Characterization of the major nuclear localization signal of the Borna disease virus phosphoprotein

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Borna disease virus (BDV) replicates and transcribes its negative-sense RNA genome in the nucleus. The BDV phosphoprotein (P) is localized in the nucleus of infected cells and cells transfected with P expression constructs. To identify the nuclear localization signal (NLS) of P, COS-7 cells were transfected with wild-type or mutant forms of P fused with green fluorescent protein (GFP). Whereas GFP alone was exclusively cytoplasmic, P or P–GFP were nuclear. Analysis of carboxy- and amino-terminal truncation mutants of P indicated that amino acids (aa) 20–37 are sufficient to promote efficient nuclear accumulation of the fusion protein. Residual nuclear import of GFP was observed with portions of P including aa 33–134 or aa 134–201, suggesting the presence of additional NLS motifs. The major NLS of P appears to be bipartite. It consists of two basic aa domains, R22RER25 and R30PRKIPR36, separated by four non-basic aa, S26GSP29.

Borna Disease virus (BDV) is a non-segmented negative-strand RNA virus that causes persistent central nervous system infection and behavioural disturbances in warm-blooded animals (Ludwig et al., 1988; Rott & Becht, 1995). It encodes at least six proteins: the nucleoprotein (N), phosphoprotein (P) (Thiedemann et al., 1992; Thiémer et al., 1992), atypical glycoprotein (gp18) (Kliche et al., 1994; Stoyloff et al., 1994), type I membrane glycoprotein (p57) (Gonzalez-Dunia et al., 1997; Schneider et al., 1997), polymerase (pol) and X-protein (Briese et al., 1994; Cubitt & de la Torre, 1994; Wehner et al., 1997). BDV replicates in the nucleus (Briese et al., 1994) and employs the cellular splicing machinery for the maturation of some of its viral transcripts (Schneemann et al., 1995). Thus, transport of viral RNAs and proteins between nucleus and cytoplasm is an essential feature of the BDV life-cycle. A functional nuclear localization signal (NLS) was recently described for the N protein (Kobayashi et al., 1998; Pyper & Gartner, 1997). Three lines of evidence indicate that P may also contain an NLS and mediate nuclear import of other BDV proteins: (1) transient expression of P results in its nuclear accumulation; (2) X interacts with P but not N; and (3) the presence of P in transfected and infected cells shifts the distribution of X from the cytoplasm to the nucleus (Schwemmle et al., 1998).

To identify regions of P that mediate nuclear localization we employed a strategy similar to that used to characterize the NLS of lymphoid enhancer factor-1 (Prieve et al., 1996). A set of plasmids encoding BDV-P, P–GFP and GFP, or P–GFP mutants lacking the amino-terminal 32 aa (NΔ32), 93 aa (NΔ93) and 133 aa (NΔ133) or the carboxy-terminal 67 aa (CΔ67), 107 aa (CΔ107) or 166 aa (CΔ166), were transiently expressed in COS-7 cells. The fusion proteins were detected by immunofluorescence after incubation with antisera to P or GFP.

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Fig. 1. Localization of P, GFP and P–GFP fusion proteins after transient expression in COS-7 cells. Plasmids encoding BDV-P, P–GFP and GFP, or P–GFP mutants lacking the amino-terminal 32 aa (NΔ32), 93 aa (NΔ93) and 133 aa (NΔ133) or the carboxy-terminal 67 aa (CΔ67), 107 aa (CΔ107) or 166 aa (CΔ166), were transiently expressed in COS-7 cells. The fusion proteins were detected by immunofluorescence after incubation with antisera to P or GFP.
of constructs was generated that encode various portions of P fused to a modified version of the green fluorescent protein (GFP), a marker protein that is confined to the cytoplasm in the absence of an exogenous NLS (Prieve et al., 1996). A summary of constructs and results of experiments described below can be found in Fig. 3(A). The corresponding cDNA regions of P [amino acids (aa) 2–201] were amplified from the plasmid P-PTRE (Schwemmle et al., 1998) by PCR and cloned into the BamHI site of the GFP expression vector (Prieve et al., 1996). P wild-type, GFP or P–GFP were expressed in COS-7 cells by transient transfection using lipofectamine (Boehringer Mannheim). After 48 h, the cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100. Thereafter, the fusion proteins were detected by immunofluorescence using either a polyclonal anti-P antibody (Kliche et al., 1996) or a polyclonal anti-GFP antibody (Clontech). Whereas both P and P–GFP accumulated in the nucleus, GFP was present only in the cytoplasm (Fig. 1).

Carboxyl- and amino-terminal deletions and point mutations were introduced into the P–GFP construct to define the region(s) of P responsible for the nuclear import of P–GFP.Transient transfection of P–GFP constructs lacking the amino-terminal 32 aa (NA32), 93 aa (NA93) or 133 aa (NA133) resulted in expression of fusion protein in both cytoplasm and nucleus (Fig. 1). In contrast, fusion proteins truncated at the carboxyl terminus of 67 aa (CA67) or 107 aa (CA107) of P were detected only in the nucleus (Fig. 1). Deletion of 166 carboxy-terminal aa of P (CA166) resulted in predominantly cytoplasmic staining (Fig. 1). These experiments indicated that the amino-terminal 35 aa of P are essential but not sufficient to direct nuclear accumulation of GFP. Thus, the P constructs CA134, CA160 and CA164 (Fig. 3A) were created to examine the importance of sequence carboxyl to the first 35 aa of the protein. Expression of each of these constructs resulted in the nuclear accumulation of the reporter protein (Fig. 2), suggesting that the nuclear localization function extends to aa 36 (R) and 37 (N).

The stretch between aa 22 and aa 36 of P includes two basic regions reminiscent of bipartite NLSs in other systems (Dingwall, 1991) (Fig. 3B). To examine the role of these regions in nuclear localization we created GFP expression constructs encoding aa 20–67 (NA19–CA134) and aa 28–67 (NA27–CA134). Whereas NA19–CA134, a fusion protein containing the first basic region (R22RER25), was found predominantly in the nucleus, NA27–CA134 was present in both cytoplasm and nucleus, suggesting that aa 20–27 contribute to the efficient nuclear accumulation of GFP (Fig. 2). The significance of the central arginine (R32) in the second basic region was examined by mutating this residue to glycine in a construct containing aa 2–41 of P (R32G–CA160). The resulting mutant fusion protein accumulated less efficiently in the nucleus indicating that R32 is an important component of the NLS. Next, the role of residues within the non-basic region that separates the two basic regions of the bipartite NLS was assessed. Serines 26 and 28, previously identified as sites for phosphorylation by PKCe (Schwemmle et al., 1997), were
that phosphorylation might influence the distribution of P in the presence of other BDV proteins.

In contrast to the nucleoprotein of BDV, which is reported to have a single continuous NLS at the amino terminus (Kobayashi et al., 1998; Pyper & Gartner 1997), the major NLS of P appears to be bipartite. Another difference may be the presence in P of additional weaker NLSs toward the carboxyl terminus. The bipartite NLS of P, spanning aa 22–36, overlaps the region of P essential to interactions with X (aa 33–115) (Schwemmle et al., 1998). The observation that coexpression of P and X results in colocalization of the two proteins in the nucleus (Schwemmle et al., 1998) is consistent with activity of weaker NLSs, when P is bound to X; however, it is equally plausible that P–X complexes are imported to the nucleus through activity of the bipartite NLS. There is precedent in influenza virus nucleoprotein (Neumann et al., 1997; Wang et al., 1997; Weber et al., 1998) and herpes simplex virus type 1 ICP27 (Mears et al., 1995) for multiple NLSs. However, we cannot discern from current information whether these NLSs between aa 33 and 201 of P are biologically significant or only potential NLS motifs that are unmasked through mutagenesis of P. Reverse genetic approaches and analysis of interactions of P with other viral and host proteins in infected cells will be essential to establish the basis for P trafficking in vivo.

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References


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