The X protein of Borna disease virus regulates viral polymerase activity through interaction with the P protein

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Borna disease virus polymerase activity is negatively regulated by the viral X protein. Using a virus minireplicon system it was found that all X mutants that no longer interacted with the viral P protein failed to exhibit significant inhibitory activity. The action of X could further be neutralized by expression of a P fragment that contained the X interaction domain but lacked all domains known to mediate interaction with other viral proteins. X thus appears to regulate the activity of the Borna disease virus polymerase by targeting the polymerase cofactor P.

The negative-strand RNA genome of Borna disease virus (BDV) encodes three proteins (N, P and L) which represent the basic components of the viral polymerase complex and a small regulatory protein, designated X or p10 (Cubitt & de la Torre, 1994; de la Torre, 2002; Schneemann et al., 2000). X can interact with P via a short leucine-rich sequence located near its N terminus (Wolff et al., 2000). The X interaction domain of the P protein is located in the N-terminal half of the molecule (Kobayashi et al., 2003). If expressed alone, X shows cytoplasmic and nuclear distribution. If expressed together with P, which usually accumulates in the nucleus, X is able to retain a fraction of P in the cytoplasm (Kobayashi et al., 1998). Cell lines constitutively expressing X exhibit a high degree of resistance to infection with BDV (Geib et al., 2003). Recent studies with viral minireplicons revealed that X is a potent negative regulator of the BDV polymerase complex (Perez et al., 2003; Schneider et al., 2003). However, the mechanism by which X exhibits its inhibitory action has to date not been resolved.

To learn more about the mode of X protein action, we investigated whether X of strain No/98, which shows only 81% sequence identity to X of strain He/80 (Nowotny et al., 2000), would inhibit the activity of a CAT reporter minireplicon system in BSR-T7 cells (Schneider et al., 2003) that is built entirely on components of strain He/80. Fig. 1 shows that X of No/98 inhibited polymerase activity in a dose-dependent manner. It was almost as potent as authentic X of strain He/80. The leucine-rich domain near the N terminus is strictly conserved between the two X proteins while the downstream sequences are not (Nowotny et al., 2000). It therefore appeared likely that the invariant 19 amino acids were involved in mediating inhibition. Since this region includes the complete P interaction motif, we hypothesized that X might act through interaction with P. We therefore employed a series of X mutants in which various amino acids near the N terminus were replaced by alanine residues (Wolff et al., 2000). Fig. 2(a) shows that, unlike wild-type X, mutants X(A8A9), X(A10A11) and X(A14A15) were unable to inhibit polymerase activity in the BDV minireplicon system. By contrast, mutants X(A6A7) and X(A18A19) exhibited inhibitory activity, although this was slightly reduced compared with wild-type X. Western blot analysis revealed that wild-type X and the various X mutants accumulated to comparable levels in transfected BSR-T7 cells (Fig. 2b), arguing against the possibility of insufficient expression. From previous yeast two-hybrid analyses, it is known that X(A6A7) and X(A18A19) interact with P, while X(A8A9), X(A10A11) and X(A14A15) cannot (Wolff et al., 2000). To reproduce this result in a mammalian system, we performed two-hybrid assays in human embryonic kidney 293T cells. With this assay, we measured interactions between a VP16-BDV-P fusion protein and wild-type or mutant forms of X, which were fused to the DNA-binding domain of Gal4. Assay conditions were basically as described by Schwemmlle et al. (1998). In agreement with the yeast two-hybrid data we found that mutants X(A6A7) and X(A18A19) interacted with P, whereas mutants X(A8A9),
Fig. 1. Divergent X proteins of BDV strains He/80 and No/98 inhibit viral polymerase activity similarly well. Using Metafectene (Biontex), BSR-T7 cells in 12-well plates were transfected with BDV minireplicon CAT reporter construct pT7-gmgA (400 ng), BDV polymerase expression construct pCA-L (200 ng), BDV-P expression construct pCA-P (25 ng), BDV-N expression construct pCA-N (250 ng) and the indicated amounts of plasmids expressing X of either BDV strain He/80 or No/98. Complete reaction mixtures lacking X expression plasmid served as a positive control (+). The transfection mixture also contained 100 ng of plasmid pBST7-luc, which served to normalize variations in transfection efficacy. Minireplicon activity in transfected cells was analysed by CAT-ELISA. Assay conditions were exactly as reported previously (Schneider et al., 2003). Extracts of cells transfected with a construct encoding measles virus polymerase L in place of BDV-L (Ø) served as a negative control. The results of one representative experiment are shown. Similar results were obtained in five additional experiments.

X(A10A11) and X(A14A15) did not (Fig. 2c). It is of interest to note that all mutants employed here except X(A10A11) are no longer able to interact strongly with importin-α (Wolff et al., 2002). Obviously, this property of the X mutants did not correlate with their ability to inhibit the BDV polymerase in the minireplicon system. If X negatively regulates BDV polymerase activity through interaction with P, it should be possible to neutralize this effect with a decoy target. We reasoned that C-terminally truncated fragments of the P protein might represent suitable decoys, because the X interaction domain is located in the N-terminal moiety of P while all other known interaction domains are located in the C-terminal moiety (Schwemmle et al., 1998). Our experiments showed that a fragment of P consisting of amino acids 1–135 (P1–135) did not disturb the BDV polymerase to any great extent even if a high amount (250 ng) of expression plasmid was transfected (Fig. 3, left columns in graph), indicating that this P fragment could be used as decoy. We therefore set up a series of minireplicon assays in which we added 25 ng of X expression plasmid and increasing amounts of plasmid encoding P1–135. The X protein exhibited a strong inhibitory effect under these conditions, provided no P1–135 was present. If 100–250 ng of the plasmid encoding P1–135 was included in the transfections, BDV polymerase activity could be rescued. In fact, in the presence of 250 ng of decoy plasmid, polymerase activity was almost as high as without X protein (Fig. 3). Interestingly, a 10-fold molar excess of P1–135 over X was required for efficient neutralization of the X inhibitory effect. Western blot analyses argued against the possibility that the decoy protein is metabolically unstable (data not shown), indicating that more than one P molecule is required to neutralize one X molecule. It remains possible, however, that the interaction between decoy and X is less efficient than the interaction between authentic P and X. Taken together, these results strongly supported the concept that X blocks BDV polymerase activity by targeting P.

A fraction of nuclear P protein was recently found to accumulate in the cytoplasm of cells that simultaneously expressed high amounts of X (Kobayashi et al., 2003). This raised the possibility that the inhibitory action of X on BDV polymerase activity might result from specific
sequestration of P in the cytoplasm. When X and P expression plasmids were co-transfected into Vero (African green monkey) cells, significant cytoplasmic retention of P was observed if equimolar amounts of X and P plasmids were used (Fig. 2d). Under these conditions, mutant X(A14A15) did not efficiently retain P in the cytoplasm, while mutant X(A18A19) did (Fig. 2d). These data suggested that cytoplasmic retention of P indeed contributes to the inhibitory effect of X. However, since cytoplasmic accumulation of P was only evident if X was abundantly present, it remains unclear whether cytoplasmic retention of P can fully explain the inhibitory effect of X on BDV polymerase activity.

From the presently available functional studies in the mini-replicon system, a picture emerges in which the X protein stands out as a key regulator of BDV polymerase activity. Results from this study indicate that X is primarily if not exclusively acting through the formation of complexes with P. X might thus sequester P away from the nuclear site of
viral genome transcription and replication. Alternatively, complex formation with X might negatively influence the interaction of P with cellular and/or viral factors. It is unclear at present whether X binding can interfere with the formation of complexes between P and the viral polymerase subunit L. We and others have shown previously that BDV is unique among viruses of the order \textit{negavirales} in that its RNA polymerase complex is tightly regulated, with high activity only observed if the N-to-P ratio is approximately 10:1 (Perez et al., 2003; Schneider et al., 2003). If we assume that BDV needs higher polymerase activity at early stages of the infection process than during persistent infection, it is not surprising that the virus has evolved an elaborate system that permits a precise fine-tuning of the polymerase activity. It looks as if BDV in that its RNA polymerase complex is tightly regulated, with high activity only observed if the N-to-P ratio is approximately 10:1 (Perez et al., 2003; Schneider et al., 2003). If we assume that BDV needs higher polymerase activity at early stages of the infection process than during persistent infection, it is not surprising that the virus has evolved an elaborate system that permits a precise fine-tuning of the polymerase activity. It looks as if BDV

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**References**


**Fig. 3.** X-mediated inhibition of the BDV polymerase can be neutralized by expression of C-terminally truncated P protein. Minireplicon assays were performed as in Fig. 1. Where indicated, we co-transfected a constant amount of pCA-X and variable amounts of a plasmid encoding the N-terminal 135 amino acids of BDV-P [pCA-P(1–135)], which include the complete X interaction domain. pCA plasmid encoding an irrelevant protein was added where necessary to keep the total DNA concentration constant in all transfections. Data are representative of three independent experiments.