Characterization of the equine infectious anaemia virus S2 protein

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S2 is an accessory protein of equine infectious anaemia virus (EIAV), the function of which is unknown. In order to gain insight into the function of S2, the intracellular localization of the protein, its interaction with viral proteins and its incorporation into viral particles have been investigated. Immunolocalization of S2 revealed punctate staining in the cytoplasm and the S2 protein co-precipitated with the EIAV Gag precursor. Despite overexpression of S2 through the use of a codon-optimized sequence, there was no preferential association of S2 with EIAV particles. These data suggest that S2 may function to organize the Gag protein during particle assembly in the cytoplasm but that it is unlikely to be involved in the early stages of the virus life-cycle.

Equine infectious anaemia virus (EIAV) replicates in all equidae, resulting in a persistent infection and chronic disease (Montelaro et al., 1993). In addition to the structural proteins encoded by gag, pol and env, EIAV has three accessory genes, tat, rev and S2 (Montelaro et al., 1993). The S2 gene is located between the second exon of tat and env, overlapping the N terminus of the Env coding region (Schiltz et al., 1992). It appears that S2 is not necessary for virus particle production, as EIAV-based vectors give similar titres on cell lines irrespective of whether they are S2 positive or S2 negative (Mitrophanous et al., 1999). EIAV that lacks S2 is able to infect and replicate in monocyte-derived macrophages with similar kinetics to S2-positive EIAV (Li et al., 1998). However, in one study, a virus lacking S2 was less pathogenic in ponies, showing a less severe platelet reduction compared with wild-type virus (Li et al., 2000). S2 is small (65 aa, 7 kDa) and basic (pI = 11.5) and contains a number of potential functional motifs (Fig. 1a). These include a nuclear localization signal, a nucleoporin motif and an SH3-binding motif (Fritz et al., 1995; Li et al., 1998) and we have also noted myristylation signals at Gly7 and Gly16, a glycosylation site at Asn52 and a number of phosphorylation sites marked in Fig. 1(a). In order to gain insight into the possible function of S2, we attempted to identify its site of action. Similar studies have been informative for other lentiviruses (Mahalingam et al., 1997; Goncalves et al., 1994; Raja et al., 1994).

Initially, we attempted to determine the localization of the wild-type S2 protein by transfecting plasmids expressing S2 into 293T cells. The constructs used for analysing S2 localization are shown in Fig. 1(b). All S2 constructs were sequenced to confirm the changes. Two kinds of S2 antibodies were generated in chickens (GenoSys) by using two synthetic polypeptides, corresponding to S2N and S2C (Fig. 1a). These antibodies were validated in Western blotting against E. coli-purified thioredoxin (Trx)–S2 fusion proteins, in which S2 protein was tagged with Trx at the C terminus. However, we did not observe any protein in 293T cells transfected with a standard S2-expressing plasmid, by either Western blot analysis or indirect immunofluorescence (data not shown). We therefore fused S2 to EGFP (enhanced green fluorescent protein) at the C and N termini (Cubitt et al., 1995). A protein of the correct molecular mass was recognized by both anti-GFP and anti-S2C antibodies when GFP was fused at the N terminus. However, when the fusion was at the C terminus of S2, the majority of the product was smaller than expected. There was a striking difference in the localization of the two fusions. The control GFP was found throughout the cells, as expected. The N-terminal fusion GFP–S2 was excluded from the nucleus, while the C-terminal fusion was found throughout the cell (data not shown). These data suggested that the precise nature of the fusion could influence localization.

We therefore used a myc epitope tag, as it is very small and therefore should not influence the localization (Wilson et al., 1996; Elefanty et al., 1996). A mycHis tag was placed at the C terminus of S2 by inserting the S2 sequence into

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(a) **EIAV Genome**

![Diagram of EIAV genome with annotations](image)

(b) **Plasmids and Protein Expression**

<table>
<thead>
<tr>
<th>Plasmid</th>
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<tr>
<td>pMyc-S2</td>
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<tr>
<td>pS2-MycHis</td>
<td>S2mycHis</td>
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<td>pOptS2\textsubscript{19}</td>
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<td>pOptS2\textsubscript{PV}</td>
<td>OptS2\textsubscript{PV}</td>
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<tr>
<td>pOptS2\textsubscript{PV}MycHis</td>
<td>OptS2\textsubscript{PV}mycHis</td>
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Fig. 1. (a) The genomic organization of EIAV and predicted S2 motifs. Locations of all genes are indicated. Two kinds of S2 sequence (depending on EIAV strain) and their potential motifs are shown. MYRISTYL, GLYCOSYL and SH3 are potential myristylation, glycosylation and SH3 motifs. S2N and S2C are regions used in generating antibodies specific for S2. CK2, Casein kinase II; PKC, protein kinase C. (b) Schematic representation of plasmid constructs used in the localization studies. CMV, Human cytomegalovirus enhancer/promoter; pA, simian virus 40 late polyadenylation signal.

pCDNA3.1MycHisA (Invitrogen). A myc tag was placed at the N terminus of S2 by inserting an appropriate synthetic oligonucleotide into pCI-S2. As shown in Fig. 2(a), both of the S2 proteins tagged with the myc epitope showed cytoplasmic localization in 293T and COS7 cells. Interestingly, the staining was punctate compared with the control lacZmycHis and the localization appeared to be biased towards the perinuclear region. This suggests an association of S2 with subcellular structures. The immunostaining was repeated with cells that were fixed without permeabilization, and no staining for S2 was observed (data not shown). This suggests that S2 is confined to the internal structures of the cytoplasm. The fact that localization was strikingly cytoplasmic irrespective of the fusion at the N or C terminus suggests that the previous data with the GFP fusion at the C terminus were artifactual.

The finding that S2 was confined to the cytoplasm was somewhat unexpected, as S2 is small enough to diffuse freely throughout the cell. Given the discrepancy with the GFP

Fig. 2. (a) Confocal microscopy of 293T and COS7 cells transiently transfected with S2 tagged with myc epitope. Cells transfected with each designated plasmid were immunostained with anti-myc MAb. COS7 cells were counterstained with the nucleic acid stain YOPRO-1. (b) Sequence comparison between wild-type S2 and OptS2\textsubscript{19}. (c) Localization of untagged S2 in D17 and COS7 cells. D17 and COS7 cells were transfected with pOptS2\textsubscript{19}, which expresses codon-optimized untagged S2 based on pSPEIAV19. The protein was probed with anti-S2C antibody (left panels) and counterstained with the nucleic acid stain YOPRO-1 (right panels). (d) COS7 cells were also transfected with pOptS2\textsubscript{PV}, which expresses codon-optimized untagged S2 based on EIAV\textsubscript{PV}. (e) COS7 cells were transfected with pOptS2\textsubscript{PV}MycHis and immunostained with anti-myc MAb. (f) COS7 cells transfected with the indicated plasmid were lysed and the supernatant and pellet were analysed by Western blot analysis. Lanes: 1, pS2MycHis; 2, pOptS2\textsubscript{PV}MycHis.
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Fig. 2. For legend see facing page.
fusion, there was concern that the myc epitope was affecting the native localization of S2. We therefore sought to improve the expression of wild-type S2. We noted that the codon usage in S2 was suboptimal for expression in mammalian cells when we compared codon usage tables of S2 with those from highly expressed human genes (Haas et al., 1996) (data not shown). This is a typical feature of lentiviruses (Haas et al., 1996). A synthetic human immunodeficiency virus (HIV) envelope gene that uses optimal codons leads to a dramatic increase in protein expression (Haas et al., 1996). We therefore constructed a synthetic S2 gene that had optimized codons (Fig. 2b). The synthetic S2 gene (OptS2\textsubscript{19}) was made from four oligonucleotide pairs designed to convert the codons in the S2 sequence of the EIAV strain pSPEIAV19 into those most frequently used in mammalian genes, as defined by Haas et al. (1996). The Kozak consensus sequence for optimal translation initiation was also included (Kozak, 1992). The localization of OptS2\textsubscript{19} was analysed in COS7 and D17 cells. With the anti-S2 antibody, significant immunofluorescence could be seen in over 40% of the COS7 cells. In all cases, the fluorescence was confined to the cytoplasm and was punctate (Fig. 2c), as observed previously.

Given that S2 could diffuse to the nucleus, its restriction to the cytoplasm must be active rather than passive. This could be achieved either by a rapid nuclear export mechanism that simply prevents detection of nuclear-localized S2 or by the binding of S2 to cytoplasmic structures. The punctate staining in the cytoplasm was suggestive of an interaction with cellular structures. Primary sequence analysis of S2 revealed a nucleoporin motif (Fig. 1a). Nucleoporin motifs mediate binding to the cytoplasmic face of the nuclear envelope. The cell culture-adapted avirulent strain pSPEIAV19 has an amino acid change in the potential nucleoporin motif and this could serve to disrupt the classical consensus nucleoporin motif (Cook et al., 1998), thereby influencing the localization of OptS2\textsubscript{19}. We therefore made OptS2\textsubscript{PV} based on the S2 sequence of EIAV strain EIAV\textsubscript{PV}, which has a potential nucleoporin motif. When the localization of this protein was studied in COS7 cells, the staining was still found in the cytoplasm and was still punctate (Fig. 2d). These data suggest that a potential nucleoporin motif in S2 does not dictate the staining pattern and imply that the attenuation of pSPEIAV19 is not due to differences in S2 sequences. This may not be surprising, as it is thought that more than one nucleoporin motif is required for localization to the nuclear membrane (Nigg, 1997).

It is possible that overexpression of S2 could influence its subcellular localization. The mycHis tag was therefore fused to OptS2\textsubscript{PV} and its localization was compared with the wild-type S2 sequence fused to mycHis. As shown in Fig. 2f, Western blot analysis using anti-myc MAbs revealed a higher level of expression of OptS2\textsubscript{PV}mycHis compared with wild-type S2. Immunofluorescence analysis of OptS2\textsubscript{PV}mycHis revealed the same localization and punctate quality of staining of S2mycHis (Fig. 2e). With S2mycHis, only 3% of the cells were stained, whereas with OptS2\textsubscript{PV}mycHis it was over 80% of the cells. We also examined whether there was any difference in the localization of S2 when it was co-expressed with other EIAV proteins. We could not detect any differences in the pattern of immunostaining, which indicates that localization of S2 may not be affected by other viral proteins (data not shown). Taken together, the data indicate strongly that S2 is naturally localized to the cytoplasm. During localization studies, it was found that the anti-S2N antibody against the region Ala\textsuperscript{12}–Pro\textsuperscript{28} could not detect any mammalian cell-expressed S2 proteins, while detecting E. coli–expressed Trx–S2. This indicates that S2 may be modified post-translationally, at least in the region of the epitope to which the anti-S2N antibody binds.

We next purified the S2 protein fused with Trx to investigate S2-interacting proteins. Equivalent amounts of Trx or Trx–S2 purified from E. coli were dialysed against coupling buffer (MOPS–NaOH pH 7.9, 0.3 M NaCl, 0.5% glycerol) and coupled with affigel-10 (Bio-Rad) according to the manufacturer’s instructions. Trx–S2-bound resin was incubated with lysates from 293T cells transfected with an EIAV gag/pol-expressing construct, pONY3.1, which expresses all EIAV proteins except envelope (Mitrophanous et al., 1999). Trx-bound resin incubated with cell lysates was used as a negative control. As shown in Fig. 3(a, b), the Gag precursor (p55) bound Trx–S2 and not Trx, which indicates that S2 interacts directly with the Gag precursor. In order to examine whether any cellular (293T) proteins bind Trx–S2 non-specifically, the Trx–S2-bound resin was incubated with a cell lysate from pCI-neo-transfected 293T cells, but no band was detected (Fig. 3a, lane 6). In order to confirm the S2–Gag interaction in vivo, co-immunoprecipitation was carried out with the codon-optimized S2 plasmid pOptS2\textsubscript{PV}MycHis and the EIAV gag/pol-expression plasmid pONY3.1AS2. The results showed that S2 interacts with the EIAV Gag precursor in vivo (Fig. 3c). The possible interaction of S2 with the EIAV envelope was also investigated, but it was found that the EIAV envelope did not co-precipitate with S2 (data not shown).

For most retroviruses, including lentiviruses, virus assembly and budding take place simultaneously on the inner face of the plasma membrane (Coffin, 1996; Joag et al., 1996) and the Gag precursor is a major mediator of virus assembly. Some accessory proteins in lentiviruses are virion associated (Bachand et al., 1999; Kondo et al., 1995; Selig et al., 1999). Given that S2 binds EIAV Gag, we further examined whether S2 is incorporated specifically into virions. The codon-optimized S2 expression plasmid pOptS2\textsubscript{19} was used in conjunction with either an EIAV (pONY3.1 + pONY4Z + pRV67) or murine leukaemia virus (MLV) (pHIT60 + pHIT111 + pRV67) vector system (Mitrophanous et al., 1999; Soneoka et al., 1995), but S2 was not found in either EIAV virions or MLV virions (data not shown). In order to increase the sensitivity, pOptS2\textsubscript{PV}mycHis was
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Fig. 3. S2 interacts with EIAV Gag precursor. (a)–(b) Equivalent amounts of Trx or Trx–S2 purified from E. coli were incubated with lysate from 293T cells transfected with EIAV gag/pol-expression construct or pCI-neo. After three washes with lysis buffer, samples were analysed by Western blot analysis with EIAV-positive horse serum (a) or anti-p26 MAb (b). Lanes: 1, 12 µg cell lysate mock-transfected 293T cells; 2, 12 µg cell lysate from 293T cells transfected with EIAV gag/pol-expression plasmid; 3, sample eluted from Trx resin incubated with input (gag/pol); 4, sample eluted from Trx–S2 resin incubated with input (gag/pol); 5, 12 µg cell lysate from 293T cells transfected with pCI-neo; 6, sample eluted from Trx–S2 resin incubated with pCI-neo. (c) Co-immunoprecipitation analysis of OptS2PVmycHis and EIAV Gag. Cell lysates of 293T cells transfected with plasmids designated as above were incubated with mouse anti-myc MAb. GammaBind Plus G Sepharose was added for immunoprecipitation (IP). Aliquots of each sample were resolved on SDS–polyacrylamide gels and examined by Western blot analysis with horse polyclonal antibodies specific for EIAV (upper panel) or anti-myc MAb (lower panel).

used, because the anti-myc MAbs are much more sensitive than the anti-S2 antibodies. A small amount of OptS2PVmycHis was incorporated into both EIAV and MLV virions, indicating that S2 is not incorporated specifically into EIAV virions (data not shown). If S2 recruitment is dependent on an interaction with Gag, it must involve the region of Gag that is well conserved between EIAV and MLV, as S2 was also incorporated into the MLV virion. However, it is more likely that S2 is incorporated passively into EIAV and MLV virions. Passive recruitment would also be consistent with the packaging of S2 into MLV virions, as this virus, which does not encode an S2-like protein, is not likely to possess a specific mechanism for S2 incorporation. Non-specific incorporation of viral proteins into virions has been reported for some of the HIV-1 accessory proteins, such as Vif and Vpu (Camaur & Trono, 1996).

In conclusion, our study shows that S2 is a cytoplasmic protein that can interact with Gag but is not incorporated preferentially into particles. This indicates that S2 may not function in the early phase of the life-cycle. The possible association of S2 with other intracellular organelles indicated by the punctate pattern of staining and the S2–Gag interaction suggests that S2 may be involved in virus particle assembly.

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References


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