JOURNAL OF PROTEOMICS XX (2014) XXX–XXX

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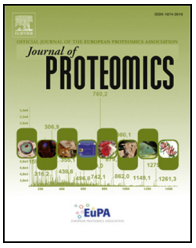

Biochemical and proteomic analysis of spliceosome factors interacting with intron-1 of human papillomavirus type-16☆

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ARTICLE INFO

Keywords:
HPV-16 intron-1
Spliceosomal factors
E6/E7$^{pre\text{-}mRNA}$
Proteomics
Alternative splicing

ABSTRACT

The human papillomavirus type 16 (HPV-16) E6/E7 spliced transcripts are heterogeneously expressed in cervical carcinoma. The heterogeneity of the E6/E7 splicing profile might be in part due to the intrinsic variation of splicing factors in tumor cells. However, the splicing factors that bind the E6/E7 intron 1 (In-1) have not been defined. Therefore, we aimed to identify these factors; we used HeLa nuclear extracts (NE) for in vitro spliceosome assembly. The proteins were allowed to bind to an RNA/DNA hybrid formed by the In-1 transcript and a 5′-biotinylated DNA oligonucleotide complementary to the upstream exon sequence, which prevented interference in protein binding to the intron. The hybrid probes bound with the nuclear proteins were coupled to streptavidin magnetic beads for chromatography affinity purification. Proteins were eluted and identified by mass spectrometry (MS). Approximately 170 proteins were identified by MS, 80% of which were RNA binding proteins, including canonical spliceosome core components, helicases and regulatory splicing factors. The canonical factors were identified as components of the spliceosomal

☆ This article is part of a Special Issue entitled: Proteomics, mass spectrometry and peptidomics, Cancun 2013.

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http://dx.doi.org/10.1016/j.jprot.2014.07.029

B-complex. Although 35–40 of the identified factors were cognate splicing factors or helicases, they have not been previously detected in spliceosome complexes that were assembled using *in vivo* or *in vitro* models.

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1. Introduction

Infection with high-risk human papillomavirus (hrHPV) causes hyper-proliferative lesions in mucosa, which in turn might be transformed into malignant neoplasias [1]. The hrHPV E6/E7 oncoproteins play a pivotal role in the malignant transformation of cervical epithelium and the underlying mechanism is well documented [2,3]. The E6/E7 oncoproteins are encoded by neighboring genes, which are expressed as polycistronic primary transcripts (pre-mRNAs) [4,5]. Interestingly, E6/E7$^{\text{pre-mRNAs}}$ are spliced in hrHPVs, but this does not occur in the low-risk HPVs [6]. HPV type 16 (HPV-16) is the most prevalent infection in cervical cancers. It is found in 60% of the cases, suggesting its major role in the development of this cancer [7]. Intriguingly, their pre-mRNAs are alternatively spliced, whereas E6/E7$^{\text{pre-mRNAs}}$ from other hrHPVs are processed by *cis* splicing [8]. Four isoforms of the HPV-16 E6 mRNA are produced by alternative splicing [9,10]. These reports suggest that the E6 splicing products might play an important role in the development of cervical cancer; however, this hypothesis is yet to be confirmed.

HPV introns are recognized and processed by the host cell splicing machinery, namely the spliceosome, which is a multi-protein complex formed by 5 small nuclear ribonucleoprotein particles (snRNPs), namely U1 to U6 snRNPs, each of which are formed with a set of specific proteins and their corresponding snRNAs [11]. Together with the snRNPs, a number of accessory factors are assembled in the pre-mRNA to constitute a catalytically active spliceosome [reviewed in 12]. The spliceosome is assembled stepwise by an ordered multi-step process and at least four intermediary particles might be fractionated by biochemical procedures, namely the E-, A-, B-, and C-complexes. The E-complex is constituted by the pre-mRNA plus the U1 snRNP; it is involved in the recognition of the splicing donor sequence. In turn, U2 snRNP and its associated factors (U2AF$^{65/35}$) bind to the branch point (BP) and the acceptor sequence, to form the A-complex. After this, the tripartite particle U4/U6°U5 snRNP is bound to form the B-complex [13]. Subsequently, ATP is necessary to produce a B active-complex (B*), which in turn gives rise to the catalytically active spliceosome, the C-complex [14]. Interestingly, evidence in yeast suggests that it may also be pre-assembled [15,16]. Regardless of the assembly mechanism, at least 300 proteins have been observed in spliceosomes [17]. Among them, heteronuclear ribonucleoproteins (hnRNP), serine-arginine rich (SR) proteins, protein kinases, cyclophillins, GTPases and RNA helicases [17,18].

The HPV-16 E6/E7 intron 1 (In-1), contains one donor splice site and three suboptimal acceptor sites. In-1 is distinct because it is embedded in the coding sequences from both, the E6 and E7 oncogenes. Thus, the coding sequences are recognized and spliced. Noteworthy, the alternatively spliced transcripts are heterogeneously expressed in cervical carcinoma cells [10,19,20]. The heterogeneous profile seems to be promoted by two conditions: intrinsic variation in the level of some splicing factors in the tumor cells [21] or a differential binding of splicing factors to polymorphic variants of the HPV-16 [22]. Unfortunately, both the mechanisms and splicing factor that facilitate this heterogeneous expression are largely unknown. Proteomic and mass spectrometry technology might be an excellent tool to identify the nuclear factor which binds the HPV-16 In-1. In fact, this technology has been recently used to analyze human Thin Prep cervical smears from normal or cancer tissues, identifying inclusive protein from different HPV types [23].

Given this background, we were prompted to investigate the identity of the nuclear factors that bind to In-1. First, we initiated the biochemical characterization of the E6/E7$^{\text{pre-mRNA}}$–protein-complexes formed during *in vitro* splicing conditions, by using HeLa nuclear extract (NE). Subsequently, the complexes were characterized by using ultraviolet (UV) cross-linking assays under different experimental conditions. Mutation/deletion analysis of the splicing donor and acceptors suggested a differential recognition of each of the acceptor sites. Moreover, these data suggested binding of multiple protein factors – including poly-U binding proteins – which seem to be important to stabilize the spliceosomal complexes. To simplify the system, we initiated the purification of factors that were bound to the shorter In-1 (sIn 1), that is, the intron sequence encompassing only the donor and the closest acceptor sequence; because, this alternative intron is used with the higher frequency in cervical carcinoma cells. A synthetic RNA probe was used to bind and to purify the nuclear factors; the probe was annealed to a 27-nt biotinylated-DNA oligonucleotide (complementary to the 5′ end of In-1) to generate a DNA/RNA hybrid. The hybrid probes bound with the proteins were coupled to streptavidin magnetic beads for purification. The proteins were eluted and subsequently identified by liquid chromatography tandem mass spectrometry (LC/MS/MS). One hundred and thirty five of these proteins corresponded to snRNP and non-snRNP splicing factors. Approximately 95 of these factors were identified as core spliceosome components. Interestingly, 35–40 of the identified factors that were cognate splicing factors or helicases were not previously reported as spliceosome components. Moreover, some of these factors are involved in enhancing weak splicing donor and acceptor recognition in mammalian mRNAs. This article discusses the significant influence of these findings in the splicing of HPV-16 In-1.

2. Materials and methods

2.1. Reagents and primers

HeLa NE containing proteins that are active in splicing were purchased from Promega Co (Madison, WI, USA). All molecular biology reagents and primers were purchased from Invitrogen