1 μg IMR-32 membrane protein-coated wells (Fig. 1B). In addition, a relatively low level of non-specific binding was recognized as compared with the negative control, and VLP binding was inhibited significantly in the presence of anti-VP1 antibody with application of 5000 HAU VLPs (Fig. 1C). Thus, it was demonstrated that VLPs bound to IMR-32 membranes in a specific manner in this immunoscreening system.

After immunoscreening of more than 600 clones, one clone, designated as "24D2," was identified as one of the antibodies showing the greatest inhibitory effect. The subtype of 24D2 was shown to be IgM using a monoclonal antibody isotype detection kit (Amersham–Pharmacia Biotech, Piscataway, NJ). The hybridoma cells were recloned to confirm the production of a monospecific antibody and cultured in a CELLine culture system (BD Biosciences, San Jose, CA) to obtain a large amount of the antibody. The concentration of the resulting supernatant was measured by semi-quantitative immunoblotting assay using mouse immunoglobulin as a standard. The antibody was separated by SDS–PAGE on a 10% gel. After blocking with 5% skim milk/PBST for 30 min, the anti-mouse immunoglobulins were diluted 1:5000 in PBST. Immunoreactive bands were detected by ECL (Amersham–Pharmacia Biotech) and analyzed with LAS-1000 Plus image analyzer (Fuji Film, Tokyo, Japan). The concentration of 24D2 was determined as the intensity relative to that of the control antibody.

Inhibition of VLP binding to IMR-32 cells by 24D2

For conjugation of purified VLPs with fluorescein isothiocyanate (FITC), aliquots of 2 mg of purified VLPs were dissolved in 0.1 M carbonate–bicarbonate buffer (pH 9.0), mixed with 156 μg FITC (Sigma), and incubated at room temperature for 2 h. To eliminate unlabeled FITC, the samples were centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was dissolved in PBS, and centrifuged at 10,000*g* overnight, and the pellet was resuspended in PBS.

Before the experiment, IMR-32 cells (2×10^4) were plated onto 8-microwell tissue culture chambers (Nalgen Nunc International) and cultured in DMEM containing 10% FBS. The cells were pre-incubated in the absence or presence of either 24D2 (50 μg/ml) or isotype control monoclonal antibody for 1 h at 37 °C. Subsequently, FITC-VLPs (1×10^5 HAU) diluted 1:10 with DMEM were added to each chamber for 1 h at 4 °C. Unbound FITC-VLPs were removed by washing with PBS, and incubated for 15 min at 37 °C, following observation with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Inhibition of JCV infection by 24D2

IMR-32 cells (5×10^4) were preincubated in the absence or presence of either 24D2 (300 μg/ml) or the isotype control monoclonal antibody for 1 h at 37 °C. The cells were then incubated with 512 HAU JCV in fresh medium with 2% FBS for 1 h at 37 °C. After washing twice with fresh medium, the cells were incubated at 37 °C for 4 days with DMEM supplemented with 2% FBS. The monolayer culture in each well was washed with PBS and lysed in 1% Triton X-100/TBS containing 2 μg/ml aprotinin. The cell lysates were centrifuged at 4 °C for 10 min at 15,000 rpm, and the supernatants were separated by SDS–PAGE on 12% gels. After transferring the separated proteins onto a PVDF membrane, the membrane was immersed in 5% skim milk/TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 30 min, and subsequently incubated with either anti-agnoprotein antibody or anti-T-antigen antibody diluted 1:1000 with TBS-T for 1 h. After washing twice with TBS-T, the membrane was incubated with HRP-conjugated $F(ab')_2$ goat anti-rabbit or HRP-conjugated $F(ab')_2$ goat anti-mouse immunoglobulins diluted 1:5000 in TBS-T for 1 h. Immunoreactive bands were detected using the ECL reagent (Amersham–Pharmacia Biotech) and analyzed using an LAS-1000 plus image analyzer. Expression of viral proteins in the cells is represented as intensity relative to that of the control sample without treatment with 24D2 or isotype control antibody. The data are presented as mean values ± SD of three independent experiments.

Analysis of the molecule recognized by 24D2

Immunoblotting. Immunoblotting was performed as described above with the following modifications. The purified IMR-32 membrane fractions were separated by SDS–PAGE on 10% gels, transferred onto PVDF membranes, and immunoblotted with the 24D2 antibody. Immunoreactive bands were analyzed as described above.

Immunocytochemistry. IMR-32 cells grown on 8-microwell chambers were fixed with methanol at −80 °C for 2 min and rehydrated in PBS for 5 min at room temperature. After incubation with blocking solution (PBS containing 3% BSA), cells were incubated with 24D2 antibody diluted 1:60 in blocking solution for 2 h. After washing three times with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgM antibody (Molecular Probes, Eugene, OR) diluted 1:500 for 1 h, and observed using a laser scanning confocal microscope (Olympus).

Immunohistochemistry. Normal human brain tissue obtained at autopsy was sectioned at a thickness of 10 μm with a Cryo 2000 cryostat (Sankyo, Tokyo, Japan) and fixed in acetone at 4 °C for 10 min. The sections were rinsed in PBS, blocked in 0.3% H_2O_2 methanol for 15 min, and incubated with 24D2 at 4 °C overnight. After incubation with biotinylated second antibody, immunoreactive products were visualized with 3,3′-diaminobenzidine. For double immunostaining of human brain tissue, the sections were rinsed twice in PBS, preincubated in PBS containing 3% BSA for 60 min, and incubated with anti-glial fibrillary acidic protein (GFAP, 1:5 dilution), anti-carbonic anhydrase-II (CA2, 1:1,000 dilution), or anti-synaptophysin (1:100 dilution) together with 24D2 (1:5) overnight. The sections were incubated with the appropriate secondary antibodies, including Alexa Fluor 488-conjugated goat anti-rabbit IgG, biotinylated anti-sheep IgG, Alexa Fluor 488-conjugated streptavidin, Alexa Fluor 488-conjugated goat anti-mouse IgG, or Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes). After rinsing with PBS for 30 min, the sections were observed by laser scanning confocal microscopy.

Results

Isolation of monoclonal antibody (24D2) which inhibits attachment of JCV

Among more than 600 clones of monoclonal antibodies against the cell membrane fraction of JCV-permissive human neuroblastoma cell line IMR-32 cells, one hybridoma clone was isolated using the immunoscreening system and was designated as "24D2" (Fig. 1A). 24D2 significantly inhibited the binding of VLPs (Fig. 2A) as well as JCV (Fig. 2B) to cell membrane fractions on the immunoplates, whereas an isotype control antibody (mouse IgM) showed no inhibitory effect (Figs. 2A and B).

The 24D2 inhibits attachment and entry of FITC-labeled VLPs into IMR-32 cells

To evaluate the inhibitory effects on attachment and entry of VLPs, which have functions in cellular attachment and entry, similar to native JCV infection

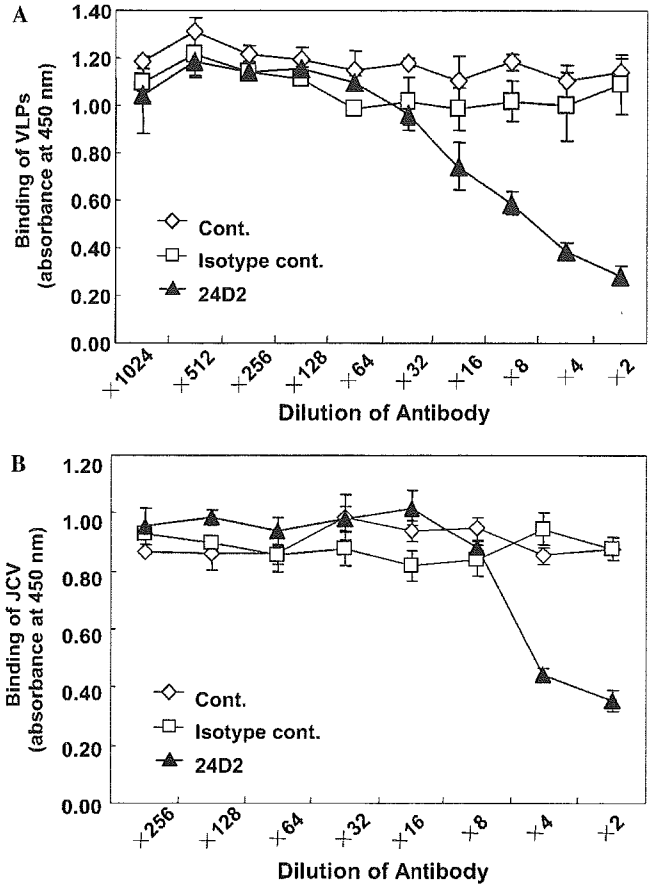

Fig. 2. Inhibition of the binding of both VLPs and JCV to IMR-32 membrane fractions on the immunoplate system by 24D2. (A,B) Inhibition of JCV and VLP binding by 24D2. IMR-32 membrane fractions were coated onto each well of the immunoplates, which were preincubated with serial dilutions of 24D2 (▲), mouse IgM (isotype control antibody, □), or without antibody (◇), and then incubated with 5000 HAU of VLPs (A) or JCV (B). Binding of VLPs or JCV was detected with anti-VP1 antibody and presented as the absorbance value. The bars represent the standard deviation of the mean of at least three independent experiments performed in duplicate.

[2,6] into IMR-32 cells, the cells were pretreated in the presence of either 24D2 or the isotype control monoclonal antibody, and incubated with FITC-labeled VLPs. 24D2 completely inhibited cellular attachment and entry of FITC-VLPs in IMR-32 cells, while the isotype control antibody had no effect on attachment or entry (Fig. 3).

Inhibition of JCV infection to IMR-32 cells by 24D2

To determine the blocking activity of 24D2 against JCV infection, IMR-32 cells were incubated with 24D2 before JCV inoculation. Four days after inoculation, expression of the viral proteins, including early and late viral proteins, T-antigen, and agnoprotein, was examined by immunoblotting. Treatment with 24D2 markedly inhibited expression of both T-antigen and agnoprotein in JCV-infected cells (Figs. 4A and B). The inhibitory effect of 24D2 against JCV infection was dose-dependent (Fig. 4C). Thus, we demonstrated that 24D2 possessed inhibition activity against JCV infection, suggesting that the molecule recognized by 24D2 plays a role as a cellular receptor for JCV.

Characterization of the molecule recognized by 24D2

We further characterized the molecule recognized by 24D2 by immunoblotting. 24D2 clearly recognized a single band with a molecular weight of around 60 kDa in membrane fractions from IMR-32 cells (Fig. 5A). Immunoblotting was also performed using various cell lines, including, HEK-293, HeLa, COS7, and SVG-A. In HEK293 cells, 24D2 recognized a single band with the same molecular weight as that of IMR-32 cells. However, in HeLa, COS7, and SVG-A cells, positive signals were observed around 30 kDa instead of 60 kDa (Fig. 5B).

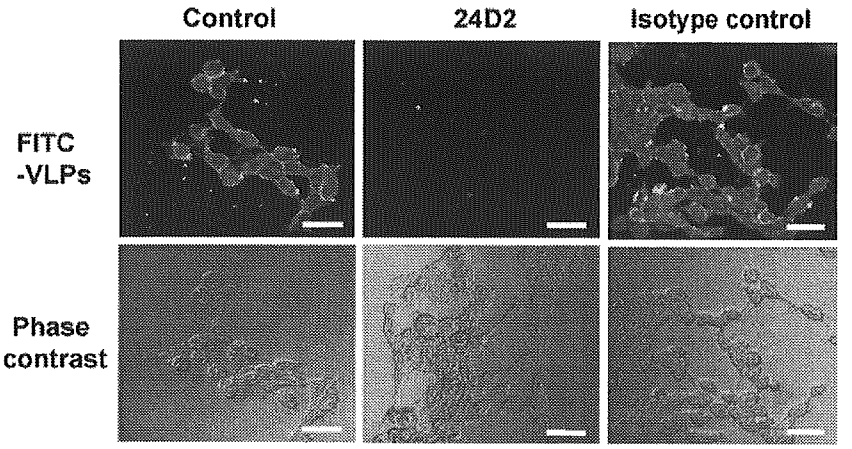

Fig. 3. Inhibitory effect of 24D2 on attachment and entry of FITC-labeled VLPs (FITC-VLPs) into IMR-32cells. After 1-h incubation with either 24D2 or the isotype control antibody, IMR-32 cells were incubated with FITC-VLPs. The immunofluorescence signal of FITC-VLPs was observed by confocal microscopy. No FITC signal was observed in cells treated with 24D2. Bars, 20 μm.

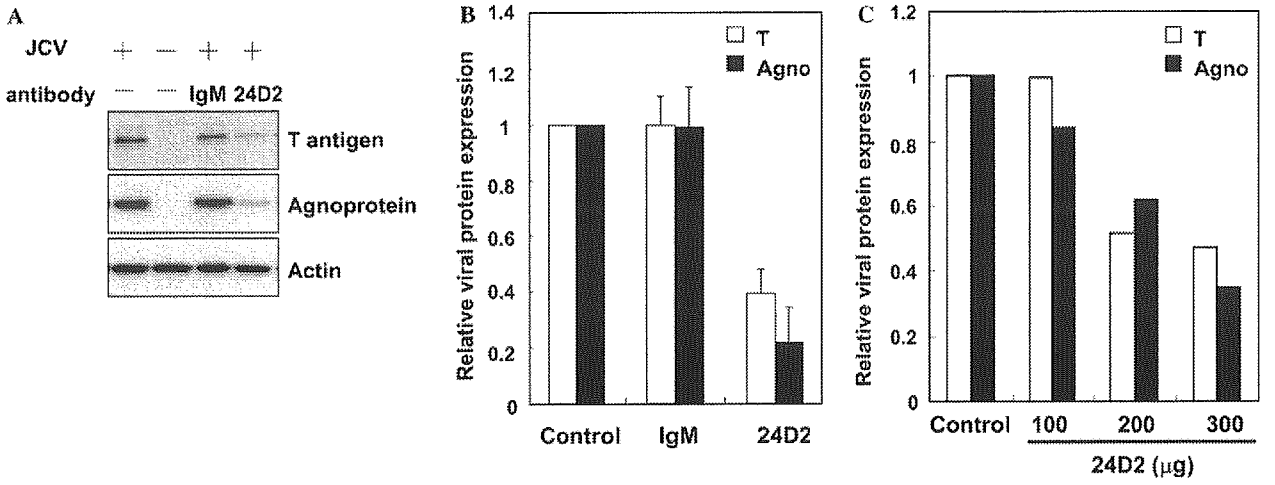

Fig. 4. Inhibition of JCV infection by treatment with 24D2. IMR-32 cells pretreated in the absence or presence of 24D2 or the isotype control antibody (IgM) were inoculated with 512 HAU of JCV for 1 h. Four days after inoculation, the cells were harvested and analyzed with immunoblotting using specific antibodies for JCV proteins (large T-antigen and agnoprotein). (A) Representative immunoblotting data with antibodies for large T-antigen and agnoprotein. Anti-actin antibody was used as a loading control. (B) Expression levels of viral proteins in IMR-32 cells treated with 24D2 or the isotype control antibody (IgM) relative to non-treated IMR-32 cells (control) at 4 days after inoculation of JCV. The bars represent mean values ± SD of at least three independent experiments. (C) The dose-dependent inhibition of JCV infection by 24D2. IMR-32 cells were incubated with different concentrations of 24D2 and incubated with 512 HAU JCV. JCV T-antigen and agnoprotein expression in IMR-32 cells was analyzed by immunoblotting 4 days after inoculation. The data are presented as amounts of viral protein expression relative to that of control infection. The assays were performed at least twice independently.

Next, we investigated the subcellular localization of positive signals with 24D2 in IMR-32 and HEK-293 cells by immunocytochemistry. The immunopositive signals for 24D2 were detected mainly on the cell surface and in the intracytoplasmic compartment (Fig. 5C). We performed the immunocytochemical examination using HeLa, COS, and SVG cells. In these cells, the immunopositive signals against 24D2 were also recognized in the cell surface and in the cytoplasm (data not shown).

The molecule recognized by 24D2 is localized mainly in glial cells in the human brain

Recently, it has been reported that JCV receptor-type α2–6-linked sialic acid was localized in oligodendrocytes and astrocytes but not in cortical neurons [13]. Although the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and intestinal tract, the major target cells of JCV infection in the CNS are glial cells, such as oligodendrocytes and astrocytes [14,15]. Therefore, we investigated the subcellular localization of the molecule recognized by 24D2 in the human brain. Positive signals for 24D2 were distributed in the glial cells of the cerebrum and cerebellum (Fig. 6A). To confirm the cellular distribution of the immunopositive signals, we performed double immunofluorescence staining using 24D2 and cell-specific markers, such as CA2, as a marker of oligodendrocytes [16–19], GFAP, a marker of astrocytes, and synaptophysin, a neuronal marker (g–i) (Fig. 6B). The immunopositive signals of 24D2 were colocalized with GFAP and CA2 (Figs. 6Ba–f). However, 24D2 failed to react with synaptophysin-positive neurons (Fig. 6Bg–i). These results indicated that the molecule recognized by 24D2 is distributed mainly in glial cells in the human brain.

Discussion

Virus attachment to cell-surface molecules is an initial event in the process of virus infection. Many viruses utilize various receptors for attachment and entry into cells. The polyomavirus, simian vacuolating virus 40 (SV 40), binds to the MHC class I molecule at the cell surface [20], but the MHC molecule is not internalized into the cells together with SV 40 [21]. The ganglioside GM1 also binds to SV 40, and is mediated to transport the virus from the plasma membrane to the endoplasmic reticulum (ER), and a role of GM1 as a functional receptor has been suggested [22]. The mouse polyomavirus (PyV) is also known to bind to the sialic acid of an as yet unidentified receptor [23]. Efforts to identify the receptor by screening for monoclonal antibodies that can protect cells from infection have been unsuccessful [24]. It has been reported that an antibody to α4β1 integrin partially inhibits PyV infection [8]. Therefore, the integrin has been suggested to act as an entry receptor in the early stages of PyV infection in fibroblasts, and PyV utilizes gangliosides as carriers from the plasma membrane to the ER [22]. Interestingly, SV40 and PyV have been shown to use clathrin-independent and caveolin-

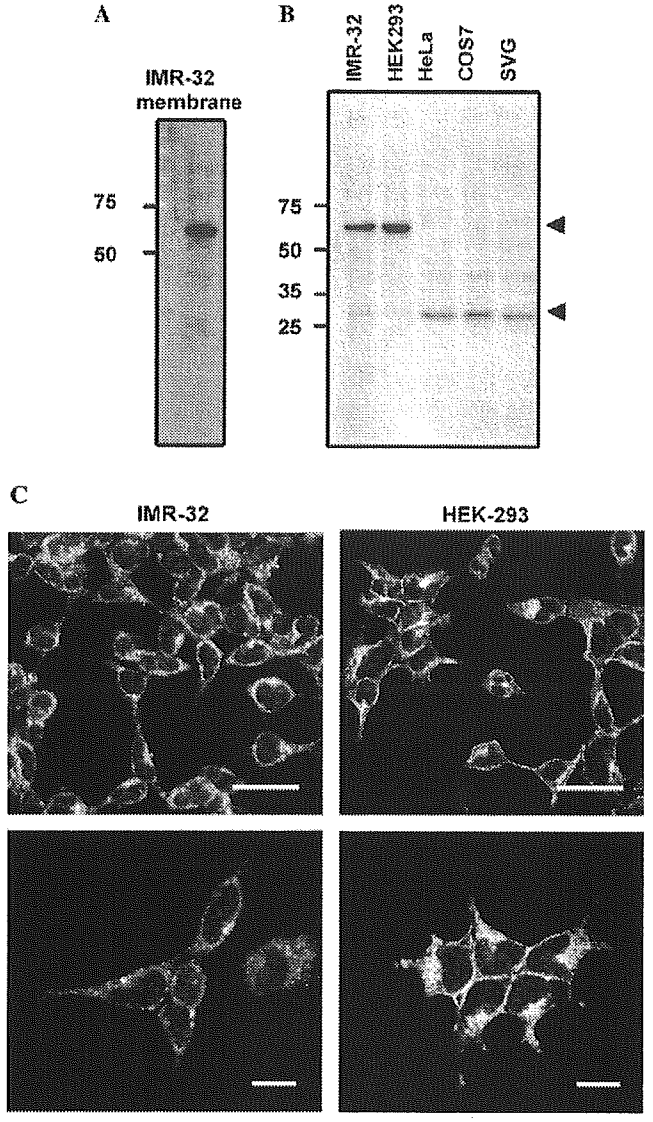

Fig. 5. Characterization of the molecule recognized by 24D2. (A) Immunoblotting analysis with membrane extracts derived from IMR-32 cells. Aliquots of 20 μg of the detergent-solubilized IMR-32 membrane fraction were loaded and immunoblotted with 24D2 at a dilution of 1:60. (B) Immunoblotting analysis with various cell lysates. The membrane fractions were prepared from IMR-32, HEK293, COS7, HeLa, and SVG-A cells. Molecular size markers are indicated on the left of the column. (C) Subcellular localization of the molecule recognized by 24D2 in IMR-32 and HEK-293 cells. Methanol-fixed cells were incubated with 24D2 at a dilution of 1:60, following Alexa Fluor 488-conjugated goat anti-mouse IgM. The immunofluorescence signal is represented as a green color. The lower panels show higher magnifications of the upper panels. Bars, 20 μm (upper panels) and 10 μm (lower panels).

dependent mechanisms to infect cells, respectively [25–27]. In contrast, JCV enters host cells by receptor-mediated clathrin-dependent endocytosis [28]. Furthermore, it has been reported that JCV does not share receptor specificity with SV 40 on human glial cells, because anti-class I antibodies failed to inhibit JCV infection [6].

It has been reported that a sialic acid-containing glycoprotein is one of the receptors of the human polyomavirus JCV in human glial cells, as neuraminidase treatment suppressed JCV attachment to the cells and subsequent infection [6].

In the present study, we have shown that the monoclonal antibody 24D2 inhibits JCV infection in IMR-32 cells. Although the major targets of JCV infection in the central nervous system are the glial cells, the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and gastrointestinal tract [29–31], and JCV can enter a wide variety of cell types [5]. The 60-kDa molecule recognized by 24D2 was detected not only in IMR-32 cells, but also in other cell lines, including the human kidney cell line, HEK293. In addition, 24D2 showed inhibition of cellular attachment and entry of FITC-VLP to HEK293 cells (data not shown). These results suggest that the molecule recognized by 24D2 also acts as a receptor in IMR-32 and HEK293 cells. As 24D2 failed to completely inhibit infection, additional molecules might participate in JCV attachment and infection.

It is interesting to note that JCV has been suggested to persistently infect the kidney, because JCV genome was detected in the urine and the renal tissue of healthy individuals [32,33]. 24D2 also recognized a molecule of 30 kDa in the membrane fractions of HeLa, COS7, and SVG-A cells. This smaller molecule might be an isotype or a proteolytic product of the larger molecule. It has been reported that a monoclonal antibody against the hyaluronan receptor recognized two distinct receptors in the cell membranes of rat liver sinusoidal endothelial cells, which were identified previously as hyaluronan-binding proteins of 175 and 300 kDa [34,35]. After reduction of disulfide bonds, the 175-kDa hyaluronan receptor was shown to be a single protein, whereas the 300-kDa molecule consisted of three subunits, i.e., α (269 kDa), β (230 kDa), and γ subunits (97 kDa) [35], and it has been reported that the 175-kDa receptor was derived from the 300-kDa receptor by proteolytic processing [36]. Similar to the hyaluronan receptors, the molecules of 30 and 60 kDa recognized by 24D2 might be isoreceptors for the same ligand or products derived from a larger receptor complex. It has been reported that the receptor with 30-kDa molecular weight for human coronavirus OC43 was isolated from the newborn mouse brain membrane fractions [37]. A 30-kDa molecule detected in HeLa, COS7, and SVG cells might be similar to a coronavirus OC43 receptor.

We also examined the localization of the putative receptor molecule in the human brain, because JCV infects mainly glial cells in the central nervous system. Positive immunoreactivity for 24D2 was colocalized with CA2-positive oligodendrocytes and GFAP-positive astrocytes, but failed to colocalize with synaptophysin-positive neurons. These findings indicated that the putative receptor molecule for JCV was localized mainly in the glial cells of the human brain. In the present study,

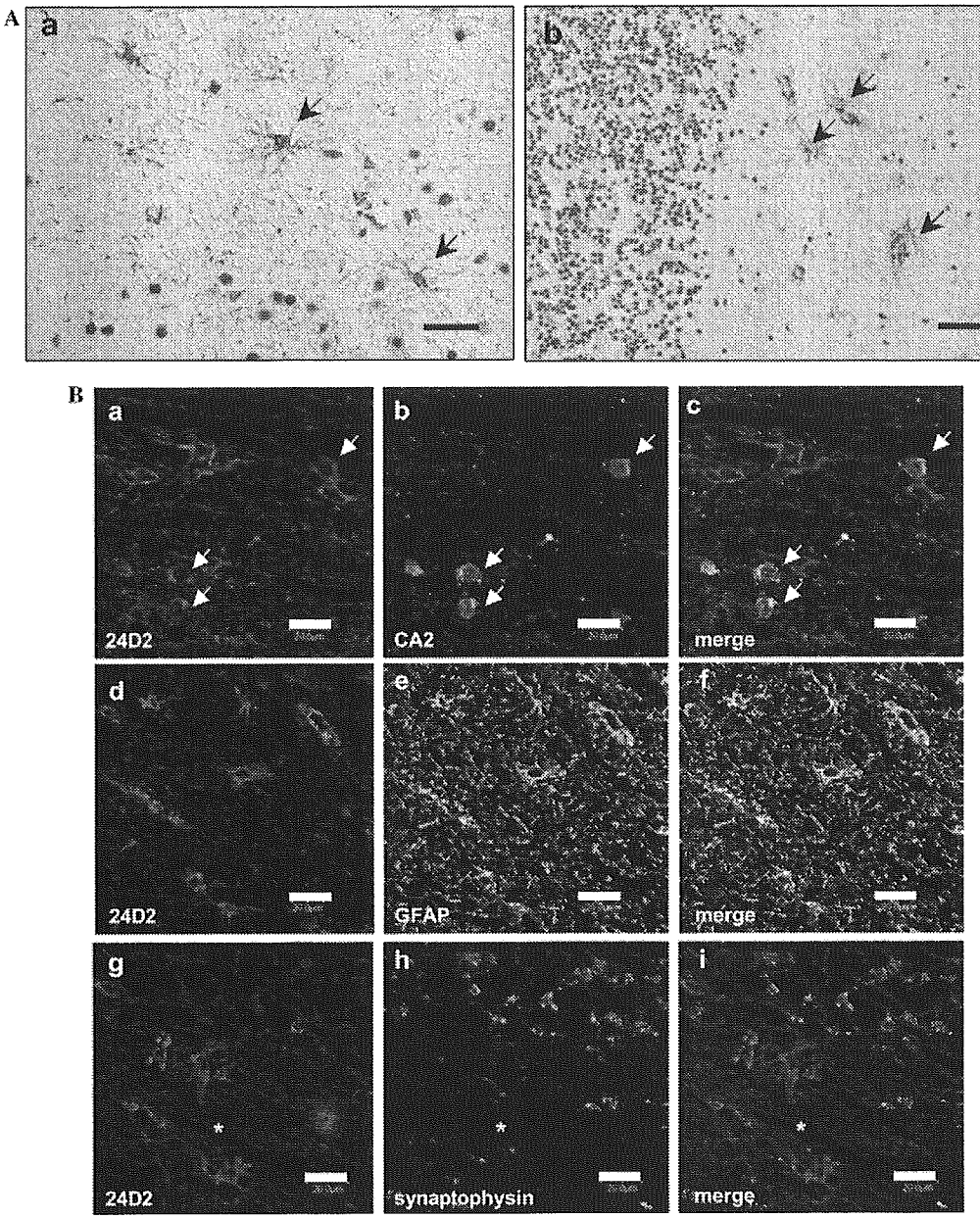

Fig. 6. Subcellular localization of the molecule recognized by 24D2 in the human brain. (A) Immunoreactivity with 24D2 in the cerebrum (a) and the cerebellum (b). Arrows indicate the immunopositive cells (a,b) in the white matter. Scale bars, 50 μm. (B) Double immunostaining using 24D2 and anti-CA2 antibody (a–c), anti-GFAP antibody (d–f), or anti-synaptophysin antibody (g–i) in the cerebrum. The merged images show that the molecule recognized by 24D2 (red color) was colocalized with CA2 and GFAP (green color). However, colocalization was not detected between 24D2 and anti-synaptophysin signals. Arrows indicate the 24D2- and CA2-double positive cells (a–c). Asterisks indicate white matter (g–i). Scale bars, 20 μm.

the neuroblastoma cell line, IMR-32, was used as the immunogen for the monoclonal antibody because these cells are highly susceptible to JCV infection [38]. Furthermore, immunization of IMR-32 cell has been shown to produce an antibody that specifically recognizes a molecule in oligodendrocytes [39]. Thus, it was suggested that IMR-32 cells possess molecules in common with brain glial cells. In Fig. 6Aa and b, the immunopositive signals against 24D2 were less than those of Fig. 6Ba, d, and g. We suppose that the difference of oligodendrocyte 24D2 immunostaining patterns among Fig. 6A and B might be due to the applied immunostaining systems. In Fig. 6A, we applied the biotinylated antibody as a second antibody, and the sections were observed with light microscopy. Meanwhile, in Fig. 6B, we observed the sections labeled with Alexa Fluor 488-conjugated second antibodies with fluorescent microscopy.

In summary, we have isolated a monoclonal antibody against the receptor molecule for JCV. By a newly established immunoscreening system, we isolated a monoclonal antibody (24D2) that inhibits cellular attachment

and infection of JCV. We also showed that the antibody specifically recognizes a membrane molecule with a molecular weight of 60 kDa, and positive signals were observed in the glial cells of the human brain. These results suggest that the molecule recognized by 24D2 acts as a JCV receptor in JCV infection. Further studies are required to characterize and determine the functional role of the molecule. A greater understanding of the molecular characteristics of the receptor molecule is necessary for the design of novel anti-JCV agents. Experiments to identify the molecules detected by 24D2 using a λ phage library are currently in progress in our laboratory.

Note added in proof

Recently, it has been reported that the antagonists and antibodies against the 5HT2 serotonergic receptors blocked JCV infection in SVG-A cells [40], suggesting that serotonin receptors act as one of the JCV receptors.

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Establishment of an immunoscreening system using recombinant VP1 protein for the isolation of a monoclonal antibody that blocks JC virus infection

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Abstract

Polyomavirus JC (JCV) infection causes the fatal human demyelinating disease, progressive multifocal leukoencephalopathy. Although the initial interaction of JCV with host cells occurs through direct binding of the major viral capsid protein (VP1) with cell-surface molecules possessing sialic acid, these molecules have not yet been identified. In order to isolate monoclonal antibodies which inhibit attachment of JCV, we established an immunoscreening system using virus-like particles consisting of the VP1. Using this system, among monoclonal antibodies against the cell membrane fraction from JCV-permissive human neuroblastoma IMR-32 cells, we isolated a monoclonal antibody designated as 24D2 that specifically inhibited attachment and infection of JCV to IMR-32 cells. The antibody 24D2 recognized a single molecule of around 60 kDa in molecular weight in the IMR-32 membrane fraction. Immunohistochemical staining with 24D2 demonstrated immunoreactivity in the cell membrane of JCV-permissive cell lines and glial cells of the human brain. These results suggested that the molecule recognized by 24D2 plays a role in JCV infection, and that it might participate as a receptor or a co-receptor in JCV attachment and entry into the cells.

Keywords: JC virus; VP1; VLP; Monoclonal antibody; PML

JC virus (JCV), which belongs to the polyomavirus family of non-enveloped DNA viruses, is known to be a causative agent of the progressive multifocal leukoencephalopathy (PML), a demyelinating disease of the central nervous system (CNS). PML affects mainly immunosuppressed patients, such as those with acquired immunodeficiency syndrome (AIDS) or following organ transplantation, and has increased in prevalence with the spread of transplantations and AIDS. However, there is still no effective treatment for PML. The major target cells of JCV infection in the CNS are the glial cells, such as oligodendrocytes and astrocytes [1]. The first step in the establishment of JCV infection is attachment of the viral capsid to receptors on host cells.

The capsid of JCV is composed of three capsid proteins, VP1, VP2, and VP3. VP1, encoded by the late region of JCV, is the major capsid protein that forms the outer surface of the virion. It has been reported that recombinant VP1 derived from *Escherichia coli* or insect cells can form virus-like particles (VLPs), and exhibit cellular attachment and intracytoplasmic trafficking similar to those of JCV virions [2–5]. In addition, VP1 has been reported to play a major function in the attachment of JCV to cells as the anti-VP1 antibody suppresses viral entry into cells and subsequent infection

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[5]. The cellular receptor molecule for JCV is unknown, but it has been reported to be an N-linked glycoprotein containing α2–6-linked sialic acids, based on the observation that α2–6-specific sialidase inhibits infection of glial cells by JCV. In addition, treatment of permissive cells with tunicamycin, which removes N-linked oligosaccharides, was shown to inhibit JCV infection in vitro [6]. These findings indicate that the interaction between the major structural protein VP1 and sialic acids is critical for JCV infectivity. However, non-enveloped viruses are unable to fuse with the cell lipid membrane bilayer, and their internalization is thought to require a specific entry receptor. Furthermore, it has been reported that infectious entry of JCV requires a ligand-inducible signal that is inhibited by genistein, a protein–tyrosine kinase inhibitor [7]. The mechanisms by which JCV binds and enters host cells and the molecules involved are not yet completely understood.

The mouse polyomavirus (PyV) is also known to bind to the sialic acid residues of a not yet identified receptor. Recently, antibodies to α4β1 integrin have been shown to partially block PyV infection. Therefore, the integrin has been suggested to act as an entry receptor for the early stages of PyV infection in fibroblasts [8].

Antibodies that react with cell-surface molecules have important applications as therapeutic agents and in the identification of viral receptors. Therefore, we attempted to isolate monoclonal antibodies to molecules on JCV-permissive cells. To isolate the monoclonal antibodies against the cell-surface molecules related to JCV infection on permissive cells, we developed an immunoscreening method and attempted to isolate blocking antibodies against JCV infection.

Using this immunoscreening system, we isolated a monoclonal antibody that recognizes the cellular-surface molecules related to JCV infection. The antibody also significantly blocked the attachment and subsequent infection of JCV. The molecule recognized by this antibody was considered to participate in virus infection, suggesting that this molecule acts as a virus receptor and/or a co-receptor. Here, we report the successful isolation and characterization of a monoclonal antibody with blocking activities toward JCV infection.

Materials and methods

Cells and antibodies

The human neuroblastoma cell line, IMR-32 (JCRB 9050), human embryonic kidney cell line, HEK293 (JCRB 9068), and human cervical carcinoma cell line, HeLa (JCRB 9004), were provided by the Health Science Research Resources Bank (Osaka, Japan). The African green monkey kidney cell line, COS-7 (#CRL 1651), was obtained from the American Type Culture Collection (Rockville, MD), and the human glial cell line, SVG-A, was kindly provided by Dr. Atwood [9]. All cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin, Sigma, St. Louis, MO) in 5% CO_2 at 37 °C. The anti-VP1 polyclonal antibody and anti-agnoprotein antibodies were prepared as described previously [5,10]. The anti-SV40T-antigen, which is known to be cross-reactive with anti-JCV T-antigen (Ab-2) monoclonal antibody, was purchased from Calbiochem (San Diego, CA). Anti-actin monoclonal antibody was purchased from Chemicon (Temecula, CA). Anti-GFAP polyclonal antibody (Nichirei, Tokyo, Japan), anti-synaptophysin monoclonal antibody (Dako, Japan), and anti-CA-2 polyclonal antibody (The Binding Site, Birmingham, UK) were prepared commercially. A mouse IgM-isotype control antibody was obtained from Sigma (St. Louis, MO).

JC virus and recombinant virus-like particles

Preparation of native JCV was performed as described previously [5]. Briefly, cell lysates from the JCV-producing cell line, JCI cells, were harvested, subjected to repeated freeze–thaw cycles, and then treated with 0.05 mg/ml neuraminidase type I (Sigma) at 37 °C for 16 h. After treatment with neuraminidase, samples were incubated at 56 °C for 30 min and centrifuged at 1000*g* for 10 min. The viral titer of virus-containing supernatant was quantified by hemagglutination (HA) assay and stored at −80 °C until use.

The preparation of virus-like particles (VLPs) was performed as described previously [5]. Briefly, the VP1 gene of JCV from pBR-Mad1 [11] was subcloned into the prokaryotic expression vector pET15-b (Novagen, Madison, WI). The plasmid was transformed into *E. coli*, BL21 (DE3)/pLys (Stratagene, La Jolla, CA). The expression of VP1 was induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30 °C, and the mixture was centrifuged at 4000*g* for 10 min. The cell pellet was resuspended in reassociation buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 1.0 mM $CaCl_2$) containing 1 mg/ml lysozyme, kept on ice for 30 min, and then sodium deoxycholate was added to a final concentration of 2 mg/ml. After 10 min on ice, the sample was lysed by five cycles of sonication in 15-s bursts. The lysate was treated with DNase I (100 U/ml) for 30 min at 30 °C and centrifuged at 10,000*g* for 10 min.

The supernatant containing the VP1 protein was subjected to 20% sucrose cushion centrifugation at 100,000*g* for 3 h at 4 °C. The pellet was resuspended with reassociation buffer and purified by CsCl gradient centrifugation. The peak fraction of VP1 (density 1.29 g/ml) was then collected and dialyzed against the reassociation buffer. The morphology of VLP, which has a diameter of 45 nm, was confirmed by electron microscopy. VLPs were quantified by HA assay, as described previously [12].

Preparation of cell membrane fraction

IMR-32 cells were harvested, washed with PBS, and centrifuged for 3 min at 1000*g*. The cells were suspended in 5 volumes of a solution containing 0.25 M sucrose and 20 mM Hepes (pH 7.5), and disrupted with a glass Dounce homogenizer. The nuclei and unbroken cells were removed by centrifugation for 7 min at 1000*g*. The supernatant was transferred to a new centrifugation tube and pelleted at 105,000*g* for 1 h in a Hitachi P70AT rotor at 4 °C. The pellet was resuspended with 3 ml of a buffer containing 2.1 M sucrose and 20 mM Hepes (pH 7.5), and purified by sucrose gradient centrifugation. The peak fraction of the membrane fraction was collected and centrifuged for 1 h at 105,000*g*. The pellet was resuspended with 1 ml of a buffer containing 20 mM Tris–HCl (pH 7.5), 400 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 1 mM PMSF in the presence of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM DTT) and incubated at 4 °C for 30 min. The protein concentration was quantified by Bio-Rad (Hercules, CA) assay using bovine serum albumin as a standard.

Production of monoclonal antibodies against IMR-32 cell-membrane protein

BALB/c mice were immunized five times with 100 μg IMR-32 cell membrane fraction, emulsified in a Freund's complete adjuvant (Sigma) for primary immunization and in a Freund's incomplete adjuvant (Sigma) for four further immunizations at 7-day intervals. Dot-blot analysis and enzyme-linked immunosorbent assay (ELISA) were performed to confirm the immunoreactivity of sera against the IMR-32 cell membrane fraction. The mice that showed recognition of IMR-32 cell membrane fraction were boosted with a final immunization, and 4 days later the spleen cells from immunized animals were fused to P3U1 myeloma cells to produce approximately 600 hybridoma-containing wells.

Development of the immunoscreening system

We developed an immunoscreening system to isolate a monoclonal antibody that recognizes the JCV receptor from antibodies raised against IMR-32 cell membranes (Fig. 1A). As it is difficult to obtain sufficient amount of native JCV, we used VLPs instead of the native virion for immunoscreening.

A 96-well microtiter plate (Nalgen Nunc International, Rochester, NY) was coated with purified IMR-32 membrane fraction (1 μg/well) in 100 mM sodium bicarbonate buffer (pH 9.5). As a negative control, the wells were coated with BSA or left uncoated. Non-specific binding was blocked with PBS (pH 7.4) containing 25% Block Ace and 0.05% NaN_3. Aliquots of 200 μl of supernatant from each hybridoma were loaded onto each membrane-coated well, and the microtiter plate was incubated overnight at 4 °C. After washing three times with PBS, a solution of purified VLPs (5000 HA) was added to each well, followed by incubation at 37 °C for 1 h. The unbound VLPs were washed out with PBS. The plates were then incubated with a 1:1000 dilution of anti-VP1 antibody in EC buffer (20 mM phosphate buffer, pH 7.0, containing 400 mM NaCl, 2 mM EDTA, 10% Block Ace, 0.2% BSA, 0.05% NaN_3, and 0.075% Chaps) at room temperature for 2 h. After washing with PBS, a 1:5000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin (Bio Source International, Camarillo, CA) in C buffer (20 mM phosphate buffer, pH 7.0, containing 400 mM NaCl, 2 mM EDTA, 10% Block Ace, 0.2% BSA, and 0.1% thimerosal) was added, and the plates were incubated for 3 h at room temperature. After washing three times, 100 μl of TMB reagent (Pierce Chemical, Rockford, IL) was added and developed for 15 min before quenching with an equal volume of 1 M phosphate buffer. The absorbance was measured at 450 nm with a microplate reader (Bio-Rad, Laboratories, Hercules, CA). In this system, antibodies that recognize VLP binding protein will inhibit binding of VLP to the membrane-coated well, while antibodies that do not recognize VLP binding protein will permit binding of VLP to the well.

To confirm the specificity of VLP binding to the IMR-32-coated wells, we examined the binding kinetics. Initially, we determined how much VLP was appropriate for detection of bound VLPs to the membrane-coated wells. The amounts of VLPs bound to the wells increased in a dose-dependent manner from 500 to 1×10^4 HAU, and reached saturation at a concentration of 1×10^5 HAU in both 0.5 and

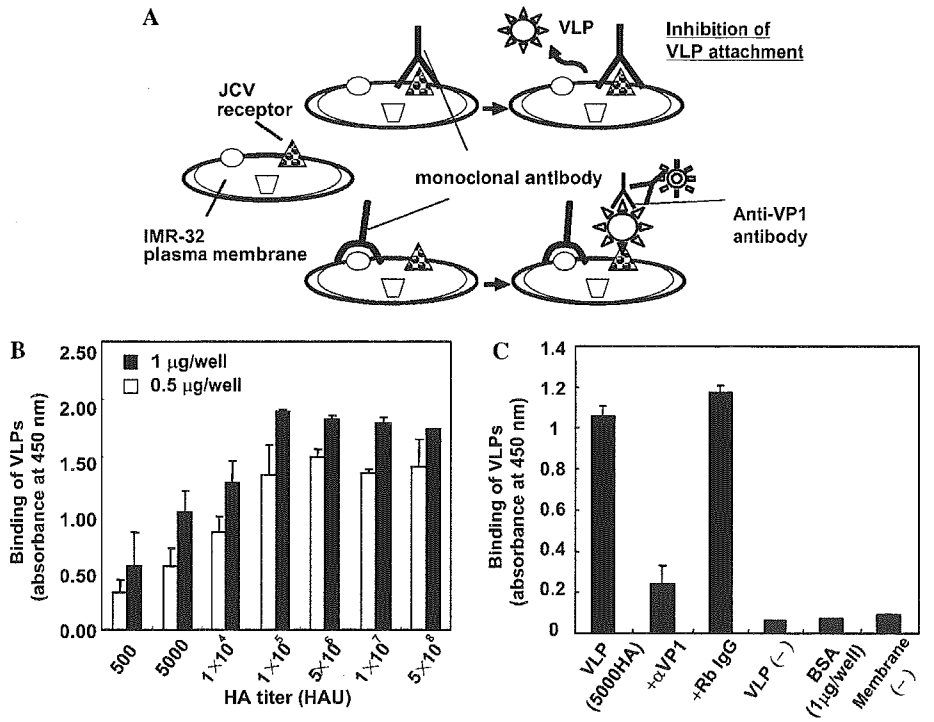

Fig. 1. Development of the immunoscreening system. (A) Strategy of the immunoscreening system using a blocking antibody against JCV infection. BALB/c mice are immunized with the membrane fraction from the human neuroblastoma cell line, IMR-32 cells. The wells of a 96-well plate are coated with the membrane fraction of IMR-32 cells, and then incubated with monoclonal antibodies derived from immunized mice before addition of VLPs. The amount of bound VLPs to the IMR-32 membrane fraction coated onto the wells is quantified with anti-VP1 antibody, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulins and TMB reagent. Absorbance was measured at 450 nm with a microplate reader. (B) The kinetics of binding of VLPs to IMR-32 membrane fractions. The amount of bound VLPs to the membrane fraction increased in a dose-dependent manner and peaked in the presence of 1×10^5 HAU VLPs. (C) Specificity of binding of VLPs to membrane-coated wells. The binding of VLPs was significantly inhibited in the presence of anti-VP1 antibody that recognizes JCV viral capsid protein, VP1, but was not affected in the presence of the isotype control rabbit normal IgG (Rb IgG). Negative controls, including VLP (−), BSA, and membrane (−), did not bind to the wells. The bars represent the standard deviation of the mean of at least three independent experiments performed in duplicate.

1 μg IMR-32 membrane protein-coated wells (Fig. 1B). In addition, a relatively low level of non-specific binding was recognized as compared with the negative control, and VLP binding was inhibited significantly in the presence of anti-VP1 antibody with application of 5000 HAU VLPs (Fig. 1C). Thus, it was demonstrated that VLPs bound to IMR-32 membranes in a specific manner in this immunoscreening system.

After immunoscreening of more than 600 clones, one clone, designated as "24D2," was identified as one of the antibodies showing the greatest inhibitory effect. The subtype of 24D2 was shown to be IgM using a monoclonal antibody isotype detection kit (Amersham–Pharmacia Biotech, Piscataway, NJ). The hybridoma cells were recloned to confirm the production of a monospecific antibody and cultured in a CELLine culture system (BD Biosciences, San Jose, CA) to obtain a large amount of the antibody. The concentration of the resulting supernatant was measured by semi-quantitative immunoblotting assay using mouse immunoglobulin as a standard. The antibody was separated by SDS–PAGE on a 10% gel. After blocking with 5% skim milk/PBST for 30 min, the anti-mouse immunoglobulins were diluted 1:5000 in PBST. Immunoreactive bands were detected by ECL (Amersham–Pharmacia Biotech) and analyzed with LAS-1000 Plus image analyzer (Fuji Film, Tokyo, Japan). The concentration of 24D2 was determined as the intensity relative to that of the control antibody.

Inhibition of VLP binding to IMR-32 cells by 24D2

For conjugation of purified VLPs with fluorescein isothiocyanate (FITC), aliquots of 2 mg of purified VLPs were dissolved in 0.1 M carbonate–bicarbonate buffer (pH 9.0), mixed with 156 μg FITC (Sigma), and incubated at room temperature for 2 h. To eliminate unlabeled FITC, the samples were centrifuged at 100,000*g* for 1 h at 4 °C. The pellet was dissolved in PBS, and centrifuged at 10,000*g* overnight, and the pellet was resuspended in PBS.

Before the experiment, IMR-32 cells (2×10^4) were plated onto 8-microwell tissue culture chambers (Nalgen Nunc International) and cultured in DMEM containing 10% FBS. The cells were pre-incubated in the absence or presence of either 24D2 (50 μg/ml) or isotype control monoclonal antibody for 1 h at 37 °C. Subsequently, FITC-VLPs (1×10^5 HAU) diluted 1:10 with DMEM were added to each chamber for 1 h at 4 °C. Unbound FITC-VLPs were removed by washing with PBS, and incubated for 15 min at 37 °C, following observation with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Inhibition of JCV infection by 24D2

IMR-32 cells (5×10^4) were preincubated in the absence or presence of either 24D2 (300 μg/ml) or the isotype control monoclonal antibody for 1 h at 37 °C. The cells were then incubated with 512 HAU JCV in fresh medium with 2% FBS for 1 h at 37 °C. After washing twice with fresh medium, the cells were incubated at 37 °C for 4 days with DMEM supplemented with 2% FBS. The monolayer culture in each well was washed with PBS and lysed in 1% Triton X-100/TBS containing 2 μg/ml aprotinin. The cell lysates were centrifuged at 4 °C for 10 min at 15,000 rpm, and the supernatants were separated by SDS–PAGE on 12% gels. After transferring the separated proteins onto a PVDF membrane, the membrane was immersed in 5% skim milk/TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 30 min, and subsequently incubated with either anti-agnoprotein antibody or anti-T-antigen antibody diluted 1:1000 with TBS-T for 1 h. After washing twice with TBS-T, the membrane was incubated with HRP-conjugated $F(ab')_2$ goat anti-rabbit or HRP-conjugated $F(ab')_2$ goat anti-mouse immunoglobulins diluted 1:5000 in TBS-T for 1 h. Immunoreactive bands were detected using the ECL reagent (Amersham–Pharmacia Biotech) and analyzed using an LAS-1000 plus image analyzer. Expression of viral proteins in the cells is represented as intensity relative to that of the control sample without treatment with 24D2 or isotype control antibody. The data are presented as mean values ± SD of three independent experiments.

Analysis of the molecule recognized by 24D2

Immunoblotting. Immunoblotting was performed as described above with the following modifications. The purified IMR-32 membrane fractions were separated by SDS–PAGE on 10% gels, transferred onto PVDF membranes, and immunoblotted with the 24D2 antibody. Immunoreactive bands were analyzed as described above.

Immunocytochemistry. IMR-32 cells grown on 8-microwell chambers were fixed with methanol at −80 °C for 2 min and rehydrated in PBS for 5 min at room temperature. After incubation with blocking solution (PBS containing 3% BSA), cells were incubated with 24D2 antibody diluted 1:60 in blocking solution for 2 h. After washing three times with PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgM antibody (Molecular Probes, Eugene, OR) diluted 1:500 for 1 h, and observed using a laser scanning confocal microscope (Olympus).

Immunohistochemistry. Normal human brain tissue obtained at autopsy was sectioned at a thickness of 10 μm with a Cryo 2000 cryostat (Sankyo, Tokyo, Japan) and fixed in acetone at 4 °C for 10 min. The sections were rinsed in PBS, blocked in 0.3% H_2O_2 methanol for 15 min, and incubated with 24D2 at 4 °C overnight. After incubation with biotinylated second antibody, immunoreactive products were visualized with 3,3′-diaminobenzidine. For double immunostaining of human brain tissue, the sections were rinsed twice in PBS, preincubated in PBS containing 3% BSA for 60 min, and incubated with anti-glial fibrillary acidic protein (GFAP, 1:5 dilution), anti-carbonic anhydrase-II (CA2, 1:1,000 dilution), or anti-synaptophysin (1:100 dilution) together with 24D2 (1:5) overnight. The sections were incubated with the appropriate secondary antibodies, including Alexa Fluor 488-conjugated goat anti-rabbit IgG, biotinylated anti-sheep IgG, Alexa Fluor 488-conjugated streptavidin, Alexa Fluor 488-conjugated goat anti-mouse IgG, or Alexa Fluor 594-conjugated goat anti-mouse IgM (Molecular Probes). After rinsing with PBS for 30 min, the sections were observed by laser scanning confocal microscopy.

Results

Isolation of monoclonal antibody (24D2) which inhibits attachment of JCV

Among more than 600 clones of monoclonal antibodies against the cell membrane fraction of JCV-permissive human neuroblastoma cell line IMR-32 cells, one hybridoma clone was isolated using the immunoscreening system and was designated as "24D2" (Fig. 1A). 24D2 significantly inhibited the binding of VLPs (Fig. 2A) as well as JCV (Fig. 2B) to cell membrane fractions on the immunoplates, whereas an isotype control antibody (mouse IgM) showed no inhibitory effect (Figs. 2A and B).

The 24D2 inhibits attachment and entry of FITC-labeled VLPs into IMR-32 cells

To evaluate the inhibitory effects on attachment and entry of VLPs, which have functions in cellular attachment and entry, similar to native JCV infection

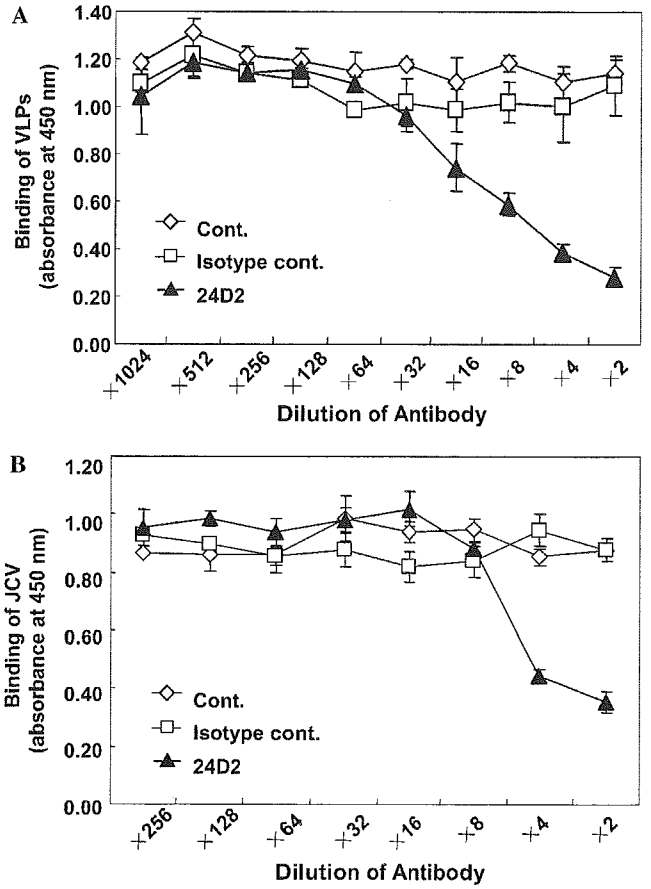

Fig. 2. Inhibition of the binding of both VLPs and JCV to IMR-32 membrane fractions on the immunoplate system by 24D2. (A,B) Inhibition of JCV and VLP binding by 24D2. IMR-32 membrane fractions were coated onto each well of the immunoplates, which were preincubated with serial dilutions of 24D2 (▲), mouse IgM (isotype control antibody, □), or without antibody (◇), and then incubated with 5000 HAU of VLPs (A) or JCV (B). Binding of VLPs or JCV was detected with anti-VP1 antibody and presented as the absorbance value. The bars represent the standard deviation of the mean of at least three independent experiments performed in duplicate.

[2,6] into IMR-32 cells, the cells were pretreated in the presence of either 24D2 or the isotype control monoclonal antibody, and incubated with FITC-labeled VLPs. 24D2 completely inhibited cellular attachment and entry of FITC-VLPs in IMR-32 cells, while the isotype control antibody had no effect on attachment or entry (Fig. 3).

Inhibition of JCV infection to IMR-32 cells by 24D2

To determine the blocking activity of 24D2 against JCV infection, IMR-32 cells were incubated with 24D2 before JCV inoculation. Four days after inoculation, expression of the viral proteins, including early and late viral proteins, T-antigen, and agnoprotein, was examined by immunoblotting. Treatment with 24D2 markedly inhibited expression of both T-antigen and agnoprotein in JCV-infected cells (Figs. 4A and B). The inhibitory effect of 24D2 against JCV infection was dose-dependent (Fig. 4C). Thus, we demonstrated that 24D2 possessed inhibition activity against JCV infection, suggesting that the molecule recognized by 24D2 plays a role as a cellular receptor for JCV.

Characterization of the molecule recognized by 24D2

We further characterized the molecule recognized by 24D2 by immunoblotting. 24D2 clearly recognized a single band with a molecular weight of around 60 kDa in membrane fractions from IMR-32 cells (Fig. 5A). Immunoblotting was also performed using various cell lines, including, HEK-293, HeLa, COS7, and SVG-A. In HEK293 cells, 24D2 recognized a single band with the same molecular weight as that of IMR-32 cells. However, in HeLa, COS7, and SVG-A cells, positive signals were observed around 30 kDa instead of 60 kDa (Fig. 5B).

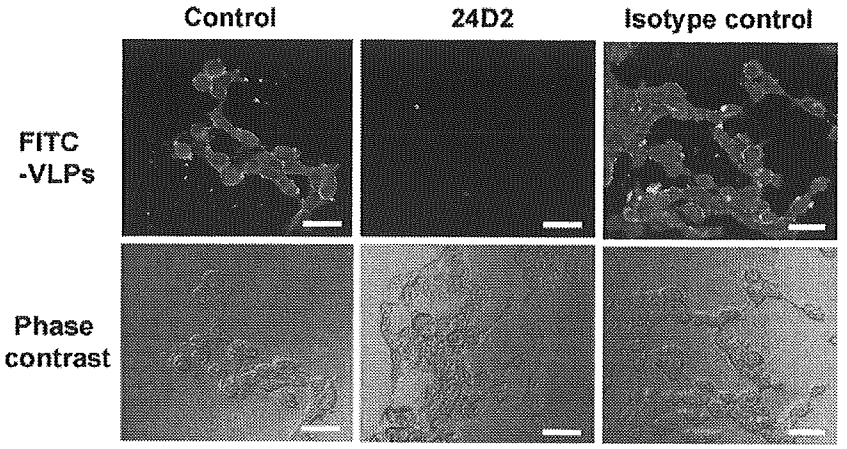

Fig. 3. Inhibitory effect of 24D2 on attachment and entry of FITC-labeled VLPs (FITC-VLPs) into IMR-32cells. After 1-h incubation with either 24D2 or the isotype control antibody, IMR-32 cells were incubated with FITC-VLPs. The immunofluorescence signal of FITC-VLPs was observed by confocal microscopy. No FITC signal was observed in cells treated with 24D2. Bars, 20 μm.

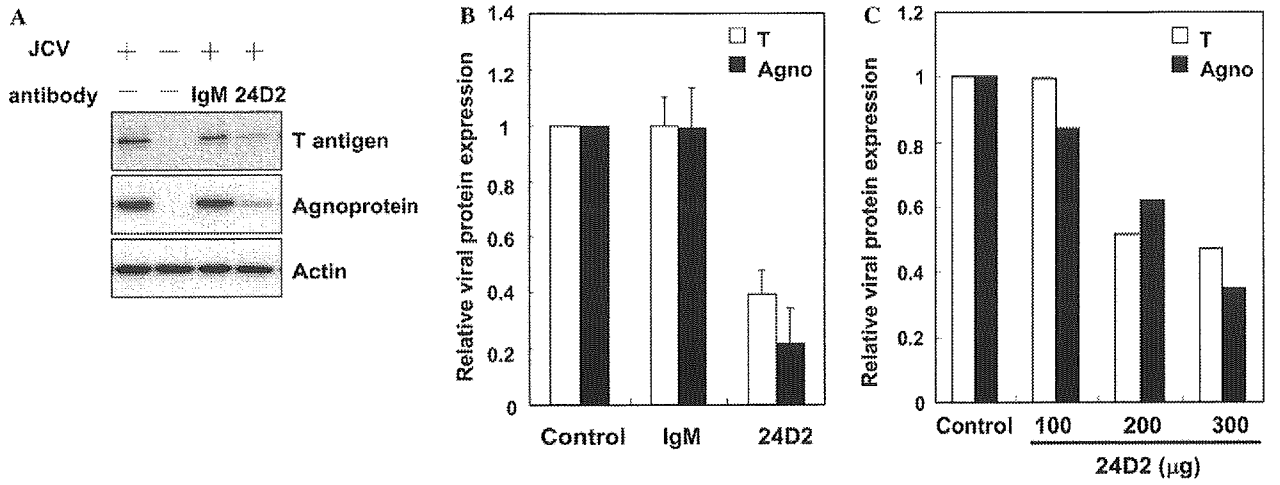

Fig. 4. Inhibition of JCV infection by treatment with 24D2. IMR-32 cells pretreated in the absence or presence of 24D2 or the isotype control antibody (IgM) were inoculated with 512 HAU of JCV for 1 h. Four days after inoculation, the cells were harvested and analyzed with immunoblotting using specific antibodies for JCV proteins (large T-antigen and agnoprotein). (A) Representative immunoblotting data with antibodies for large T-antigen and agnoprotein. Anti-actin antibody was used as a loading control. (B) Expression levels of viral proteins in IMR-32 cells treated with 24D2 or the isotype control antibody (IgM) relative to non-treated IMR-32 cells (control) at 4 days after inoculation of JCV. The bars represent mean values ± SD of at least three independent experiments. (C) The dose-dependent inhibition of JCV infection by 24D2. IMR-32 cells were incubated with different concentrations of 24D2 and incubated with 512 HAU JCV. JCV T-antigen and agnoprotein expression in IMR-32 cells was analyzed by immunoblotting 4 days after inoculation. The data are presented as amounts of viral protein expression relative to that of control infection. The assays were performed at least twice independently.

Next, we investigated the subcellular localization of positive signals with 24D2 in IMR-32 and HEK-293 cells by immunocytochemistry. The immunopositive signals for 24D2 were detected mainly on the cell surface and in the intracytoplasmic compartment (Fig. 5C). We performed the immunocytochemical examination using HeLa, COS, and SVG cells. In these cells, the immunopositive signals against 24D2 were also recognized in the cell surface and in the cytoplasm (data not shown).

The molecule recognized by 24D2 is localized mainly in glial cells in the human brain

Recently, it has been reported that JCV receptor-type α2–6-linked sialic acid was localized in oligodendrocytes and astrocytes but not in cortical neurons [13]. Although the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and intestinal tract, the major target cells of JCV infection in the CNS are glial cells, such as oligodendrocytes and astrocytes [14,15]. Therefore, we investigated the subcellular localization of the molecule recognized by 24D2 in the human brain. Positive signals for 24D2 were distributed in the glial cells of the cerebrum and cerebellum (Fig. 6A). To confirm the cellular distribution of the immunopositive signals, we performed double immunofluorescence staining using 24D2 and cell-specific markers, such as CA2, as a marker of oligodendrocytes [16–19], GFAP, a marker of astrocytes, and synaptophysin, a neuronal marker (g–i) (Fig. 6B). The immunopositive signals of 24D2 were colocalized with GFAP and CA2 (Figs. 6Ba–f). However, 24D2 failed to react with synaptophysin-positive neurons (Fig. 6Bg–i). These results indicated that the molecule recognized by 24D2 is distributed mainly in glial cells in the human brain.

Discussion

Virus attachment to cell-surface molecules is an initial event in the process of virus infection. Many viruses utilize various receptors for attachment and entry into cells. The polyomavirus, simian vacuolating virus 40 (SV 40), binds to the MHC class I molecule at the cell surface [20], but the MHC molecule is not internalized into the cells together with SV 40 [21]. The ganglioside GM1 also binds to SV 40, and is mediated to transport the virus from the plasma membrane to the endoplasmic reticulum (ER), and a role of GM1 as a functional receptor has been suggested [22]. The mouse polyomavirus (PyV) is also known to bind to the sialic acid of an as yet unidentified receptor [23]. Efforts to identify the receptor by screening for monoclonal antibodies that can protect cells from infection have been unsuccessful [24]. It has been reported that an antibody to α4β1 integrin partially inhibits PyV infection [8]. Therefore, the integrin has been suggested to act as an entry receptor in the early stages of PyV infection in fibroblasts, and PyV utilizes gangliosides as carriers from the plasma membrane to the ER [22]. Interestingly, SV40 and PyV have been shown to use clathrin-independent and caveolin-

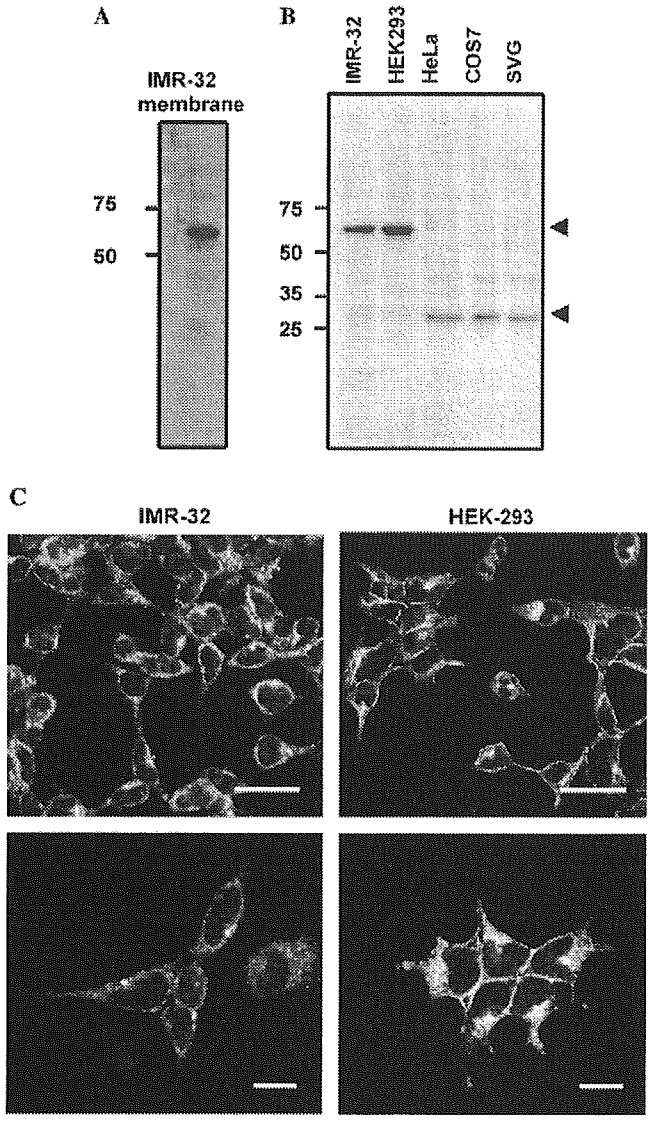

Fig. 5. Characterization of the molecule recognized by 24D2. (A) Immunoblotting analysis with membrane extracts derived from IMR-32 cells. Aliquots of 20 μg of the detergent-solubilized IMR-32 membrane fraction were loaded and immunoblotted with 24D2 at a dilution of 1:60. (B) Immunoblotting analysis with various cell lysates. The membrane fractions were prepared from IMR-32, HEK293, COS7, HeLa, and SVG-A cells. Molecular size markers are indicated on the left of the column. (C) Subcellular localization of the molecule recognized by 24D2 in IMR-32 and HEK-293 cells. Methanol-fixed cells were incubated with 24D2 at a dilution of 1:60, following Alexa Fluor 488-conjugated goat anti-mouse IgM. The immunofluorescence signal is represented as a green color. The lower panels show higher magnifications of the upper panels. Bars, 20 μm (upper panels) and 10 μm (lower panels).

dependent mechanisms to infect cells, respectively [25–27]. In contrast, JCV enters host cells by receptor-mediated clathrin-dependent endocytosis [28]. Furthermore, it has been reported that JCV does not share receptor specificity with SV 40 on human glial cells, because anti-class I antibodies failed to inhibit JCV infection [6].

It has been reported that a sialic acid-containing glycoprotein is one of the receptors of the human polyomavirus JCV in human glial cells, as neuraminidase treatment suppressed JCV attachment to the cells and subsequent infection [6].

In the present study, we have shown that the monoclonal antibody 24D2 inhibits JCV infection in IMR-32 cells. Although the major targets of JCV infection in the central nervous system are the glial cells, the viral genome was detected in various organs, such as the kidney, lymphoid tissue, lung, liver, and gastrointestinal tract [29–31], and JCV can enter a wide variety of cell types [5]. The 60-kDa molecule recognized by 24D2 was detected not only in IMR-32 cells, but also in other cell lines, including the human kidney cell line, HEK293. In addition, 24D2 showed inhibition of cellular attachment and entry of FITC-VLP to HEK293 cells (data not shown). These results suggest that the molecule recognized by 24D2 also acts as a receptor in IMR-32 and HEK293 cells. As 24D2 failed to completely inhibit infection, additional molecules might participate in JCV attachment and infection.

It is interesting to note that JCV has been suggested to persistently infect the kidney, because JCV genome was detected in the urine and the renal tissue of healthy individuals [32,33]. 24D2 also recognized a molecule of 30 kDa in the membrane fractions of HeLa, COS7, and SVG-A cells. This smaller molecule might be an isotype or a proteolytic product of the larger molecule. It has been reported that a monoclonal antibody against the hyaluronan receptor recognized two distinct receptors in the cell membranes of rat liver sinusoidal endothelial cells, which were identified previously as hyaluronan-binding proteins of 175 and 300 kDa [34,35]. After reduction of disulfide bonds, the 175-kDa hyaluronan receptor was shown to be a single protein, whereas the 300-kDa molecule consisted of three subunits, i.e., α (269 kDa), β (230 kDa), and γ subunits (97 kDa) [35], and it has been reported that the 175-kDa receptor was derived from the 300-kDa receptor by proteolytic processing [36]. Similar to the hyaluronan receptors, the molecules of 30 and 60 kDa recognized by 24D2 might be isoreceptors for the same ligand or products derived from a larger receptor complex. It has been reported that the receptor with 30-kDa molecular weight for human coronavirus OC43 was isolated from the newborn mouse brain membrane fractions [37]. A 30-kDa molecule detected in HeLa, COS7, and SVG cells might be similar to a coronavirus OC43 receptor.

We also examined the localization of the putative receptor molecule in the human brain, because JCV infects mainly glial cells in the central nervous system. Positive immunoreactivity for 24D2 was colocalized with CA2-positive oligodendrocytes and GFAP-positive astrocytes, but failed to colocalize with synaptophysin-positive neurons. These findings indicated that the putative receptor molecule for JCV was localized mainly in the glial cells of the human brain. In the present study,

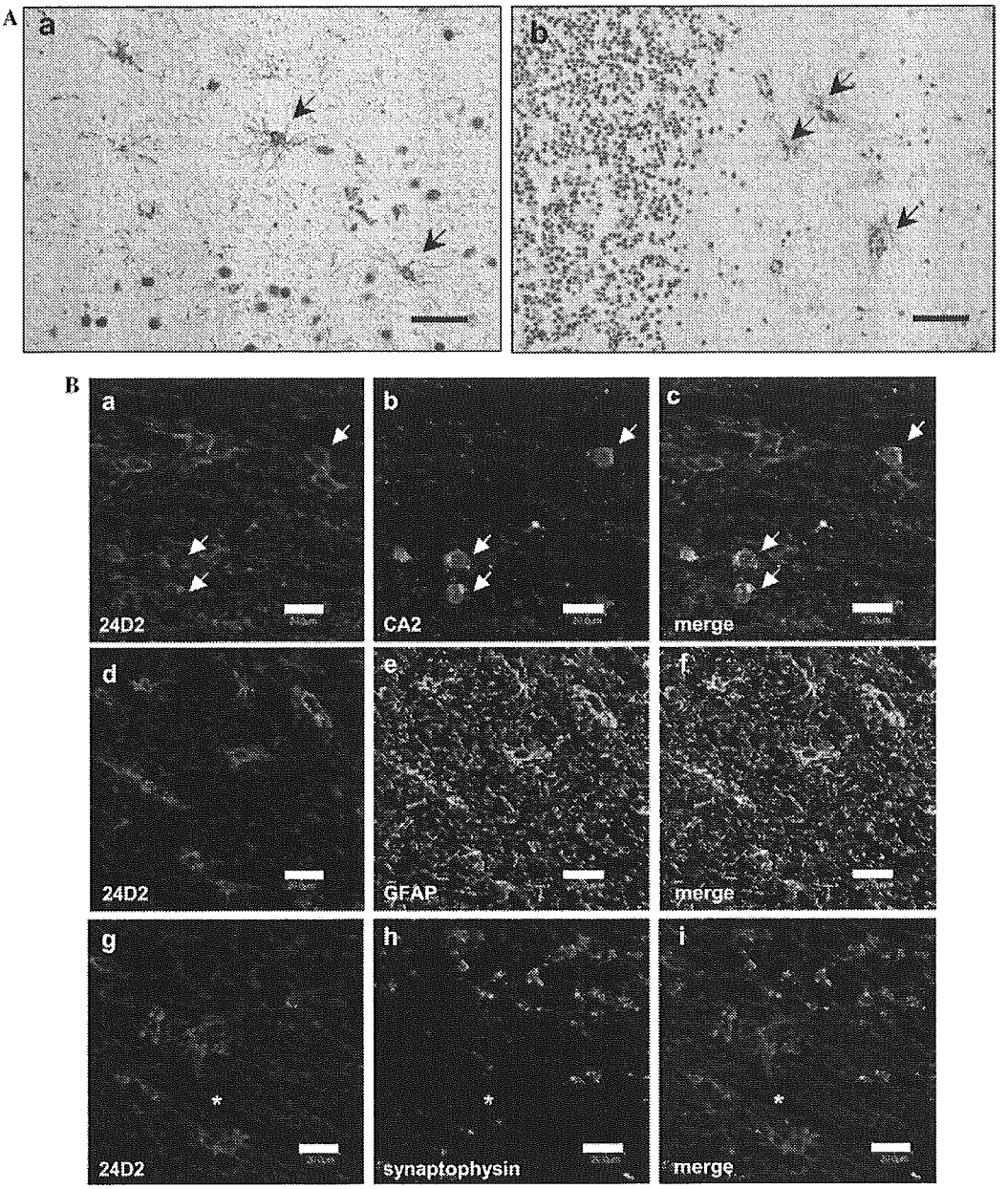

Fig. 6. Subcellular localization of the molecule recognized by 24D2 in the human brain. (A) Immunoreactivity with 24D2 in the cerebrum (a) and the cerebellum (b). Arrows indicate the immunopositive cells (a,b) in the white matter. Scale bars, 50 μm. (B) Double immunostaining using 24D2 and anti-CA2 antibody (a–c), anti-GFAP antibody (d–f), or anti-synaptophysin antibody (g–i) in the cerebrum. The merged images show that the molecule recognized by 24D2 (red color) was colocalized with CA2 and GFAP (green color). However, colocalization was not detected between 24D2 and anti-synaptophysin signals. Arrows indicate the 24D2- and CA2-double positive cells (a–c). Asterisks indicate white matter (g–i). Scale bars, 20 μm.

the neuroblastoma cell line, IMR-32, was used as the immunogen for the monoclonal antibody because these cells are highly susceptible to JCV infection [38]. Furthermore, immunization of IMR-32 cell has been shown to produce an antibody that specifically recognizes a molecule in oligodendrocytes [39]. Thus, it was suggested that IMR-32 cells possess molecules in common with brain glial cells. In Fig. 6Aa and b, the immunopositive signals against 24D2 were less than those of Fig. 6Ba, d, and g. We suppose that the difference of oligodendrocyte 24D2 immunostaining patterns among Fig. 6A and B might be due to the applied immunostaining systems. In Fig. 6A, we applied the biotinylated antibody as a second antibody, and the sections were observed with light microscopy. Meanwhile, in Fig. 6B, we observed the sections labeled with Alexa Fluor 488-conjugated second antibodies with fluorescent microscopy.

In summary, we have isolated a monoclonal antibody against the receptor molecule for JCV. By a newly established immunoscreening system, we isolated a monoclonal antibody (24D2) that inhibits cellular attachment

and infection of JCV. We also showed that the antibody specifically recognizes a membrane molecule with a molecular weight of 60 kDa, and positive signals were observed in the glial cells of the human brain. These results suggest that the molecule recognized by 24D2 acts as a JCV receptor in JCV infection. Further studies are required to characterize and determine the functional role of the molecule. A greater understanding of the molecular characteristics of the receptor molecule is necessary for the design of novel anti-JCV agents. Experiments to identify the molecules detected by 24D2 using a λ phage library are currently in progress in our laboratory.

Note added in proof

Recently, it has been reported that the antagonists and antibodies against the 5HT2 serotonergic receptors blocked JCV infection in SVG-A cells [40], suggesting that serotonin receptors act as one of the JCV receptors.

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Identification of FEZ1 as a Protein That Interacts with JC Virus Agnoprotein and Microtubules

ROLE OF AGNOPROTEIN-INDUCED DISSOCIATION OF FEZ1 FROM MICROTUBULES IN VIRAL PROPAGATION*

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The human polyomavirus JC virus (JCV) is the causative agent of a fatal demyelinating disease, progressive multifocal leukoencephalopathy, and encodes six major proteins, including agnoprotein. Agnoprotein colocalizes with microtubules in JCV-infected cells, but its function is not fully understood. We have now identified fasciculation and elongation protein zeta 1 (FEZ1) as a protein that interacted with JCV agnoprotein in a yeast two-hybrid screen of a human brain cDNA library. An *in vitro* binding assay showed that agnoprotein interacted directly with FEZ1 and microtubules. A microtubule cosedimentation assay revealed that FEZ1 also associates with microtubules and that agnoprotein induces the dissociation of FEZ1 from microtubules. Agnoprotein inhibited the promotion by FEZ1 of neurite outgrowth in PC12 cells. Conversely, overexpression of FEZ1 suppressed JCV protein expression and intracellular trafficking in JCV-infected cells. These results suggest that FEZ1 promotes neurite extension through its interaction with microtubules, and that agnoprotein facilitates JCV propagation by inducing the dissociation of FEZ1 from microtubules.

The human polyomavirus JC virus (JCV)[1] is the causative agent of progressive multifocal leukoencephalopathy, a fatal demyelinating disease. The genome of JCV comprises a double-stranded circular DNA molecule that contains three functional regions: the viral early and late genes and the noncoding regulatory sequence (1). The early region of the JCV genome encodes the large T and small t antigens, which are responsible for the initiation of viral DNA replication and activation of late gene transcription (2–4). The late coding region encodes three structural proteins (VP1, VP2, and VP3) that are components of the viral capsid (5). In addition, the leader sequence of late transcripts encodes agnoprotein, a viral auxiliary protein that contains 71 amino acids (6).

The auxiliary proteins of various eukaryotic viruses have diverse effects on different stages of infection, including transcription (7, 8), viral assembly (9), and the release of viral particles (10, 11). They can also affect host cell functions and thereby contribute to the pathogenesis of viral-induced disease (12). The agnoprotein of simian vacuolating virus 40 (SV40), which belongs to the same polyomavirus family as does JCV, contributes to various stages of the viral lytic cycle. Mutation of the agnoprotein of SV40 was found to result in a moderate growth defect that was attributable to impairment of the viral maturation pathway (13–15). Immunofluorescence analysis revealed that SV40 agnoprotein facilitates the localization of VP1, the major capsid protein, to the nucleus and perinuclear region of infected cells (16). Furthermore, the lack of agnoprotein led to inefficient release of mature SV40 virions from infected cells and impaired the ability of the virus to propagate in monkeys (14).

In addition to SV40, JCV is closely related to other polyomaviruses, including human BK virus. These viruses exhibit marked nucleotide sequence similarity, especially in the coding regions (1). All JCV proteins with the exception of agnoprotein are localized predominantly in the nuclei of infected cells (17, 18), whereas JCV agnoprotein is largely restricted to the perinuclear region of the cytoplasm (19), as is SV40 agnoprotein (20). We previously showed that deletion of the agnogene of JCV results in a viral growth defect (21). Furthermore, a small interfering RNA (siRNA) specific for agnoprotein mRNA was found to inhibit JCV infection (22). These observations suggest that the agnoprotein of JCV, like that of SV40, plays an important role in viral propagation, although the molecular mechanism of this action remains unknown.

We have now identified fasciculation and elongation protein zeta 1 (FEZ1) as a protein that interacted with JCV agnoprotein in a yeast-two hybrid assay. FEZ1 is a brain-specific coiled-

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[1] The abbreviations used are: JCV, JC virus; FEZ1, fasciculation and elongation protein zeta 1; GST, glutathione *S*-transferase; GFP, green fluorescent protein; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; NGF, nerve growth factor; RT, reverse transcription; CC coiled-coil; Dox, doxycycline; PKC, protein kinase C; siRNA, small interfering RNA; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); His-Agno, histidine-tagged agnoprotein; MES, 4-morpholineethanesulfonic acid.

coil protein that comprises 392-amino acid residues and is expressed in neurons (23). It is related to *Caenorhabditis elegans* UNC-76, which is required for normal axonal bundling and outgrowth. We further demonstrate that agnoprotein inhibited the function of FEZ1 apparently by blocking the association of FEZ1 with microtubules, and overexpression of FEZ1 inhibited the intracellular spread of JCV. Our results suggest that agnoprotein promotes the intracellular translocation of viral particles on microtubules by inducing the dissociation of FEZ1.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—A full-length FEZ1 cDNA was amplified by the PCR from an adult human brain cDNA library and was subcloned into pCXN2-FLAG (24), pGEX6P1 (Amersham Biosciences), and pEGFP-N1 (Clontech, Palo Alto, CA); the resulting expression vectors were designated pFLAG-FEZ1 (for expression of FEZ1 with an NH_2-terminal FLAG tag), pGST-FEZ1 (for expression of FEZ1 fused at its NH_2 terminus with glutathione *S*-transferase), and pFEZ1-GFP (for expression of FEZ1 fused at its COOH terminus to green fluorescent protein), respectively. Complementary DNAs for deletion mutants of FEZ1 were generated by PCR from pFLAG-FEZ1 and subcloned into pGEX6P1. For expression of JCV agnoprotein in mammalian cells, the agnoprotein cDNA was amplified by PCR from a plasmid containing the JCV genome, pJC1->4pJCV (HSRRB, Osaka, Japan), and subcloned into either pcDNA4HisMax (Invitrogen) or the bicistronic expression vector pERedNLS (kindly provided by M. Matsuda).

Yeast Two-hybrid Assay—The yeast two-hybrid assay was performed with a Matchmaker System 3 and an adult human brain cDNA library obtained from Clontech. Yeast AH109 cells were transformed with both the brain cDNA library and a full-length cDNA for JCV agnoprotein subcloned into the yeast shuttle vector pGBKT7. Plasmids isolated from positive colonies were introduced into *Escherichia coli* DH5α and sequenced, and the DNA sequence data were compared with sequences in the NCBI data base with the BLAST program.

Cell Culture and Virus Preparation—Human embryonic kidney 293 (HEK293) cells (HSRRB), SV40-transformed human glial SVG-A cells (kindly provided by W. J. Atwood) (25), and JCV-producing (JCI) cells (26) were maintained under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin (Sigma). HEK293 cells that express JCV agnoprotein in an inducible manner (293AG cells) were established with the T-REx system (Invitrogen); agnoprotein expression was induced by exposure of the cells to doxycycline (Invitrogen) at a final concentration of 1 μg/ml. To establish 293AG cells that stably express FEZ1-GFP, we transfected 293AG cells with pFEZ1-GFP and cultured the transfectants in the presence of Geneticin (Invitrogen) at 400 μg/ml. Several clones were expanded in the selection medium, after which expression of FEZ1-GFP was confirmed by immunoblot analysis. Control 293AG cell lines were similarly established by stable transfection with pEGFP-N1. To establish SVG-A cells that stably express FLAG-FEZ1, we transfected SVG-A cells with pFLAG-FEZ1 and cultured the transfectants in the presence of Geneticin (100 μg/ml). Expression of FLAG-FEZ1 in selected cells was confirmed by immunoblot analysis. Again, control SVG-A cell lines were similarly established by stable transfection with pCXN2-FLAG. PC12 cells stably expressing either FEZ1-GFP or GFP were kindly provided by T. Fujita (27) and were maintained in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin, streptomycin, and Geneticin (400 μg/ml). For virus preparation, JC virus-infected SVG-A cells were cultured for 2 weeks, harvested, and suspended in 10 mM Tris-HCl (pH 7.5) containing 0.2% bovine serum albumin. The cells were frozen and thawed three times and then treated for 16 h at 37 °C with neuraminidase type V (Sigma) at 0.05 unit/ml. After an additional incubation for 30 min at 56 °C, the cell lysate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was assayed for JCV by a hemagglutination assay (28) and stored at −80 °C until use.

Protein Preparation—Recombinant baculovirus bearing Agno was constructed using the Gateway system (Invitrogen). Briefly, the coding region of Agno was amplified by PCR using a specific primer set 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGGATGGTTCTTCCGCCAGCTGTC-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTATGTAGCTTTTGGTTCA-3′ and pBR-Mad1 (30) as a template. PCR products were subcloned into pDONR201 (Invitrogen), following cloning into pDEST10 (Invitrogen). Recombinant baculoviruses were prepared according to manufacturer's instruction. The histidine-tagged agnoprotein (His-Agno) was purified as follows: a 500-ml culture of Sf9 cells infected with the recombinant baculovirus for 3 days was harvested by centrifugation at 500 × *g* for 10 min, and the cell pellet was resuspended in 5 volumes of the lysis buffer (600 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% (v/v) Triton X-100, 1 mM PMSF, and 0.05 mg/ml DNase) using the Teflon homogenizer. After rotation for 30 min at 4 °C, the extract was clarified by centrifugation at 24,000 × *g* for 20 min at 4 °C. The supernatant was loaded onto the nickel-chelated Cellulofine (Seikagaku Corp., Tokyo, Japan) column pre-equilibrated with the lysis buffer without PMSF and DNase, and the column was washed with same buffer. After extensively washing with the solution (600 mM NaCl, 20 mM imidazole-HCl, pH 7.5), the bound proteins were eluted with the buffer (600 mM NaCl and 200 mM imidazole-HCl, pH 7.5). The anti-Agno antibody-conjugated Sepharose resin was added into the eluate, and the mixture was rotated overnight at 4 °C. The resin was transferred into an empty column and washed with TBS containing 0.05% (w/v) Tween 20 (TBST). The bound proteins were eluted with the solution (0.1 M glycine-HCl, pH 2.8), and the elute was immediately neutralized by addition of 0.1 volume of 1 M Tris-base. The fractions containing His-Agno were pooled and dialyzed against TBS. The purified His-Agno was stored at 4 °C until use.

Primary Antibodies—Mouse monoclonal antibodies to large T antigen (Ab-2), to α-tubulin, to pan-actin (MAB1501R), and to MAP2 (clone AP-20) were obtained from Oncogene Research Products (Uniondale, NY), Sigma, Chemicon International (Temecula, CA), and Roche Diagnostics, respectively. Goat polyclonal antibodies to GST were from Amersham Biosciences. Rabbit polyclonal antibodies to JCV agnoprotein and to JCV VP1 were produced as described previously (19, 29). Rabbit polyclonal antibodies to enhanced GFP were kindly provided by N. Mochizuki. Mouse monoclonal antibodies to FLAG (M2) and horseradish peroxidase-conjugated mouse monoclonal antibodies to FLAG (M2) were from Sigma.

Transfection, Immunoblot Analysis, and Immunoprecipitation—Cell transfection was performed with Lipofectamine 2000 (Invitrogen). For immunoblot analysis, cells were harvested 24–48 h after transfection, lysed in radioimmune precipitation assay buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), and mixed with Complete protease inhibitor mixture (Roche Diagnostics) (30). The cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene difluoride filter (Millipore, Bedford, MA). The filter was incubated with primary antibodies, and immune complexes were then detected with horseradish peroxidase-conjugated secondary antibodies and ECL reagents (Amersham Biosciences). The FLAG epitope was detected directly with horseradish peroxidase-conjugated primary antibodies.

For detection of *in vivo* interaction between agnoprotein and FEZ1, 293AG cells were transfected with pFLAG-FEZ1, and, 48 h after transfection, lysed in buffer C (50 mM Hepes-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 0.05% Triton X-100, 5 mM PMSF), mixed with Complete protease inhibitor mixture, and subjected to immunoprecipitation. For detection of *in vivo* interaction between FEZ1 and tubulin, 293AG cells stably expressing FEZ1-GFP were lysed in PTN buffer (100 mM PIPES-NaOH (pH 6.3), 30 mM Tris-HCl, 50 mM NaCl, 1 mM EGTA, 1.25 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 10 mM PMSF), mixed with Complete protease inhibitor mixture, and subjected to immunoprecipitation.

Immunoprecipitation was performed by incubation of cell lysates at 4 °C first for 1 h with protein G-Sepharose FF beads (Amersham Biosciences) and then, after removal of the beads, for 4 h with antibody-coupled protein G-Sepharose FF beads. After washing with cell lysis buffer, the bead-bound proteins were subjected to immunoblot analysis.

Immunofluorescence Analysis—Cells were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS, and incubated at room temperature with 5% dried skim milk in PBS (31). For detergent extraction, cells were washed once with PBS and twice with PHEM buffer (60 mM PIPES, 25 mM HEPES, pH 6.9; 10 mM EGTA, 2 mM $MgCl_2$) before extraction with 0.2% Saponin in PHEM buffer for 3 min on ice. The detergent-insoluble cell components remaining on the coverslips were then washed with PHEM buffer and fixed with methanol for 4 min at −20 °C. The cells were then incubated first with primary antibodies and then with Alexa 488- or Alexa 594-labeled goat antibodies to rabbit immunoglobulin G or with Alexa 594-labeled goat antibodies to mouse immunoglobulin G (Molecular Probes, Eugene, OR). Nuclei were counterstained with propidium iodide (0.2 μg/ml), and the cells

were then observed with a confocal laser-scanning microscope (Olympus, Tokyo, Japan).

GST Precipitation Assay—GST fusion proteins of FEZ1 or its deletion mutants were expressed in *Escherichia coli* AD494 DE3 and purified with the use of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). For *in vitro* GST precipitation assays, GST or GST fusion proteins (50 pmol) were mixed with 10 μl of the histidine-tagged agnoprotein in a final volume of 500 μl with binding buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.5 mM PMSF) and Complete protease inhibitor mixture and then incubated for 1 h at 4 °C. After the addition of 10 μl of 50% (v/v) glutathione-Sepharose 4B, the mixture was incubated for another 3 h at 4 °C. The beads were separated by centrifugation and washed with binding buffer, and the bound proteins were subjected to immunoblot analysis with antibodies to agnoprotein.

Microtubule Cosedimentation Assay—Microtubule binding assays were performed as previously described (32–35). In brief, GST fusion proteins of FEZ1 or its deletion mutants (0.5 μg) in 50 μl of a MES-based buffer (100 mM MES-NaOH (pH 6.8), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM GTP) supplemented with 50 μM Taxol (Sigma) were centrifuged at 100,000 × *g* for 1 h at 25 °C. Purified tubulin (Sigma) at 5 mg/ml in the MES-based buffer was polymerized by incubation for 30 min at 37 °C in the presence of 50 μM Taxol and GFP at a final concentration of 2.5 mM, and the polymerized microtubules were separated by centrifugation at 100,000 × *g* for 30 min at 25 °C. The supernatants containing the GST fusion proteins were then incubated with the polymerized microtubules (50 μg) for 30 min at 37 °C, after which the mixtures were each layered on top of 100 μl of 30% sucrose in the MES-based buffer and centrifuged at 30,000 × *g* for 30 min at 25 °C. The resulting supernatants and pellets (washed once with the MES-based buffer) were subjected to immunoblot analysis.

For examination of the interaction between agnoprotein and microtubules, JCI cells were lysed in PTN buffer supplemented with Complete protease inhibitor mixture, and the cell lysate was centrifuged at 100,000 × *g* for 30 min at 25 °C. The resulting supernatant (60 μl containing 100 μg of protein) was added to a pellet of polymerized microtubules (50 μg) and incubated for 30 min at 37 °C in the presence of 50 μM Taxol. Samples were then layered over 100 μl of 30% sucrose in the MES-based buffer and centrifuged at 30,000 × *g* for 30 min at 25 °C. The resulting supernatants and pellets (washed once with the MES buffer) were subjected to immunoblot analysis.

Assay of Neurite Extension in PC12 Cells—PC12 cells stably expressing either FEZ1-GFP or GFP were transfected with pERedNLS-agno or the empty vector. At 24 h after transfection, the cells were incubated for 4 h in serum-free medium and then maintained for 48 h in serum-free medium supplemented with nerve growth factor (NGF) (Sigma) at a concentration of 50 ng/ml. The cells were washed with PBS, fixed with 3% paraformaldehyde at room temperature for 10 min, and then washed three times with PBS. Cells expressing agnoprotein were identified on the basis of their DsRed-positive nuclei. A change in cell morphology characterized by neurite outgrowth, cell flattening, and an increase in the size of the cell body was defined previously (27, 36, 37).

RT-PCR Analysis—For RNA extraction, cells grown in 6-well plates were washed once with PBS and harvested with trypsin-EDTA. Total RNA was isolated with the use of an RNeasy Mini kit (Qiagen, Valencia, CA), and portions (500 ng) were treated with 2 units of DNase I (Invitrogen) for 1 h at 37 °C in a 10-μl reaction mixture before incubation with 2.5 mM EDTA for 15 min at 65 °C. The treated RNA (4 μl) was subjected to RT with a Superscript first-strand synthesis system (Invitrogen). The absence of contamination with genomic DNA was verified by the addition of RNase-free water instead of Superscript II reverse transcriptase as a negative control for each sample. PCR was performed in a final volume of 50 μl containing cDNA, Gene *Taq* universal buffer, 2.5 mM of each deoxynucleoside triphosphate, Gene *Taq* polymerase (Nippon Gene, Tokyo, Japan), and primers specific either for FEZ1 (5′-CACTGGTGAGTCTGGATG-3′ and 5′-CGAGGTCCTCCATGGACTTGAAG-3′) or for β-actin (5′-TTGCCGACAGGATGCAGAA-3′ and 5′-GCCGATCCACACGGAGTACT-3′). The reaction mixtures were incubated at 94 °C for 1 min and then subjected to 38 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s with a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). All reactions were confirmed in at least three independent experiments.

siRNA Preparation—The following stealth RNA duplexes were synthesized by Invitrogen: FEZ1–301 sense, 5′-GAGAAGCUCAAUGUCUGCUUUCGGA-3′ and antisense, 5′-UCCGAAAGCAGACAUUGAGCUUCUC-3′; FEZ1–352 sense, 5′-GCUCCCGUGAAGAACCAGUUACAGA-3′ and antisense, 5′-UCUGUAACUGGUUCUUCACGGGAGC-3′; Scramble sense, 5′-GCAUCGUACAGACAAUCUUCAGUUU-3′ and antisense, 5′-AAACTGAAGAUUGUCUGUACGAUGC-3′.

Virus Inoculation and siRNAs Transfection—SVG-A sells were grown to 50% confluence on a 6-well plate and incubated with 1000 hemagglutination units of JCV in Dulbecco's minimal essential medium containing 2% fetal bovine serum for 24 h, At 24 h post inoculation of JCV, transfection of siRNA (100 pmol) were performed with Lipofectamine 2000 (Invitrogen) to the JCV-infected SVG-A cells. The cells were harvested and analyzed by Western blot analysis at 96 h post transfection. The results were confirmed by at least three independent experiments.

RESULTS

Identification of FEZ1 as an Agnoprotein-binding Protein—We first performed a yeast two-hybrid assay with full-length JCV agnoprotein as the bait to identify proteins that interact with agnoprotein. Several positive clones were isolated from a human brain cDNA library and were found to encode a portion of FEZ1 that contains the three poly-glutamic acid regions and the coiled-coil (CC) domain (Fig. 1*A*). The largest cDNA clone encoded amino acids 32–392 of human FEZ1.

To examine the interaction between agnoprotein and FEZ1, we established a cell line, designated 293AG, that was derived from HEK293 cells and that expresses JCV agnoprotein (tagged with the hexahistidine and Myc epitopes at its NH_2 terminus) under the control of a tetracycline-responsive promoter. All 293AG cells expressed the recombinant agnoprotein (molecular size, ~14 kDa) within 3 h of exposure to doxycycline (Dox). In the absence of Dox, we failed to detect agnoprotein in the cells by immunoblot or immunocytofluorescence analysis (data not shown). Immunoprecipitation and immunoblot analysis of 293AG cells transfected with an expression vector for FLAG epitope-tagged FEZ1 revealed that FLAG-FEZ1 coprecipitated with agnoprotein (Fig. 1*B*).

The subcellular localization of exogenously expressed agnoprotein and FEZ1 in transfected HEK293 cells was examined by immunocytofluorescence analysis. Confocal microscopy revealed that agnoprotein immunoreactivity was present in the perinuclear region and extended into the cytoplasm in a mesh-like pattern (Fig. 1*C*). In most cells, FEZ1 was detected throughout the cytoplasm and colocalized with agnoprotein only in the perinuclear region (Fig. 1*C*, *upper panels*). However, in some cells the localization of FEZ1 is mirrored by that of agnoprotein and well colocalized with agnoprotein (Fig. 1*C*, *lower panels*). This suggests that FEZ1 was recruited to the same location as agnoprotein as a result of interactions between FEZ1 and agnoprotein.

Association of Agnoprotein with Microtubules—Agnoprotein colocalized with microtubules in the perinuclear region of JCV-infected SVG-A cells (Fig. 2*A*), consistent with our previous observations (38). To confirm the association between agnoprotein and microtubules, we performed a microtubule cosedimentation assay with lysates of JCI cells and microtubules polymerized in the presence of Taxol. Agnoprotein was indeed detected in the sedimented fraction only in the presence of microtubules (Fig. 2*B*). In contrast, the large T antigen of JCV, which was detected in the nucleus of JCV-infected cells (19), was present exclusively in the supernatant fraction even in the presence of microtubules (Fig. 2*B*), suggesting that the association of agnoprotein with microtubules is specific. However, it was not clear if agnoprotein binds directly to microtubules, because the cosedimentation assays were performed on cell extracts. To confirm the direct binding of agnoprotein to microtubules, we performed the assay using recombinant histidine-tagged agnoprotein (His-Agno) from the insect cells infected with the recombinant baculovirus encoding His-Agno cDNA (Fig. 2*C*). We incubated the His-Agno in the MES-based buffer in the absence (Fig. 2*D*, *lanes 1* and *6*) or presence of 40 μM