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B23/nucleophosmin interacts with bovine immunodeficiency virus Rev protein and facilitates viral replication

Ana Maria Passos-Castilho, Claude Marchand, Denis Archambault*

Department of Biological Sciences, University of Quebec at Montreal, Quebec, Canada H3C 3P8

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ABSTRACT

The bovine immunodeficiency virus (BIV) Rev shuttling protein contains nuclear/nucleolar localization signals and nuclear import/export mechanisms that are novel among lentivirus Rev proteins. Several viral proteins localize to the nucleolus, which may play a role in processes that are essential to the outcome of viral replication. Although BIV Rev localizes to the nucleoli of transfected/infected cells and colocalizes with one of its major proteins, nucleophosmin (NPM1, also known as B23), the role of the nucleolus and B23 in BIV replication remains to be determined. Here, we demonstrate for the first time that BIV Rev interacts with nucleolar phosphoprotein B23 in cells. Using small interfering RNA (siRNA) technology, we show that depletion of B23 expression inhibits virus production by BIV-infected cells, indicating that B23 plays an important role in BIV replication. The interaction between Rev and B23 may represent a potential new target for the development of antiviral drugs against lentiviruses.

1. Introduction

Bovine immunodeficiency virus (BIV) is a lentivirus of the *Retroviridae* family with high homology to human immunodeficiency virus type 1 (HIV-1) and other animal lentiviruses. The 8.960 kb in length BIV provirus DNA presents a typical retroviral genomic structure containing the *gag*, *pol*, and *env* genes flanked by long terminal repeats at the 5' and 3' termini. Additional open reading frames in proximity to the *pol-env* junction encode nonstructural regulatory/accessory proteins, such as the Rev protein (Gonda et al., 1987, 1994; St-Louis et al., 2004).

Although the pathogenicity of BIV remains controversial, persistent lymphocytosis, neurological disorders, weight loss, diminished milk production, lymphoid hyperplasia, and opportunistic bacterial infections have been associated with BIV infection (Van der Maaten et al., 1972; Martin et al., 1991; Carpenter et al., 1992; Onuma et al., 1992; Brujeni et al., 2010). Interestingly, BIV-infected rabbits show symptoms similar to HIV infection (Kalvatchev et al., 1995, 1998). Furthermore, BIV cell infection can also be inhibited by HIV inhibitors, indicating that BIV and HIV share similar inhibitor targets (Tobin et al., 1996). Studies on BIV can serve, therefore, as a safe way to screen potential new targets for the development of antiviral therapeutics for more pathogenic lentiviruses (Yao et al., 2010).

The BIV Rev protein is a 23-kDa (186 amino acid [aa]-long) shuttling phosphoprotein that mediates the nuclear export of partially spliced and unspliced viral RNAs encoding structural proteins and is essential for viral replication (Oberste et al., 1991). Although both BIV and HIV-1 Rev localize to the nucleus and nucleoli of transfected/infected cells and colocalize with the nucleolar protein nucleophosmin (NPM1, also known as B23) (Oberste et al., 1993; Gomez Corredor and Archambault, 2009), we have recently demonstrated that BIV Rev is remarkably different from HIV-1 Rev. Moreover, BIV Rev contains nuclear import and export signals and mechanisms that are novel among the Rev proteins characterized so far in lentiviruses. BIV Rev has a bipartite nuclear localization signal (NLS) with an atypical 20 aa spacer sequence that contains the nucleolar localization signal (NoLS) (Gomez Corredor and Archambault, 2009) and an AMP-dependent protein kinase inhibitor-type nuclear export signal (Gomez Corredor and Archambault, 2012).

The nucleolus is the largest compartment of the cell nucleus and the most comprehensively studied nuclear domain. In addition to its well-known role as a site of rRNA transcription, processing, and assembly into ribosomal subunits, nucleoli have been increasingly recognized as dynamic structures involved in cell cycle regulation, viral replication, and stress responses (Boisvert et al., 2007; Couté et al., 2006). Recent studies have also clearly demonstrated that several viruses induce important alterations of the nucleoli, which may have a direct role in processes that are essential for the outcome of infection, such as viral replication, virus assembly, and the control of intracellular trafficking (for reviews see references Hiscox et al., 2010; Rawlinson and Moseley, 2015).

* Correspondence to: University of Quebec at Montreal, Department of Biological Sciences, P.O. Box 8888, Succursale Centre-Ville, Montreal, Quebec, Canada H3C 3P8.
E-mail address: archambault.denis@uqam.ca (D. Archambault).

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B23 is a 37-kDa (294 aa-long), highly phosphorylated, multifunctional protein, mainly localized in the nucleoli, that participates greatly in RNA regulatory mechanisms, including transcription, ribosome assembly and biogenesis, mRNA stability, translation and microRNA processing (Colombo et al., 2011). B23 shuttles between the nucleus and the cytoplasm and has been implicated in different stages of viral infection through its interaction with several viral proteins, such as HIV-1 Rev, the adenovirus/Japanese encephalitis virus/hepatitis B and C virus core, encephalomyocarditis viruses 2A and 3BCD, Newcastle disease virus M, herpes simplex virus type 1 UL24 and US11, adeno-associated virus (AAV) Rep, Epstein Barr virus (EBV) EBNA1-3, and human papillomaviruses E6 and E7 (Aminev et al., 2003; Mai et al., 2006; Tsuda et al., 2006; Bevington et al., 2007; McCloskey et al., 2010; Lymberopoulos et al., 2011; Samad et al., 2012; Dichamp et al., 2014; Duan et al., 2014; Jeong et al., 2014; Malik-Soni and Frappier, 2014; Rawlinson and Moseley, 2015). While the exact mechanism by which B23 regulates viral replication remains unknown for some of these interactions, examples of established mechanisms include regulation of adenovirus chromatin structure at the late infection stages (Samad et al., 2012), interaction with adenovirus basic core proteins, functioning as a histone chaperone and stimulation of viral replication (Samad et al., 2007), contributions to the transcriptional activation of EBV EBNA1 (Malik-Soni and Frappier, 2014), modulation of hepatitis delta virus replication through complex formation with small HDAg (Huang et al., 2001), and enhancement of AAV replication by interaction with a G-quadruplex DNA sequence (Satkunanathan et al., 2017).

HIV-1 Rev colocalizes and interacts with B23 in the nucleolus of transfected/infected cells, and reduction of virus production has been reported in cells treated with a synthetic peptide that competes with Rev by binding B23 with high-affinity (Fankhauser et al., 1991; Miyazaki et al., 1996; Nouri et al., 2015). However, the role of the nucleolus and one of its major proteins, B23, in BIV replication remains to be determined. Here, we demonstrate for the first time that BIV Rev interacts with the nucleolar phosphoprotein B23. Using small interfering RNA (siRNA) technology, we show that low levels of B23 impair virus production by BIV-infected cells.

2. Results and discussion

2.1. BIV Rev interacts with B23 in vivo

Since BIV Rev localizes mainly to the nucleolus and colocalizes with the nucleolar protein B23 (Gomez Corredor and Archambault, 2009), we wished to determine whether BIV Rev interacts with B23. To address this, we performed an immunoprecipitation (IP) assay using HEK293T cells transfected to express BIV Rev fused to enhanced green fluorescent protein (EGFP). As shown in Fig. 1A, B23 co-precipitated with EGFP-BIV Rev but not with EGFP alone. Similar results were observed when BIV-permissive Cf2Th (an adherent epithelial line, derived from normal fetal canine thymus) (Bouillant et al., 1989), transformed bovine peritoneal macrophages (BoMac) (Stabel and Stabel, 1995) or Vero cells were used (data not shown).

To confirm the interaction of BIV Rev with B23, we performed reciprocal IP experiments using HEK293T cells cotransfected to express EGFP-BIV Rev and B23 fused to red fluorescent protein from Discosoma sp. (dsRed). EGFP-BIV Rev but not EGFP alone co-precipitated with overexpressed B23 (Fig. 1B).

These results agree with studies showing the interaction of viral proteins with B23, including HIV-1 Rev (Fankhauser et al., 1991; Hiscox et al., 2010).

2.2. Interaction between BIV Rev and B23 is independent of RNA

To determine the nature of the interaction between BIV Rev and B23 and to assess whether this interaction occurs through RNA intermediates, IP experiments were performed using RNase A-treated lysates from cells transfected with the EGFP-BIV Rev expression vector. Samples treated with RNase A did not show any difference in interaction between EGFP-BIV Rev and B23 compared to the untreated samples (Fig. 1C). This result indicated that, although both BIV Rev and B23 can bind to RNA (Dumbar, 1989; Cullen, 2003), they form a specific protein-protein type interaction and that RNA is not required as a binding intermediate.

2.3. The BIV Rev bipartite NLS is essential and sufficient for interaction with B23

BIV Rev contains an arginine- and lysine-rich region encompassing aa 71–110, which contains a bipartite NLS and a separate NoLS (Fig. 2A). As B23 is known to bind arginine/lysine-rich domains of proteins, we hypothesized that BIV Rev interacts with B23 through this identified aa 71–110 region. Thus, to determine the region of BIV Rev required for B23 interaction, we performed an IP analysis using HEK293T cells transfected to express EGFP-BIV $Rev_{71–110}$ (containing only aa 71–110) and an alanine-substitution mutant, EGFP-BIV $Rev_{K/O-NLS/NoLS}$, in which all arginine and lysine residues within the aa 71–101 sequence (which contains the aa composing the NLS and NoLS) have been substituted by alanine residues. IP analysis identified co-precipitation of B23 with EGFP-BIV $Rev_{71–110}$ but not EGFP-BIV $Rev_{K/O-NLS/NoLS}$ (Fig. 2B). This result correlates with the fact that, as previously shown in HEK293T cells (Gomez Corredor and Archambault, 2009), the EGFP-fused mutant BIV $Rev_{71–110}$ accumulated in the nucleoli of BIV-permissive Cf2Th cells, and colocalized with B23, similarly to the EGFP-fused wild-type (WT) BIV Rev. In contrast, EGFP-BIV $Rev_{K/O-NLS/NoLS}$ presented a diffuse cytoplasmic distribution with no accumulation in the nucleus or nucleoli (Fig. 2C). Therefore, we concluded that the region between aa 71 and 101, which contains the bipartite NLS and the NoLS, is necessary and sufficient for the interaction between BIV Rev and B23. This result is in accordance with other studies that showed the binding of B23 with arginine/lysine-rich domains of viral proteins, such as HIV-1 Rev and Tat (Fankhauser et al., 1991; Marasco et al., 1994), Newcastle disease virus matrix protein (Duan et al., 2014) and herpes simplex virus US11 (Nouri et al., 2015).

2.4. Multiple domains within BIV Rev bipartite NLS interact with B23

To map the binding domains between the BIV Rev NLS and B23, we performed IP analysis of HEK293T cells expressing EGFP-fused Rev half NLS deletion mutants, BIV $Rev_{\Delta 71-90}$ (lacking aa 71–90) and BIV $Rev_{\Delta 91-110}$ (lacking aa 91–110). As shown in Fig. 2C, both these mutants were excluded from the nucleus and localized solely to the cytoplasm of Cf2Th cells. Interestingly, IP analysis identified the co-precipitation of both these half NLS deletion mutants with B23 (Fig. 2B). These results suggest that both the first and second arginine/lysine-rich NLS clusters could interact with B23. Nevertheless, the impact of aa 79–80 and aa 88 residues that compose the NoLS and are part of the half NLS deletion mutants, cannot be excluded. Our results are in agreement with herpes simplex virus US11 protein, which has also been shown to possess multiple binding sites for B23 (Nouri et al., 2015).

2.5. Interaction between BIV Rev and B23 is independent of nucleolar localization of Rev

To investigate whether the NoLS region and the nucleolar localization of BIV Rev are essential for the interaction with B23, we performed IP analysis with HEK293T cells expressing an alanine mutant, EGFP-BIV $Rev_{K/O-NoLS}$ in which two arginines of the NoLS at positions 79 and 80 that were essential for nucleolar localization have been substituted by alanine (Gomez Corredor and Archambault, 2009). Confocal microscopy of Cf2Th cells showed that EGFP-BIV $Rev_{K/O-NoLS}$ localizes to the nucleus but is excluded from the nucleolus (Fig. 2C). IP analysis showed that EGFP-BIV $Rev_{K/O-NoLS}$ precipitates with B23, thus