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JC virus agnoprotein enhances large T antigen binding to the origin of viral DNA replication: Evidence for its involvement in viral DNA replication

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ABSTRACT

Agnoprotein is required for the successful completion of the JC virus (JCV) life cycle and was previously shown to interact with JCV large T-antigen (LT-Ag). Here, we further characterized agnoprotein's involvement in viral DNA replication. Agnoprotein enhances the DNA binding activity of LT-Ag to the viral origin (Ori) without directly interacting with DNA. The predicted amphipathic α-helix of agnoprotein plays a major role in this enhancement. All three phenylalanine (Phe) residues of agnoprotein localize to this α-helix and Phe residues in general are known to play critical roles in protein–protein interaction, protein folding and stability. The functional relevance of all Phe residues was investigated by mutagenesis. When all were mutated to alanine (Ala), the mutant virus (F31AF35AF39A) replicated significantly less efficiently than each individual Phe mutant virus alone, indicating the importance of Phe residues for agnoprotein function. Collectively, these studies indicate a close involvement of agnoprotein in viral DNA replication.

Introduction

The integrity and proper functioning of living cells depend on highly organized and sophisticated interactions among their macromolecules including proteins. Upon infection, viruses often hijack these interactions for their own benefit. To understand the roles of JCV agnoprotein in an infected cellular environment, it is central to identify and characterize its interaction parameters with its partners. Agnoprotein encoded by JCV late genome was previously shown to interact with a number of viral and cellular proteins, including JCV large T-antigen (LT-Ag) (Safak et al., 2001), small t-antigen (Sm t-Ag) (Sariyer et al., 2008), p53 (Darbinyan et al., 2002), YB-1 (Safak et al., 2002), FEZ1 (Suzuki et al., 2005) and HP1-α (Okada et al., 2005). JCV agnoprotein is a small (71 aa), highly basic phosphoprotein (Khalili et al., 2005; Sariyer et al., 2006) and has been implicated in many different aspects of the JCV life cycle, including viral replication (Safak et al., 2001), transcription (Safak et al., 2001), encapsidation (Sariyer et al., 2006), functioning as viroporin (Suzuki et al., 2010b) and promotion of the transport of virions from nucleus to cytoplasm (Okada et al., 2005; Suzuki et al., 2005) indicating that it is a multifunctional protein. In addition, it has also been shown to deregulate cell cycle progression in that cells which stably express agnoprotein largely accumulate at the G2/M phase of the cell cycle (Darbinyan et al., 2002). Despite these previous reports and intense research, the regulatory role of this protein in viral replication cycle has yet to be fully understood.

The agnoprotein sequences from other polyomaviruses, including SV40 and BK virus (BKV) show high identity and similarity to that of JCV agnoprotein (Safak et al., 2001). For example, there is 82% identity and 93% similarity between JCV and BKV agnoprotein sequences; and these are 60% and 79%, respectively between JCV and SV40 agnoproteins (Altschul et al., 1997). Like JCV agnoprotein, the agnoprotein of SV40 and BKV was also previously implicated to be involved in different aspects of viral replication cycle, including transcription, translation, virion production and maturation of the viral particles (Alwine, 1982; Haggerty et al., 1989; Hay et al., 1982; Hou-Jong et al., 1987; Johannessen et al., 2008; Margolskee and Nathans, 1983; Moens et al., 2011; Myhre et al., 2010; Ng et al., 1985; Unterstab et al., 2010).

Besides agnoprotein, JCV encodes a limited number of regulatory proteins, including LT-Ag, Sm t-Ag and T′-proteins (Bollag et al., 2000; Frisque et al., 1984; Saribas et al., 2010) and investigation of their regulatory roles is critically important for understanding the progression of the fatal human brain disease, progressive multifocal encephalopathy (PML). PML results from the lytic infection of oligodendrocytes, a subset of glial cells in the central nervous system (CNS), by JCV. This virus is a ubiquitous

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polyomavirus and most of the human population (70–80%) is asymptomatically infected by this virus in early childhood worldwide. The virus undergoes a latency period after initial infection and reactivation mainly occurs in patients with immunosuppressive conditions (1–5%), including in those with AIDS (Berger, 2011; Major et al., 1992). However, recently scientific community was also surprised by the emergence of PML in a very small percent of the immune disorder patients including those of multiple sclerosis (MS) and severe psoriasis (Crohn's disease) who underwent an immunosupressive immune therapy—treating patients with specific monoclonal antibodies (natalizumab and efalizumab, respectively) directed against certain cell surface receptors of T and B cells (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005; Van Assche et al., 2005). Occurrence of all these new PML cases in such patient population indicates that alterations in either arm of immune system may provide with a supporting environment for reactivation of JCV virus in certain MS and Crohn's disease patients. The rationale behind using these antibodies in MS and Crohn's disease cases was to prevent extravasation of T cells into the brain and infiltration of B cells into the layers of the skin, respectively by blocking several cell surface receptors on each cell type. It was expected to down-regulate the harmful effects of these immune cells in target organs. Natalizumab, for example, targets α4 integrin, which forms heterodimer complexes when complexed with integrin b1 (α4β1, also known as VLA-4) or with integrin β7 (α4β7) on both B and T cells. Both complexes serve as attachment ligands for vascular cell adhesion molecules (VCAM) on endothelial cells, thus preventing T cells from extravasation into the brain or gut. Another monoclonal antibody, efalizumab, binds to CD11α, an integrin molecule on B and T cells and blocks the attachment of both cell types to the intercellular adhesion molecules (ICAM) on endothelial cells and prevents their infiltration into the layers of the skin (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005; Saribas et al., 2010; Van Assche et al., 2005). As a result, treatment of T and B cells with specific antibodies appears to cause harmful effects on these immune cell populations and subsequently fosters conditions for reactivation of JCV in certain immunocompromised patients and leads to the onset of PML.

Agnoprotein is a mainly cytoplasmic protein with high concentrations accumulated around the perinuclear area of the infected cells and a small amount of it is consistently found in the nucleus of the infected cells (Safak et al., 2002). This suggests regulatory roles for this protein in the nucleus. Characterization of agnoprotein's interactions with its targets is essential for understanding of its roles in viral replication cycle. Such detailed characterizations will provide us new opportunities to develop novel therapeutics against this protein. Our previous studies characterized the interaction of agnoprotein with LT-Ag and demonstrated that agnoprotein targets the helicase domain of JCV LT-Ag (Safak et al., 2001) and does not require DNA or RNA for interaction with LT-Ag. Recently, we also showed that the JCV and SV40 with null mutants of agnoprotein cannot sustain their replication cycle although the mutant viruses were shown to be successfully released from the infected cells (Sariyer et al., 2011). It was interesting to observe, however, that the majority of the released virions were deficient in DNA content, which may explain, at least in part, why agnoprotein null mutants were unable to continue to their viral propagation cycle (Sariyer et al., 2011). More recently, we discovered that agnoprotein forms highly stable, SDS-resistant homodimers and oligomers; and the 17–42 amino acid region of agnoprotein is responsible for this property (Saribas et al., 2011). The crystal structure of JCV agnoprotein is unknown. However, 3D computer modeling studies suggests that the 17–42 amino acid region is involved in forming an amphipathic α-helical structure (Saribas et al., 2011). This region contains a Leu/Ile/Phe-rich domain (aa 28–39), mainly consisting of Leu, Ile and Phe. Interestingly, all three Phe residues (Phe31, Phe35 and Phe39) of agnoprotein localize to this Leu/Ile/Phe-rich domain. The negatively charged residues, Glu34 and Asp38, are also interspersed within this domain.

Phenylalanine residues have been reported to play diverse but important regulatory roles in functions of many different proteins through hydrophobic interactions [pi(π)–pi(π) stacking] (Bowden et al., 2008; Brinda et al., 2002; Dhe-Paganon et al., 2004; King et al., 2011; Milardi et al., 2011). These residues were also found to be involved in "cation-π" interactions which could take place between Phe and charged residues (Arg or Lys or His) (Gallivan and Dougherty, 1999; Pless et al., 2008; Pletneva et al., 2001; Shi et al., 2002a, 2002b). Recent reports also indicate that Phe residues can be involved in "anion-π" interactions (Jackson et al., 2007; Philip et al., 2011). All these interactions mediated by Phe residues are known to contribute to (i) the protein-protein interactions at the protein interfaces, (ii) protein folding and stability (Gallivan and Dougherty, 1999; Pless et al., 2008; Pletneva et al., 2001; Shi et al., 2002a, 2002b). Based on the reported contribution of the phenylalanine residues in the literature and the positioning of all three Phe residues within the hydrophobic pocket of agnoprotein, we reasoned that they may also play important structural and functional roles in the biology of agnoprotein in the infected cells.

In this report, we show that agnoprotein induces DNA binding activity of LT-Ag to *Ori* without directly interacting with DNA and that the predicted main α-helix domain of the protein plays a major role in this induction. Upon mutation of each Phe residue to Ala, agnoprotein mostly lost its ability to enhance DNA binding activity of LT-Ag. Protein-protein interaction studies (GST-pull down) demonstrated that interaction of each agnoprotein mutant (F31A, F35A and F39A) with LT-Ag significantly decreased compared to that of WT, which is consistent with our findings from the DNA binding studies. More importantly, the level of the viral DNA replication significantly diminished when all three Phe residues were simultaneously mutated to Ala compared to a slight decrease, which was observed for individual mutants, indicating the importance of a combinatorial effect of Phe residues on agnoprotein function. Additionally, results from immunocytochemistry studies suggest that Phe residues also contribute to agnoprotein function by assisting to its strategic distribution in the infected cells, mostly accumulating around the perinuclear region.

Results

Localization of agnoprotein in infected cells

Agnoprotein is a mainly cytoplasmic protein with high concentrations accumulating in the perinuclear region of the infected cells. However, it has been consistently observed that a small amount of this protein can be detected in the nucleus with conventional fluorescence microscopy studies (Safak et al., 2002). Prediction studies show that agnoprotein has a weak bipartite nuclear localization signal (Dingwall and Laskey, 1986; Dingwall and Laskey, 1991; Sigrist et al., 2010) localized to the N-terminus region of the protein which supports our previous observations (Safak et al., 2002) (Fig. 1A). To confirm its nuclear localization, we examined the co-localization of agnoprotein with VP1 and LT-Ag by employing deconvolution microscopy. This technique allows us to examine different cross sections of the infected cells similar to those obtained using confocal fluorescence microscopy studies. For this purpose, SVG-A cells were infected with JCV Mad-1 strain, fixed with cold