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Chimeric Feline Coronaviruses That Encode Type II Spike Protein on Type I Genetic Background Display Accelerated Viral Growth and Altered Receptor Usage▽

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Persistent infection of domestic cats with feline coronaviruses (FCoVs) can lead to a highly lethal, immunopathological disease termed feline infectious peritonitis (FIP). Interestingly, there are two serotypes, type I and type II FCoVs, that can cause both persistent infection and FIP, even though their main determinant of host cell tropism, the spike (S) protein, is of different phylogeny and displays limited sequence identity. In cell culture, however, there are apparent differences. Type II FCoVs can be propagated to high titers by employing feline aminopeptidase N (fAPN) as a cellular receptor, whereas the propagation of type I FCoVs is usually difficult, and the involvement of fAPN as a receptor is controversial. In this study we have analyzed the phenotypes of recombinant FCoVs that are based on the genetic background of type I FCoV strain Black but encode the type II FCoV strain 79-1146 S protein. Our data demonstrate that recombinant FCoVs expressing a type II FCoV S protein acquire the ability to efficiently use fAPN for host cell entry and corroborate the notion that type I FCoVs use another main host cell receptor. We also observed that recombinant FCoVs display a large-plaque phenotype and, unexpectedly, accelerated growth kinetics indistinguishable from that of type II FCoV strain 79-1146. Thus, the main phenotypic differences for type I and type II FCoVs in cell culture, namely, the growth kinetics and the efficient usage of fAPN as a cellular receptor, can be attributed solely to the FCoV S protein.

Coronaviruses are enveloped positive-stranded RNA viruses that infect a wide range of vertebrate species including livestock, companion animals, and humans. Coronaviruses are associated mainly with respiratory and enteric infections that can cause, in conjunction with systemic and/or persistent infection, severe diseases in animals and humans (17, 18, 32). Many coronaviruses have adopted a pronounced tropism for monocytic cells such as conventional dendritic cells (cDCs) and macrophages that can be attributed to particular receptor specificities of coronavirus surface (S) glycoproteins (1, 18, 22, 28, 33). On the cellular level, infection of macrophages results in a host cell transcriptional response that virtually lacks type I interferon (IFN) expression but is characterized mainly by a markedly increased level of expression of inflammatory cytokines (6, 18, 20, 25, 28). These inflammatory stimuli initiate and contribute to the pathogenesis of coronavirus-induced severe diseases. For example, overshooting inflammatory cytokine responses are associated with (i) mouse hepatitis virus-induced acute viral hepatitis in mice (6), (ii) lethal feline coronavirus (FCoV)-induced infectious peritonitis that is characterized by widespread granulomatous inflammatory lesions in multiple organs (8, 13), and (iii) severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV)-induced extensive tissue damage to the lung parenchyma of fatal SARS cases (16, 23, 28).

Feline infectious peritonitis (FIP) is a highly lethal, immunopathological disease accompanied by the development of vasculitis, anemia, neutrophilia, lymphopenia, and multifocal granulomatous inflammatory lesions in various organs (8). It is now widely accepted that the causative agent, feline infectious peritonitis virus (FIPV), represents a highly pathogenic FCoV mutant that arises during persistent, mostly benign FCoV infection. Notably, there are two serotypes of FCoVs, namely, type I and type II FCoVs, and both can infect wild and domestic *Felidae*. Both FCoV serotypes are genetically and antigenetically closely related to the group 1 coronaviruses canine coronavirus (CCoV) and porcine transmissible gastroenteritis virus (TGEV) (2, 8, 15). Furthermore, both FCoV serotypes have been associated with the development of pathogenic FIPV, although type I FCoV prevails in the field, with a seropositivity of 20 to 60% for domestic cats and up to 90% for animal shelters or multicat households. Consequently, type I FCoV-derived FIPVs are responsible for about 70 to 85% of FIP cases for domestic cats (14). Accumulating genetic evidence supports the idea that type II FCoVs have arisen by homologous recombination between type I FCoV and CCoV,

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and as a result of this recombination, the spike (S) gene and adjacent regions of type I FCoV were replaced by the corresponding region of the CCoV genome (2, 8, 9, 15). Genetic analyses of several type II FIPV isolates revealed that the 5′ and 3′ recombination sites for different type II isolates can vary slightly, strongly suggesting that these type II variants have originated from independent recombination events and that interspecies transmission of FCoV and/or CCoV may occur frequently (8, 9).

The coronavirus S protein is the main determinant of host and cell type specificity, and thus, it remains enigmatic how type I and type II FCoVs encoding S proteins of different phylogenies and limited sequence identity (<50%) can cause the same disease. It is accepted that type II FCoVs employ feline aminopeptidase N (fAPN) as a cellular receptor, and it was previously proposed that type I FCoV host cell entry is also facilitated, although inefficiently, by fAPN (29). However, it is important that only type II FCoV infection, but not type I FCoV infection, can be blocked by the fAPN-specific monoclonal antibody (MAb) R-G-4, a result which questioned whether type I FCoVs indeed use fAPN or another, yet-to-be-identified, cellular receptor (11). It is also noteworthy that in cell culture, type II FCoV isolates usually replicate reasonably well, whereas type I FCoV isolates grow less efficiently, if at all, and therefore, much of our knowledge of basic aspects of FCoV replication and pathogenesis is based on investigations of type II strains (8).

Recently, we have established a reverse genetic system based on type I FCoV strain Black (27). Here we report the generation of recombinant FCoVs encoding the type II FCoV S protein derived from highly virulent type II FCoV strain 79-1146 on the genetic background of type I FCoV strain Black. Our phenotypic analysis *in vitro* revealed that the 79-1146 S protein gave rise to a large-plaque phenotype, increased peak titers and, surprisingly, also accelerated replication kinetics similar to those of type II FCoV strain 79-1146. Furthermore, infections by recombinant type II S protein-expressing FCoV but not those of parental recombinant type I FCoV strain Black could be blocked by fAPN-specific MAb R-G-4 in feline FCWF-4 cells and $CD14^+$ feline monocytes.

MATERIALS AND METHODS

Viruses and cells. *Felis catus* whole-fetus 4 (FCWF-4) and baby hamster kidney (BHK-21) cells were originally purchased from the American Type Culture Collection and obtained from the diagnostic laboratory of our institute. Monkey kidney (CV-1) cells were purchased from the European Collection of Cell Cultures. D980R cells were a kind gift from G. L. Smith, Imperial College, London, United Kingdom. BHK-Tet/ON cells were a kind gift from N. Tautz, University of Lübeck, Germany. All cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). FCoV strain Black and FCoV strain 79-1146 were a kind gift from R. J. de Groot, University of Utrecht, The Netherlands. FCoV strain 79-1146, FCoV strain Black, and recombinant FCoVs were propagated on FCWF-4 cells. Recombinant vaccinia viruses were propagated, titrated, and purified as described previously (27).

Plasmid construction and generation of recombinant vaccinia viruses. In order to generate recombinant vaccinia viruses, plasmids pS1, pS2, pS2-GFP, and pSc based on pGem-T (Promega) were generated. Plasmid pS1 contains sequences corresponding to nucleotides (nt) 19890 to 20440 of the FCoV strain Black genome (GenBank accession number EU186072), followed by nt 20436 to 20960, corresponding to the FCoV strain 79-1146 genome (GenBank accession number AY994055). Plasmids pS2 and pS2-GFP contain sequences corresponding to nt 24303 to 24794 of FCoV strain 79-1146 followed by either nt 24810 to 25300, corresponding to the FCoV strain Black genome (pS2), or the sequence coding for green fluorescent protein (GFP) (pS2-GFP). Plasmid pSc contains the complete coding sequence of the FCoV strain 79-1146 S gene (nt 20436 to 24795). Plasmid pGPT-S1-2 is based on pGPT-1 (10), and fragments S1 and S2, derived from plasmids pS1 and pS2, respectively, were cloned downstream and upstream of the guanosine-phosphoribosyltransferase (GPT) gene encoded in pGPT-1 (10). Plasmid pGPT-S1-2-GFP is based on pGPT-1, and fragments S1 and S2-GFP, derived from plasmids pS1 and pS2-GFP, respectively, were cloned downstream and upstream of the GPT gene in pGPT-1.

For the generation of recombinant vaccinia viruses containing the type II FCoV strain 79-1146 S gene on the genetic background of type I FCoV strain Black, two previously described (27) vaccinia viruses (vrecFCoV and vrecFCoV-GFP) were used for vaccinia virus-mediated homologous recombination. Vaccinia virus vrecFCoV contains the full-length cDNA of type I FCoV strain Black, and vaccinia virus vrecFCoV-GFP contains type I FCoV strain Black cDNA where the accessory genes 3a to 3c were replaced by the GFP gene (27). Two steps of recombination using GPT positive selection first and GPT negative selection second were performed as described previously (7, 10, 27). To construct vaccinia virus vrecFCoV-SII, plasmid pGPT-S1-2 was used for vaccinia virus-mediated recombination with vrecFCoV. The recombinant GPT-containing vaccinia virus obtained after GPT positive selection was then used for vaccinia virus-mediated recombination with plasmid pSc to obtain recombinant vaccinia virus vrecFCoV-SII after GPT negative selection. To construct vaccinia virus vrecFCoV-GFP-SII, plasmid pGPT-S1-2-GFP was used for vaccinia virus-mediated recombination with vrecFCoV-GFP. The recombinant GPT-containing vaccinia virus obtained after GPT positive selection was then used for vaccinia virus-mediated recombination with plasmid pSc to obtain recombinant vaccinia virus vrecFCoV-GFP-SII after GPT negative selection. The sequences of all generated plasmid DNAs and the recombined regions within the recombinant vaccinia viruses were verified at the nucleotide level by standard sequencing analysis.

Recovery of recombinant FCoV. To recover recombinant FCoVs, vaccinia virus DNA derived from vrecFCoV-GFP, vrecFCoV-SII, or vrecFCoV-GFP-SII was prepared, cleaved with ClaI, and used as a template for *in vitro* transcription, as described previously (7, 27). Ten micrograms of *in vitro*-transcribed RNA was electroporated into BHK cells, which express the type I FCoV strain Black nucleocapsid protein (BHK-FCoV-N), as described previously (7, 27). The electroporated cells were cocultivated with FCWF-4 cells, and after 48 h of incubation, the supernatant containing recombinant FCoVs was harvested for further analysis. The identity of recombinant FCoVs was verified by reverse transcription (RT)-PCR and sequencing analysis.

Generation of BHK-fAPN cells. For the generation of BHK-21 cells stably expressing feline aminopeptidase N (fAPN), the fAPN gene was amplified from FCWF-4 cells by RT-PCR. The resulting PCR product was cloned into plasmid E88A10 (kindly provided by D. Wentworth, University of Albany and Wadsworth Center, Albany, NY) (34) to replace the human APN gene encoded in E88A10 using XhoI and SacII cleavage sites. The resulting plasmid, designated pCMV-fAPN, encodes the fAPN gene under the control of the cytomegalovirus (CMV) promoter. To generate the fAPN-expressing cell line BHK-fAPN, plasmid pCMV-fAPN was transfected into BHK-21 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, and 48 h after transfection, the cells were placed under G418 (Invitrogen) selection. G418-resistant colonies were expanded and analyzed for fAPN expression by immunofluorescence microscopy using MAb R-G-4.

IFA and flow cytometry. An immunofluorescence assay (IFA) was used to detect fAPN expression on the cell surface. Cells were fixed with 2% paraformaldehyde and incubated with hybridoma cell culture supernatant containing MAb R-G-4 (kindly provided by T. Hohdatsu, Kitasato University, Japan). Incubation with R-G-4 MAb was followed by incubation with secondary antibody Cy3 goat anti-mouse (1:500) (Jackson ImmunoResearch Laboratories). Immunolabeled cells were analyzed by using a Leica DM IL fluorescence microscope (Leica Microsystems). Flow cytometry analysis of GFP expression in FCWF-4 cells was done by using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). To assess fAPN surface expression, FCWF cells or feline monocytes were detached from the culture dish by using ice-cold 2 mM EDTA–phosphate-buffered saline (PBS). Cells were incubated at 4°C with supernatant of the hybridoma cell line containing MAb R-G-4, washed twice with PBS–5% fetal calf serum (FCS)–2 mM EDTA, and subsequently stained with a fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig antibody (Chemicon, Australia) as a secondary reagent. After three washing steps, the cells were acquired with a FACS Canto II flow cytometer (BD Biosciences, Allschwil, Switzerland), and data were analyzed with FlowJo 7.2.5 (Treestar, Ashland, OH).

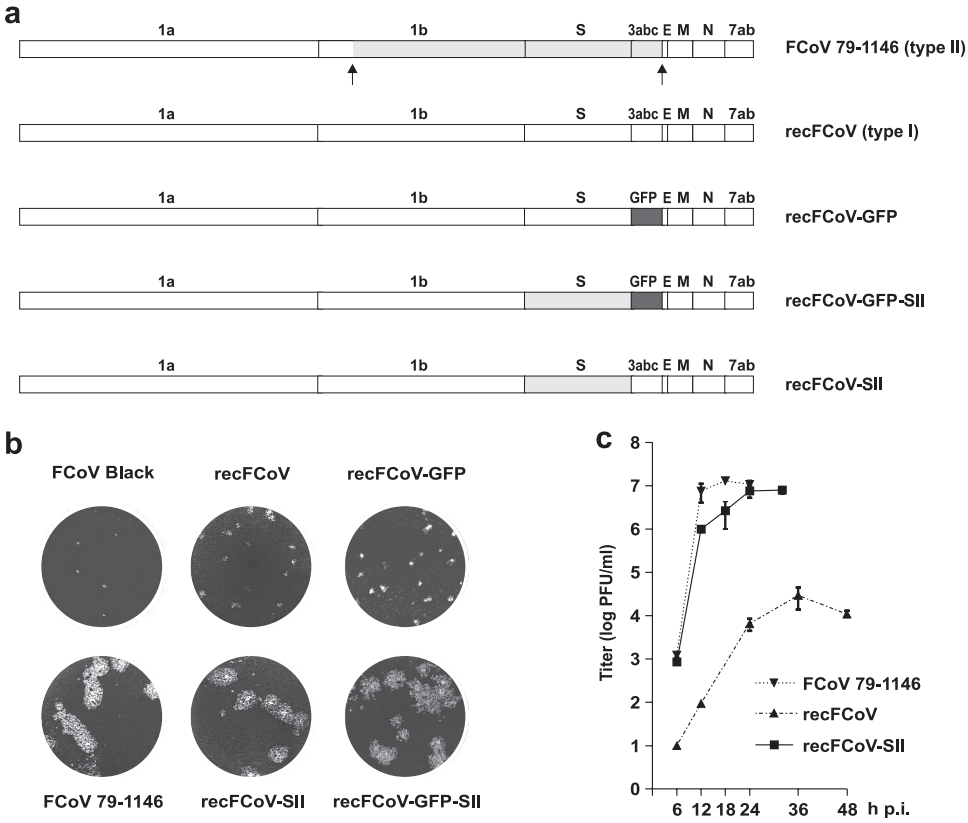

FIG. 1. Generation of recombinant FCoVs and phenotypic analysis using FCWF-4 cells. (a) Schematic representation of the genome organization of type II FCoV strain 79-1146, recombinant type I FCoVs recFCoV and recFCoV-GFP, and recombinant chimeric FCoVs recFCoV-GFP-SII and recFCoV-SII. CCoV-derived sequences in the type II FCoV strain 79-1146 genome are indicated in gray, and approximate recombination sites are depicted by arrows. Type I FCoV strain Black-derived sequences are indicated in white, and GFP is indicated in black. Sequences derived from type II FCoV strain 79-1146 in the chimeric viruses recFCoV-GFP-SII and recFCoV-SII are indicated in gray. (b) Plaque morphology of FCoV strain Black, recFCoV, recFCoV-GFP, FCoV strain 79-1146, recFCoV-SII, and recFCoV-GFP-SII 48 h after infection of FCWF-4 cells. (c) Growth kinetics of recFCoV, recFCoV-SII, and FCoV strain 79-1146 after infection of FCWF-4 cells (MOI = 0.1).

Blockade of FCoV infection with MAb R-G-4. The blockade of fAPN-mediated FCoV infection of BHK-fAPN, FCWF-4, or $CD14^+$ feline monocytes was done in a 6-well format by incubating the cells for 30 min at 37°C with 1 to 50 μl/well of hybridoma cell culture supernatant containing MAb R-G-4. Following incubation, the cells were washed with PBS and used for further analysis.

Isolation of blood monocytes. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Paque separation from 15 ml heparinized blood obtained from four specific-pathogen-free (SPF) cats. Anti-human CD14 magnetic beads (Miltenyi Biotec) were used to obtain $CD14^+$ feline monocytes as described previously (27).

RESULTS

Generation of recombinant type I FCoV expressing the type II FCoV spike protein. In order to replace the type I FCoV strain Black S gene by the type II FCoV strain 79-1146 S gene, we used our recently developed reverse genetic system that is based on the full-length cDNA of type I FCoV-Black cDNA cloned into vaccinia virus (27). Vaccinia virus vrecFCoV, containing FCoV strain Black cDNA, was modified by two rounds of vaccinia virus-mediated homologous recombination to exactly replace the type I FCoV strain Black S gene with the type II FCoV strain 79-1146 S gene. Similarly, we modified GFP-encoding recombinant vaccinia virus vrecFCoV-GFP (that contains the GFP gene instead of the FCoV genes 3a to c) to replace the type I FCoV strain Black S gene with the type II FCoV strain 79-1146 S gene. The resulting recombinant vaccinia viruses vrecFCoV-SII and vrecFCoV-GFP-SII were used to rescue the recombinant FCoVs, designated recFCoV-SII and recFCoV-GFP-SII, respectively. The structural relationship of the type I, type II, and chimeric type I/type II FCoVs used in this study is shown in Fig. 1a.

The type II spike protein determines large plaque size and accelerated replication kinetics of FCoV. After recovery of the recombinant FCoVs, we immediately noticed a phenotypic shift of recFCoV-SII and recFCoV-GFP-SII toward type II FCoV strain 79-1146 in cell culture. First, we noticed that upon passaging, a full cytopathic effect (CPE) became apparent within 16 to 24 h postinfection (p.i.) of FCWF-4 cells with recFCoV-SII and recFCoV-GFP-SII, whereas FCoVs encoding the type I S protein induced full CPE only after 48 h p.i. To analyze the phenotype of type II S protein-encoding FCoVs in more detail, we performed a plaque assay with FCWF-4 cells. As shown in Fig. 1b, the large-plaque phenotype of recFCoV-SII and recFCoV-GFP-SII was indistinguishable from that of type II FCoV strain 79-1146. In contrast, infection of FCoVs encoding the type I S protein gave rise to tiny plaques, and notably, those plaques were visible only at 48 h p.i., again in contrast to the large plaques of type II S protein-encoding viruses, which were already visible at 24 h p.i. Second, we

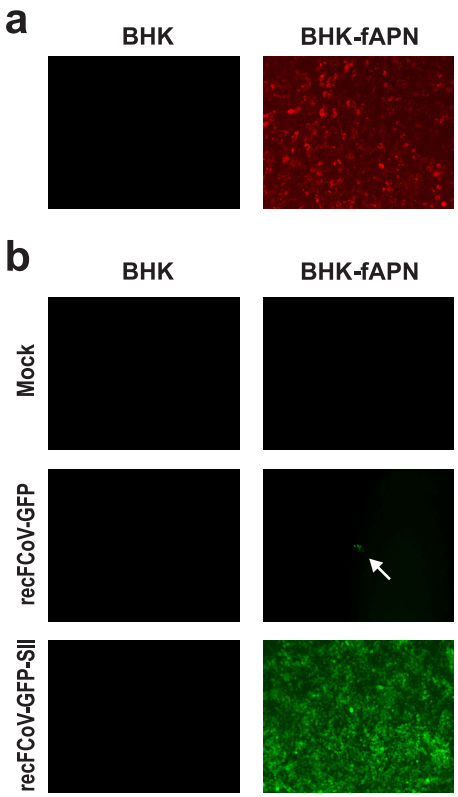

FIG. 2. Generation and infection of fAPN-expressing BHK-21 cells. (a) Immunofluorescence analysis of BHK-21 (left) and BHK-fAPN (right) cells. (b) GFP expression analyzed by fluorescence microscopy of mock-, recFCoV-GFP-, or recFCoV-GFP-SII-infected BHK-21 or BHK-fAPN cells at 36 h p.i. (MOI = 0.1). The white arrow depicts patches of green fluorescent BHK-fAPN cells after recFCoV-GFP infection.

analyzed the growth kinetics of recFCoV-SII on FCWF-4 cells after infection with a multiplicity of infection (MOI) of 0.1. As shown in Fig. 1c, recFCoV-SII replication on FCWF-4 cells, as determined by titration of cell culture supernatant at various time points p.i., was similar to that of type II FCoV strain 79-1146, with peak titers of up to 10^7 PFU/ml already at 24 h p.i. As described above, recFCoV reached much lower peak titers of 5×10^4 PFU/ml only after 36 h p.i. Taken together, the integration of the type II FCoV strain 79-1146 S gene into the genetic background of type I FCoV strain Black greatly accelerated virus replication kinetics, increased peak titers by about 2.5 orders of magnitude, and gave rise to a large-plaque phenotype indistinguishable from that of type II FCoV strain 79-1146.

FCoV type II spike confers entry into fAPN-expressing cells. We were also interested to know to which extent fAPN facilitates host cell entry of FCoVs expressing either the type I or the type II S protein. To address this question we first produced an expression plasmid containing the fAPN gene [amplified by using poly(A) RNA from FCWF-4 cells as an RT-PCR template] and used this construct to establish a BHK-21-derived cell line, designated BHK-fAPN, that stably expresses fAPN. As shown in Fig. 2a, fAPN expression on BHK-fAPN cells was readily detectable by IFA using fAPN-specific MAb R-G-4. Next, we infected parental BHK-21 and fAPN-expressing BHK-fAPN cells with the GFP-expressing viruses recFCoV-GFP and recFCoV-GFP-SII. As shown in Fig. 2b, we could confirm that BHK-21 cells are not susceptible to infection with either virus. However, BHK-fAPN cells were efficiently infected by type II S protein-expressing recFCoV-GFP-SII, as shown by the abundant green fluorescence throughout the culture. When we studied infection of BHK-fAPN cells with type I S protein-expressing recFCoV-GFP, we repeatedly detected only very few green fluorescent cells, suggesting that fAPN may indeed enable the host cell entry of recFCoV-GFP albeit inefficiently. This observation agrees well with results from a previous study in which infection of fAPN-expressing BHK-21 cells with type I FCoV strain UCD-1 was observed with low efficiency (29).

Blockade of fAPN in BHK-fAPN and FCWF-4 cells. MAb R-G-4 was previously described to block fAPN-mediated infection of type II FCoVs but not to inhibit infection by various type I FCoVs, including FCoV strain Black (11). Therefore, we analyzed whether MAb R-G-4 can block the infection of a recombinant virus that is based on a type I genetic background but encodes a type II S protein. As shown in Fig. 3a, the infection of FCWF-4 cells with recFCoV-GFP-SII (MOI = 0.1) was efficiently blocked by MAb R-G-4 in a dose-dependent manner. In contrast, the infection of FCWF-4 cell with recFCoV-GFP could not be blocked by MAb R-G-4, even at the highest antibody concentration used in this experiment. To further corroborate this result, we quantified the number of green fluorescent FCWF-4 cells after recFCoV-GFP or recFCoV-GFP-SII infection by flow cytometry. Again, increasing MAb R-G-4 concentrations led to decreasing numbers of GFP-expressing cells following infection with recFCoV-GFP-SII, whereas the number of GFP-expressing cells following recFCoV-GFP infection remained the same independently of the concentration of MAb R-G-4 used (Fig. 3b). Finally, we infected BHK-fAPN cells with recFCoV-GFP and again observed very few green fluorescent cells even in the presence of high MAb R-G-4 concentrations (Fig. 3c). Thus, we conclude that fAPN-specific MAb R-G-4 that was previously shown to block type II FCoVs but not type I FCoVs is also able to block a recombinant FCoV that is based on a type I genetic background but expresses the type II S protein from FCoV strain 79-1146.

Effect of fAPN blockade on FCoV infection of feline monocytes. To investigate whether the above-described differences in receptor usage between recFCoV-GFP and recFCoV-GFP-SII are also apparent in natural target cells of FCoV, $CD14^+$ feline monocytes were prepared as described previously (27). These monocytes were treated with MAb R-G-4 or left untreated and were then infected with GFP-expressing recombinant FCoVs (MOI = 0.1). We first tested recFCoV-GFP and FCoV-GFP-SII infection of $CD14^+$ feline monocytes derived from four different donors without MAb R-G-4 treatment. As described previously (27), recFCoV-GFP infection gave rise to only very few green fluorescent $CD14^+$ feline monocytes (Fig. 4a) irrespective of from which donor the cells were derived. When we infected $CD14^+$ feline monocytes derived from the same donors with recFCoV-GFP-SII, we detected only very few green fluorescent cells for $CD14^+$ feline monocytes derived from three donors, similar to recFCoV-GFP infection (data not shown). A significantly increased number of green fluorescent $CD14^+$ feline monocytes was observed for only one donor (cat 7) (Fig. 4a). In order to determine whether fAPN

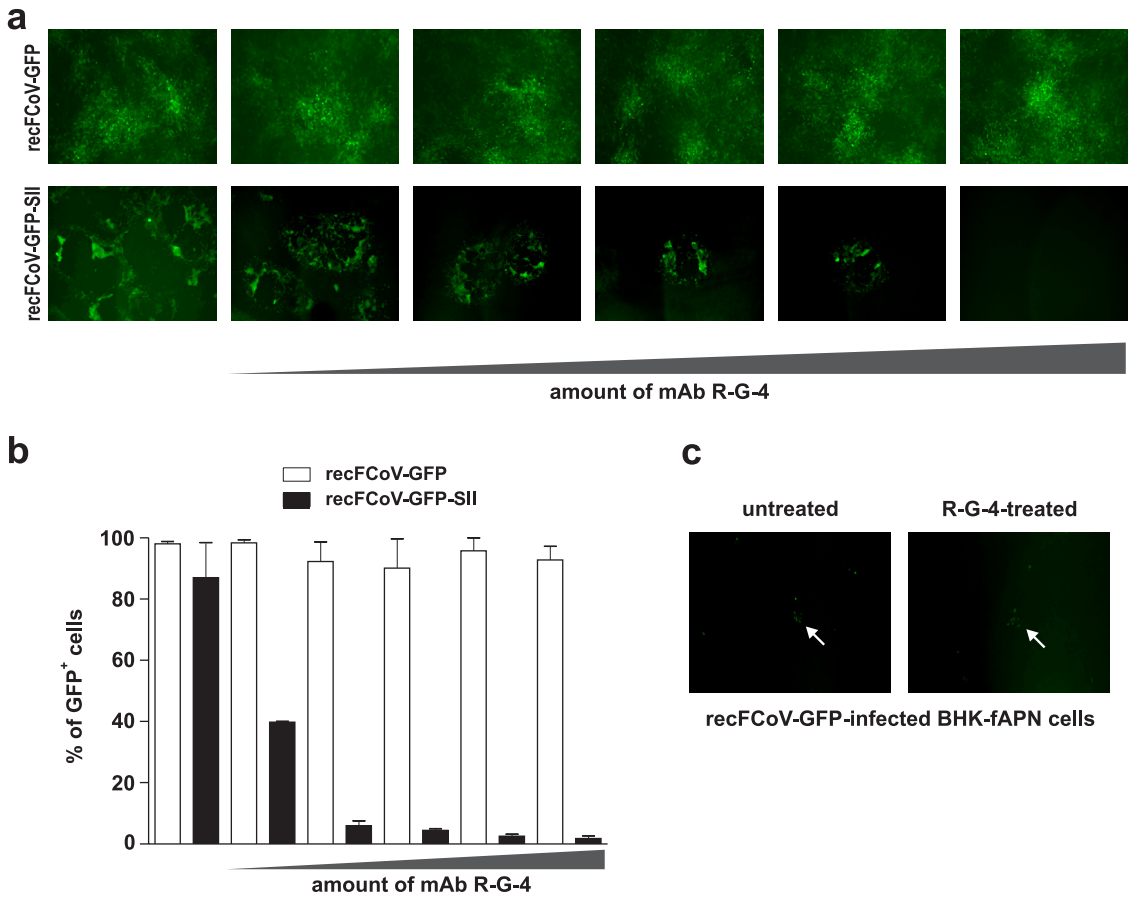

FIG. 3. Effect of MAb R-G-4 treatment on FCoV infection of FCWF-4 and BHK-fAPN cells. (a) Analysis of GFP expression of recFCoV-GFP (top row)- or recFCoV-GFP-SII (bottom row)-infected FCWF-4 cells by fluorescence microscopy at 36 h p.i. (MOI = 0.1). The leftmost panels show infected FCWF-4 cells without MAb R-G-4 treatment, and the following panels from left to right show FCWF-4 cells that have been treated with increasing amounts of MAb R-G-4 (1, 3, 5, 10, and 50 μl/per well). (b) Fluorescence-activated cell sorter (FACS) analysis of GFP-expressing FCWF-4 cells at 36 h p.i. with recFCoV-GFP (white bars) or recFCoV-GFP-SII (black bars) (MOI = 0.1). Cells were left untreated or were treated with MAb R-G-4 as described above (a). (c) GFP expression analyzed by fluorescence microscopy of recFCoV-GFP-infected BHK-fAPN cells at 36 h p.i. (MOI = 0.1). Cells were either left untreated or were treated with MAb R-G-4 (50 μl/per well). The white arrow depicts patches of green fluorescent cells that were also detectable in the presence of MAb R-G-4.

surface expression differs, we analyzed feline monocytes from different donors by flow cytometry using fAPN-specific MAb R-G-4. As shown in Fig. 4b, fAPN expression was readily detectable on FCWF cells but not on feline monocytes. Thus, it remains elusive whether fAPN is expressed only on very few $CD14^+$ feline monocytes and whether there is a difference among donors. Nevertheless, MAb R-G-4 treatment of such $CD14^+$ feline monocytes before infection resulted in a markedly decreased number of green fluorescent cells after recFCoV-GFP-SII infection, whereas no difference was observed after recFCoV-GFP infection. Thus, fAPN-specific MAb R-G-4 did block recFCoV-GFP-II but not recFCoV-GFP infection of primary feline $CD14^+$ monocytes. In addition, we analyzed viral titers from supernatants of recFCoV-GFP- and recFCoV-GFP-SII-infected feline $CD14^+$ monocytes from two representative donors (MOI = 0.1). As shown in Fig. 4c, recFCoV-GFP infection of feline $CD14^+$ monocytes derived from donor 4 or donor 7 was not productive with or without MAb R-G-4 treatment, and viral titers were below the detection limit at 36 h p.i. However, after recFCoV-GFP-SII infection of feline $CD14^+$ monocytes, we observed different outcomes. First, when feline $CD14^+$ monocytes derived from donor 4 were infected without MAb R-G-4 treatment, we detected constant but low titers until 36 h p.i., followed by a drop below the detection limit at 48 h p.i. R-G-4 treatment resulted in slightly reduced titers and a drop below the detection limit already at 36 h p.i. This phenotype is representative of feline $CD14^+$ monocytes derived from the three donors that showed only very few green fluorescent cells by immunofluorescence analysis. Second, when feline $CD14^+$ monocytes derived from donor 7 were infected, we detected productive infection with peak titers of 10^4 PFU/ml at 36 to 48 h p.i. Notably, this productive infection could be blocked by MAb R-G-4 treatment (Fig. 4c).

DISCUSSION

Although type I and type II FCoVs are both known to cause persistent infection in cats, which can, upon selection of pathogenic FIPV variants, culminate in lethal FIP, they are also known to display substantial phenotypic differences in cell cul-

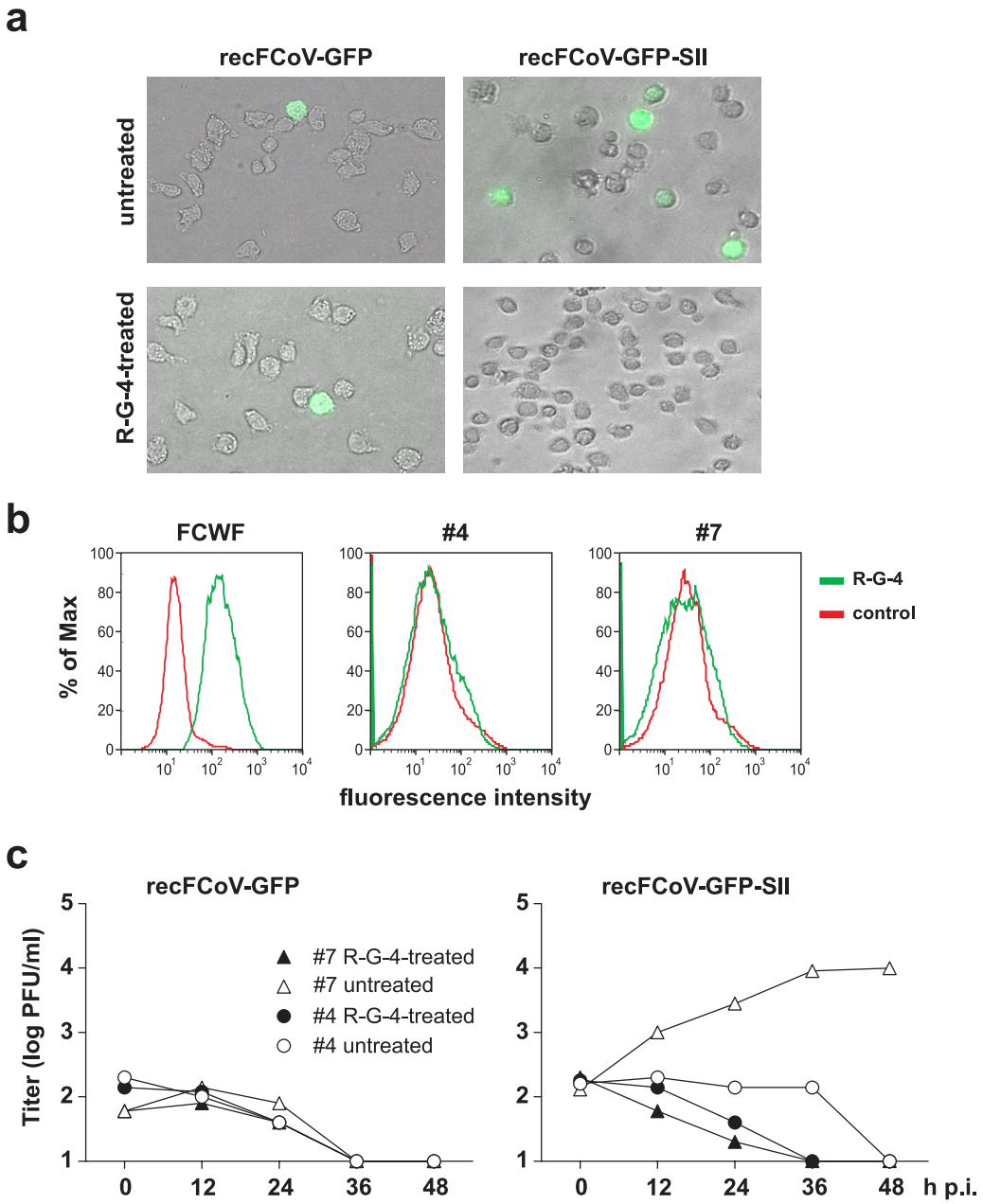

FIG. 4. Effect of MAb R-G-4 treatment on FCoV infection of CD14$^+$ feline monocytes. (a) Detection of GFP expression in untreated or MAb R-G-4-treated (50 μl/per well) CD14$^+$ feline monocytes derived from donor 7 after infection with recFCoV-GFP or recFCoV-GFP-SII (MOI = 0.1) at 36 h p.i. (b) fAPN surface expression by FACS was assessed for FCWF cells and monocytes of donors 4 and 7 as indicated. Cells were incubated with MAb R-G-4, followed by a secondary FITC-labeled goat anti-mouse Ig antibody (green lines) or incubated with the FITC-labeled goat anti-mouse Ig antibody alone (red lines) (control). (c) Growth kinetics of recFCoV-GFP or recFCoV-GFP-SII after infection of untreated or MAb R-G-4-treated (50 μl/per well) CD14$^+$ feline monocytes derived from donor 4 or 7 as indicated (MOI = 0.1).

ture. Here we have used a reverse genetic approach based on cloned type I FCoV strain Black to dissect the impact of FCoV S proteins on FCoV receptor usage and growth properties in cell culture. Based on our data we can draw two main conclusions. First, the FCoV type II spike protein is the main determinant for efficient fAPN-mediated host cell entry, whereas the FCoV type I S protein can utilize fAPN only very inefficiently. This result corroborates data from previous studies that proposed that type I FCoVs use an alternative host cell receptor(s) (5, 11). However, it also refines data from those previous studies, since our conclusions are based on chimeric FCoVs that express a type II S protein on the genetic background of a type I FCoV, and thus, the phenotypic differences concerning receptor usage can be attributed solely to the S protein. Second, and quite unexpectedly, our data revealed that the FCoV S protein is also a major determinant of FCoV replication kinetics in cell culture.

The latter conclusion is very important because a major

obstacle to the study of type I FCoVs is their poor growth in cell culture. According to our data, type I FCoVs are apparently capable of replicating efficiently in cell culture once they encode an appropriate spike protein. Therefore, we can exclude that differences within regions that had recombined to give rise to type II FCoV genotypes, i.e., the replicase gene region encoding nsp12 to nsp16 and the accessory genes 3a, 3b, and 3c, account for the poor growth of type I FCoVs in cell culture. Accordingly, it should be feasible in future studies to generate a panel of chimeric viruses based on cloned type I FCoV strain Black encoding spike proteins derived from known type I and type II FCoVs in order to analyze whether growth kinetics of such chimeric viruses correlate strictly with the corresponding parental FCoVs in cell culture. It is even conceivable to generate chimeric viruses encoding S proteins derived from FCoV field isolates that are difficult to propagate in cell culture and for which only S gene sequences are available. Thus, we expect that the reverse genetic approach presented in this study will facilitate the establishment of an improved cell culture system for type I FCoVs and will furthermore allow the analysis of FCoV S protein function in the context of virus infection.

Clearly, the analysis of recombinant FCoV S gene chimeras and the elucidation of optimal growth conditions for type I FCoVs in cell culture are closely linked to the question of FCoV receptor usage. We show here that recombinant FCoVs based on a type I genetic background can efficiently use fAPN as a receptor if they express the type II FCoV strain 79-1146 S protein. Notably, fAPN can also serve as a functional receptor for other group I coronaviruses, such as the closely related CCoV and TGEV, but also for the more distantly related human coronavirus 229E (HCoV-229E) (29, 33). However, conversely, human APN and porcine APN cannot be utilized by FCoVs for host cell entry. Detailed analyses by Tusell and colleagues (30) using chimeric feline and murine APN molecules revealed that short stretches of amino acids within the fAPN molecule (amino acids [aa] 732 to 746 and aa 761 to 788), and probably posttranslational modifications within these regions, are critical for FCoV (type II), CCoV, and TGEV infection, whereas another short stretch within fAPN (aa 288 to 290) is critical for HCoV-229E infection. Notably, MAb R-G-4, which was previously shown to bind within aa 251 to 582 of fAPN (30), can block fAPN-dependent infection of these viruses, suggesting that the different regions that are critically involved in HCoV-229E, CCoV, TGEV, and FCoV infection lie close together on the fAPN surface. Since MAb R-G-4 cannot block infection by type I FCoV (e.g., recFCoV-GFP in this study), we and others proposed that type I FCoVs use another main receptor for host cell entry (5, 11). It should be kept in mind, however, that we repeatedly observed a small number of green fluorescent cells following infection of BHK-fAPN cells with type I recFCoV-GFP, whereas no green fluorescent cells were detected after infection of the parental BHK-21 cell line. This result concurs with those for fAPN-expressing BHK-21 cells that were infected with type I FCoV strain UCD (29) and suggests that fAPN can serve as a receptor for type I FCoV host cell entry although very inefficiently. Since this residual susceptibility of fAPN-BHK cells to type I FCoV infection cannot be blocked by the fAPN-specific antibody R-G-4, it is possible that type I FCoV binding to fAPN occurs at a different region on the fAPN molecule compared to that for the fAPN binding of type II FCoV, HCoV-229E, CCoV, and TGEV. Alternatively, fAPN may represent a coreceptor that facilitates type I FCoV binding or entry. Interestingly, we observed that the human hepatoma Huh7 cell line is susceptible to recFCoV-GFP infection, with about 90% of green fluorescent cells at 48 to 72 h p.i. (B. Bank-Wolf and H.-J. Thiel, unpublished data), although it was demonstrated that human APN is not a functional receptor for FCoV infection. To add yet another puzzling detail, about 10% of Huh7 cells displayed green fluorescence following FCoV-GFP-SII infection (Bank-Wolf and Thiel, unpublished). Thus, the question of FCoV receptor usage appears to be more complex than initially thought since type I and type II FCoVs apparently can use a receptor(s) for host cell entry that is not restricted to feline cells (21).

The use of recombinant GFP-expressing FCoVs also greatly facilitates the analysis of feline $CD14^+$ monocyte infection (27), and the data presented in this study corroborate phenotypic differences concerning type I and type II FCoV receptor usages in these primary cells. It was previously suggested that the ability to efficiently infect macrophages and monocytes might be a distinguishable factor between highly pathogenic (i.e., FIP-causing) and less pathogenic FCoVs (4, 22, 24). Since this observation is based mainly on analyses of type II FCoV variants, it remains to be determined if this correlation also holds true for virulent versus avirulent type I FCoVs. The spike genes used in this study are both derived from FIP isolates, suggesting that, as part of the pathogenic potential of a given virus, the phylogeny of the spike protein (type I versus type II spike proteins) might impact the ability of FCoVs to infect feline monocytes. However, it is too early to draw any general conclusion, and therefore, also in this context, future analyses using a panel of recombinant S gene chimeric viruses are highly desirable. Our data also revealed that the host genetic background is of importance with regard to the susceptibility of feline monocytes to FCoV infection. We observed that monocytes derived from donor 7 were repeatedly most susceptible to recFCoV-GFP-SII infection, whereas monocytes from the three other donors were less susceptible. Our analysis of surface fAPN expression by flow cytometry revealed that, if at all, only a small number of feline monocytes express fAPN, and no differences between monocytes derived from donor 7 and those derived from other donors were detectable. Interestingly, Takano and colleagues previously reported a similar small number of feline macrophages expressing fAPN (26). These macrophages displayed a substantial upregulation of fAPN after incubation with the inflammatory cytokine TNF-α, suggesting that the susceptibility of feline macrophages to FCoV type II infection increases under inflammatory conditions. Differences in the susceptibilities of feline monocytes and macrophages to FCoV infection have also been described by several other reports, and it is known that upon experimental infection of cats with type II FIPV, some animals may not show any clinical signs or may even remain seronegative (3, 4, 12, 13, 19, 31). Therefore, it would be most interesting to study whether the susceptibility of monocytic cells to FCoV infection is related solely to efficient receptor usage or if other host cell factors play a role.

In conclusion, we have shown that the FCoV S protein is of

major importance with regard to receptor usage and viral growth kinetics. The knowledge that recombinant viruses based on a type I FCoV background can replicate to high titers in cell culture if they express an appropriate S protein raises the expectation that the elaboration of improved cell culture conditions for the efficient propagation of type I FCoV isolates and the identification of additional FCoV host cell receptors may be achievable in the near future. Furthermore, the reverse genetic approach to generate chimeric type I/type II FCoVs will also facilitate studies of FCoV target cell tropism and important virus-host interactions and will eventually help us to understand how type I and type II FCoVs cause the same disease in the natural host.

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