In addition to BST2, several factors induced by type I IFN contribute to viral restriction during the late stages of virus assembly and release. For example, the ubiquitin-like protein ISG15 specifically inhibits the ubiquitination of Gag and its association with Tsg101, resulting in the restriction of HIV-1 budding (*40*). A tripartite motif (TRIM) protein, TRIM22, is another ISG that blocks HIV-1 assembly by disrupting the proper trafficking of Gag to the plasma membrane (*41*). These restrictions are Vpu-independent mechanisms because these factors either interact with Gag or modulate its function. Our data demonstrate that type I IFN can also trigger the dephosphorylation of Vpu through SCYL2 and thereby facilitate BST2-mediated viral restriction even in the presence of Vpu, implying a regulatory role for SCYL2 among the array of IFN-mediated antiviral host measures. In conclusion, our current study provides evidence that the IFN-mediated antiviral response targets the posttranslational modification of Vpu and contributes to the inhibition of HIV-1 release from infected cells. The molecular machinery underlying the posttranslational modification of Vpu may thus be an attractive therapeutic target for the treatment of HIV infection.

MATERIALS AND METHODS

In vitro protein production

A total of 412 complementary DNAs (cDNAs) encoding human protein kinases were constructed as described previously (*69*). The protein production method has been described previously (*42*, *70*, *71*). Briefly, DNA templates containing a biotin-ligating sequence were amplified by split-PCR with cDNAs and corresponding primers and then used with the GenDecoder protein production system (CellFree Science). For synthesis of HIV-1 Vpu protein, *vpu* genes derived from the pNL4-3 proviral plasmid (*72*) were generated by split-PCR and used as templates with the Wheat Germ Expression Kit (CellFree Science) in accordance with the manufacturer's instructions.

AlphaScreen-based protein-protein interaction assays

FLAG-tagged Vpu proteins were mixed with biotinylated kinases in 15 μl of reaction buffer [20 mM tris (pH 7.6), 5 mM $MgCl_2$, 1 mM dithiothreitol] in a well of 384-well optiplates (PerkinElmer) and incubated at 26°C for 1 hour. The mixture was then added to AlphaScreen buffer containing anti–immunoglobulin G (protein A) acceptor beads and streptavidin-coated donor beads (0.1 μl each; PerkinElmer) and the anti-FLAG M2 antibody (5 μg/ml; Sigma) and further incubated at 26°C. One hour later, AlphaScreen signals from the mixture were detected with an EnVision device (PerkinElmer) with the AlphaScreen signal detection program.

Cells and transfections

HEK 293T, HeLa, LLC-MK2, Jurkat, and H9 cells were cultured under standard conditions. Plasmid or siRNA transfection into adherent cells was performed with the Effectene or Hiperfectamine transfection reagent (Qiagen), respectively, in accordance with the manufacturer's instructions. Transfection of suspension cells with plasmids was performed with the Neon Transfection System (Invitrogen) according to the manufacturer's protocol.

Plasmids and viruses

Human SCYL2 (GenBank accession no. BC063798) full-length sequences (1 to 929 amino acid residues) and deletion inserts (1 to 375, 1 to 697, and 376 to 929 amino acids) were amplified from pCMV6-XL4-SCYL2 (Origene) with primer pairs containing restriction enzyme sites and a stop codon. These inserts were subcloned into pCMV-HA/pCMV-Myc vector (Clontech). Alternatively, on the basis of bioinformatic predictions, the 3.3-kb fragment upstream of the SCYL2-encoding gene was amplified from HEK 293T cDNA and subcloned into the pGL4.10 firefly luciferase reporter vector (Promega). A human codon-optimized HIV-1 Vpu expression vector (*73*) and a Vpu-deleted HIV-1 molecular clone (*74*) were provided by K. Strebel [National Institutes of Health (NIH), Bethesda, MD]. The Vpu(S52,56A) mutants were constructed with standard molecular cloning procedures. The construct encoding KSHV-K5 (*75*) was provided by P. Cannon (University of Southern California, Los Angeles, CA). The plasmids expressing PP2A/A and $PP2A_C$ (*76*) were provided by A. Yamashita (Yokohama City University, Kanagawa, Japan). The VSVG-pseudotyped virus stocks were produced by transient transfection of HEK 293T cells with the pNL4-3 proviral clone and vectors expressing VSVG at a molar ratio of 3:1. After 48 hours, the culture supernatants containing virus were collected, filtered through a 0.45-μm Millex-HV filter (Millipore), and immediately stored at −80°C until required.

siRNA, IFN treatments, and RT-PCR

An array of siRNAs targeting the genes shown in Fig. 1D, as well as BST2, CHC, and PP2A/A, was obtained from Qiagen and used as a mixture of three different targeting sequences. In experiments involving SCYL2 knockdown, we used at least two SCYL2-specific Stealth RNAi constructs (oligo ID

HSS124796 and HSS183194, Invitrogen) to avoid off-target effects. Stealth RNAi Luciferase Reporter Control (Invitrogen) was used as the negative control. For detection of SCYL2 mRNA in IFN-treated cells, total RNA was extracted with the RNeasy Mini Kit (Qiagen) from cells treated with or without recombinant human IFN-α (1000 U/ml; Sigma) or IFN-β (1000 U/ml; PBL Biomedical Laboratories) for 6 hours before harvesting. The cDNA was generated with ReverTra Ace (Toyobo) and an oligo$(dT)_{20}$ primer, and then PCR was performed with Ex Taq (Takara Bio). The PCR primers used were as follows: *SCYL2*, 5′-gggaatcagcaaattgacaaagttt-3′ (forward) and 5′-agtccttagtctgtttaacagtagc-3′ (reverse); *ACTB*, 5′-ggacttcgagcaagagatgg-3′ (forward) and 5′-agcactgtgttggcgtacag-3′ (reverse).

Single-cycle virus release assays

For transfection-based assays, cells in 12-well plates were cotransfected with pNL4-3 or pNL4-3ΔVpu (100 ng) and either an SCYL2 expression vector or an empty vector (0, 50, and 100 ng) and cultured for 2 days. For infection-based assays, cells were infected with the VSVG-pseudotyped HIV-1 at an MOI of 0.01 or 0.2 for 8 hours and cultured for 2 days (HeLa cells) or 4 days (H9 cells). In experiments with siRNA, cells were transfected with a pool of siRNAs 24 hours before being either transfected with pNL4-3 or infected with virus. Virus-containing supernatants were harvested and filtered to remove debris, and viral p24 antigens were measured with an ELISA kit (Zepto Metrix). The cell lysates were prepared with HBST buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5% Triton X-100] containing protease inhibitor cocktail (Roche). Western blotting analysis and the antibodies used have been described previously (*77*). In some experiments, OA (Calbiochem) was added 18 hours before harvesting. In experiments with IFN-β, virus-producing cells were washed twice with phosphate-buffered saline (PBS) and treated with IFN-β for 8 hours before being harvested. The culture supernatants and cell lysates were subjected to p24 ELISA measurement or Western blotting analysis, as described earlier.

Flow cytometry

Cells in 12-well plates were transfected with vectors encoding Vpu (100 ng), green fluorescent protein (GFP) (100 ng), and either HA-SCYL2 or empty plasmid (0, 1, and 2 μg). Alternatively, vector encoding KSHV-K5 was used instead of vector encoding Vpu. Eighteen hours later, cells were harvested in PBS containing 5 mM EDTA and washed with PBS containing 1% bovine serum albumin (BSA). The cells were blocked with 10% normal goat serum and then stained with an anti-BST2 antibody (a gift from Chugai Pharmaceutical Co.) and a phycoerythrin (PE)–conjugated secondary antibody (Beckman Coulter) or a PE/Cy7-conjugated anti-CD4 antibody (BioLegend). All samples were fixed with 4% paraformaldehyde and analyzed with a FACSCanto II instrument (BD Biosciences) and FlowJo software (Treestar) gated for the GFP-positive fraction.

Detection of pVpu

For phosphate-affinity (Phos-tag) gel analysis, cells in 12-well plates were transfected with vectors encoding either wild-type Vpu or the Vpu(S52,56A) mutant (100 ng) together with plasmid encoding SCYL2 (at 0, 500, or 1000 ng). Two days later, cell lysates were loaded onto an 8% polyacrylamide gel containing 50 μM $MnCl_2$ and 25 μM Phos-tag acrylamide (Wako). After electrophoresis, gels were soaked in a general transfer buffer with 1 mM EDTA for 10 min to eliminate Mn^{2+} ions and then subjected to Western blotting analysis with anti-Vpu antibody (a gift from K. Strebel). A phospho-specific polyclonal antibody against Vpu phosphorylated at Ser^{52} and Ser^{56} was produced and purified by Scrum Inc.

Microscopy

One day before transfection, cells were seeded onto glass-bottomed dishes (Matsunami). At 48 hours after transfection, the cells were fixed and stained as described previously (*77*). Microscopic imaging was performed with an FV1000-D confocal laser scanning microscope (Olympus) equipped with a 60× oil-immersion objective.

In vitro and in vivo binding assays

For in vitro GST pull-down assays, biotinylated SCYL2 was incubated with either GST-Vpu or GST–dihydrofolate reductase (DHFR) at 26°C for 2 hours before being co-incubated with glutathione-Sepharose beads (GE Healthcare) at 4°C for 3 hours. The beads were then washed three times, and bound proteins were analyzed by Western blotting with streptavidin–horseradish peroxidase (HRP) conjugate (GE Healthcare). For immunoprecipitation analysis, cells expressing epitope-tagged proteins were lysed with HBST buffer containing protease inhibitor cocktail (Roche). Cell lysates were immunoprecipitated with EZview Red Affinity Gel (Sigma), and bound proteins were analyzed by Western blotting.

Quantitative phosphatase assays

Phosphorylated or unphosphorylated Vpu peptides including Ser^{52} and Ser^{56} ($AEDS_{52}GNES_{56}EGE$) were chemically synthesized by Scrum Inc. Cells in six-well plates were transfected with pCMV-HA-SCYL2. Two days later, cell lysates were prepared with HBST buffer containing protease inhibitor cocktail alone or with 10 nM OA. Cell lysates were immunoprecipitated with an anti-HA antibody and incubated with 10 μl of 1 mM of either phosphorylated or unphosphorylated Vpu peptide in 50 μl of reaction buffer [50 mM imidazole (pH 7.2), 200 μM EGTA, 0.25% β-mercaptoethanol, BSA (0.1 mg/ml)] at 37°C for 8 hours. To stop the reactions, 50 μl of 20 mM ammonium molybdate was added to the mixture. The amounts of free phosphate ion were measured by absorbance at 630 nm. Standard curves were obtained with a Ser/Thr phosphatase assay kit (Promega) according to the manufacturer's instructions.

Luciferase reporter assays

Cells in 24-well plates were transfected with the pGL4-based luciferase reporter plasmid and with pGL4-TK-Rluc (Promega) as a transfection control. Two days later, cells were treated with IFN-β (1000 U/ml) for 8 hours before being harvested. Luciferase activity was determined with a Dual-Luciferase Reporter assay system (Promega) and normalized to *Renilla* luciferase activity.

Statistical analysis

All graphs present the means and SDs. Statistical significance of differences between two groups was tested by two-tailed unpaired *t* test with Prism 6 software (GraphPad). In cases where multiple comparisons within an experiment were made, one-way analysis of variance (ANOVA) was used. A P value of <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/5/245/ra73/DC1

Fig. S1. Schematic representation of the initial screening method.

Fig. S2. The CKII inhibitor DRB phenocopies the effect of increased SCYL2 abundance.

Fig. S3. SCYL2 fails to inhibit SIV_{GSN} Vpu–induced counteraction of BST2.

Fig. S4. SCYL2 inhibits Vpu function through a phosphorylation-dependent mechanism.

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Molecular Analysis of Genome of the Pandemic Influenza A(H1N1) 2009 Virus Associated with Fatal Infections in Gunma, Tochigi, Yamagata, and Yamaguchi Prefectures in Japan during the First Pandemic Wave

Masatsugu Obuchi[1]*, Shoichi Toda[2], Hiroyuki Tsukagoshi[3], Teruko Oogane[4], Chieko Abiko[5], Keiji Funatogawa[4], Katsumi Mizuta[5], Komei Shirabe[2], Kunihisa Kozawa[3], Masahiro Noda[6], Hirokazu Kimura[6], and Masato Tashiro[1]

[1]*Influenza Virus Research Center and*
[6]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011;*
[2]*Yamaguchi Prefectural Institute of Public Health and Environment, Yamaguchi 753-0821;*
[3]*Gunma Prefectural Institute of Public Health and Environmental Science, Gunma 371-0052;*
[4]*Tochigi Prefectural Institute of Public Health and Environmental Science, Tochigi 329-1196; and*
[5]*Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, Japan*

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In May 2009, a novel swine-origin pandemic influenza A(H1N1) 2009 virus [A(H1N1)pdm09] was first identified in Osaka and Hyogo prefectures of Japan, which then spread to other prefectures in the next several weeks (1). During the summer, pandemic influenza activity remained low; however, it subsequently increased and reached a peak in November 2009 (2). In most cases, infection by A(H1N1)pdm09 caused mild disease, though there have been sporadic cases with severe or fatal outcomes (2). One hundred and ninety-eight fatal cases were reported during the first pandemic wave in Japan (2). Several countries report that an amino acid substitution of aspartic acid by glycine at position 222 (D222G) in hemagglutinin (HA) is associated with disease severity; however, many A(H1N1)pdm09 isolates without this mutation have been identified in severe and fatal cases (3-5). To further explore the molecular determinants of A(H1N1)pdm09 that are associated with severity, we performed whole genome analysis on virus isolates obtained from the fatal cases identified in Gunma, Tochigi, Yamagata, and Yamaguchi prefectures between May 2009 and March 2010.

Nasal swabs were collected from hospitalized influenza patients and sent to the district's prefectural public health institute for diagnosis and viral strain surveillance. Clinical specimens were first inoculated onto Madin-Darby canine kidney (MDCK) cells for virus isolation. Viral RNA was then extracted from the virus culture supernatant using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction was carried out using the One Step RNA PCR Kit (AMV) (TaKaRa Bio Inc., Otsu, Japan) and virus-specific primers established by the National Institute of Technology and Evaluation and the National Institute of Infectious Diseases, Japan (6). Amplicons were purified using ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA). The sequence data were assembled and analyzed using the Sequencher for Macintosh V4.9 software (Hitachi Software Engineering Co., Tokyo, Japan) and GENETYX-Mac Ver. 13.0.17 (Genetyx Corp., Tokyo, Japan).

In Gunma, Tochigi, Yamagata, and Yamaguchi prefectures, a total of 12 fatal cases were reported to the Ministry of Health, Labour and Welfare of Japan during the first pandemic wave. A(H1N1)pdm09 isolates were successfully obtained from 9 fatal cases; the patients were aged between 4 and 85 years (Table 1). Of these patients, 2 developed pneumonia (Patients 6 and 9) and 1 patient developed multiple organ failure (Patient 3). Patient 8 suffered a comorbidity, namely a subarachnoid hemorrhage. Moreover, 5 patients had underlying diseases: 3 had chronic obstructive pulmonary disease, 1 had multiple myeloma and diabetes mellitus, and 1 had lung cancer. Of the 7 patients taking medication, 4 were taking oseltamivir and 3 zanamivir. In this study, no pandemic influenza-related deaths were reported in pregnant women. For comparison, we obtained 6 isolates from influenza patients presented with mild conditions (Patients 10-15).

Nucleotide sequencing of entire viral genome segments (i.e., polymerase basic 2 [PB2], polymerase basic 1 [PB1], polymerase acidic [PA], HA, nucleoprotein [NP], neuraminidase [NA], matrix [M] protein, and nonstructural [NS] protein genes) was conducted for all 15 viral isolates. The amino acid sequences of viral proteins were deduced from the nucleotide sequences. Table 2 shows the amino acid differences between the virus isolates analyzed in this study and A(H1N1)pdm09 isolates (as consensus sequences) collected worldwide between April 2009 and March 2010. The consensus

*Corresponding author: Present address: Department of Virology, Toyama Institute of Health, 17-1 Nakataikoyama, Imizu-shi, Toyama 939-0363, Japan. Tel: +81-766-56-8143, Fax: +81-766-56-7326, E-mail: masatsugu.obuchi@pref.toyama.lg.jp

Table 1. Fatal and mild cases of A(H1N1)pdm09 infection and virus isolates in this study

Patient	Sex/age (yr)	Clinical feature	Severity	Medication	Underlying disease	Sampling date	Virus isolate	GenBank accession no.
1	M/85	Influenza	Fatal	Oseltamivir	None	Nov. 25, 2009	A/Gunma/287/2009	AB704443-AB704450
2	M/60	Influenza	Fatal	Zanamivir	Multiple myeloma, diabetes mellitus	Dec. 15, 2009	A/Gunma/293/2009	AB704451-AB704458
3	M/83	Multiple organ failure	Fatal	Oseltamivir	COPD	Oct. 29, 2009	A/Tochigi/350/2009	AB704459-AB704466
4	F/8	Influenza	Fatal	Zanamivir	None	Nov. 24, 2009	A/Tochigi/445/2009	AB704467-AB704474
5	M/56	Influenza	Fatal	None	None	Jan. 13, 2010	A/Tochigi/2/2010	AB704475-AB704482
6	F/62	Pneumonia	Fatal	Oseltamivir	COPD	Nov. 9, 2009	A/Yamagata/473/2009	AB704491-AB704498
7	F/13	Influenza	Fatal	Zanamivir	COPD	Nov. 21, 2009	A/Yamaguchi/217/2009	AB704507-AB704514
8	M/4	Subarachnoid hemorrhage	Fatal	None	None	Dec. 2, 2009	A/Yamaguchi/247/2009	AB704515-AB704522
9	M/60	Pneumonia	Fatal	Oseltamivir	Lung cancer	Dec. 5, 2009	A/Yamaguchi/248/2009	AB704523-AB704530
10	M/10	Influenza	Mild	None	None	Nov. 19, 2009	A/Gunma/262/2009	AB704419-AB704426
11	M/9	Influenza	Mild	Zanamivir	None	Nov. 16, 2009	A/Gunma/263/2009	AB704427-AB704434
12	M/4	Influenza	Mild	Oseltamivir	None	Nov. 25, 2009	A/Gunma/267/2009	AB704435-AB704442
13	M/56	Influenza	Mild	Unknown	None	Jan. 12, 2010	A/Tochigi/10/2010	AB704483-AB704490
14	F/50	Influenza	Mild	None	None	Nov. 27, 2009	A/Yamagata/674/2009	AB704499-AB704506
15	M/7	Influenza	Mild	Oseltamivir	None	Dec. 18, 2009	A/Yamaguchi/273/2009	AB704531-AB704538

COPD, chronic obstructive pulmonary disease.

sequences of each segment consist of 2728 (PB2), 2312 (PB1), 2366 (PA), 4733 (HA), 2440 (NP), 4401 (NA), 3449 (MP), and 2376 (NS) nucleotide sequences, which were downloaded from the Global Initiative on Sharing All Influenza Data EpiFlu database (http://platform.gisaid.org/epi3/frontend#34c9cf). Amino acid substitutions, namely S203T in HA, V100I in NP, V106I and N248D in NA, and I123V in NS1, which are known as specific markers for cluster 2, were present in all the analyzed sequences, indicating that these virus isolates belonged to the cluster containing a large majority of circulating A(H1N1)pdm09 strains in Japan, during the peak phase of the pandemic (7). There was no reassortment with other seasonal (either H1N1 or H3N2), swine, or avian influenza A viruses. A total of 39 unique amino acid differences were found in 9 isolates obtained from fatal cases: 5 in PB2, 4 in PB1, 9 in PA, 6 in HA, 2 in NP, 6 in NA, 3 in M2, 2 in NS1, and 2 in nuclear export protein (NEP). Of these differences, only V19I in HA was common to 2 of the isolates (i.e., A/Yamaguchi/217/2009 and A/Yamaguchi/248/2009). A marker for oseltamivir resistance, H275Y in NA, was identified in A/Yamaguchi/248/2009 that was derived from an oseltamivir-treated patient. Frequently observed changes in the fatal cases, defined as more than 3 out of 9 isolates, were commonly observed in mild cases (i.e., T257A, I435V, and N537S in PB1; S69L, D274N, and E374K in HA; and G41E in NA), suggesting that these amino acid substitutions are unlikely to be associated with the level of severity.

The amino acid location at position 222 in the receptor binding site of HA predicts that alterations to this position would influence the binding specificity of viruses. Previous studies have reported that a D222G substitution confers enhanced binding to α2,3-linked (avian-like) rather than α2,6-linked (human-like) sialic acids, suggesting an augmented ability to bind to lung cells in the lower respiratory tract in humans and cause an exacerbation of the disease (3). No D222G substitution in the HA was observed in any of the isolates analyzed in this study. Alternatively, a D222E substitution was found in 1 of the isolates, A/Yamaguchi/247/2009, which was derived from a fatal infection. This substitution, however, seems to be unrelated to the disease severity of the A(H1N1)pdm09 as previously reported (4,8). Another V132E amino acid mutation in the receptor binding site was found in 2 isolates, a fatal case-strain (i.e., A/Tochigi/2/2010) and a mild case-strain (i.e., A/Yamaguchi/273/2009); however, the precise impact of this mutation is unclear. A mixed population of viruses possessing 163K/E in an antigenic site was found in the isolate A/Yamaguchi/217/2009, which was also derived from a fatal infection. However, the antigenicity of this isolate was similar to that of the vaccine strain, A/California/07/2009 (data not shown).

Virus isolates derived from patients with fatal infection manifested sporadic amino acid changes in the PB2 and PA proteins more frequently than those derived from patients with mild infections (Table 2). Notably, 6 of the 9 isolates from fatal cases had 1 or 2 amino acid substitutions in the PA, e.g., E2K, A70V, P325L, V387I, S405A, V432F, L589I, S594G, and A598P. The RNA-dependent RNA polymerase of influenza viruses is a complex of 3 viral proteins, PB1, PB2, and PA, and

Table 2. Amino acid differences in the viral proteins of A(H1N1)pdm09 isolates obtained from fatal and mild cases

Virus isolate[1]	Viral protein[2]																																		
	PB2									PB1										PA															
	121	251	368	495	588	616	660	673	682	36	76	94	257	383	393	435	537	609	652	2	70	224	325	387	405	432	578	589	594	598					
Consensus	K	R	R	V	T	I	K	G	G	T	D	F	T	E	R	I	N	V	A	E	A	S	P	V	S	V	G	L	S	A					
A/Gunma/287/2009					I				R			L														A			I							
A/Gunma/293/2009							R	R	R				A																	P						
A/Tochigi/350/2009																V	S																		
A/Tochigi/445/2009																V	S																		
A/Tochigi/2/2010							R						A							K					F										
A/Yamagata/473/2009			K													V	S																		
A/Yamaguchi/217/2009				I																			L												
A/Yamaguchi/247/2009	R													D					V					I											
A/Yamaguchi/248/2009		K											A			V		A			V							G							
A/Gunma/262/2009							R																												
A/Gunma/263/2009																V	S																		
A/Gunma/267/2009								R	R		N				T	V	S																		
A/Tochigi/10/2010							R			M																									
A/Yamagata/674/2009							R																												
A/Yamaguchi/273/2009						V	R						A														S								
A/California/07/2009																						P													

Virus isolate	Viral protein																								
	HA																	NP							
	19	69	83	132	163	171	197	203	222	258	274	297	304	321	339	374	393	31	100	119	297	363			
Consensus	V	S	S	V	K	K	A	T	D	E	D	P	P	V	G	E	V	R	I	V	Y	V			
A/Gunma/287/2009													S		R						H	I			
A/Gunma/293/2009																K									
A/Tochigi/350/2009		L									N					K									
A/Tochigi/445/2009		L									N					K									
A/Tochigi/2/2010				E												K									
A/Yamagata/473/2009		L									N					K									
A/Yamaguchi/217/2009	I				K/E																				
A/Yamaguchi/247/2009									E			S					I								
A/Yamaguchi/248/2009	I																								
A/Gunma/262/2009						R	T													I					
A/Gunma/263/2009		L									N					K									
A/Gunma/267/2009		L								V	N					K									
A/Tochigi/10/2010						R	T								R										
A/Yamagata/674/2009						R	T											K							
A/Yamaguchi/273/2009				E												K									
A/California/07/2009			P					S						I					V						

Table 2. Continued

Virus isolate	Viral protein																														
	NA															M1	M2				NS1							NEP			
	41	62	76	82	106	110	140	221	248	275	351	381	382	416	465	64	39	50	51	63	2	64	93	100	112	122	123	2	89	105	
Consensus	G	V	A	S	I	S	L	N	D	H	F	T	G	D	F	F	I	C	I	P	D	I	M	D	I	A	V	D	A	L	
A/Gunma/287/2009						C											V	Y		A		T									
A/Gunma/293/2009																							I								
A/Tochigi/350/2009	E																														
A/Tochigi/445/2009	E							S																							
A/Tochigi/2/2010																							I								
A/Yamagata/473/2009	E																													I	
A/Yamaguchi/217/2009																													V		
A/Yamaguchi/247/2009												N			L											T					
A/Yamaguchi/248/2009				P						Y																					
A/Gunma/262/2009		I					I						E	N		Y															
A/Gunma/263/2009	E																														
A/Gunma/267/2009	E																														
A/Tochigi/10/2010													E	N		Y					G			N				G			
A/Yamagata/674/2009													E	N		Y			T						M						
A/Yamaguchi/273/2009			T																				I								
A/California/07/2009					*V*				*N*		*Y*																*I*				

1): Virus isolates obtained from fatal cases and their amino acid substitutions are indicated with a bold font. The vaccine strain for the 2009–2010 influenza season and its amino acid substitutions are indicated in an italicized font.

2): PB2, polymerase basic protein 2; PB1, polymerase basic protein 1; PA, polymerase acidic protein; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M1, matrix protein 1; M2, matrix protein 2; NS1, nonstructural protein 1; NEP, nuclear export protein. Fatal case-specific substitutions are indicated with opened boxes. Positions of antigenic and receptor binding sites in the HA are indicated with gray and dotted backgrounds, respectively.

plays a key role in viral growth within the mammalian host cells. Recent studies demonstrated that mutations within PB2 or PA lead to increased pathogenicity of the A(H1N1)pdm09 in mice (9–11). A limitation of this study is the relatively small size of the analyzed data set. In addition, the pathogenetic role of the observed viral mutations remains to be elucidated.

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Conflict of interest None to declare.

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Short Communication

Molecular Epidemiology of Human Metapneumovirus from 2009 to 2011 in Okinawa, Japan

Minoru Nidaira[1]*, Katsuya Taira[1], Hirotsune Hamabata[2], Tatsuyoshi Kawaki[3], Kazuo Gushi[4], Youko Mahoe[1], Noriyuki Maeshiro[1], Yasuhito Azama[1], Shou Okano[1], Hisako Kyan[1], Jun Kudaka[1], Hiroyuki Tsukagoshi[5], Masahiro Noda[6], and Hirokazu Kimura[6]

[1]*Department of Biological Science, Okinawa Prefectural Institute of Health and Environment, Okinawa 901-1202;*
[2]*Awase Daiichi Clinic, Okinawa 904-2172;*
[3]*Aozora Pediatric Clinic, Okinawa 901-1302;*
[4]*Gushi Kodomo Clinic, Okinawa 901-0244;*
[5]*Gunma Prefectural Institute of Public Health and Environmental Sciences, Gunma 371-0052; and*
[6]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011, Japan*

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SUMMARY: To clarify the molecular epidemiology of human metapneumovirus (HMPV) in Okinawa Prefecture, located in a subtropical region of Japan, we performed genetic analysis of the *F* gene in HMPV from patients with acute respiratory infection from January 2009 to December 2011. HMPV was detected in 18 of 485 throat swabs (3.7%). Phylogenetic analysis showed that 17 strains belonged to subgroup A2 and 1 strain belonged to subgroup B1. We did not observe seasonal prevalence of HMPV during the investigation period. A high level of sequence identity was observed in the strains belonging to subgroup A2 (>95%), and no amino acid substitution was found compared with other strains detected in Japan and other countries. The pairwise distance values among the present strains belonging to subgroup A2 were short. Our results suggest that the predominant HMPV strains belonging to A2 are highly homologous and seasonal epidemics were not seen in Okinawa during the investigation period.

Human metapneumovirus (HMPV) is a member of the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Metapneumovirus*, and is an important causative agent of acute respiratory infection (ARI) in humans (1). Recent studies suggest that HMPV affects both children and adults, including the elderly (2,3). In addition, recurrent HMPV infection occurs throughout life (4,5).

Okinawa Prefecture is located in a subtropical region of Japan, and the prevalence of various respiratory viruses might be unique to the area. For example, the prevalent season of respiratory syncytial virus (RSV) infection in Okinawa differs from that of mainland Japan (6). In addition, the epidemic pattern of influenza virus (Flu) is distinct (7-9). However, the epidemiology of HMPV in subtropical regions such as Okinawa remains unknown. Here, we analyzed the *F* gene detected in patients with ARI to address the molecular epidemiology of HMPV in Okinawa.

From January 2009 to December 2011, 485 throat swab samples were collected from 417 patients with ARI at Awase Daiichi Clinic (located in the central region of Okinawa island), Aozora Pediatric Clinic (located in a southern region of Okinawa island), and Gushi Kodomo Clinic (also in a southern region of Okinawa island). This was conducted as part of a collaboration with the local health authority of Okinawa Prefecture for the surveillance of viral diseases in Japan. Three hundred sixty-nine of the 485 samples were single samples from 369 patients. The remaining 116 samples were collected as follows: 36 patients were samples twice (72 samples), 7 patients were sampled 3 times (21 samples), 3 patients were sampled 4 times (12 samples), 1 patient was sampled 5 times (5 samples), and 1 patient was sampled 6 times (6 samples). Samples were not collected from the same patient in the same month and year. Informed consent was obtained from all patients or the parents/guardians of underage patients for the donations of throat swab samples used in the study.

Patients were aged from 0 to 73 years (5.9 ± 12.2 years; mean ± standard deviation [SD]) and were residents of Okinawa Prefecture, Japan. Of the 485 samples, 195 were collected from patients diagnosed with upper respiratory infection (URI) and 290 with lower respiratory infection (LRI), including bronchitis and pneumonia. One hundred seventy-two of the 290 LRI patients were diagnosed with wheezy LRI, and 118 were diagnosed with non-wheezy LRI.

Viral RNA was extracted from 140 μL of samples using the QIAamp Viral RNA Mini Kit (Qiagen, Tokyo, Japan) and suspended in DNase/RNase-free water. After RNA extraction, cDNA was synthesized using a PrimeScript RT reagent kit (Takara, Shiga, Japan), and

*Corresponding author: Mailing address: Department of Biological Science, Okinawa Prefectural Institute of Health and Environment, 2085 Ozato, Nanjo-shi, Okinawa 901-1202, Japan. Tel: +81-98-945-0785, Fax: +81-98-945-9366, E-mail: nidairam@pref.okinawa.lg.jp

PCR was performed using the primers MPVF1f and MPVF1r, as described previously (10). Amplification products were separated by electrophoresis on a 2.0% (w/v) agarose gel stained with ethidium bromide. Amplicons were purified using a QIAquick PCR Purification Kit (Qiagen), and nucleotide sequences were determined by direct sequencing. Sequence data were registered under accession numbers AB683044-AB683047 and AB683238-AB683251 at DDBJ/EMBL/GenBank. Phylogenetic analysis of the partial nucleotide (nt) sequences (321 nt) of the *F* region of HMPV was achieved using Molecular Evolutionary Genetics Analysis (MEGA) software, version 5 (11). Evolutionary distances were estimated using Kimura's two-parameter method, and phylogenetic trees were constructed using the neighbor-joining method (12). The reliability of the tree was estimated using 1000 bootstrap replications. In addition, the pairwise distances for the present strains were calculated to assess the frequency distribution, as previously described (13).

We detected other respiratory viruses in the present samples using (RT)-PCR methods for RSV (14), human parainfluenza viruses type 1-3 (HPIV1-3) (15), human rhinoviruses (HRV) (16,17), and human bocaviruses (HBoV) (18). In addition, to isolate adenoviruses (AdV), enterovirus (EV), and Flu (subtype A to C), we applied cell culture methods using 3 cell lines (HEp-2, RD18S, and MDCK cells), as previously described (19). The cells were checked daily for cytopathic effect (CPE), and culture supernatant fluids were harvested when CPE was clearly observed. The culture supernatants were examined for AdV and EV by (RT)-PCR methods, as previously described (16,17,20).

Statistical analysis was performed by a χ^2 test using Statcel (OMS, Tokyo, Japan). A P value of <0.05 was regarded as statistically significant.

Eighteen HMPV strains were detected in the 485 samples (3.7%); the 18 HMPV positive samples were collected from 18 different patients. In addition, other respiratory viruses such as RSV, HPIV1-3, HRV, and HBoV were detected or AdV and EV strains were isolated (Table 1). All 18 HMPV strains were detected in the 389 samples collected from patients aged 0-6 years (4.6%). Four of 18 strains were detected in 117 samples collected from patients under 1 year old (3.4%), and 4 were detected in 165 samples collected from patients aged 1 year (2.4%), and 5 were detected in 58 samples collected from patients aged 2 years (8.6%), and 3 were detected in 21 samples collected from patients aged 3 years (14.3%), and 1 was detected in 12 samples collected from patients aged 5 years (8.3%), and 1 was detected in 5 samples collected from patients aged 6 years (20.0%). HMPV strains were not detected in the 96 patients aged >6 years. Five HMPV strains were detected in 195 URI patients (2.6%), and 13 HMPV strains were detected in 290 LRI patients (4.5%). There was no significant difference between the detection rates in URI and LRI (χ^2 test; $P = 0.27$). Of the 13 HMPV strains detected in LRI patients, 9 strains were detected in the 172 wheezy LRI patients (5.2%) and 4 strains were detected in the 118 non-wheezy LRI patients (3.4%). There was no significant difference between the detection rates (χ^2 test; $P = 0.45$). HMPV alone was detected in 16 of the 18 patients, while HMPV plus HRV was detected in 1 patient and HMPV plus EV in the remairing patient. In each month, 0-3 HMPV strains were detected (0-15%) (Fig. 1). There was no significant difference between the detection rates (χ^2 test; $P > 0.05$), and no seasonal prevalence was found during the investigation periods.

On the phylogenetic tree, 17 strains were classified into subgroup A2 and a single strain detected in May 2011 was classified into subgroup B1 (Fig. 2). Strains belonging to subgroup A2 were subdivided into 3 clusters: A2a, A2b, and A2c. Cluster A2a included 2 strains detected in March 2009 and 1 strain detected in July 2009. Cluster A2b included 1 strain detected in July 2009 and another in April 2011. Cluster A2c included 9 strains detected in 2010, 1 strain detected in March

Table 1. Detection of the respiratory viruses in the present study

	No. of detection (%)
HMPV	18 (3.7)
RSV	55 (11.3)
HPIV1-3	28 (5.8)
HRV	25 (5.2)
HBoV	18 (3.7)
AdV	39 (8.0)
EV	24 (4.9)
Flu	0 (0)

HMPV, human metapneumovirus; RSV, respiratory syncytial virus; HPIV1-3, human parainfluenza virus type 1-3; HRV, human rhinovirus; HBoV, human bocavirus; AdV, adenovirus; EV, enterovirus; Flu, influenza virus.

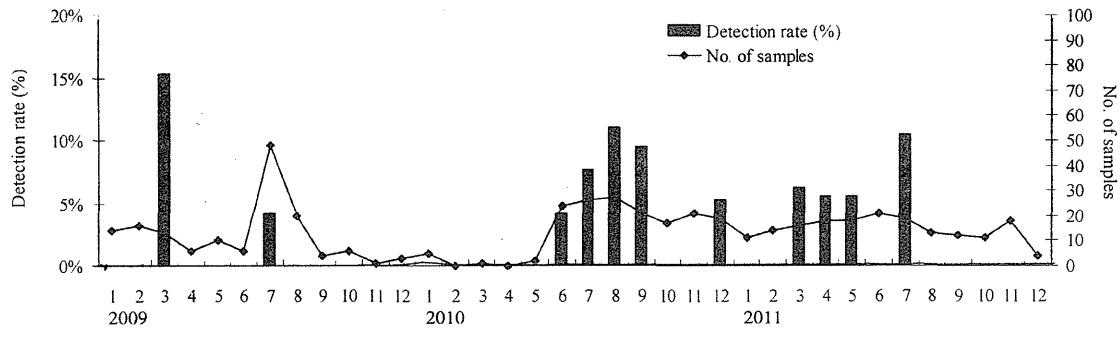

Fig. 1. Monthly distribution of HMPV strains detected in Okinawa Prefecture, Japan, from January 2009 to December 2011.

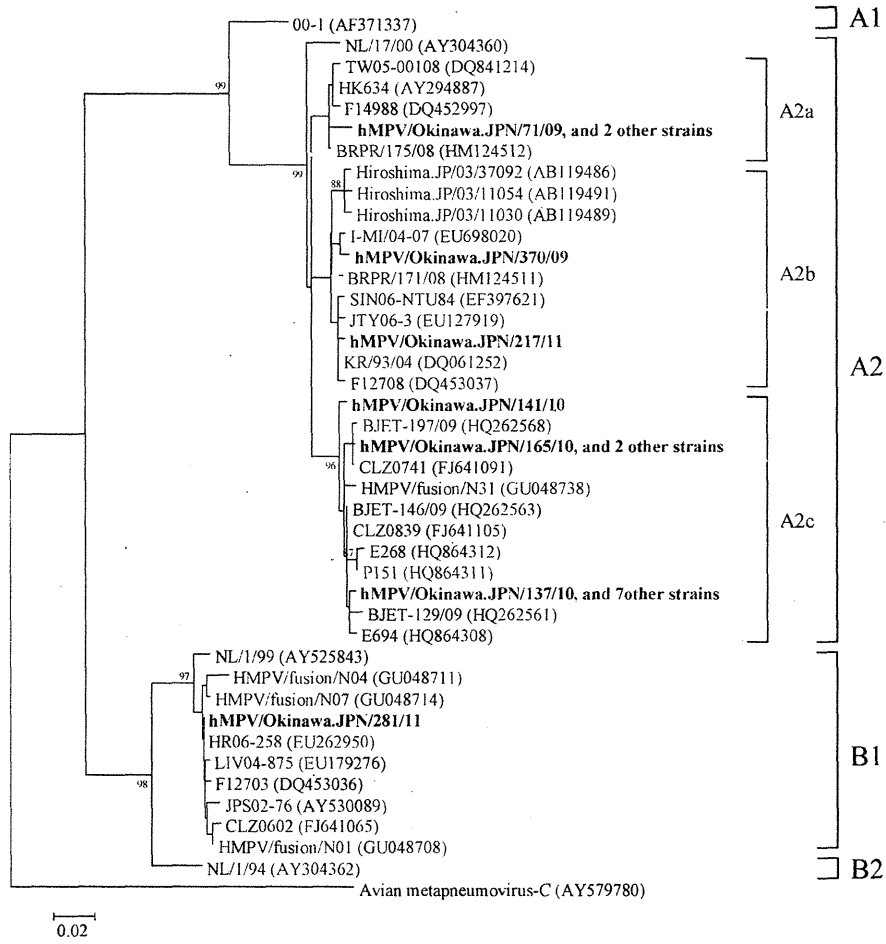

Fig. 2. Phylogenetic tree of HMPV based on the *F* gene sequences. Avian metapneumovirus-C was used as an outgroup. The phylogenetic tree was constructed after neighbor-joining analysis based on the nucleotide sequences of the partial *F* region (321 nt) with 1000 bootstrap replicates. Bootstrap values >70% are indicated at each node. A1, A2, B1, and B2 are the subgroups. A2a, A2b, and A2c are the clusters indicated in this study. Bar indicates nucleotide substitutions per site.

2011, and 2 strains detected in July 2011. Nucleotide identity levels were high (>95%) among the 17 present strains belonging to subgroup A2 and, in comparison with other strains detected in Japan and other countries, no amino acid substitutions were found. The pairwise distances among the present strains belonging to subgroup A2 did not exceed 0.05. These values were not significant compared with those of Japanese and overseas strains.

We detected HMPV in around 4% of samples from patients with ARI in Okinawa from 2009 to 2011 in this study, although a seasonal prevalence was not observed. The phylogenetic tree showed that the HMPV strains detected belonged to subgroups A2 and B1, and A2 was predominant in Okinawa during the investigation period. The present strains belonging to subgroup A2 were subdivided into 3 clusters. Cluster A2a included HMPV strains detected in 2009, and cluster A2c included HMPV strains detected from 2010 to 2011. However, cluster A2b included HMPV strains detected in 2009 and 2011 and the bootstrap value was less than 50% at the nodes of these clusters. Nucleotide identity levels among the present strains belonging to subgroup A2 were high. No amino acid substitution was found, and our results are compatible with earlier reports (21,22). In addition, pairwise distance values were short. The results indicated that the predominant HMPV strains were highly homologous during the investigation period.

Seroepidemiological studies have indicated that in some countries almost all children show evidence of HMPV infection by the age of 5 (23,24). In Japan, Ebihara et al. reported that around 40% of children aged from 4 months to 5 years were seropositive for HMPV (25), and Mizuta et al. reported that the isolation rate of HMPV in patients aged <5 years was higher than that in patients aged ≥5 years (21). In another report from China, HMPV was mainly detected in patients aged <5 years and around 10% of patients were co-infected with other ARI viruses such as HPIV, Flu, AdV, and RSV (26). Moreover, A2 and B2 were the predominant subgroups in Yamagata Prefecture from 2004 to 2009 (21), while A2 was predominant in Yamaguchi Prefecture in 2009 (22). In the present study, 18 HMPV strains were detected in patients aged 0-6 years. In addition, HRV and EV were detected in 2 of these patients (11.1%). Thus, other respiratory viruses might be detected at a steady rate in patients with ARI due to HMPV. These

findings suggested that the molecular epidemiology of HMPV in Okinawa is similar to that of other areas.

Some longitudinal studies suggest that the high season for HMPV in the Northern Hemisphere is from winter to spring (between January and May) and the low season is in the fall (around September and October) (2,21). The high season for HMPV in tropical and subtropical areas varies—winter to spring in Brazil (27), spring and/or summer in Taiwan (28,29), and the rainy season in Vietnam (30). In this study, HMPV did not appear to have a high season in Okinawa. Although the reasons are not yet known, this trend may differ from that of other tropical and subtropical areas. However, our investigation periods may be short. Thus, to clarify the prevalence season of HMPV in Okinawa, more longitudinal studies may be needed. In addition, the present samples were collected from only 3 different areas. Thus, to rigorously investigate the prevalence of HMPV in our prefecture, additional samples from different areas should be tested.

In conclusion, the predominant HMPV strain showed a high degree of genetic homology throughout the investigation period in Okinawa. However, additional studies, including those designed to investigate the clinical features of HMPV infection, are needed to further understand the epidemiology of the virus in Okinawa.

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Conflict of interest None to declare.

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Detection of Human Coronavirus NL63 and OC43 in Children with Acute Respiratory Infections in Niigata, Japan, between 2010 and 2011

Miyako Kon[1], Kaori Watanabe[1], Takashi Tazawa[1], Kanako Watanabe[1], Tsutomu Tamura[1], Hiroyuki Tsukagoshi[2], Masahiro Noda[3], Hirokazu Kimura[3], and Katsumi Mizuta[4]*

[1]*Niigata Prefectural Institute of Public Health and Environmental Sciences, Niigata 950-2144;*
[2]*Gunma Prefectural Institute of Public Health and Environmental Sciences, Gunma 371-0052;*
[3]*Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 208-0011; and*
[4]*Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, Japan*

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Human coronavirus (HCoV) is a member of the respiratory viruses that includes HCoV-229E, HCoV-OC43, and the severe acute respiratory syndrome (SARS) CoV (1). Recently, new types of HCoVs, such as NL63 and HKU-1, have also been described (1). Historically, HCoV research has been hampered by poor growth and lack of cytopathic effect in cell culture (1). The development of polymerase chain reaction (PCR) technology had allowed the field of coronavirology to develop widely and rapidly (1). However, according to the Infectious Agents Surveillance Report, only 54 and 59 HCoV-positive cases, including 18 cases from Niigata, were reported in Japan in 2010 and 2011, respectively (2). Although we have succeeded in isolating HCoV-229E viruses from children with nasopharyngitis using CaCo-2 cells in March 2008 and April 2010, we failed to isolate any further HCoVs thereafter (3). We therefore used reverse-transcription PCR (RT-PCR) methods in a screening analysis for HCoV in order to clarify the epidemiology of this virus in Niigata, Japan.

Between August 2010 and July 2011, 507 throat and nasal swab specimens were collected from patients with upper or lower acute respiratory infections at pediatric clinics working in collaboration with the Niigata Prefectural Health authorities as part of the national surveillance of viral diseases in Japan. Specimens were transported to the Virology Section of the Niigata Prefectural Institute of Public Health and Environmental Sciences for virus isolation. We were able to isolate respiratory viruses, including influenza virus, parainfluenza virus, RS virus, human metapneumovirus, rhinovirus, adenovirus, and enterovirus, from 376 specimens using 6 cell lines (MDCK, LLC-MK2, CaCo-2, HEp-2, Vero9013, and RD-18S). Therfore, we investigated the presence of HCoV in the 131 specimens from which no other respiratory virus was isolated.

Viral nucleic acid was extracted from the specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA), suspended in AVE buffer, and applied to RT reactions using a PrimeScript™ RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Then, we screened for the amplification of 4 HCoVs (HCoV-229E, HCoV-OC43, NL63, and HKU-1) by performing multiplex PCR using outer sense and antisense primers and by heminested PCR, using inner sense and outer antisense primers, as a previously reported method (4) with some modifications. Direct sequencing was used to determine the nucleic acid sequence of the 443-bp and 328-bp PCR products for identifying HCoV-OC43 and NL63, respectively. When the screening identified a HCoV-positive sample, we also amplified a portion of the spike glycoprotein region by performing PCR using our original primers to construct a phylogenetic tree. We prepared the following primer pairs: 1st PCR primers (OC-SP1F: 5′-ATGGTGGATAATGTTACTAGGCT-3′ and OC-SP1R: 5′-TAGTACCTGCAGGACAAGTG C-3′) and 2nd PCR primers (OC-SP2F: 5′-ATAATGT TACTAGGCTGCATGA-3′ and OC-SP2R: 5′-CAGG ACAAGTGCCTATACCA-3′) for HCoV-OC43 based on the reference strain (AY391777) and 1st PCR primers (NL-SP1F: 5′-TGAGTTTGATTAAGAGTGGTAGG-3′ and NL-SP1R: 5′-CAAACTGCAAGTGCTCACA C-3′) and 2nd PCR primers (NL-SP2F: 5′-GATTAA GAGTGGTAGGTTGTTG-3′ and NL-SP2R: 5′-GCT

*Corresponding author: Mailing address: Department of Microbiology, Yamagata Prefectural Institute of Public Health, Tokamachi 1-6-6, Yamagata, Yamagata 990-0031, Japan. Tel: +81-23-627-1373, Fax: +81-23-641-7486, E-mail: mizutak@pref.yamagata.jp

Table 1. Clinical characteristics of patients and detected human coronaviruses

Patient	Sex	Age[1]	Sampling date	Clinical condition and diagnosis	NL63 strain	GenBank accession no.	
						Spike glycoprotein	Nucloeocapsid protein
1	M	7 Y	1 Dec. 2010	Fever, URI[2]	HCoV-NL63/Niigata.JPN/10-1575	AB695183	AB695176
2	M	1 Y	13 Dec. 2010	Fever, bronchitis	HCoV-NL63/Niigata.JPN/10-1606	AB695184	AB695177
3	F	4 M	22 Dec. 2010	Fever, URI	HCoV-NL63/Niigata.JPN/10-1697	AB695185	AB695178
4	F	2 M	22 Dec. 2010	URI	HCoV-NL63/Niigata.JPN/10-1698	AB695186	AB695179
5	M	7 Y	27 Dec. 2010	Fever, URI	HCoV-NL63/Niigata.JPN/10-1708	AB695187	AB695180
6	M	8 M	4 Jan. 2011	Fever, otitis media	HCoV-NL63/Niigata.JPN/11-22	AB695188	AB695181
7	F	1 Y	19 Jan. 2011	Fever, URI	HCoV-NL63/Niigata.JPN/11-119	AB695189	AB695182

Patient	Sex	Age[1]	Sampling date	Clinical condition and diagnosis	OC43 strain	GenBank accession no.	
						Spike glycoprotein	Membrane and nucloeocapsid protein
8	M	1 Y	15 Feb. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11-286	AB695078	AB695068
9	F	2 Y	21 Feb. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11-335	AB695079	AB695069
10	M	2 Y	23 Feb. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11-343	AB695080	AB695070
11	M	2 M	28 Feb. 2011	URI	HCoV-OC43/Niigata.JPN/11-400	AB695081	AB695071
12	M	3 Y	29 Mar. 2011	Fever, URI, otitis media	HCoV-OC43/Niigata.JPN/11-564	AB695082	AB695072
13	F	3 Y	20 May 2011	Fever, URI, FC[3]	HCoV-OC43/Niigata.JPN/11-764	AB695083	AB695073
14	M	2 Y	25 May 2011	Fever, bronchitis	HCoV-OC43/Niigata.JPN/11-768	AB695084	AB695074
15	F	8 M	25 May 2011	Fever, bronchitis	HCoV-OC43/Niigata.JPN/11-769	AB695085	AB695075
16	M	9 Y	27 Jun. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11-833	AB695086	AB695076
17	F	1 Y	27 Jul. 2011	Fever, URI	HCoV-OC43/Niigata.JPN/11-981	AB695087	AB695077

[1]: Y, year old; M, month old.
[2]: URI, upper respiratory infection.
[3]: FC, febrile convulsion.

CACACTGCAACTTTTCA-3′) for NL63 based on the reference strain (AY518894). Sequence data for the spike glycoprotein from the HCoV-OC43 and NL63 strains were added to the DNA Data Bank of Japan under the accession numbers AB695078–AB695087 and AB695183–AB695189, respectively.

We detected NL63 in 7 and HCoV-OC43 in 10 of the 131 specimens (Table 1). Neither HCoV-229E nor HKU-1 was detected. The spike glycoprotein regions of all 17 NL63- or HCoV-OC43-positive specimens were successfully sequenced.

The age distribution of the HCoV-positive patients was between 2 months and 9 years (average, 2.3 years), and most of the patients (14/17; 82%) were less than 4 years old. Clinically, 15 patients presented with fever and 13 had upper respiratory infections.

We found 5 and 2 NL63-positive cases in December 2010 and January 2011, respectively. In 2011, we found 4, 1, 0, 3, 1, and 1 HCoV-OC43-positive patients in February, March, April, May, June, and July, respectively. Although HCoV infections are generally seen from midwinter to early spring, the seasonality varies from year to year (1). Interestingly, the NL63-positive cases were all observed in winter, whereas the HCoV-OC43-positive cases were observed later. According to Gaunt et al. (4) and Kaida et al. (5), the detection rate of HCoVs decreases in the following order: HCoV-OC43 > NL63 > HKU-1 > HCoV-229E. Although we presume that no patient was infected with HKU-1 or HCoV-229E during the study period, further investigation will be required to clarify the seasonality of HCoV infections in Japan.

Phylogenetic analysis indicated that the NL63 strains from patients No. 1–No. 6, detected between December 2010 and January 2011, were closely related to the 25222/2004/SWE strain (DQ231160). Only 1 strain, from patient No. 7, was closely related to the 41687/2007/SWE strain (FJ656160) (Fig. 1A). Four HCoV-OC43 strains, detected in February 2011, were closely related to the 89996 Belgium 2003 strain (AY903454), and 6 strains, detected between March and July 2011, were closely related to the 34364 Belgium 2004 strain (AY903455) (Fig. 1B). Interestingly, these findings suggest that the NL63 and HCoV-OC43 viruses changed over time. Similarly, Vijgen et al. reported that 2 genetically distinct HCoV-OC43 strains circulated in 2003 and 2004 in Belgium (6).

We attempted to isolate 16 of the 17 HCoV-positive specimens using the CaCo-2 cell line, with which we had previously isolated HCoV-229E (3), and the LLC-MK2 cell line, which is commonly used to isolate HCoV (7). Crystal trypsin (final concentration, 0.5 μg/ml) was added to the maintenance media and cells were passaged 5 times. However, we failed to observe any cytopathic effect or to amplify the HCoV genome. HCoVs tend to induce subtle cytopathic effects and many cell types are not susceptible (7). We noticed that HCoV-229E does not grow in the CaCo-2 cell line if trypsin is not present in the maintenance media (3); this finding was a milestone in our attempts to isolate one of the HCoVs. Further study will be required to elucidate the cell lines and the proper maintenance media necessary to isolate different HCoVs.

According to the national surveillance in Japan (2), HCoV detection has been reported from only 8 prefectures, including Niigata, Osaka City, and Mie (5).

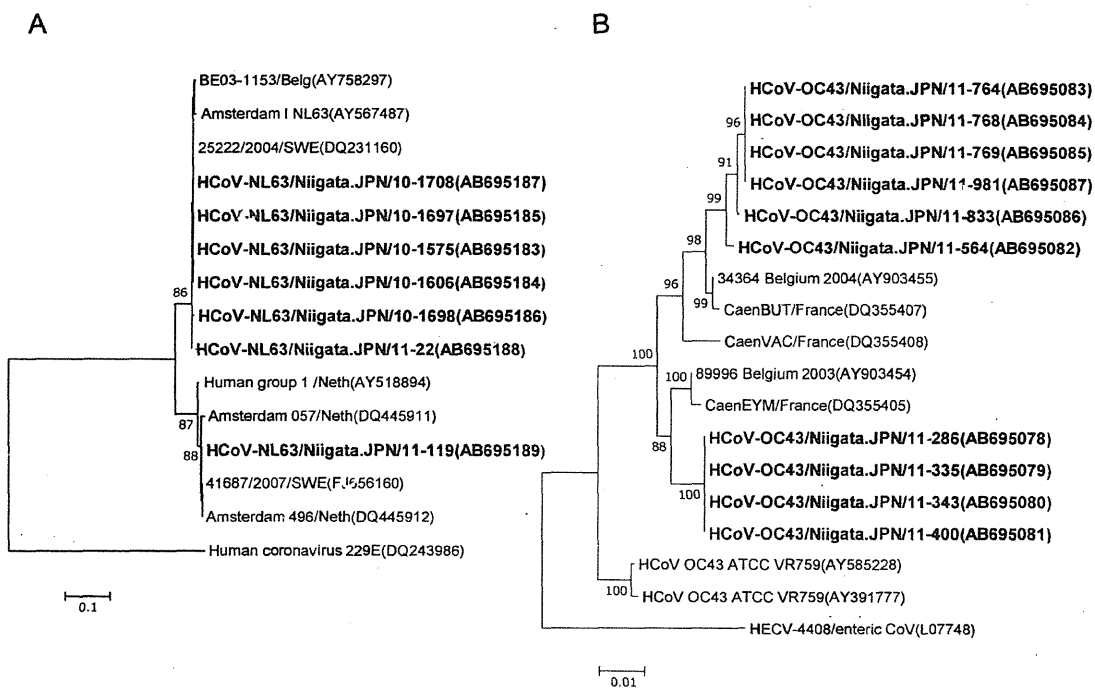

Fig. 1. Phylogenetic trees of the partial spike glycoprotein gene of the human coronavirus NL63 (A) and OC43 (B) strains. The trees of the NL63 (A) and HCoV-OC43 (B) strains were based on 576 bp and 1488 bp nucleotides, respectively. Evolutionary distance was calculated using the Maximum Composite Likelihood method, and the trees were plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. The scales of NL63 (A) and HCoV-OC43 (B) indicate 10% and 1% nucleotide differences, respectively. The present strains are represented in bold type.

Therefore, the epidemiology of HCoV in Japan is not well understood and HCoV surveillance may have been ineffective. In this study, we failed to isolate HCoV, but succeeded in detecting the HCoV genome in 13% of specimens (17/131) using RT-PCR. These findings suggest that the difficulties associated with HCoV isolation underscore the importance of HCoV as a causative agent of acute respiratory infections in the national surveillance. Together with parainfluenza virus type 4 and rhinovirus, HCoV is considered one of the most difficult respiratory viruses to identify, despite the fact that these viruses are responsible for a large proportion of viral respiratory tract infections (8). The use of molecular methods may assist in the study of HCoV epidemiology, and our primers designed for sequencing the HCoV-OC43 and NL63 spike glycoprotein gene will be useful for future molecular epidemiological analyses. This does not diminish the need to develop virus isolation technologies and to stock clinical isolates in public health laboratories in order to develop such viral disease control strategies as vaccine development and longitudinal epidemiological studies (8). To conclude, we should continue to clarify the etiology and epidemiology of HCoV infection using a combination of virus isolation techniques and molecular methods such as RT-PCR.

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Conflict of interest None to declare.

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