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Distribution and function of JCV agnoprotein

Yuki Okada,[1,2] Shuichi Endo,[1] Hidehiro Takahashi,[3] Hirofumi Sawa,[1] Takashi Umemura,[2] and Kazuo Nagashima[1,4]

[1]Laboratory of Molecular and Cellular Pathology, School of Medicine, Hokkaido University, Sapparo, Japan; [2]Laboratory of Comparative Pathology, Graduate School of Veterinary Medicine, Hokkaido University, Sapparo, Japan; [3]Department of Pathology, Institute of Infections Diseases, Tokyo, Japan; and [4]Core Research for Evolutional Science and Technology, Japan Science and Technology, Japan

JC virus (JCV), the causative agent of progressive multifocal leukoencephalopathy (PML), encodes six major proteins including agnoprotein, the function of which is unknown. To explore its function, we initially studied the expression and localization of agnoprotein in both cultured cells and PML brain using immunohistochemical methods. Employing a specific polyclonal antibody, agnoprotein was found mostly in the cytoplasm of persistently infected JCI cells and in the finely elaborated cytoplasmic processes of oligodendroglial cells in PML brain. The immunohistochemistry indicated that the cytoplasm of oligodendroglial cells was relatively well-preserved in the demyelinated foci. Agnoprotein coprecipitated with tubulin in immunoprecipitation assays and the colocalization of agnoprotein with cytoplasmic tubulin was verified by double immunostaining with confocal microscopy. Transfection of an agnogene deleted JCV Mad1 strain (Mad1$_{\Delta agno}$) into the susceptible cell line failed to produce not only agnoprotein but also VP1 and large T mRNAs, whereas the wild-type JCV Mad1 resulted in the expression of both large T and VP1 mRNAs. The cytoplasmic agnoprotein was phosphorylated and when coexpressed with GST-EGFP, was also localized in the cytoplasm. Inhibition of protein kinase A by its inhibitor H-89, however, reversed the cytoplasmic localization of agnoprotein to the nuclear compartment. Our results suggest that JCV agnoprotein may "shuttle" between the nucleus and cytoplasm in a phosphorylation-dependent manner during viral replication. *Journal of NeuroVirology* (2001) **7,** 302–306.

Keywords: JC virus; agnoprotein; immunohistochemistry; agno-deficient virus; phosphorylation

The JCV genome encodes six proteins: the oncogenic early protein, large T antigen, small t antigen, the viral capsid proteins VP1, VP2, VP3, and agnoprotein, although the presence of three additional early proteins, T'135, T'136, and T'165, has also been demonstrated (Trowbridge and Frisque, 1995). The genome for agnoprotein, which shares 58% and 54% of identity with those of SV40 and BKV, respectively, is located at the 5′ end of the JCV late region as the smallest open-reading frame, encoding a polypeptide of 71 amino acids.

The agnoproteins of SV40 and BKV have been reported to be confined to the perinuclear region and the cytoplasm of virus-infected cells (Nomura *et al*, 1983; Rinaldo *et al*, 1998). SV40 agnoprotein is not present in native virions, but it contributes to viral replication, such as in transcription of viral genes (Alwine, 1982; Hay *et al*, 1982; Hay and Aloni, 1985), translation of late viral proteins, processing of viral proteins, and assembly of virions (Stacy *et al*, 1989). Furthermore, SV40 agnoprotein facilitates nuclear translocation of the major capsid protein VP1 (Carswell and Alwine, 1986; Ishii *et al*, 1996), and it is also known as a DNA-binding protein (Jay *et al*, 1981). The agnoprotein of BKV is phosphorylated *in vivo* and may bind to several host-cell proteins

Address correspondence to Dr. Kazuo Nagashima, Laboratory of Molecular and Cellular Pathology, Hokkaido University School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapparo, 060-8638, Japan. E-mail: knagashi@med.hokudai.ac.jp

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(Rinaldo *et al*, 1998). These data suggest that the polyomavirus agnoproteins may play an important role in viral proliferation within host cells. However, there have been very few reports relating to the function of JCV agnoprotein (Safak *et al*, 2001). In this paper, we show the localization of JCV agnoprotein of JCV infected cells, reveal the transcriptional efficiency of an agnogene-deleted mutant JCV, and demonstrate the nuclear and cytoplasmic translocation of agnoprotein, depending on its phosphorylation.

Results

Intracellular localization of JCV agnoprotein

To analyze the role of JCV agnoprotein, we have generated a polyclonal antibody against agnoprotein. The specificity of the antibody was verified by Western blotting, which labeled a protein band at approximately 8 kDa. Immunohistochemical analysis using this antibody, demonstrated that JCV agnoprotein is largely located in the cytoplasm of the JCV-infected cell line, JCI (Nukuzuma *et al*, 1995). In PML brains, there were many enlarged basophilic nuclei (Figure 1a) that were labeled with VP1 and large T antibodies (Shintaku *et al*, 2000). By contrast, agnoprotein labeled the cytoplasm of infected oligodendroglia with enlarged nuclei being unstained (Figure 1b). In addition, extended cytoplasmic processes were stained in a punctate, granular, or beaded network within the regions of demyelinating lesion (Figure 1b). Occasionally, nuclear and perinuclear staining were also observed.

By double immunostaining, agnoprotein was largely detected in the cells without glial fibrillary acidic protein (GFAP), but on rare occasions, i.e., in those with GFAP, double immunostaining indicated that the astrocytes as well as oligodendrocytes expressed agnoprotein. More detailed analysis using immunoelectron microscopy confirmed that major capsid protein, VP1, is localized on the virus particles within the nuclei, whereas agnoprotein is recognized mainly within the cytoplasm. Only a small fraction was detected in the nuclei and in association with the nuclear membrane.

By immunoprecipitation and confocal microscopic observation using double immunostaining, it could be demonstrated that in the JCV-infected cells, agnoprotein was colocalized with tubulin in the cytoplasm (Okada *et al*, 2001). Punctated or beaded localization in the long, slender cytoplasmic processes was also visualized by confocal microscopy. *In vitro* mutagenesis analysis revealed that JCV agnoprotein has both nuclear localization signals (NLS) and the nuclear export signals (NES), suggesting that it might function as shuttle protein.

Effects of JCV agnoprotein on the virus transcription

Because mutation of the SV40 genome encoding the agnoprotein has been reported to result in a considerable reduction and delay in the yield of virus (Mertz *et al*, 1983; Ng *et al*, 1985), we have examined the effect of JCV agnoprotein on proliferation and propagation of the virus. For this purpose, both the agnoprotein deletion mutant ($Mad1_{\Delta agno}$) with a point mutation at the initiation codon of the protein and the wild-type ($Mad1_{WT}$) JCV DNAs were transfected into the susceptible neuroblastoma cell line IMR-32 (Akatani *et al*, 1994), and mRNA expression levels of the viral proteins by reverse transcriptase (RT)-PCR analysis were examined. Total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan), and was reverse transcribed using Superscript II (Life Technologies, Rockville, MD) according to the manufacturer's instructions. PCR amplification was carried out on the reverse-transcribed cDNA with three sets of primers. Primers are designed according to the published report (Krynska *et al*, 1999) as follows: PEP1 and PEP2 used to amplify the N-terminal region encoding the Rb pocket-binding domain of JCV T antigen (nucleotides 4,427–4,255); VP2 and VP3 used to amplify a portion of the VP1 gene (nucleotides 2,578–2,797); AG1 and AG2 used to amplify the entire agnogene (nucleotides 277–492) for both wild-type or ΔAgno JCV. Amplified DNAs were electrophoresed on 2% agarose gels and quantified using an Imaging Densitometer (Bio-Rad, Hercules, CA).

It could be shown that in the $Madl_{WT}$-transfected cells, mRNAs of T-antigen and VP1 could be detected from day 7 onward, whereas the mRNAs of these proteins were significantly decreased in the $Mad1_{\Delta agno}$-transfected cells (Figure 2). With wild-type virus, expression of T-antigen (T-Ag), agnoprotein, and VP1 increased gradually after day 14. Dense signals of agnoprotein and VP1 detected as early as day 7 could be due to technical variation. These data suggest that JCV agnoprotein may be involved in viral transcription. To investigate the role of agnoprotein in viral transcription, the promoter activities of JCV Mad1 strain were evaluated in the presence of JCV agnoprotein in the cytoplasm or nucleus. In the presence of nuclear agnoprotein, JCV promotes both early and late transactivation, suggesting that agnoprotein could be a transactivator for the virus promoter (data not shown).

Phosphorylation and translocation of JCV agnoprotein

JCV agnoprotein shares approximately 50% identity with SV40 and BKV agnoproteins, which are known to be phosphorylated (Jackson and Chalkley, 1981; Nomura *et al*, 1983). JCV agnoprotein has five potential phosphorylation sites, two of which are in consensus sequence being phosphorylated by protein

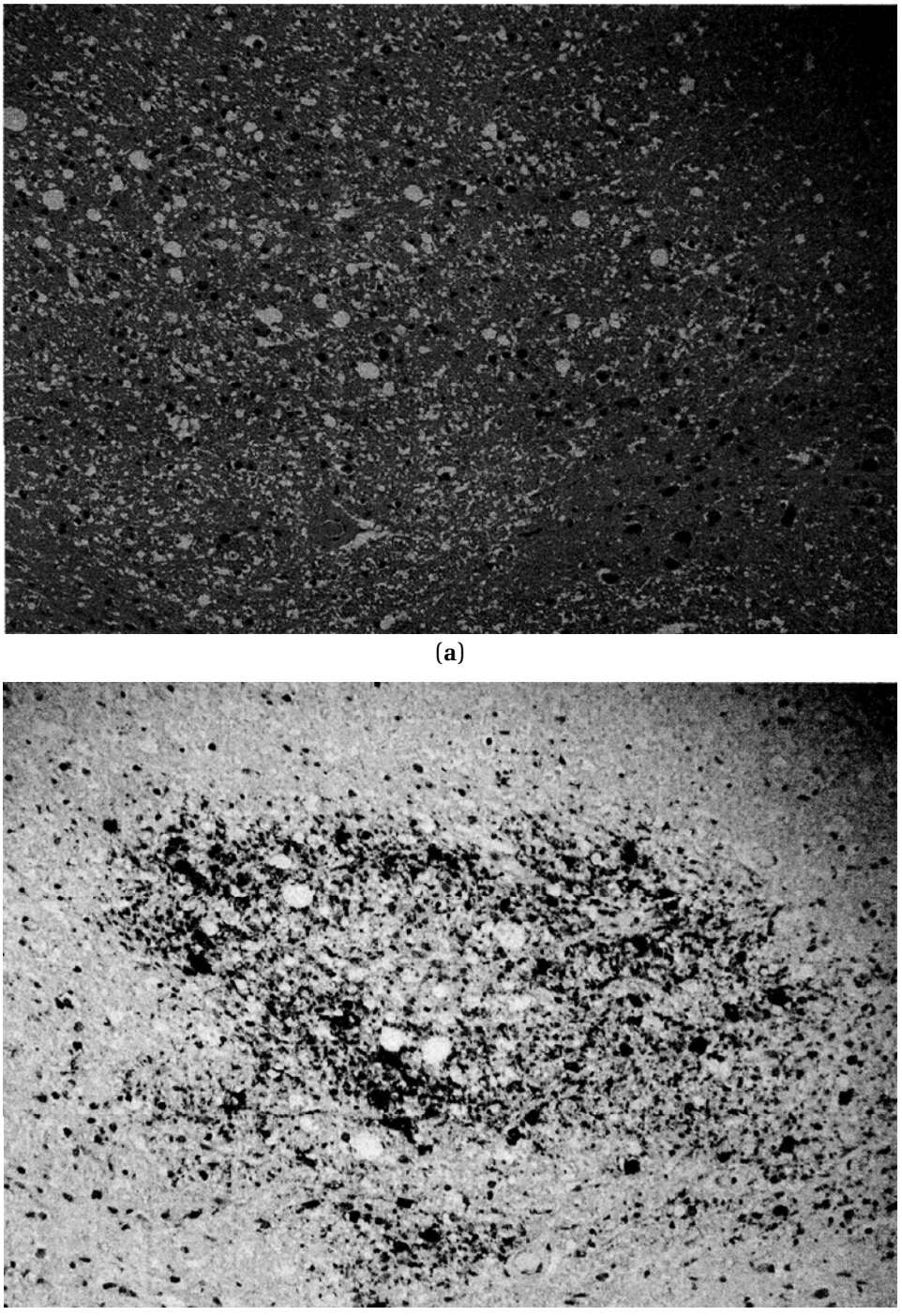

Figure 1 Photomicrograph of an early demyelinated focus of PML. Serial sections were stained with hematoxylin and eosin (**a**) and immunostained with anti-agnoprotein antibody (**b**). Presence of a network of cytoplasmic processes was demonstrated by agnoprotein immunohistochemistry, visualized as a brown color.

kinase C, and that are also conserved in SV40 and BKV. Immunoprecipitation with anti-JCV agnoprotein antibody revealed a radioactive band of approximately 8 kDa, corresponding to phosphorylated agnoprotein detected in the metabolically labeled JCV-infected cell lysates. It has been reported that phosphorylation modulates the nucleo-cytoplasmic translocation of some proteins, such as cyclin, IκBα, and several other viral proteins (Toyoshima *et al*, 1998; Johnson *et al*, 1999). To examine whether the phosphorylation is related to the translocation of agnoprotein, we first established a GST and green

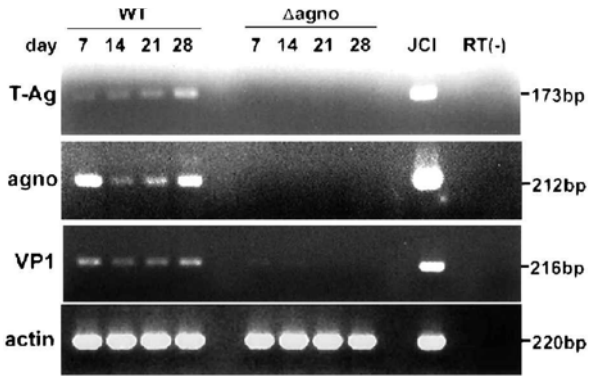

Figure 2 Effect of JCV agnoprotein on the viral propagation. RT-PCR assays for the JCV proteins after transfection with $Mad1_{WT}$ or $Mad1_{\Delta agno}$ DNA into IMR-32 cells. The analysis was performed in three independent transformants with $Mad1_{WT}$ or $Mad1_{\Delta agno}$. Note that the amplification of T-antigen (173 bp), agnogene (212 bp), and VP1 (216 bp) was observed only in $Mad1_{WT}$. Human β-actin (220 bp) was used as an internal control. JCI; the JCV-infected cell line, used as positive control. RT(**–**); a negative control, total RNA sample without reverse transcriptase treatment.

fluorescence protein-linked agnoprotein (agno-GST-EGFP) to avoid passive diffusion through the nuclear pore complex (NPC) because of its small molecular size. It was demonstrated that agno-GST-EGFP located in the cytoplasm moved to the nucleus in more than a half of cells after treatment with protein kinase A inhibitor (H-89) (data not shown). Thus, the protein kinase A-induced phosphorylation may play an important role in the translocation of agnoprotein.

Discussion

In this study, it was shown that agnoprotein is expressed, and located mainly within the cytoplasm, in a similar manner to SV40 and BKV agnoproteins. To our surprise, the presence of a network of cytoplasmic processes was first visualized in the demyelinated foci of the PML brain by agnoprotein immunohistochemistry. This suggests that the cytoplasm of the infected oligodendroglia remained intact in the demyelinated foci in spite of loss of myelin sheaths. In the cytoplasm, agnoprotein colocalized with tubulin, but it remains to be established whether agnoprotein functions to protect the cytoplasm from degeneration or to enhance cellular damage in JCV infection.

Agnoprotein binds to the large T-antigen (Safak *et al*, 2001), which is always located in the nuclei in JCV-infected cells, and was demonstrated in a few nuclei in the infected cells and PML brain. This suggests that agnoprotein may be a shuttle protein, because it contains the motifs of both NLS and NES. Because the molecular weight of agnoprotein is small enough to freely translocate between the nucleus and cytoplasm, we expressed agnoprotein as a fusion protein with GST-EGFP (agno-GST-EGFP) in the mammalian cells. Although the fusion protein was localized in the cytoplasm, agno-GST-EGFP with mutated NES motif remained in the nuclei. Similarly, mutation of an NLS motif reversed the localization of agnoprotein, demonstrating that both motifs of agnoprotein function in infected cells.

To examine the function of agnoprotein, we introduced a mutation in the initial start codon of the agnogene. In the cells transfected with agnogene-mutant viral DNA, expression of mRNAs of VP1 as well as large T were significantly inhibited in contrast to those of transfected with the wild-type JCV DNA. This suggests that agnoprotein, a leader protein of the late gene transcript, also influences the transcription of the early T-antigen. However, our system consisting of IMR-32 cell and JCI (Mad-4 type JCV) requires a long incubation time of usually up to 4 weeks. To reduce this time delay, we should use a much more rapid system of transfection and proliferation. To date, the precise role of JCV agnoprotein is unknown; however, in this study, we clearly demonstrate that agnoprotein is indispensable for virus proliferation.

According to the results of the translocation of agnoprotein in the presence of the inhibitor of protein kinase A, dephosphorylated agnoprotein may exist in the nuclei and transfer into the cytoplasm after phosphorylation. This hypothesis may explain the findings that agnoprotein is localized mainly in the cytoplasm and phosphorylated in the infected cells. It is also hypothesized that dephosphorylated agnoprotein may act as transactivator of JCV and that the phosphorylated form may play a role in preservation of the structure of the host cell in cooperation with tubulin.

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