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Human polyomavirus JC small regulatory agnoprotein forms highly stable dimers and oligomers: Implications for their roles in agnoprotein function

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ABSTRACT

JC virus (JCV) encodes a small basic phosphoprotein from the late coding region called agnoprotein, which has been shown to play important regulatory roles in the viral replication cycle. In this study, we report that agnoprotein forms highly stable dimers and higher order oligomer complexes. This was confirmed by immunoblotting and mass spectrometry studies. These complexes are extremely resistant to strong denaturing agents, including urea and SDS. Central portion of the protein, amino acids spanning from 17 to 42 is important for dimer/oligomer formation. Removal of 17 to 42 aa region from the viral background severely affected the efficiency of the JCV replication. Extracts prepared from JCV-infected cells showed a double banding pattern for agnoprotein *in vivo*. Collectively, these findings suggest that agnoprotein forms functionally active homodimer/oligomer complexes and these may be important for its function during viral propagation and thus for the progression of PML.

Introduction

Dimer and higher order oligomer formation of proteins is common, and is frequently a necessary phenomenon for biological systems. Subunit interactions are essential processes for regulation of the functions of many proteins including enzymes, ion channels, receptors and transcription factors (Marianayagam et al., 2004). Dimer/oligomer formation can also help to minimize the genome size of the organisms by allowing multiple combinations of active protein molecules. On the contrary, such formations may have harmful consequences for a biological system when non-native oligomers associated with pathogenic states are generated. For example, β-amyloid deposition in the brains of affected individuals results in Alzheimer's disease. This protein forms fibrillar structures through the self-association of the monomers (Serpell et al., 1997; Sunde and Blake, 1997; Sunde et al., 1997).

There are many examples of the self-association and oligomerization of viral proteins. Examples include polyomavirus large T antigen, (LT-Ag) and human deficiency virus 1 (HIV-1) small proteins, Rev and vpr. LT-Ag binds to its target sequences as a double hexamer in the origin of replication and initiates the viral DNA replication (Auborn et al., 1988; Lynch and Frisque, 1990; Simmons et al., 1990). Rev, a small HIV-1 protein, is involved in the transport of incomplete spliced RNA molecules from nucleus to cytoplasm. This protein binds to Rev response elements present in the intron region of the viral transcripts and was found to function as both stable dimers and oligomers as evidenced by 3D structural studies (Daugherty et al., 2008, 2010a, 2010b; DiMattia et al., 2010). Like Rev, Vpr also forms stable dimers, arrests cells at G2/M phase transition and induces apoptosis (Bolton and Lenardo, 2007; Cui et al., 2006; Fritz et al., 2008, 2010; Godet et al., 2010; Iordanskiy et al., 2004; Poon et al., 2007). Vpr 3D structure has been determined by NMR, showing that the carboxy-terminal helix is responsible for its dimer formation (Bourbigot et al., 2005). An Ebola virus-specific transcription factor, VP30 was also found to form homodimers. A region within this protein spanning amino acid residues 94–112 is essential for oligomerization, where a cluster of four leucine residues was shown to have a critical importance. Mutation of only one of these leucine residues resulted in a molecule that no longer forms oligomers and therefore supports EBOV-specific transcription (Hartlieb et al., 2003). Some viral proteins undergo nucleic acid-induced polymerization process in a sequence specific manner. One example of those is the Borna disease virus (BDV) nucleoprotein which requires the presence of 5′-specific BDV RNA for its oligomerization process (Hock et al., 2010).

JC virus (JCV), a member of the polyomaviridae family of viruses is known to be the etiologic agent of fatal disease, progressive

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multifocal leukoencephalopathy (PML) (Frisque and White, 1992; Major et al., 1992). The viral genome encodes several regulatory proteins, including large T antigen (LT-Ag), small t antigen (Sm t-Ag) T'_{165}, T'_{136}, T'_{135} and agnoprotein (Bollag et al., 2006, 2010; Khalili et al., 2005, 2008; Major et al., 1992; Safak et al., 2001). Agnoprotein is a small basic protein, expressed by all three JCV, BK virus (BKV) and simian virus 40 (SV40) polyomaviruses. Its expression during the viral lytic cycle has been demonstrated by biochemical and immunocytochemical methods in infected cells (Gilbert et al., 1981; Rinaldo et al., 1998; Safak et al., 2002) and in tissue sections (Okada et al., 2002). It is a predominantly cytoplasmic protein localized to the perinuclear region of infected cells, however, a small fraction of it was also found to be localized to the nucleus. This is supported by a recent report by Unterstab et al., where it was found that agnoprotein becomes nuclear when both amino acids 25 (Ala) and 39 (Phe) are mutated to aspartic acid (Asp) and glutamic acid (Glu) respectively (Unterstab et al., 2010). Amino acid sequence alignment of the agnoproteins for JCV, BKV and SV40 shows about 70% sequence identity among these functionally related proteins (Khalili et al., 2005; Safak et al., 2001). While the amino-terminal and central regions of each agnoprotein exhibit considerable sequence identity with one another, the sequences toward the carboxy-terminal region are more divergent.

It has been recently shown that amino terminus of agnoprotein is targeted for phosphorylation by a well-characterized protein kinase, protein kinase C (PKC). This modification plays a significant role in the function of this protein during the viral replication cycle of BK virus and JC virus (Johannessen et al., 2008; Sariyer et al., 2006). SV40 agnoprotein was also previously reported to be phosphorylated but no function was assigned to it (Nomura et al., 1983). Agnoprotein has also been previously found to functionally interact with viral and cellular proteins, including LT-Ag (Safak et al., 2001) and Sm t-Ag (Sariyer et al., 2008), Y-box binding protein, Yb-1 (Safak et al., 2002), p53 (Darbinyan et al., 2002) and HP1α (Suzuki et al., 2005). Mutational analysis of agnoprotein from the closely related virus SV40 suggested that it may have effects on various aspects of the viral lytic 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; Margolskee and Nathans, 1983; Ng et al., 1985a, 1985b).

Based on amino acid sequence, the predicted molecular weight of JCV agnoprotein is ~8.00 kDa. However, this protein has been previously detected as two discrete bands in extracts prepared from both transfected and infected cells (Del Valle et al., 2002; Merabova et al., 2008). These findings are also consistent with recent observations by Suzuki et al.(2010), who demonstrated the homodimer and homo-oligomer formation of agnoprotein by intermolecular fluorescence resonance energy transfer (FRET) analysis and chemical crosslinking studies. It was also previously observed that bacterially produced GST-fusion protein of agno was found to induce multiple higher molecular weight protein complexes (Safak et al., 2001). Those complexes were initially thought to be bacterial proteins that strongly interact and co-purify with agnoprotein during the affinity purification process (Safak et al., 2001). Previously, it was not clear that they result from the dimer/oligomer formation property of agnoprotein itself. In this report, we have shown that bacterially produced MBP and GST fusion agnoproteins form highly stable homodimers and oligomers *in vitro*, mapped the dimerization domain of agnoprotein to amino acids 17–42 and investigated the functional consequences of the deletion of the dimerization domain from the viral background, where deletion of this domain severely affected the replication cycle of JCV. Finally, analysis of agnoprotein, expressed in infected cells, showed two discrete bands on immunoblots suggesting that agnoprotein may also form dimers and perhaps oligomers *in vivo* and functions accordingly during the JCV infection cycle.

Results

Detection of agnoprotein-induced high molecular weight complexes in affinity purified fusion protein fractions

Our previous analysis of GST-agno fusion protein by SDS-polyacrylamide gel consistently revealed the presence of additional agnoprotein-induced higher molecular weight complexes (Safak et al., 2001; Sariyer et al., 2006). These complexes were originally presumed to be bacterial proteins that strongly interact and thereby co-purify with agnoprotein during affinity purification process (Safak et al., 2001; Sariyer et al., 2006). In this report, we sought to further investigate the nature of these complexes, by determining whether complex formation only occurs when it is fused to GST (glutathione-S-transferase). To address this question, agnoprotein was fused to a maltose binding protein (MBP), then expressed in *Escherichia coli* and purified by affinity chromatography. As shown in Figs. 1A and B, the expected size of monomeric GST-Agno is near 32 kDa and that of MBP-Agno is 51 kDa. However, in both cases, additional bands were observed in the respective lanes, running at significantly higher levels than the expected sizes of each agno fusion protein (GST

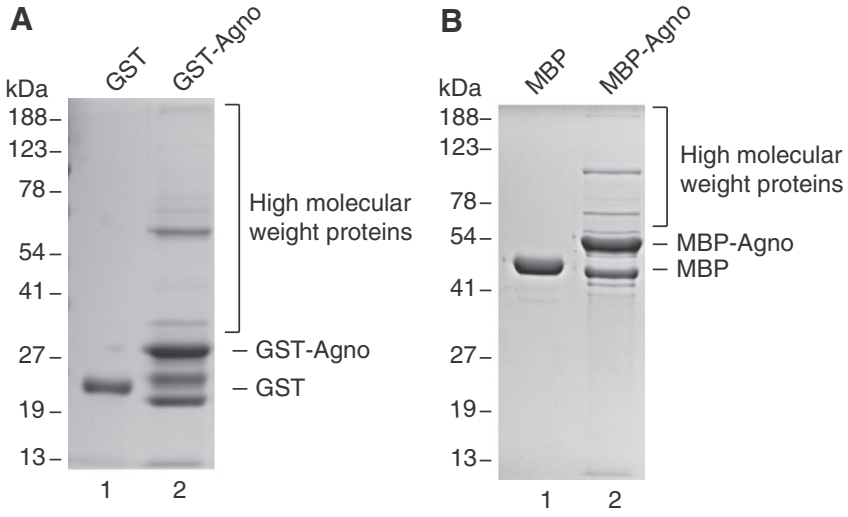

Fig. 1. Expression of agnoprotein as GST and MBP fusion protein. (A) GST alone (5 μg) and GST-Agno (5 μg) were expressed in *E. coli*, affinity purified using GSH-Sepharose 4B resin, separated on a 10% SDS-polyacrylamide gel and analyzed by coomassie staining. (B) MBP alone (5 μg) and MBP-Agno (5 μg) were expressed in *E. coli*, affinity purified using amylose FF resin, separated on a 10% SDS-polyacrylamide gel and analyzed by coomassie staining.