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Binding to retinoblastoma pocket domain does not alter the inter-domain flexibility of the J domain of SV40 large T antigen ☆

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ABSTRACT

Simian Virus 40 uses the large T antigen (Tag) to bind and inactivate retinoblastoma tumor suppressor proteins (Rb), which can result in cellular transformation. Tag is a modular protein with four domains connected by flexible linkers. The N-terminal J domain of Tag is necessary for Rb inactivation. Binding of Rb is mediated by an LXCXE consensus motif immediately C-terminal to the J domain. Nuclear magnetic resonance (NMR) and small angle X-ray scattering (SAXS) were used to study the structural dynamics and interaction of Rb with the LXCXE motif, the J domain and a construct (N_{260}) extending from the J domain through the origin binding domain (OBD). NMR and SAXS data revealed substantial flexibility between the domains in N_{260}. Binding of pRb to a construct containing the LXCXE motif and the J domain revealed weak interactions between pRb and the J domain. Analysis of the complex of pRb and N_{260} indicated that the OBD is not involved and retains its dynamic independence from the remainder of Tag. These results support a 'chaperone' model in which the J domain of Tag changes its orientation as it acts upon different protein complexes.

Introduction

Simian Virus 40 (SV40[1]), a double stranded DNA polyomavirus, encodes the multi-functional multi-domain large T antigen (Tag) protein. Tag is required at several stages of viral productive infection, and is necessary and sometimes sufficient, to induce cellular transformation [1]. During viral infection, Tag is required for the initiation and elongation steps of viral DNA replication, transcriptional repression of the late viral promoter, regulating cellular transcription, and virion assembly. SV40 transforms cells by evasion, inactivation, and deregulation of the growth pathways of the host [2]. It performs these actions primarily through the action of Tag.

The amino-terminus of Tag is a J domain (Fig. 1A), and genetic and biochemical studies have established that Tag is a DnaJ molecular chaperone [3]. DnaJ chaperones work in concert with DnaK family chaperones. Tag binds the mammalian DnaK homologue Hsc70, and like other DnaJ–DnaK interactions, the binding of Tag to Hsc70 leads to activation of the Hsc70 ATPase activity. The J domain and its interaction with Hsc70 are essential for Tag mediated cellular transformation [4].

One well defined role for the J domain in transformation is the disruption of the interaction between retinoblastoma (Rb) tumor suppressor proteins and transcriptional activator E2F proteins [5]. The Rb family contains pRb, p107, and p130 proteins. These multi-domain proteins (Fig. 1B) have been implicated in transcriptional activation and several other cellular processes [6]. One function of Rb proteins is to regulate the cell cycle by inhibiting proliferation. Rb is bound to the E2F/DP1 in G1 phase and represses its activity [7]. In late G1 phase, Rb is inactivated via phosphorylation by cyclin dependent kinases, which in turn releases E2F/DP1 and enables entry into the S phase and progression of the cell cycle [8]. Inactivation of Rb causes deregulated E2F

☆ Experimental and theoretical scattering profiles, and $P(r)$ functions, will be deposited in the BIOISIS database (www.bioisis.net).

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[1] *Abbreviations used:* BME, beta-mercapto ethanol; DTT, dithiothreotol; IPTG, isopropyl β-d-1-thiogalactopyranoside; OBD, origin binding domain; HD, helicase domain; Hsc70, heat shock cognate protein 70; NMR, nuclear magnetic resonance; $P(r)$, probability of distribution; Rb, retinoblastoma tumor suppressor protein; R_g, radius of gyration; SAXS, small angle X-ray scattering; SEC, size exclusion chromatography; SV40, Simian Virus 40, Tag, large T-antigen.

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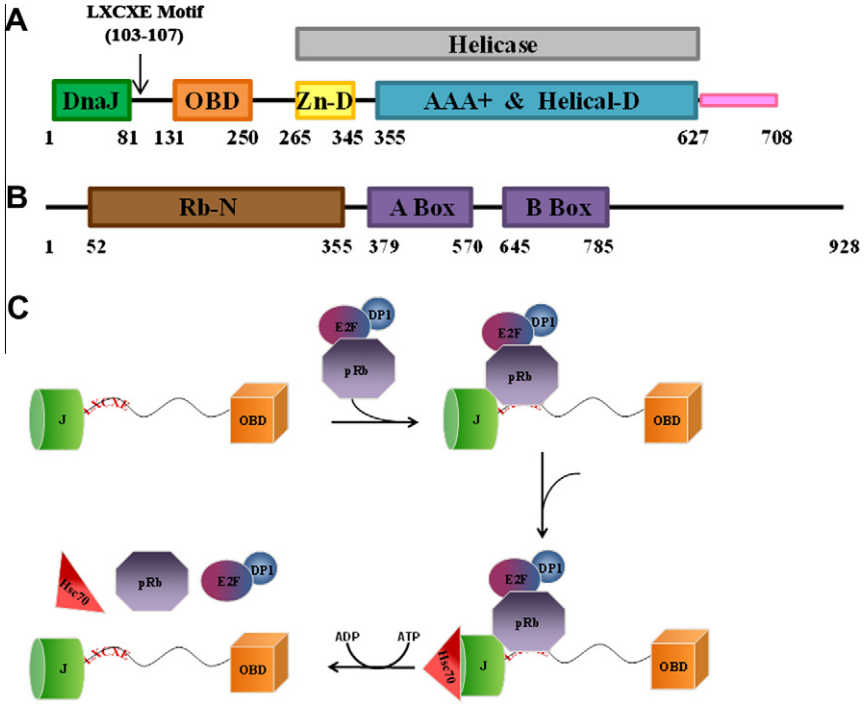

Fig. 1. Domain structure of SV40 Tag (A) and pRb (B), and an overview of the chaperone model (C). Tag bindsRb/E2F complexes primarily via the LXCXE motif, recruits its co-chaperone Hsc70, ATP hydrolysis occurs and the complex is disassembled.

activity, inappropriate cellular proliferation and may potentiate progression to tumorigenesis [9].

During both productive infection and cell transformation Tag induces the release of E2F transcription factors from the three retinoblastoma proteins, pRb, p107, and p130 [5,10]. The release of E2F from Rb is dependent on a functional J domain and Hsc70-mediated ATP hydrolysis [3]. These observations led to a "chaperone" model in which Tag first binds to Rb–E2F complexes, and then energy derived from ATP hydrolysis by Hsc70, recruited by the J domain, is used to liberate E2F from Rb (Fig. 1C) [11].

The chaperone model involves Tag recruiting Hsc70 and multiprotein complexes so that the chaperone machinery can alter specific protein–protein interactions. In this model, Tag can alter the target complex by: (1) dislodging specific proteins from the complex; (2) altering the conformation and thus the activity of proteins in the complex; (3) targeting specific proteins in the complex for post-translational modification and/or degradation. This model is consistent with studies of Tag action on pRb/E2F complexes, in which it has been shown that energy derived from Hsc70-mediated ATP hydrolysis is used to release E2F4 from its association with p130 [5]. In the absence of ATP hydrolysis, Tag associates with both Hsc70 and p130/E2F4 complexes [5]. Hence, the assembly of Tag and Rb is a biologically important intermediate of the chaperone reaction.

If this model is correct, Hsc70 must have the ability to act on different cellular complexes, each containing a unique set of proteins. For example, genetic studies indicate that, in addition to targeting Rb–E2F complexes bound near the amino-terminus, the J domain acts on targets bound in the carboxy-terminal portion of Tag [12]. Thus, in each case the J domain must be in position so that Hsc70 is correctly oriented relative to the target complex. This suggests that the J domain must be able to adopt multiple orientations relative to the other Tag domains (Fig. 1). This hypothesis is supported by cryo-EM studies of Tag [13], in which the absence of the J domain in the structure is presumed to arise from it having different orientations in different molecules on the grid.

Tag binds Rb proteins primarily through a consensus LXCXE binding motif (Tag 103–107) that resides in the linker between the OBD and the J domain (Fig. 1) [14]. However, the details of how Tag coordinates the protein interactions involved in inactivation of Rb remains poorly understood. A crystal structure has been determined of the N-terminal region of Tag including the J domain and the LXCXE motif (N_{117}), in complex with the pocket domain of Rb (pRbA/B) [15]. The interaction interface of Tag in this structure primarily involves the LXCXE motif, but a few contacts between the J domain and pRbA/B were also observed. The chaperone model requires that the J domain be able to adopt different orientations in order to correctly position Hsc70 for action on its multiple targets. In contrast, the crystal structure suggests the J domain and pRb are in a fixed orientation. We propose that flexibility must exist in the linker between the J and OB domains of Tag to provide the variation in positioning required for function. To test this hypothesis we used a combination of NMR and small angle X-ray scattering (SAXS) experiments to characterize the relative flexibility of the linker between J and OB domains, and to determine if this flexibility is restricted upon binding to the Rb substrate.

Materials and methods

DNA constructs

A plasmid expressing SV40 Tag construct N_{260} (4–260) obtained from Xiaojiang Chen (University of Southern California) was subcloned into an in-house kanamycin resistant pbG101 vector (L. Mizoue, Center for Structural Biology) containing an H3C cleavable N-terminal 6xHis-GST tag. The Tag constructs N_{117} (4–117) and N_{102} (4–102) were subcloned from N_{260} into the ampicillin resistant pET15b vector containing an N-terminal 6xHis tag. pRbA/B (379–570/645–772) in an ampicillin resistant pGex-kg vector containing a thrombin cleavable GST tag was used in these studies because it