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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; Thierer *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.*, 1992; Cubitt *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

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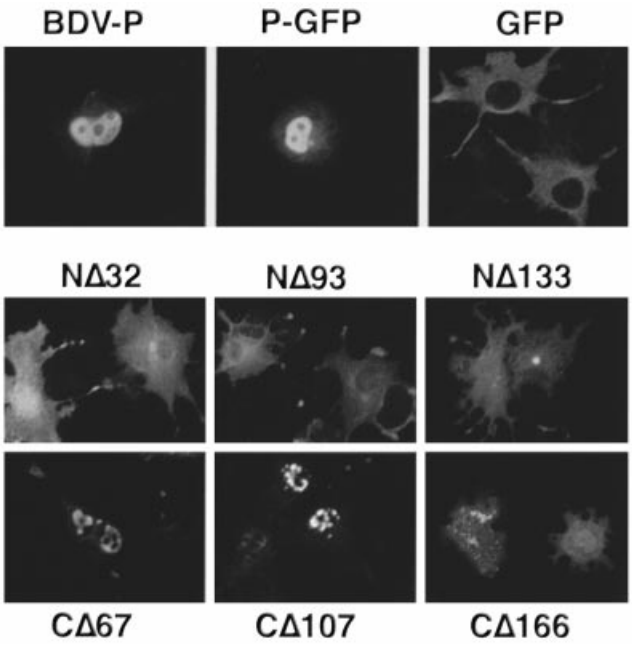

Fig. 1. Localization of P, GFP and P–GFP fusion proteins after transient expression in COS-7 cells. Plasmids encoding BDV-P, P–GFP and GFP, or P–GFP mutants lacking the amino-terminal 32 aa (NΔ32), 93 aa (NΔ93) and 133 aa (NΔ133) or the carboxy-terminal 67 aa (CΔ67), 107 aa (CΔ107) or 166 aa (CΔ166), were transiently expressed in COS-7 cells. The fusion proteins were detected by immunofluorescence after incubation with antisera to P or GFP.

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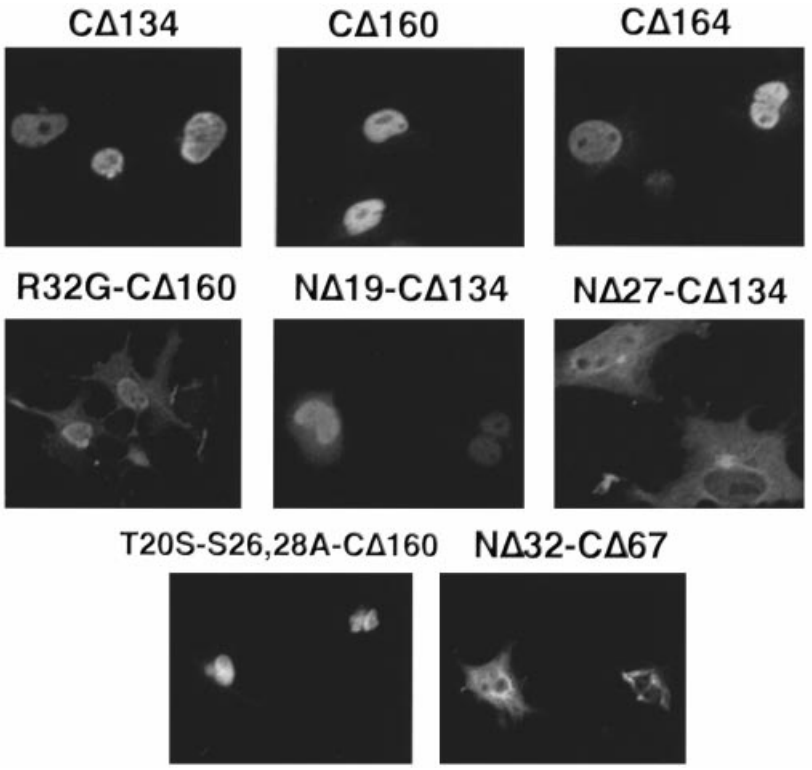

Fig. 2. Mapping of the major NLS of P. Plasmids encoding a selection of P–GFP mutants were transiently expressed in COS-7 cells. Fusion proteins were detected by immunofluorescence using antisera to P. Upper panels: carboxy-terminal deletion of 134 aa (CΔ134), 160 aa (CΔ160) and 164 aa of P(CΔ164) of P. Middle panels: carboxy-terminal deletion of 160 aa with an additional substitution at aa position 32 (R32G–CΔ160), deletion of carboxy-terminal 134 aa and 19 (NΔ19–CΔ134) or 27 amino-terminal aa (N27–C134). Lower panels: carboxy-terminal deletion of 160 aa with three aa substitutions (T20S-S26,28A–CΔ160), amino- and carboxy-terminal deletion of 32 aa and 67 aa (NΔ32–CΔ67). See Fig. 3 (A) for details of plasmid construction.

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 *Bam*HI 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 (NΔ32), 93 aa (NΔ93) or 133 aa (NΔ133) 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 (CΔ67) or 107 aa (CΔ107) of P were detected only in the nucleus (Fig. 1). Deletion of 166 carboxy-terminal aa of P (CΔ166) 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 CΔ134, CΔ160 and CΔ164 (Fig. 3 A) 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. 3 B). To examine the role of these regions in nuclear localization we created GFP expression constructs encoding aa 20–67 (NΔ19–CΔ134) and aa 28–67 (NΔ27–CΔ134). Whereas NΔ19–CΔ134, a fusion protein containing the first basic region (R22RER25), was found predominantly in the nucleus, NΔ27–CΔ134 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–CΔ160). 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 PKCε (Schwemmle *et al*., 1997), were

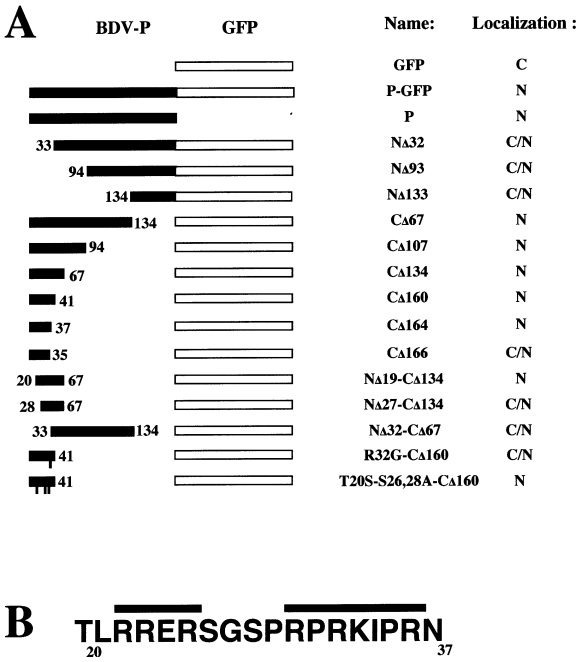

Fig. 3. (A) Schematic representation of the P–GFP fusion proteins transiently expressed in COS-7 cells. The distribution of fusion protein 48 h after transfection is indicated as follows: C, cytoplasmic; N, nuclear; C/N, cytoplasmic and nuclear. (B) Sequence of the bipartite nuclear localization signal of BDV-P. Residues contained in the two basic regions that constitute the bipartite signal are indicated by black bars.

mutated to alanines, and threonine 20 was mutated to serine (T20S-S26,28A–CΔ160). These mutations outside of the two basic domains that constitute the putative bipartite NLS had no impact on GFP distribution, providing further support for the presence of a strong bipartite NLS (Fig. 2).

The observation that P–GFP fusion proteins truncated at the amino terminus of 32, 93 or 133 aa (NΔ32, NΔ93 and NΔ133) were primarily but not exclusively cytoplasmic (Fig. 1) suggests the presence of weak NLS sequences in portions of P other than those that constitute the bipartite NLS. To address this possibility, a construct was created in which GFP was fused to aa 33–134 of P (NΔ32–CΔ67). Proteins expressed from NΔ32–CΔ67 (Fig. 2) were present predominantly in the cytoplasm; however, signal was also detected in the nucleus. In concert with results obtained with NΔ133, these data are consistent with the presence of at least two potential NLSs: one between aa 33 and 134, and a second carboxyl to residue 134.

The rate of nuclear import of some viral proteins, for example SV40 large T-antigen (Rihs *et al.*, 1991) and influenza virus nucleoprotein (Neumann *et al.*, 1997), is dependent on phosphorylation by cellular protein kinases. In studies reported here mutation of PKC phosphorylation sites within the bipartite NLS (serines at aa 26 and 28) (Schwemmle *et al.*, 1997) had no impact on the nuclear localization of P–GFP fusion protein (Fig. 2); however, we did not examine either the kinetics of nuclear import in PKC mutants or the possibility 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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