Short Communication

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.*, 1995; Tomonaga *et al.*, 2002). X and P are synthesized from overlapping open reading frames of a single viral mRNA. They are both abundantly present in virus-infected cells. X is localized in the cytoplasm of infected cells as well as in characteristic nuclear dots, which seem to contain all components of the BDV polymerase complex and therefore presumably represent the sites of virus replication (Wolff *et al.*, 2000). X can interact with P via a short leucine-rich sequence located near its N terminus (Wolff *et al.*, 2000), which is highly conserved (Nowotny *et al.*, 2000; Staeheli *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 Schwemmle *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),

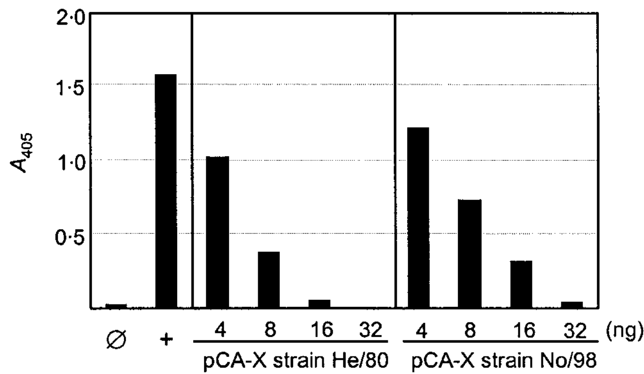


Fig. 1. Divergent X proteins of BDV strains He/80 and No/98 inhibit viral polymerase activity similarly well. Using Metafectene (Biontex), BSR-T7 cells in 12-well plates were transfected with BDV minireplicon CAT reporter construct pT7-gmgA (400 ng), BDV polymerase expression construct pCA-L (200 ng), 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 (∅) 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.

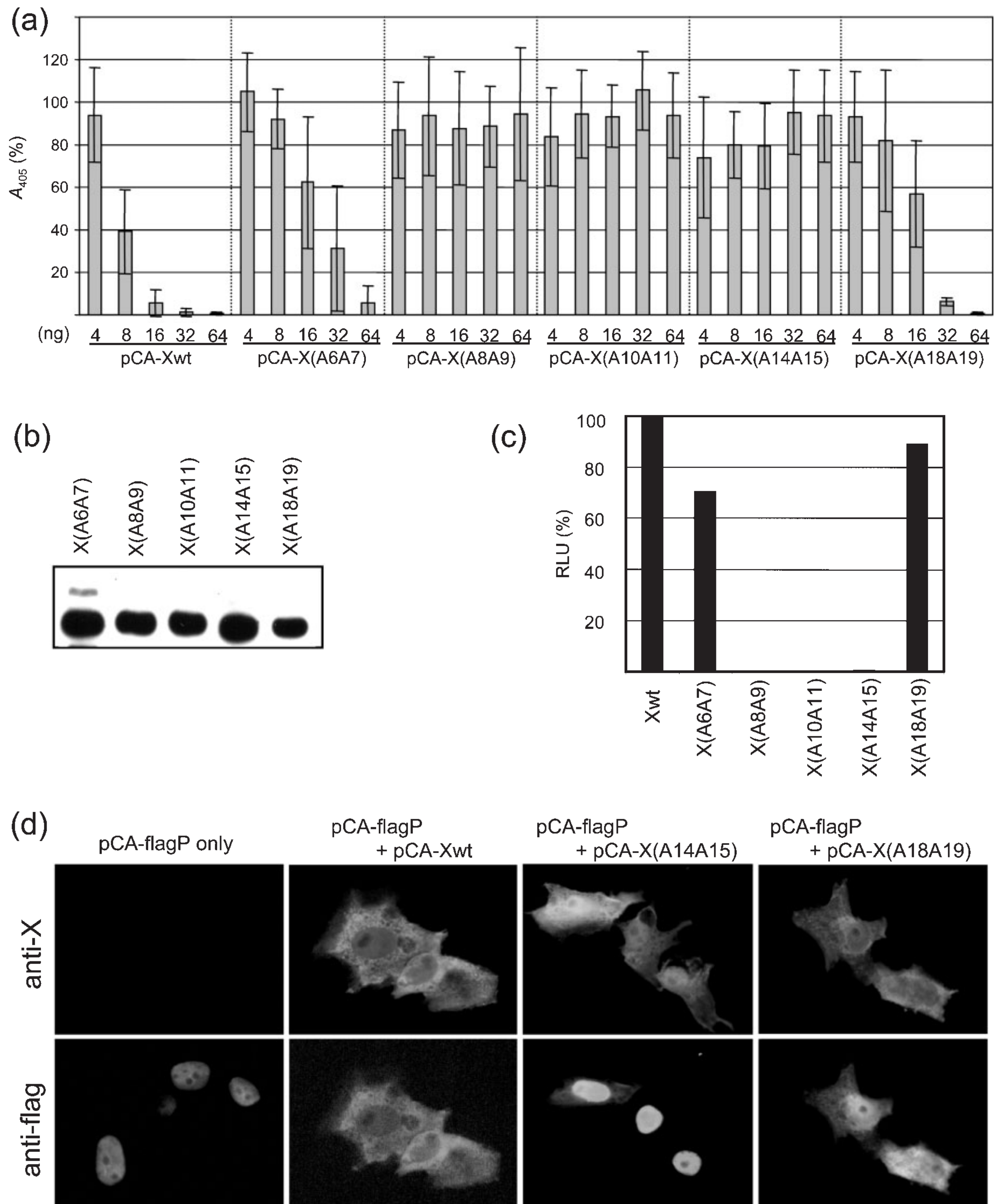
If X negatively regulates BDV polymerase activity through interaction with P, it should be possible to neutralize this effect with a decoy target. We reasoned that C-terminally truncated fragments of the P protein might represent suitable decoys, because the X interaction domain is located in the N-terminal 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 (P_{1-135}) 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 P_{1-135}. The X protein exhibited a strong inhibitory effect under these conditions, provided no P_{1-135} was present. If 100–250 ng of the plasmid encoding P_{1-135} 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 P_{1-135} 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

Fig. 2. Ability of X protein variants to inhibit BDV polymerase activity correlates with ability to interact with viral P protein. (a) Polymerase activity in the presence of various concentrations of plasmids encoding the indicated X mutants of He/80. Minireplicon assay conditions were as in Fig. 1. Polymerase activity in the absence of X was set to 100 %. Data represent the mean values ± SD of at least four independent experiments. (b) Similar metabolic stability of various X mutant proteins. Expression plasmids encoding the indicated X mutant proteins were transfected into 293T cells. Cell extracts were prepared at 72 h post-transfection and samples were analysed by Western blotting using a monospecific rabbit antiserum against BDV-X (Schwemmle *et al.*, 1998). (c) Interaction of various X mutants with BDV-P determined by mammalian two-hybrid assays. A construct encoding a VP16–BDV-P fusion protein was transfected into 293T cells together with constructs encoding Gal4–BDV-X fusion proteins carrying alanine substitutions at the indicated amino acid positions of the X open reading frame. Interactions were recorded by determining firefly luciferase reporter gene activity as described (Schwemmle *et al.*, 1998). The transfection mixtures also contained 100 ng of a constitutively expressed *Renilla* luciferase reporter gene, which was used to correct for variations in transfection efficacy. Data are representative of two independent experiments. RLU, relative light units. (d) Cytoplasmic retention of BDV-P by wild-type and mutant X proteins. Expression plasmid pCA-flagP (encoding N-terminally flag-tagged BDV-P) was transfected into Vero cells either alone or together with identical amounts of expression plasmids encoding wild-type or mutant X proteins as indicated. The intracellular localization of X and P was analysed with rabbit anti-X and mouse anti-flag antibodies, respectively. As reported previously (Kobayashi *et al.*, 2003), a significant fraction of P, which usually accumulates almost exclusively in the nucleus, was retained in the cytoplasm if wild-type X or mutant X(A18A19) was co-expressed. This effect was much less pronounced if mutant X(A14A15), which is no longer capable of interacting with P, was used.

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



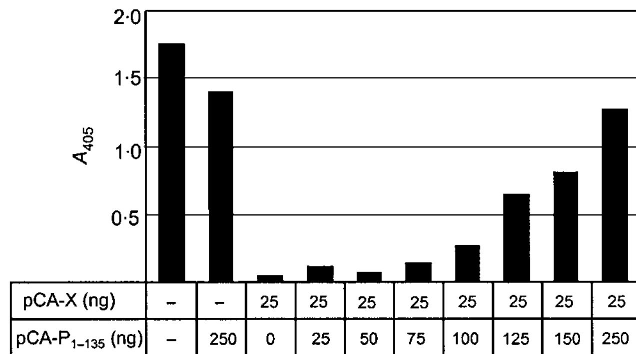


Fig. 3. X-mediated inhibition of the BDV polymerase can be neutralized by expression of C-terminally truncated P protein. Minireplicon assays were performed as in Fig. 1. Where indicated, we co-transfected a constant amount of pCA-X and variable amounts of a plasmid encoding the N-terminal 135 amino 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.

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 *Mononegavirales* 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 chose to use the N-to-P ratio as a molecular switch. This switch might only function reliably well if buffered against minor fluctuations in P levels. Our data suggest that X is serving precisely this buffering function, by temporarily reducing the intracellular concentration of biologically active P protein. Finally, we wish to point out that in infected cells the buffering effect of X might have different consequences than in the minireplicon system. Rather than being simply inhibitory, X may facilitate the switch of the viral polymerase from transcription to replication mode.

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