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Brief communication

Monitoring HPV-16 E7 phosphorylation events

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ABSTRACT

HPV-16 E7 is one of the key proteins that, by interfering with the host metabolism through many protein-protein interactions, hijacks cell regulation and contributes to malignancy. Here we report the high resolution investigation of the CR3 region of HPV-16 E7, both as an isolated domain and in the full-length protein. This opens the way to the atomic level study of the many interactions in which HPV-16 E7 is involved. Along these lines we show here the effect of one of the key post-translational modifications of HPV-16 E7, the phosphorylation by casein kinase II.

1. Introduction

Human papillomaviruses (HPVs) constitute a family of more than 100 viruses, which infect the mucosa and the squamous epithelia (Bernard et al., 2010). HPVs are classified as Low Risk or High Risk depending on their propensity to cause either benign or dysplastic lesions which can ultimately lead to cancer (Bernard et al., 2010; zur Hausen, 1996; de Villiers et al., 2004). Within the HR group, HPV-16 and HPV-18 are the types most often found in cervical cancer, which is the second most common cancer in women worldwide (zur Hausen, 1996, 2002).

HPVs have a small circular double stranded DNA (about 8 kb) encoding only a few proteins. Among them, two proteins, E6 and E7, play an important role in cancer development in the oncogenic variants (Münger et al., 2004). Both proteins work in cooperation to hijack cell regulation by interacting with many proteins of the host, such as the tumor suppressors p53, in case of E6, and pRb in case of E7 (Barbosa, 1996; Werness et al., 1990; Dyson et al., 1989). However, the presence of E7 is sufficient to immortalize epithelial cells even in the absence of E6 (Halbert et al., 1991) and in transgenic mouse models E7 plays a major role in cervical cancer development (Riley et al., 2003). This shows the importance of E7 in the development of HPV-related malignancy.

HPV-16 E7 is a heterogeneous protein in terms of its structural and dynamic properties. It is 98 amino acids long, with three conserved regions (CR) (Phelps et al., 1992). CR1 and CR2 are located in the N-terminal half, which is intrinsically disordered and is characterized by high flexibility (Calçada et al., 2013; Ohlenschläger et al., 2006). CR3 is in the C-terminal part of the protein, which appears to be more structured and which contains two CXXC zinc-binding motifs separated by 29 amino acids (Fig. 1A). This domain is also responsible for the formation of a homodimer as shown by the 3D structure of E7 CR3 from HPV-1A (Liu et al., 2006) and HPV-45 (Ohlenschläger et al., 2006). In contrast, the 3D structure of analogous constructs from HPV-16 could not be determined (Ohlenschläger et al., 2006; Liu et al., 2006; Todorovic et al., 2011).

E7 acts as a hub protein, interacting with several partners that are involved in many different processes, such as cell cycle control and apoptosis (Davies et al., 1993; McIntyre et al., 1996; Funk et al., 1997; Jones et al., 1997a; Massimi et al., 1996). One of the most important and well described interactions for HPV-16 E7 is with the retinoblastoma tumor suppressor (pRb), which binds through the LXCXE motif present in CR2 (Fig. 1A) (Dyson et al., 1989; Lee et al., 1998). However, optimal interaction with pRb also requires residues in the CR3 domain (Chemes et al., 2010; Todorovic et al., 2012; Jansma et al., 2014). Indeed, CR3 is involved in many other interactions that contribute to HPV-mediated oncogenesis such as, for example, interactions with the cyclin-dependent kinase inhibitors p21 and p27, and transcription factors like the TATA box-binding protein (TBP), p300/CBP and E2F. Some of these interactions are strongly modulated by post-translational modifications (PTMs) of E7. In particular, the phosphorylation of Ser31 and Ser32 by casein kinase II (CKII) has

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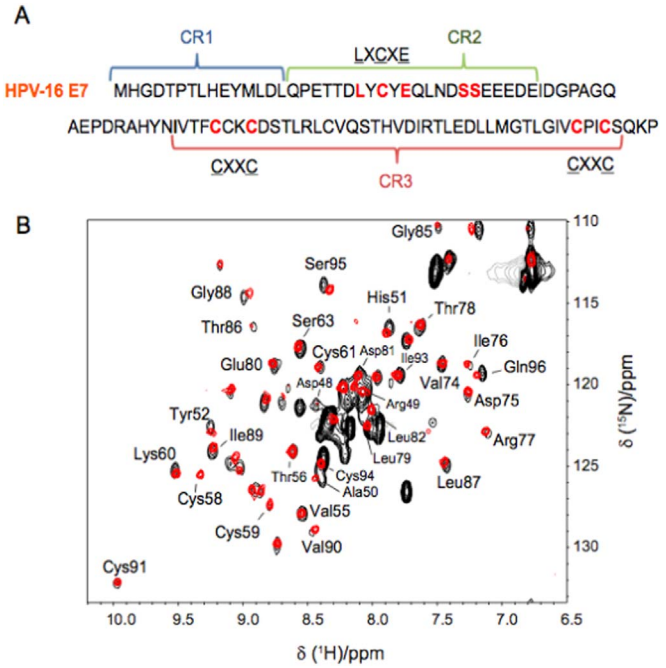

Fig. 1. A) Amino acid sequence of HPV-16 E7, where specific motifs are highlighted in red: the LXCXE motif for pRb binding, the CKII phosphorylation site in CR2, and the zinc-binding motifs CXXC in CR3. B) $^{1}H-^{15}N$ SOFAST-HMQC spectra recorded for HPV-16 E7 (black) and for HPV-16 E7 CR3 (red). The resulting assignment is reported in the figure, indicating the amino acid residue. The experiments were recorded at 11.7 T on samples 0.30 mM E7 CR3 and 0.34 mM E7.

been shown to play an important role in modulating many of E7's activities (Firzlaff et al., 1989, 1991). For example CKII phosphorylation of E7 plays an important role in the ability of E7 to transform cells and for its ability to interact with TBP (Massimi et al., 1996; Barbosa et al., 1990; Firzlaff et al., 1991). Recent studies also suggest that the acidic domain that comprises the CKII recognition site, which lies downstream of the LXCXE motif, also contributes towards the recognition of pRb (Chemes et al., 2010; Dick and Dyson, 2002; Singh et al., 2005). Furthermore, an intact CKII phosphorylation site was also found to be important for E7 to be able to target the pRb and the related p130 pocket proteins for degradation (Jones et al., 1997b; Genovese et al., 2008). Recent studies have also shown that increased levels of phosphorylation of a variant HPV-16 E7 also increases interaction with TBP and pRb (Zine El Abidine et al., 2017). However, what effect phosphorylation of E7 by CKII has upon the structural and dynamic properties of E7 is still unknown. Whilst recent studies have used nuclear magnetic resonance (NMR) spectroscopy to characterize the N-terminal domain of HPV-16 E7 (Calçada et al., 2013) and structures of the C-terminal region have been reported for HPV-1a (Liu et al., 2006) and HPV-45 E7 (Ohlenschläger et al., 2006), there is currently no information on how phosphorylation of E7 by CKII might modulate this structure.

Here we describe the atomic resolution characterization of E7 CR3 from HPV-16. Moreover we investigated the effect of phosphorylation of E7 through time-resolved NMR experiments. This provides the basis for detailed studies of the properties of the phosphorylated forms of E7 and how this modulates its interactions with many cellular proteins.

2. Results and discussion

For any detailed analysis of E7 at the molecular level, including the effect of post-translational modifications (PTMs) and protein-protein interactions, it is necessary to have the sequence-specific assignment of the polypeptide. The NMR analysis of the full-length protein had provided only the assignment of the N-terminal portion, comprising

Table 1
Sequence specific assignment of H^N, N, C', C^α, and C^β in HPV-16 E7 CR3. Proton resonances were calibrated with respect to the signal of 2,2-dimethylsilapentane-5-sulfonic acid (DSS). Carbon and nitrogen resonances were referenced indirectly to the ^{1}H standard using the conversion factor derived from the ratio of NMR frequencies according to IUPAC convention. The data have been deposited in the BMRB with accession number 26069.

Residue n°	AA	H^N	N	C^α	C^β	C'
45	A			52.30	19.27	173.93
46	E	8.36	121.88	54.54		
47	P			62.94	31.79	176.40
48	D	8.44	120.64	54.50	40.98	175.90
49	R	8.10	120.19	55.09	32.63	174.79
50	A	8.49	125.49	50.81	21.35	174.72
51	H	7.91	116.60	55.83	32.51	175.29
52	Y	9.28	122.75	5752	40.04	
53	N			52.80		174.27
54	I	9.09	124.90	60.07		174.77
55	V	8.56	127.65	61.82		175.27
56	T	8.65	123.83	58.42	69.48	170.94
57	F	8.27	119.93	55.31	41.73	175.14
58	C	9.36	125.22	59.12		178.16
59	C	8.85	127.14	59.73	27.53	174.05
60	K	9.57	125.28	57.62	33.69	177.29
61	C	8.46	118.68	59.16		176.07
62	D	8.19	119.83	56.92	40.67	175.22
63	S	8.65	117.43	60.24		
...						
73	H			59.41	30.22	177.14
74	V	7.52	118.44	65.41		178.25
75	D	7.31	120.29	57.16		177.98
76	I	7.32	118.53	60.74		176.69
77	R	7.16	122.67	58.16	29.18	178.53
78	T	7.66	116.15	66.40	67.98	176.32
79	L	8.10	122.29	58.28	41.59	177.84
80	E	8.81	118.52	60.45	29.65	179.28
81	D	8.14	119.12	57.33	39.81	180.10
82	L	8.07	121.24	58.10		
83	L					
84	M					176.43
85	G	7.54	110.03	45.32		174.71
86	T	8.97	116.20	61.73	68.89	173.84
87	L	7.50	124.52	55.80		174.48
88	G	8.99	114.12	44.11		171.80
89	I	9.28	123.74	59.82	44.16	174.21
90	V	8.57	127.66	60.88	33.80	175.65
91	C	10.2	131.87	58.09		
92	P			65.40	32.21	178.92
93	I	7.85	119.22	63.85	37.30	179.30
94	C	8.45	124.64	64.15	29.36	178.05
95	S	8.34	113.84	60.40	63.40	174.49
96	Q	7.24	119.19	55.71	29.03	
97	K	7.62	122.56	54.37		
98	P					

the CR1 and the CR2 regions (Calçada et al., 2013), since the weak intensity and the broadening of the signals from the CR3 region in the 2D and 3D NMR spectra of E7 did not allow detection of the correlations necessary to achieve sequence-specific assignment. Therefore, the isolated E7 CR3 domain was expressed, purified and analyzed by NMR spectroscopy.

The overlay of the $^{1}H-^{15}N$ correlation spectra acquired from the full length protein (E7) with that from the CR3 domain (E7 CR3) clearly shows that the structural properties of the CR3 domain are quite similar in both polypeptides, since there are only minor changes in the chemical shift between the two spectra (Fig. 1B). This also means that the assignment obtained for the E7 CR3 can be easily transferred to the full length E7.

On the shorter construct a series of 3D NMR experiments could be acquired with sufficient sensitivity and resolution to achieve the sequence-specific assignment of the majority of the E7 CR3 signals. A few cross peaks in the 2D $^{1}H-^{15}N$ correlation spectra, most likely deriving from the 64–72 fragment, could not be assigned because of