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Structural basis for the regulation of nuclear import of Epstein-Barr virus nuclear antigen 1 (EBNA1) by phosphorylation of the nuclear localization signal

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ABSTRACT

Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is expressed in every EBV-positive tumor and is essential for the maintenance, replication, and transcription of the EBV genome in the nucleus of host cells. EBNA1 is a serine phosphoprotein, and it has been shown that phosphorylation of S385 in the nuclear localization signal (NLS) of EBNA1 increases the binding affinity to the nuclear import adaptor importin-α1 as well as importin-α5, and stimulates nuclear import of EBNA1. To gain insights into how phosphorylation of the EBNA1 NLS regulates nuclear import, we have determined the crystal structures of two peptide complexes of importin-α1: one with S385-phosphorylated EBNA1 NLS peptide, determined at 2.0 Å resolution, and one with non-phosphorylated EBNA1 NLS peptide, determined at 2.2 Å resolution. The structures show that EBNA1 NLS binds to the major and minor NLS-binding sites of importin-α1, and indicate that the binding affinity of the EBNA1 NLS to the minor NLS-binding site could be enhanced by phosphorylation of S385 through electrostatic interaction between the phosphate group of phospho-S385 and K392 of importin-α1 (corresponding to R395 of importin-α5) on armadillo repeat 8.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpes virus associated with a diverse range of tumors of both lymphoid and epithelial origin [1]. EBV nuclear antigen 1 (EBNA1) is a DNA-binding protein expressed in every EBV-positive tumor. EBNA1 plays an essential role in the maintenance and replication of the episomal EBV genome through its direct interaction with sequences in the EBV latent origin of replication (oriP) [2–5], and also acts as a transcriptional regulator [6,7]. It has been shown that EBNA1 induces B cell lymphomas in transgenic mice [8], enhances cell survival [9], and induces genetic instability [10], indicating that EBNA1 might contribute directly to oncogenesis. EBNA1 is phosphorylated at multiple serine residues when expressed in human and insect cells [11–14]. Although the physiological significance of EBNA1 phosphorylation remains incompletely understood, it has been suggested that phosphorylation of EBNA1 serine residues contributes to segregation and maintenance of the EBV genome, transcriptional activation, and nuclear import of EBNA1 [15–18].

Transport of macromolecules between the cytoplasm and nucleus occurs through nuclear pore complexes (NPCs) [19]. Eukaryotic cells control and finely tune many biological processes by regulating nuclear transport [20]. Phosphorylation of cargoes has emerged as one of the important mechanisms to regulate a multitude of nuclear transport pathways [20], including the importin (Imp) α:β-dependent nuclear import pathway [21,22]. The Impα adaptor proteins bind cargo proteins possessing the nuclear localization signal (NLS), and heterodimerize with Impβ through the N-terminal Impβ-binding (IBB) domain, forming the heterotrimeric Impα:β:NLS-cargo complexes that permeate through NPCs and deliver NLS-cargoes into the nucleus [19–22]. Mammalian cells have at least seven Impα isoforms, whose expression is tightly regulated depending on cell type and developmental stage [23,24]. Each isoform of Impα has different substrate specificity, and many cellular and viral cargoes have been shown to associate preferentially with specific isoforms of Impα [23,24].

Previous cell biological and biochemical studies identified the

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NLS of EBNA1 (^{379}KRPRSPSS^{386}) [25] and demonstrated that EBNA1 binds to the nuclear import adaptor Impα1 [26–28] as well as Impα5 [28]. The amino-terminal K379 and R380 of the EBNA1 NLS are essential for nuclear translocation [18]. Although the serine residues (S383, S385, and S386) are not essential for the EBNA1 NLS, both S383 and S385 are important for nuclear translocation [18]. Phosphorylation of S385 increases nuclear import efficiency [18] and also increases the binding affinity of the EBNA1 NLS to Impα1 as well as Impα5 [18,29]. In this study, we used X-ray crystallography to elucidate how phosphorylation of EBNA1 NLS can regulate its interaction with Impα.

2. Materials and methods

2.1. Preparation of protein-peptide complexes for crystallization

N-terminally His_6-and S-tagged ΔIBB Impα1 (mouse, residues 70–529) was expressed from pET30a (Novagen) [30] in the *E. coli* host strain BL21-CodonPlus(DE3)RIL (Stratagene), and was purified over Ni-NTA (Novagen) and gel filtration over Superdex200 (GE Healthcare). S385-phosphorylated EBNA1 NLS peptide ^{378}EKRPRPRSP-pS-S^{386} (pS stands for phosphoserine) and non-phosphorylated EBNA1 NLS peptide ^{378}EKRPRPRSPSS^{386} were synthesized by GenScript. Prior to crystallization, ΔIBB Impα1 and the NLS peptide were mixed in a molar ratio of 1:3 in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM 2-mercaptoethanol.

2.2. X-ray crystallography

Crystals of ΔIBB Impα1 bound to S385-phosphorylated EBNA1 NLS peptide were grown at 20 °C from 0.2 mM ΔIBB Impα1 and 0.6 mM NLS peptide by hanging drop vapor diffusion against 0.1 M MES (pH 6.5), 0.9 M sodium citrate, and 10 mM DTT. Crystals of ΔIBB Impα1 bound to non-phosphorylated EBNA1 NLS peptide were grown at 20 °C from 0.2 mM ΔIBB Impα1 and 0.6 mM NLS peptide by hanging drop vapor diffusion against 0.1 M MES (pH 6.5), 0.8 M sodium citrate, and 10 mM DTT. Crystals were cryoprotected using mother liquor containing 23% glycerol, and flash-cooled in liquid nitrogen. X-ray diffraction datasets were collected at 95 K at Photon Factory beamline BL-17A using a Pilatus3 S6M detector. Diffraction data were processed using MOSFLM and CCP4 programs [31]. The structures were solved by molecular replacement using MOLREP [32] using the structure of ΔIBB Impα1 bound to Bimax1 NLS peptide (PDB code, 3UKW) [33] as a search model. The structures were refined by iterative cycles of model building using COOT [34] and refinement using REFMAC5 [35] and PHENIX [36]. MolProbity [37] was used to validate the final models. Structural figures were produced using CCP4MG [38] and PyMOL [39]. Coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with accession codes 5WUM (ΔIBB Impα1 bound to S385-phosphorylated EBNA1 NLS peptide) and 5WUN (ΔIBB Impα1 bound to non-phosphorylated EBNA1 NLS peptide).

3. Results and discussion

3.1. Crystal structure of Impα1 bound to S385-phosphorylated EBNA1 NLS peptide

We obtained crystals of the NLS-binding armadillo (ARM) repeat domain of Impα1 bound to the S385-phosphorylated NLS peptide of EBNA1, and determined the structure at 2.0 Å resolution by molecular replacement (Table 1). The structure was refined to free and working *R*-factor values of 21.2% and 18.6%, respectively. Residues 378–385 and 378–383 of the NLS peptide bound to the minor and major NLS-binding sites, respectively, were identified unambiguously in the electron density map (Fig. 1A and B). Notably, the electron density of the phosphorylated side chain of pS385 was clearly visible at the minor site (Fig. 1A), but this phosphoserine was not visible at the major site (Fig. 1B). The NLS peptides bound along the inner concave surface of Impα1 in almost fully extended conformations (Fig. 2A) and formed an extensive network of interactions with the surface residues of Impα1 (Fig. 3A–D).

Table 1
Crystallographic statistics.

Crystal	ΔIBB Impα1 bound to S385-phosphorylated NLS of EBNA1	ΔIBB Impα1 bound to non-phosphorylated NLS of EBNA1
Data collection		
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions		
a, *b*, *c* (Å)	77.91, 90.30, 99.63	78.51, 90.18, 98.89
α, *β*, *γ* (degree)	90, 90, 90	90, 90, 90
Wavelength (Å)	0.98	0.98
X-ray source	Photon Factory BL-17A	Photon Factory BL-17A
Resolution range (Å)[a]	41.97–2.00 (2.05–2.00)	28.80–2.20 (2.27–2.20)
No. of measured reflections[a]	288684 (16841)	218370 (16032)
No. of unique reflections[a]	48181 (3485)	36317 (3083)
Completeness (%)[a]	99.9 (99.3)	99.9 (97.3)
R_{merge} (%)[a]	5.9 (86.3)	8.7 (99.0)
Mean $I/\sigma(I)$[a]	19.0 (1.8)	14.1 (1.8)
Mean *I* half-set correlation CC(1/2)[a]	0.999 (0.653)	0.998 (0.599)
Multiplicity[a]	6.0 (4.8)	6.0 (5.2)
Refinement		
Resolution range (Å)[a]	41.97–2.00 (2.04–2.00)	28.80–2.20 (2.26–2.20)
R_{work} (%)[a]	18.6 (28.2)	17.7 (27.6)
R_{free} (%)[a]	21.2 (33.0)	20.4 (31.6)
No. of atoms		
Protein	3363	3340
Water	258	174
No. of amino acids	440	437
Mean *B* factor (Å^2)		
ΔIBB Impα1	42.6	48.2
EBNA1 NLS (major site)	50.4	57.4
EBNA1 NLS (minor site)	63.5	61.9
Water	48.5	50.1
RMSD from ideality		
Bond lengths (Å)	0.004	0.004
Bond angles (degree)	0.630	0.683
Protein geometry[b]		
Rotamer outliers (%)	0	0
Ramachandran favored (%)	99.1	98.4
Ramachandran outliers (%)	0	0
Cβ deviations > 0.25 Å (%)	0	0
MolProbity score (percentile)	1.28 (99)	1.15 (100)
PDB code	5WUM	5WUN

[a] Values in parentheses are for the highest-resolution shell.
[b] MolProbity [37] was used to analyze the structures.

At the minor NLS-binding site (Fig. 3A, C, and D), the positively charged side chains of EBNA1 fitted into three acidic pockets on Impα1: K379^{EBNA1} made hydrogen bonds with the side chain of T328^{Impα1} and the main-chain carbonyls of V321^{Impα1} and N361^{Impα1}; R380^{EBNA1} was sandwiched between the indole rings of W399^{Impα1} and W357^{Impα1} and formed a salt bridge with E396^{Impα1}; R382^{EBNA1} made a hydrogen bond with N319^{Impα1} and a bidentate salt bridge with E354^{Impα1}. Although pS385^{EBNA1} was not in direct contact with Impα1 residues, the negatively charged

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