世界アレナウイルスも，クラスリン依存的エンドサイトーシスによって細胞内に侵入する[21]．更に，侵入後に細胞内小胞輸送を司るRabファミリー分子の依存性についても解析され，TfR1を利用するアレナウイルスはRab5およびRab7に依存的に細胞侵入するのに対し，LCMVやラッサウイルスはこれらの分子に非依存的に細胞侵入することが明らかにされている[21]．

アレナウイルスの膜融合に関しては，低pH環境下においてGP2が構造変換を起こすことで誘導される．GP2は，レトロウイルスやパラミクソウイルスなどのウイルスエンベロープ蛋白質と同様のクラスⅠに分類される膜融合蛋白質である．またアレナウイルスに共通した特徴として，ウイルス間で保存された長い58アミノ酸からなるシグナルペプチドがある．このシグナルペプチド（stable signal peptide; SSP）は，N末端のグリシンがミリスチン化されていて2箇所の疎水性領域を持つ[15]．さらにSSPは，GP1，GP2と共にエンベロープ蛋白質の一部として構成される構造蛋白でもあり，N末端側の疎水性領域直後の細胞外領域にある保存された33位のリジンがGP2の膜融合に重要な役割を果たしている．GP2-SSPの特徴的な膜融合における作用をターゲットとした低分子阻害薬の開発も進められており，アレナウイルスの治療薬としても期待されている[15,36]．

アレナウイルスのリバースジェネティクス

約30年前にポリオウイルスのリバースジェネティクスが確立されて以降，プラス鎖，マイナス鎖を問わず多くのウイルス種でリバースジェネティクスが確立されてきた．リバースジェネティクスが確立できると，そのウイルス種の細胞レベル，分子レベルでの生活環を解析できるだけでなく，病原性の解明や治療薬の開発に至るまで非常に有用性の高いツールとして応用できる．アレナウイルスにおいても，2000年にLCMVのミニゲノムシステムが開発され，続けてラッサウイルス，ピチンデウイルス，タカリベウイルス，フニンウイルスで確立されている[37]．その後，完全長のクローンからの感染性ウイルスの回収もLCMV，ピチンデウイルス，フニンウイルスで可能となっている[37]．最近，ラッサウイルスでもcDNAから感染性ウイルスを回収することに成功し，様々な非翻訳領域の変異体を用いてウイルス複製や病原性の影響を検証することに応用されている[38]．またミニゲノムを利用して，エンベロープ蛋白質を他種のウイルスに置き換えたキメラウイルスも作製されており，ワクチン候補としての研究や，ラッサウイルス等BSL4のウイルスを本来のウイルスに近い形で比較的安全かつ簡便に取り扱えるために，様々な解析に利用されている[39,40,41]．ごく最近，2008年に新規に同定されたルジョウイルスのリバースジェネティクスも確立され，今後ルジョウイルスの基礎研究も進展すると思われる[42]．

アレナウイルスの実験室診断

出血熱の原因となるアレナウイルスは，BSL4研究施設でのみ扱うことの可能な病原体であるため，ウイルス分離検査や抗体検出用ウイルス抗原の調整にはBSL4研究施設が必要である．こうした環境下では，血液，咽頭スワブ，尿サンプルなどからVero細胞株や乳飲み仔マウス脳内接種等によりウイルスを分離できる．アレナウイルスは，通常Vero細胞などでは細胞変性効果を示さないので，特異抗体を用いてウイルスを同定する．感染病理学的検査によりウイルス抗原を検出することも可能となる[43]．

我が国では現在まで，国立感染症研究所にある高度封じ込め研究施設をBSL4として稼働していないために，BSL4に分類されるラッサウイルスなどは培養できない．そのため我々は，RT-PCR法によるウイルスRNAの検出の他，株間での抗原性の交差反応性が最も高いと考えられているNPの組換え蛋白質を用いたウイルスの抗体検出法，組換えNPに対するモノクローナル抗体を用いた抗原検出ELISAなどを開発している．現在までに，ラッサウイルス[5]およびフニンウイルス[6]については，検査，診断体制が整えられており，他のアレナウイルス種においても現在取り組まれている．また，近年開発されたレトロウイルスやVSVを基盤としたシュードタイプウイルスも，感染患者血清中に存在するウイルスに対する中和抗体価を測定するのに有用なツールとなっている．これら基盤となるウイルスには緑色蛍光蛋白質やルシフェラーゼ，分泌型ヒトアルカリフォスファターゼなどのマーカー遺伝子が搭載され，シュードタイプウイルスの感染性を簡便に評価することができる．我々も既に各種アレナウイルスのエンベロープ蛋白質を外套し，感染性を保持したシュードタイプVSVを作製しており，感染疑い患者血清の中和抗体の測定に応用している．最近までの我々のアレナウイルスに関する実験室診断法については，より詳細に記した最新の総説[7]を参考にしていただきたい．

ワクチン開発

ワクチン開発に関しては，患者数が多いラッサ熱とアルゼンチン出血熱において積極的に研究が進められている[44,45]．それぞれの原因ウイルスであるラッサウイルスとフニンウイルスは，共に再感染による重篤化が認められていないこともあり，一度感染し回復すれば防御免疫が持続すると考えられる[46]．アルゼンチン出血熱では，弱毒化フニンウイルスCandid #1株を用いた弱毒生ワクチンが開発されており[44]，既にアルゼンチンでは1991年から使用され患者発生が激減している．一方，ラッサ熱では承認されたワクチンは存在しない．実験的には，ラッサウイルスに近縁で病原性の弱いモペイアウイルスや，モペイアウイルスとラッサウイルスのリアソータントであるML29株を免疫す

ることによる防御効果が，モルモットやマーモセットなどを用いた動物実験において確認されている[47,48,49]．また，リバースジェネティクス等の技術を利用したDNAベースの組換えウイルスを用いたワクチン開発も進められており，組換え黄熱ワクチン17D株にラッサウイルスのGPCを組み込んで発現する組換えウイルスのワクチンは，第II相，第III相試験まで行われているが[50]，組換え体の性状や安定性にまだ問題がある[51,52]．VSVでも他の多くのウイルスでの応用[53,54,55]と同様に，VSVのエンベロープ遺伝子GをラッサウイルスのGPCに置き換えた自立複製型の組換えVSVを接種することで，霊長類を含む動物実験においてラッサウイルスの感染からの防御に有効であることが報告されている[56,57]．その他，ベネズエラ馬脳炎ウイルスのレプリコンにGPCを組み込んだ非複製型組換えウイルス[58]やGPC, NP, Z蛋白質を培養細胞に発現させて作製されたウイルス様粒子[59]，GPCやNPを発現するプラスミドDNA[60]を用いたワクチン開発も進められている．しかしながら，こうしたワクチン候補は，ラッサウイルスの流行地であるアフリカの環境状態や経済状態を鑑みると，ヒトへの効果も含めて，実際に臨床現場で応用できるようになるにはまだ多くの課題が残されていると思われる．

おわりに

出血熱を引き起こすアレナウイルスは，ヒトが罹患した際の重篤度や危険性から，国際的にも病原体取り扱いレベルでは最高度のBSL4病原体に分類され，また国内における感染症法の分類で最も危険性が高いとされる1類感染症に指定されている．日本では，国立感染症研究所にBSL4施設が設置されているにも関わらず，BSL4としては稼働されていない．ラッサ熱をはじめとするウイルス性出血熱は，我が国では現在まで稀な感染症として対策も希薄になりがちではあるが，昨今，映画やマスメディアによる影響もあり，いったん国内で発生が確認されると大混乱を招く恐れもある．そのため，こうした事態が起こっても正しく対応できる診断体制や治療・予防方法の確立が求められている．ウイルスそのものを理解するための基礎研究やそれを基にした治療法やワクチン開発などの応用研究，そして，国内において感染者が発生した際に診断，対応が迅速に行えるように，他のウイルス同様，アレナウイルスを含む出血熱ウイルスに関してもハード面を含めた研究環境が整うことが期待される．

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Arenavirus infections

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Arenaviruses are the collective name for viruses, which belong to the family *Arenaviridae*. They replicate in the cytoplasm of cells, and were named after the sandy (Latin, *arenosus*) appearance of the ribosomes often seen in thin sections of virions under electron microscope. Several arenaviruses, such as Lassa virus in West Africa, and Junin, Guanarito, Sabia, Machupo, and Chapare viruses in South America, cause sever viral hemorrhagic fevers (VHF) in humans and represent a serious public health problem. These viruses are categorized as category 1 pathogens thus should be handles in a BSL4 laboratory. Recently, Lujo virus was isolated as a newly discovered novel arenavirus associated with a VHF outbreak in southern Africa in 2008. Although, we have no VHF patients caused by arenaviruses in Japan, except for a single imported Lassa fever case in 1987, it is possible that VHF patients occur as imported cases as for other VHF in the future. Therefore, it is necessary to develop the diagnostics and therapeutics in consideration of patient's severe symptoms and high mortality even in the disease-free countries. In this review, we will broadly discuss the current knowledge from the basic researches to diagnostics and vaccine developments for arenavirus diseases.

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Analysis of Lujo Virus Cell Entry using Pseudotype Vesicular Stomatitis Virus

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ABSTRACT

Several arenaviruses are known to cause viral hemorrhagic fever (VHF) in sub-Saharan Africa and South America, where VHF is a major public health and medical concern. The biosafety level 4 categorization of these arenaviruses restricts their use and has impeded biological studies, including therapeutic drug and/or vaccine development. Due to difficulties associated with handling live viruses, pseudotype viruses, which transiently bear arenavirus envelope proteins based on vesicular stomatitis virus (VSV) or retrovirus, have been developed as surrogate virus systems. Here, we report the development of a pseudotype VSV bearing each envelope protein of various species of arenaviruses (AREpv), including the newly identified Lujo virus (LUJV) and Chapare virus. Pseudotype arenaviruses generated in 293T cells exhibited high infectivity in various mammalian cell lines. The infections by New World and Old World AREpv were dependent on their receptors (human transferrin receptor 1 [hTfR1] and α-dystroglycan [αDG], respectively). However, infection by pseudotype VSV bearing the LUJV envelope protein (LUJpv) occurred independently of hTfR1 and αDG, indicating that LUJpv utilizes an unidentified receptor. The pH-dependent endocytosis of AREpv was confirmed by the use of lysosomotropic agents. The fusion of cells expressing these envelope proteins, except for those expressing the LUJV envelope protein, was induced by transient treatment at low pH values. LUJpv infectivity was inhibited by U18666A, a cholesterol transport inhibitor. Furthermore, the infectivity of LUJpv was significantly decreased in the Niemann-Pick C1 (NPC1)-deficient cell line, suggesting the necessity for NPC1 activity for efficient LUJpv infection.

IMPORTANCE

LUJV is a newly identified arenavirus associated with a VHF outbreak in southern Africa. Although cell entry for many arenaviruses has been studied, cell entry for LUJV has not been characterized. In this study, we found that LUJpv utilizes neither αDG nor hTfR1 as a receptor and found unique characteristics of LUJV glycoprotein in membrane fusion and cell entry. Proper exclusion of cholesterol or some kinds of lipids may play important roles in LUJpv cell entry.

Arenaviruses belong to the family *Arenaviridae* and are classified into two complexes, New World and Old World arenaviruses, based on serological, genetic, and geographical relationships and the rodent hosts (1). New World arenaviruses are further divided into 3 major clades (A, B, and C). Clade B contains 5 hemorrhagic fever-causing arenaviruses that are known to cause South American hemorrhagic fever in humans: Junin virus (JUNV), Guanarito virus (GTOV), Sabia virus (SABV), Machupo virus (MACV), and Chapare virus (CHPV) (2). Of the Old World arenaviruses, Lassa virus (LASV) is endemic in western Africa and is known to cause viral hemorrhagic fever (VHF) in humans (3). Recently, another Old World arenavirus, Lujo virus (LUJV), was identified as a cause of VHF with a high case fatality rate of 80% (4).

Arenaviruses are enveloped negative-strand RNA viruses with a genome that is bisegmented into S and L segments. The S segment encodes a nucleocapsid protein (NP) and an envelope glycoprotein precursor (GPC); the L segment encodes a matrix protein (Z) and an RNA-dependent RNA polymerase (L). The GPC is synthesized as a single polypeptide and undergoes processing by the host cell signal peptidase (SPase) and subtilisin-like kexin isozyme-1/site-1-protease (SKI-1/S1P), yielding typical receptor-binding (G1), transmembrane fusion (G2), and stable signal peptide (SSP) subunits, respectively (5). Viral entry into target cells is initiated by the binding of G1 to appropriate cell surface receptors. The first cellular receptor for arenavirus to be identified was α-dystroglycan (αDG), a ubiquitous receptor for extracellular matrix proteins (6). αDG is a binding receptor for Old World arenaviruses, such as LASV and lymphocytic choriomeningitis virus (LCMV), and some of the clade C New World arenaviruses (7). Among the New World arenaviruses, many pathogenic viruses use human transferrin receptor 1 (hTfR1) as a receptor for cell entry (8, 9). A number of additional receptor candidates for JUNV, LASV, and LCMV have recently been reported, including the C-type lectin family, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), liver and lymph node sinusoidal endothelial calcium-dependent lectin (LSECtin), and two members of the TAM family (Axl and Tyro3)

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(10–13). In the case of LUJV, the receptor candidate (or candidates) is at this time largely unknown.

The categorization in many countries of VHF-causing arenaviruses as biosafety level 4 (BSL4) pathogens means that they may only be handled in specific institutions that are equipped with BSL4 facilities. This has impeded analysis of the life cycle and pathogenesis of these pathogenic arenaviruses and the development of therapeutic agents and vaccines for arenaviral hemorrhagic fevers. The analysis of the initial steps of viral infection, including identification of the entry receptors, is important for understanding the life cycle of these viruses and for further developing entry inhibitors. Several alternative research tools for the viruses have therefore been developed. A cell fusion assay was established to examine the membrane fusion activities of viral envelope proteins (14). The assay is sensitive and can easily determine cell fusion using reporter genes. Pseudotype virus systems based on vesicular stomatitis virus (VSV) or lentivirus and retrovirus have also been established to examine viral entry mechanisms and to identify putative entry receptors (15). These systems are beneficial in the study of highly pathogenic arenavirus infections.

In the present study, we generated pseudotype VSVs bearing envelope proteins of several arenaviruses, including LUJV. In particular, the characteristics of envelope proteins of LUJV were determined for the first time with respect to their glycosylation, fusion activities, and utilization of known arenaviral receptors and the involvement of cholesterol or sphingolipid in cell entry.

MATERIALS AND METHODS

Plasmids and cells. The cDNAs of the SABV, MACV, CHPV, and LUJV GPCs were obtained by chemical synthesis (Codon Devices, Cambridge, MA). The GPC cDNAs of JUNV (strain MC2) and LASV (strain Josiah) were supplied by V. Romanowski (Universidad Nacional de La Plata) and C. J. Peters (University of Texas Medical Branch), respectively. The GenBank accession numbers of the nucleotide sequences of the SABV, MACV, CHPV, LUJV, JUNV, and LASV GPC genes are NC_006317, NC_005078, NC_010562, FJ952384, U70799, and J04324, respectively. The GPC cDNAs of SABV, MACV, CHPV, LUJV, JUNV, and LASV were cloned into the expression vector pKS336 (16). The resulting plasmids were designated pKS-SABV-GP, pKS-MACV-GP, pKS-CHPV-GP, pKS-LUJV-GP, pKS-JUNV-GP, and pKS-LASV-GP. Each GP sequence of the plasmids was confirmed to be identical to the original cDNA sequence using an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). The plasmid pKS-EBOV-GP (Reston) was prepared as described previously (17).

FLAG/One-STrEP (FOS)-tagged fusion protein expression vectors were also constructed. cDNAs of the CHPV-GP, LUJV-GP, JUNV-GP, and LASV-GP with the stop codon deleted were synthesized by PCR from each of the above-described cDNAs. The PCR products were cloned into pCAG-MCS2-FOS, which expresses carboxyl-terminally FOS-tagged fusion protein (provided by E. Morita, Osaka University). The resulting plasmids were designated pCAG-CHPV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-JUNV-GP-FOS, and pCAG-LASV-GP-FOS. The cDNA encoding G protein (G) of VSV was amplified from pCAG-VSV-G by PCR and cloned into pCAG-MCS2-FOS to construct pCAG-VSV-G-FOS in order to express carboxyl-terminally FOS-tagged VSV G protein.

A plasmid carrying hTfR1 cDNA was generated. Total RNAs were isolated from 293T cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription with Superscript III reverse transcriptase (Invitrogen) using random hexamer primers (Invitrogen). The cDNA encoding hTfR1 was amplified by PCR using an Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN) with cDNA synthesized from the 293T RNAs. The amplified cDNA was cloned into pKS336. The resulting plasmid was designated pKS-hTfR1. The cloned sequence was confirmed to be identical to the hTfR1 cDNA sequence using an ABI Prism 3100-Avant Genetic Analyzer.

Hamster (BHK and CHO), mouse (NIH 3T3, NMuLi, and P388), rabbit (PK15), monkey (VeroE6, COS7, and MARC), and human (Huh7, HepG2, Hep3B, 293T, HeLa, Saos-2, Raji, U937, Molt-4, and Jurkat) cell lines were obtained from the American Type Culture Collection (Manassas, VA) or DS Pharma Biomedical Co. Ltd. (Osaka, Japan). All of the cell lines, with the exceptions of Raji, Molt-4, P388, and Jurkat cells, were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS). Raji, Molt-4, P388, and Jurkat cells were grown in RPMI 1640 (Sigma-Aldrich) containing 10% FBS. To establish a CHO cell line that stably expresses hTfR1 (CHO/hTfR1), CHO cells were transfected with pKS-hTfR1 using Fugene HD (Roche) reagent. The transfected CHO cells were selected with DMEM containing 10% FBS and 2 μg/ml of Blasticidin S-HCl (Invitrogen). When clusters of the cells appeared, some clusters of the cells were cloned and subcultured to establish CHO/hTfR1. The αDG knockout embryonic stem (ES) cell clone 354.B11 was provided by K. P. Campbell (Howard Hughes Medical Institute, University of Iowa) and was cultured using Esgro Complete Plus Clonal Grade Medium (Merck Millipore, Darmstadt, Germany). A CHO cell mutant defective in the NPC1 gene (CHO/A101) and CHO/A101 cells stably expressing FLAG-tagged NPC1 (CHO/A101/NPC1-KI) had been previously generated and were maintained in Ham's F-12 containing 10% FBS (18).

Generation of pseudotype VSVs. Pseudotype VSVs bearing the GPC of arenaviruses (AREpv), GP of ebolavirus (EBOpv), G of VSV (VSVpv), and murine leukemia virus envelope protein (MLVpv) were generated as described previously (17, 19, 20). Briefly, 293T cells were grown to 70% confluence on collagen-coated tissue culture plates and then transfected with pKS-LASV-GP, pKS-JUNV-GP, pKS-LUJV-GP, pKS-CHPV-GP, pKS-SABV-GP, pKS-MACVGP, pKS-EBOV-GP, pCAG-CHPV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LASV-GP-FOS, pCAG-VSV-G, pCAG-VSV-G-FOS, or pFBASALF (which expresses murine leukemia virus envelope proteins; provided by T. Miyazawa, Kyoto University). After 24 h of incubation, the transfected cells were infected with G-complemented (*G) VSVΔG/Luc (*G-VSVΔG/Luc) (21) at a multiplicity of infection (MOI) of 0.1. The virus was adsorbed and then extensively washed four times with serum-free DMEM. After 24 h of incubation, the culture supernatants containing pseudotype VSVs were centrifuged to remove cell debris and stored at −80°C until use. The infectious titers of the pseudotype VSVs were also determined by a focus-forming assay, as described below, and were measured as focus-forming units (FFU). AREpv, EBOpv, VSVpv, and MLVpv infectivities were assessed by luciferase activity. The relative light unit (RLU) value of luciferase was determined using a Bright-Glo luciferase assay system (Promega Corporation, Madison, WI), according to the manufacturer's protocol.

Focus-forming assay. Huh7 or Vero cells were transfected with pCAG-VSV-G. At 24 h posttransfection, the cells infected with the pseudotype viruses were cultured with 0.8% methylcellulose in 10% FBS DMEM for 48 h. FFU were determined by counting visible foci.

Immunofluorescence assays. For immunofluorescence staining of the cells with antibodies, Huh7 cells transfected with pCAG-LASV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-CHPV-GP-FOS, or pCAG-VSV-G-FOS or CHO/hTfR1 cells were fixed with acetone at room temperature for 5 min. To stain the FOS-tagged protein and hTfR1, the fixed transfected Huh7 and CHO/hTfR1 cells were incubated with mouse monoclonal anti-FLAG primary antibody (Sigma) and mouse monoclonal anti-hTfR1 antibody (BD Biosciences, San Jose, CA), respectively. The CHO cells were also prepared and stained as hTfR1 negative-control cells. All of the cells were rinsed with phosphate-buffered saline (PBS) and incubated with goat anti-mouse Alexa Fluor 488 (Invitrogen). After washing with PBS, the stained cells were observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

Immunoblotting. 293T cells were transfected with each of the plasmids pCAG-CHPV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LASV-GP-FOS, and pCAG-VSV-G-FOS. At 24 h posttransfection, the cells were collected and lysed in PBS containing 1% NP-40. The lysates were centrifuged to separate insoluble pellets from supernatants. The supernatants were used as samples. Pseudotype VSVs bearing FOS-tagged GPCs generated as described above were pelleted through a 20% (wt/vol) sucrose cushion at 25,000 rpm for 2 h in an SW28 rotor (Beckman Coulter, Tokyo, Japan). The pellets were resuspended in PBS and used as additional samples. Each sample, boiled in loading buffer, was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to a methanol-activated polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) and reacted with mouse monoclonal anti-FLAG antibody (Sigma). Immune complexes were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) and detected by an LAS3000 analyzer (Fuji Film, Tokyo, Japan).

The lysates of CHO/hTfR1 and CHO cells were also treated as described above. To detect the bands of hTfR1 and β-actin, mouse monoclonal anti-hTfR1 antibody (BD Biosciences) and mouse monoclonal anti-β-actin antibody (Sigma), respectively, were used as described above.

Effects of treatment with antiserum on LUJpv infectivity. To characterize the infection of LUJpv, the pseudotype was preincubated with serially diluted anti-LUJV-GP polyclonal antibody for 1 h at 37°C and then inoculated into Huh7 cells. Anti-LUJV-GP polyclonal antibody was prepared by immunization of rabbits with the expression plasmid pKS-LUJV-GP, as described previously (22). After 2 h of adsorption at 37°C, the cells were washed with DMEM containing 10% FBS, and infectivity was determined after 24 h of incubation at 37°C.

Involvement of hTfR1 in AREpv infections. To determine the involvement of hTfR1 in viral entry, Huh7 or U937 cells were pretreated with various concentrations of anti-hTfR1 for 1 h at 37°C and inoculated with a series of AREpv, VSVpv, or MLVpv at an MOI of 1. After 1 h of adsorption at 37°C, the cells were washed and cultured for 24 h at 37°C. Pseudotype infectivities were determined by measuring luciferase activities after 24 h of incubation at 37°C.

The CHO and CHO/hTfR1 cells were infected with a series of AREpv or VSVpv, respectively, at an MOI of 1. Pseudotype infectivities were determined by measuring luciferase activities after 24 h of incubation at 37°C. AREpv infectivity for CHO/hTfR1 cells was normalized to the infectivity for CHO cells.

Huh7 cells were incubated at 37°C for 2 h in the presence of various concentrations of ferric ammonium citrate (FAC), which is rich in iron and is known to decrease TfR mRNA in the cells (23). The cells were then infected with the series of AREpv or VSVpv, as described above. Pseudotype infectivities were determined by measuring luciferase activities after 24 h of incubation at 37°C.

Involvement of αDG in AREpv infection. To examine the involvement of αDG in viral entry, Raji, Jurkat, or αDG knockout ES cells expressing like-acetylglucosaminyltransferase (LARGE) or αDG were prepared by infection with lentiviral vectors encoding LARGE, DG, or control (fCD2) genes as constructed previously (11). The cells were infected with a series of AREpv or VSVpv at an MOI of 1. Pseudotype infectivities were determined by measuring luciferase activities after 24 h of incubation at 37°C.

Effects of enzymes, chemicals, and low-pH exposure on AREpv infection. To examine the effects of oligosaccharide modification of arenavirus GPs or VSV-G, cell lysates and purified pseudotype virions were digested with endoglycosidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F) (Roche) in accordance with the manufacturer's protocol and analyzed by immunoblotting.

To examine the effects of endosomal acidification of cells on viral entry, Huh7 cells were treated with various concentrations of inhibitors of endosomal acidification—bafilomycin A_1 from *Streptomyces griseus* (Sigma), ammonium chloride (Sigma), and chloroquine (Sigma)—for 1 h at 37°C. The cells were then infected with the series of AREpv, VSVpv, or MLVpv at an MOI of 1. Pseudotype infectivity was determined by measuring luciferase activities after 24 h of incubation at 37°C.

To examine the pH dependence of AREpv cell entry, the series of AREpv or VSVpv was exposed to citrate-phosphate buffer (0.1 M citric acid, 0.2 M sodium dihydrogenorthophosphate) adjusted to the indicated pH level (pH 7, 6, 5, 4, or 3) for the indicated time (0, 15, 30, 60, 90, or 120 s). After pH neutralization with a 100× volume of DMEM containing 10% FBS, the viruses were inoculated into Huh7 cells. After 24 h of incubation at 37°C, residual infectivity was determined by measuring luciferase activities and comparison with the infectivities of the pseudotypes exposed to the buffer adjusted to pH 7.

To examine the involvement of cholesterol and sphingolipids in viral entry, Huh7 cells were treated with various concentrations of chlorpromazine, imipramine, desipramine, amitriptyline, or U18666A for 1 h at 37°C. The cells were then infected with the series of AREpv, EBOpv, VSVpv, or MLVpv at an MOI of 1. Pseudotype infectivity was determined by measuring luciferase activities after 24 h of incubation at 37°C.

To examine the involvement of NPC1 in viral entry, wild-type CHO (CHO/wt), CHO/A101, and CHO/A101/NPC1-KI cells were infected with the series of AREpv, EBOpv, or VSVpv at an MOI of 1. Pseudotype infectivity for these cells was determined by measuring luciferase activities after 24 h of incubation at 37°C. Pseudotype infectivity for CHO/A101 and CHO/A101/NPC1-KI cells was normalized to the infectivity for CHO/wt cells.

Syncytium formation and quantitative reporter assays for cell fusion. To examine whether syncytium formation of the cells expressing arenavirus GPC is induced by low-pH exposure, Huh7 cells were transfected with pCAG-CHPV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LASV-GP-FOS, or pCAG-VSV-G-FOS. At 24 h posttransfection, the cells were rinsed once with PBS and then incubated with citrate-phosphate buffers adjusted to the indicated pH value (pH 7, 6, 5, 4, or 3) for 2 min. The citrate-phosphate buffers were then replaced with DMEM containing 10% FBS and incubated for 24 h for LASV-GP/JUNV-GP- and LUJV-GP/CHPV-GP-expressing cells or 8 h for VSV-G-expressing cells. The cell monolayers were then observed for the induction of cell fusion under a phase-contrast microscope.

To quantify cell fusion induced by arenavirus GPs, a reporter assay was carried out as follows. 293T cells were grown on 35-mm tissue culture plates and transfected with pCAG-CHPV-GP-FOS, pCAG-LUJV-GP-FOS, pCAG-JUNV-GP-FOS, pCAG-LASV-GP-FOS, or pCAG-VSV-G-FOS, together with pCAGT7pol (provided by Y. Matsuura, Osaka University), an expression plasmid carrying the T7 RNA polymerase gene under the control of the CAG promoter (14). The Huh7 target cells were grown on 35-mm tissue culture plates and transfected with pT7EMCVLuc (provided by Y. Matsuura), a reporter plasmid carrying a firefly luciferase gene under the control of the T7 promoter. At 24 h posttransfection, the target cells were collected by trypsinization and regrown in a 96-well plate. The 293T cells were treated with 0.05% EDTA in PBS and suspended in DMEM containing 10% FBS. The 293T cells were overlaid onto the target Huh7 cells and incubated for 4 h. The cocultured cells were bathed in citrate-phosphate buffers adjusted to the indicated pH values for 2 min and then incubated with DMEM containing 10% FBS for 12 h. Cell fusion activity was quantitatively determined by measuring luciferase gene expression in the lysates of the cocultured cells. The RLU values of luciferase were determined using a Bright-Glo luciferase assay system and normalized to the values of cells treated with pH 7 buffer.

RESULTS

Production and characterization of AREpv. LASpv, JUNpv, LUJpv, CHPpv, and VSVpv were generated in 293T cells transiently expressing the carboxyl-terminally FOS-tagged envelope glycoproteins of LASV, JUNV, LUJV, CHAPV, and VSV, respectively, upon infection of *G pseudotype VSV, as previously reported (21, 24). To examine the properties of the arenaviral GPs

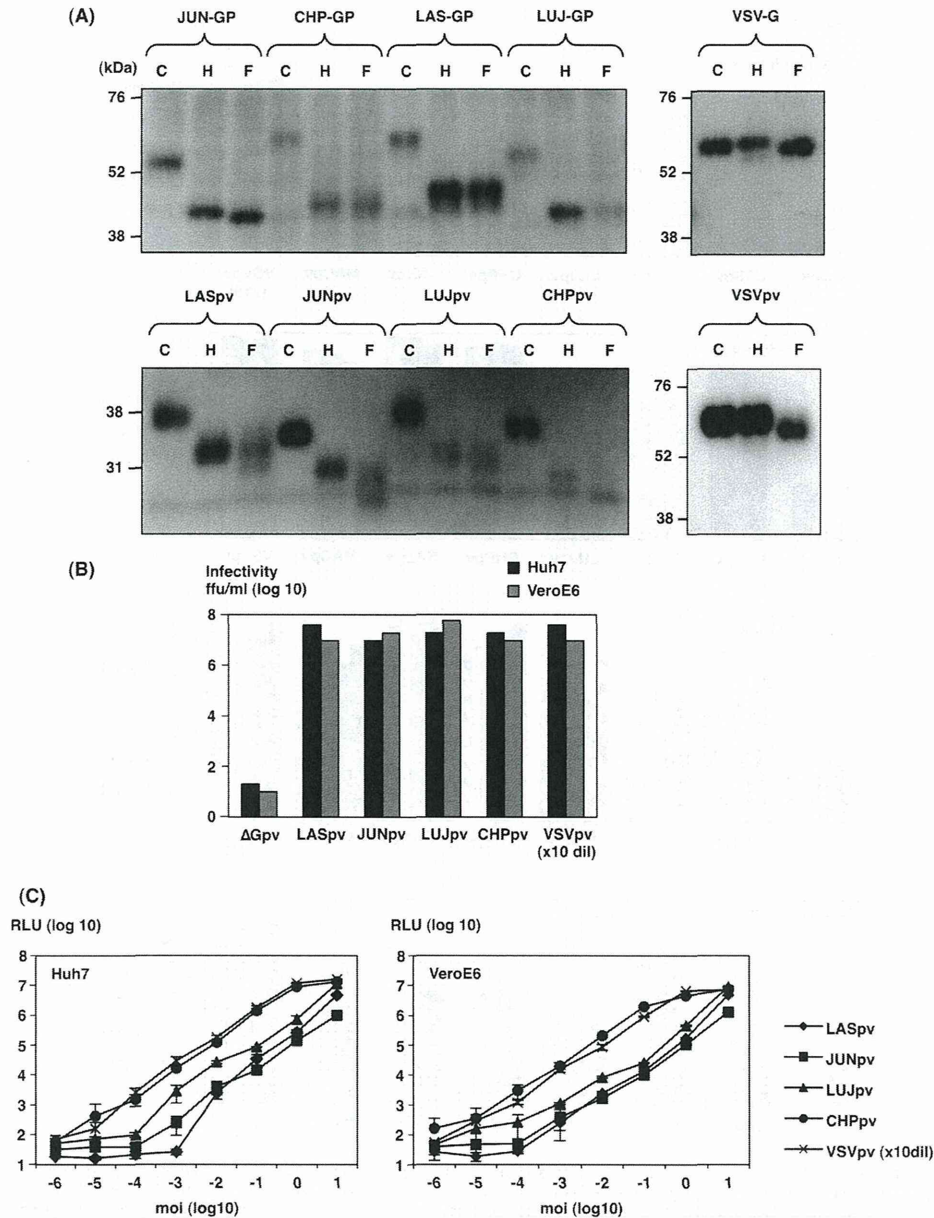

FIG 1 Glycosylation of glycoproteins in AREpv. (A) The GPC or G2 proteins of arenaviruses (LAS-GP, JUN-GP, LUJ-GP, and CHP-GP) or G protein of VSV expressed in 293T cells (top) and incorporated into the particles of AREpv (LASpv, JUNpv, LUJpv, and CHPpv) or VSVpv (bottom) were either untreated (lanes C) or treated with endoglycosidase H (lanes H) or peptide-*N*-glycosidase F (lanes F). Following fractionation on SDS-PAGE gels, the glycoproteins were detected by immunoblotting using anti-FLAG monoclonal antibody. (B) Infectivities of AREpv generated in 293T cells were determined in Huh7 and VeroE6 cells by focus-forming assay. (C) Dose-(MOI)-dependent relative luciferase activities (RLU) were determined in Huh7 and VeroE6 cells by luciferase assay. The MOI of each virus was determined on the basis of the titer in a focus-forming assay in Huh7 cells. The results shown are from three independent assays, with error bars representing standard deviations. VSV without envelope (ΔG) was used as a negative control. VSVpv (VSV) was used at 10-fold dilution (dil).

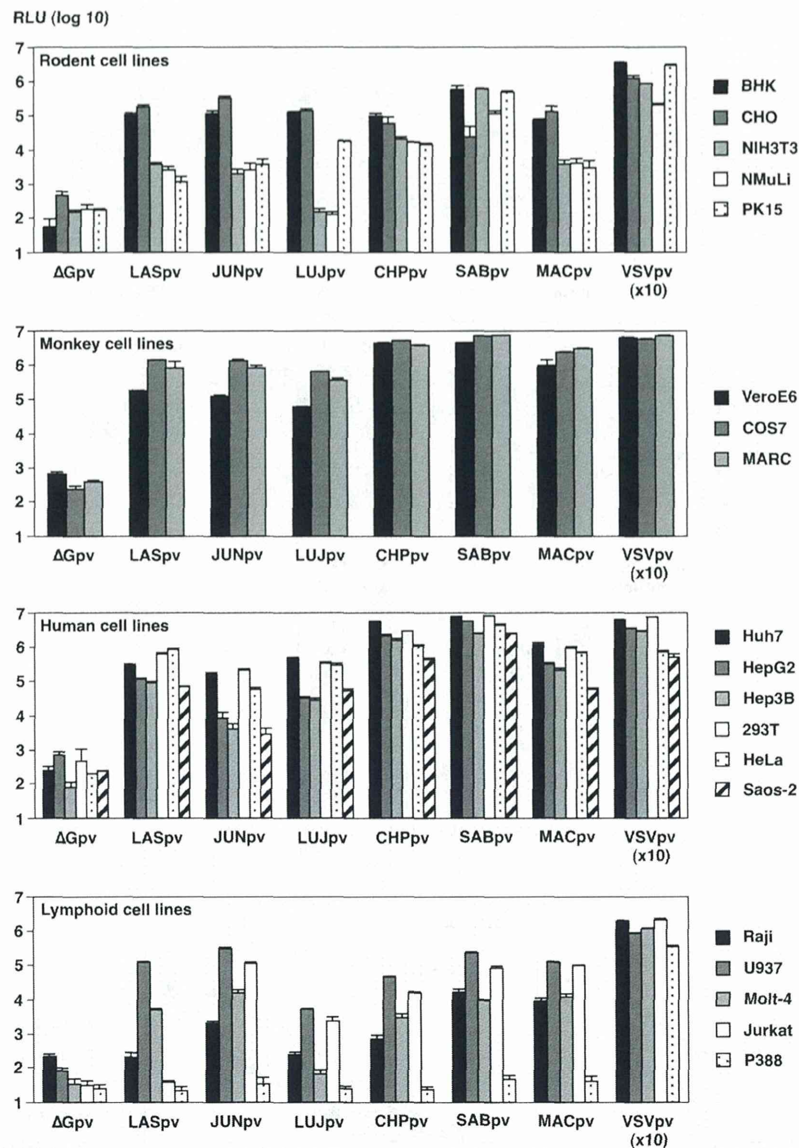

FIG 2 Efficiency of gene transduction into various mammalian cell lines by AREpv. The AREpv (LASpv, JUNpv, LUJpv, CHPpv, SABpv, and MACpv) generated by 293T cells were inoculated into the indicated cell lines at an MOI of 1. The MOI of each virus was determined on the basis of the titer in a focus-forming assay in Huh7 cells. At 24 h postinfection, the infectivities of the viruses were determined as RLU. The results shown are from three independent assays, with error bars representing standard deviations. ΔG was used as a negative control, and VSV was used at 10-fold dilution.

incorporated into AREpv particles, the arenaviral GPs expressed in 293T cells and incorporated into the viral particles were digested with Endo H and PNGase F and then examined by immunoblotting using anti-FLAG monoclonal antibody (Fig. 1A). Since the arenaviral GPCs were FOS tagged at the carboxyl terminus, the GPC and processed G2 were detected in the immunoblotting. All of the GPCs in the lysates of the cells transfected with GPC-expressing plasmids and G2 incorporated into the viral particles that were examined in the present study were sensitive to both Endo H and PNGase F treatments, suggesting that both immature and mature GPs exhibited high-mannose-type glycosylation. In contrast, the sugar chains of VSV-G protein in the cells and on the