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Original article

Heat shock cognate protein 70 controls Borna disease virus replication via interaction with the viral non-structural protein X

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Abstract

Borna disease virus (BDV) is a non-segmented, negative-sense RNA virus and has the property of persistently infecting the cell nucleus. BDV encodes a 10-kDa non-structural protein, X, which is a negative regulator of viral polymerase activity but is essential for virus propagation. Recently, we have demonstrated that interaction of X with the viral polymerase cofactor, phosphoprotein (P), facilitates translocation of P from the nucleus to the cytoplasm. However, the mechanism by which the intracellular localization of X is controlled remains unclear. In this report, we demonstrate that BDV X interacts with the 71 kDa molecular chaperon protein, Hsc70. Immunoprecipitation assays revealed that Hsc70 associates with the same region of X as P and, interestingly, that expression of P interferes competitively with the interaction between X and Hsc70. A heat shock experiment revealed that BDV X translocates into the nucleus, dependent upon the nuclear accumulation of Hsc70. Furthermore, we show that knockdown of Hsc70 by short interfering RNA decreases the nuclear localization of both X and P and markedly reduces the expression of viral genomic RNA in persistently infected cells. These data indicate that Hsc70 may be involved in viral replication by regulating the intracellular distribution of X.

Keywords: Borna disease virus; Protein X; Heat shock cognate protein 70; Nuclear localization; Virus replication

1. Introduction

Borna disease virus (BDV) belongs to the Bornaviridae family within the non-segmented negative-strand RNA viruses, Mononegavirales, which are characterized by highly neurotropic and noncytopathic infection of a wide variety of host species, including humans. Among the animal-derived mononegaviruses [1–4], BDV has several distinguishing features. One of the most striking characteristics is the cellular localization of its replication [5,6]. BDV RNA is transcribed and replicated in the nucleus, while other negative-strand RNA viruses replicate in the cytoplasm. Furthermore, unique among RNA viruses, BDV establishes a long-lasting persistent infection in both transformed and animal brain cell nuclei [5,6], suggesting that the virus uses mechanisms for RNA replication previously unknown in eukaryotic cells.

BDV encodes at least six different proteins. Of these, the nucleoprotein (N) and phosphoprotein (P) are major components of BDV ribonucleoproteins (RNPs) and are expressed abundantly in infected cells [7]. In addition to the other viral structural proteins, such as matrix and the envelope

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glycoprotein, BDV produces a 10-kDa non-structural protein X, encoded by an open reading frame (ORF) which overlaps the P ORF by 215 nucleotides [7]. Previous studies using BDV minireplicon systems revealed that X strongly inhibits the polymerase activity of BDV, suggesting that it is a negative regulator of viral replication [8,9]. On the other hand, studies using reverse genetics have shown that a moderate level of X is required in infected cells for productive viral replication [10,11]. These observations indicate that X may control viral polymerase activity at the level needed to maintain a persistent infection in the nucleus. Although the precise mechanism by which X controls viral replication remains unclear, we reported previously that interaction of X with P promotes nuclear export of P, resulting in the cytoplasmic accumulation of both proteins [12,13]. P was retained in the cytoplasm of BDV-infected cells only when expression of X was detected in the same cell [12], suggesting that interaction between X and P reduces the level of P in the nucleus and thus prevents the formation of an active polymerase complex. Furthermore, Schwardt et al. demonstrated that BDV X may regulate viral polymerase activity via its direct incorporation into viral RNPs in the nucleus, because apparently X is present in BDV-specific nuclear dot structures where replication of BDV RNPs may occur [14]. All these observations suggest that the intracellular localization, as well as nuclear transport, of X may be important for the regulation of viral polymerase activity in infected cells. Although X is known to contain a non-canonical nuclear localization signal (NLS) within the N-terminal 20 amino acid domain and by itself localizes in the nucleus [15], it remains to be determined how a protein with a molecular mass of 10 kDa, which may be excluded by the nuclear pore, is maintained in the cell nucleus and then regulates viral polymerase activity.

In this study, we present data showing that BDV X interacts with the constitutive heat shock cognate 70 protein (Hsc70) in BDV-infected cells. We found that the N-terminal 16 amino acids of X are important for the interaction with Hsc70 and, interestingly, that their association is interfered with competitively by the expression of P. Heat shock stress of BDV-infected cells revealed that BDV X translocates rapidly into the nucleus in association with nuclear accumulation of Hsc70. Furthermore, analysis using short interfering RNA (siRNA) for Hsc70 demonstrated that knockdown of Hsc70 decreases the replication of the BDV genomic RNA, as well as the nuclear distribution of both X and P, in persistently infected cells. Our results suggested that Hsc70 may be involved in the nuclear localization of X and thereby regulate viral replication in the nucleus.

2. Materials and methods

2.1. Cell lines and virus

The OL cell line, derived from a human oligodendroglioma, was cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (4.5%) supplemented with 5% fetal bovine serum (FBS) and 4 mM glutamine. HEK293T (293T; human embryonic kidney) cells were cultured in DMEM-low glucose (1.0%) supplemented with 5% FBS. BDV-infected OL cells, a cell line persistently infected with strain huP2br [16], were cultured using the same conditions as the parental cell line.

2.2. Plasmid construction

Constructions of expression vectors encoding Flag-tagged BDV P and X have been previously described [12]. To generate the eukaryotic expression plasmid encoding Flag-tagged N, BDV N cDNA was amplified by PCR and inserted into *Kpn* I and *Xho* I sites of pcDNA3 plasmid (Invitrogen). The hemagglutinin (HA)-tagged Hsc70 expression plasmid, pcHA-Hsc70, was constructed by the insertion of Hsc70 cDNA amplified from total RNA extracted from OL cells into *Kpn* I and *Xho* I sites of pcDNA-HA vector. The tandem tagged BDV X expression vector (pTAP-X) was generated as follows. The BDV cDNA corresponding to BDV X ORF was amplified with pgX plasmid [12] and digested with *Eco*R I and *Xho* I enzymes. The resultant fragment was inserted into the tandem affinity purification (TAP) assay vector (a kindly gift of Dr. Matsuura, Osaka University), which contain a HA-TEV (tobacco etch virus protease cleavage site)-Flag cassette sequence at the multiple cloning site of pcDNA3 vector.

2.3. TAP assay

Two days before transfection, 2.5×10^5 BDV-infected or uninfected OL cells were seeded in 60-mm culture dishes and transfected with pTAP-X by lipofectamine 2000 (Invitrogen). At 72 h post-transfection, the cells were harvested and lysed with TNE buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1 or 0.5% Nonidet P-40 [NP-40], and complete protease inhibitor) on ice for 60 min. After centrifugation, the soluble fraction was immunoprecipitated with anti-HA resin (Sigma–Aldrich) overnight at 4 °C. The resin was washed five times with TNE buffer and the tagged protein was released from the resin by digestion with AcTEV protease (Invitrogen) in 150 μL of TEV buffer containing 10 U protease for 3 h at 16 °C. TNE buffer was added to the supernatant from the centrifugation to increase the volume to 1 ml and the preparation was immunoprecipitated with anti-Flag M2 resin (Sigma–Aldrich) for 4 h at 4 °C. After washing five times with TNE buffer, the resin was incubated with 100 μl of TNE buffer containing 450 ng/μl of 3× Flag peptide (Sigma–Aldrich) for 3 h at 4 °C. The eluates were analyzed by SDS-PAGE and visualized by silver staining (Wako).

The protein bands of interest were excised, digested in-gel with trypsin, and analyzed by nanocapillary reversed-phase LC–MS/MS using a C18 column (φ 75 μm) on a nano LC system (Ultimate, LC Packing) coupled to a quadrupole time-of-flight mass spectrometer (QTOF Ultima, Waters). Direct injection data-dependent