Distinct domains in herpes simplex virus type 1 US11 protein mediate post-transcriptional transactivation of human T-lymphotropic virus type I envelope glycoprotein gene expression and specific binding to the Rex responsive element

Nathalie Schaerer-Uthurralt,1 Monique Erard,2 Karine Kindbeiter,1 Jean-Jacques Madjar1 and Jean-Jacques Diaz1

1 Immuno-Virologie Moléculaire et Cellulaire CNRS UMR5537, Faculté de Médecine Lyon-R.T.H. Laennec, Rue Guillaume Paradin, 69372 Lyon CEDEX 08, France
2 Laboratoire de Biologie Moléculaire des Eukaryotes CNRS, 118 route de Narbonne, 31062 Toulouse CEDEX, France

Herpes simplex virus type 1 (HSV-1) US11 protein is an RNA-binding protein which is able to mediate post-transcriptional transactivation of human T-lymphotropic virus type I (HTLV-I) envelope glycoprotein gene expression by interacting with the Rex responsive element (XRE) located at the 3' end of the env mRNA. In view of this functional activity, and because US11 protein is capable of substituting for HTLV-I Rex protein, it was hypothesized that US11 protein should exhibit at least two functional domains, an RNA-binding domain for specific interaction with the target RNA, and an effector domain involved in transport and translation of this mRNA. Recombinant US11 wild-type and deleted proteins were tested for their ability (i) to bind to the XRE and to HSV-1 UL34 RNA, the natural target of US11 protein, and (ii) to transactivate HTLV-I env gene expression. The C-terminal half of US11 protein, consisting of 20–24 XPR repeats, was necessary and sufficient to mediate RNA-binding with a high affinity and specificity. Structure prediction analyses showed the likely conformation of this domain to be that of a polyproline type II helix. Localized within the first 40 amino acids of the N-terminal region of US11 protein was the effector domain, deletion of which created US11(Δ1–40), a trans-dominant negative mutant. These results demonstrate structural differences between US11 protein and proteins like Rex and Rev, despite their functional similarities.

Introduction

The herpes simplex virus type 1 (HSV-1) genome contains at least 79 genes, grouped according to the function of their products into those which are essential for viral DNA synthesis and assembly of infectious virus particles, and those allowing virus replication in cells under various conditions (Ward & Roizman, 1994). Many of the latter appear to be dispensable for virus growth in cell culture (Roizman & Sears, 1993). For this reason, it is very likely that the cellular counterparts of these so-called non-essential genes should complement the defective viruses (Ward & Roizman, 1994). HSV-1 US11 true late gene is one of these non-essential genes (Johnson et al., 1986; Longnecker & Roizman, 1987). Even though US11 protein may be dispensable in certain conditions, it appears to be essential for HSV-1 replication in cells submitted to thermal stress, probably by playing a role in post-transcriptional regulation of gene expression (Diaz et al., 1996; Diaz-Latoud et al., 1997; Roller & Roizman, 1991). Since the first identification of US11 protein as a DNA-binding protein, strong evidence has been brought showing that this protein also exhibits RNA-binding activity which appears to be essential for its biological function (Bayliss et al., 1975; Dalziel & Marsden, 1984; Diaz et al., 1996; MacLean et al., 1987; Roller et al., 1996; Roller & Roizman, 1990, 1991). In vitro interaction of US11 protein with a synthetic RNA was found to be sequence- and secondary structure-specific. However, the RNA bound by US11 corresponded mostly to an antisense sequence of the 5' untranslated region of US11 mRNA and therefore probably does not exist in infected cells (Roller & Roizman, 1990). It was later demonstrated that, in fact, US11 protein bound specifically to HSV-1 UL34 mRNA, at least in vitro. The UL34 gene is essential for HSV-1 and its expression appears to be regulated.
by US11 protein through an unknown mechanism (Roller & Roizman, 1991). However, no clear function has been ascribed to US11 protein in the HSV-1 life cycle (Roller & Roizman, 1991, 1992, 1994).

As a true late gene product, US11 protein is packaged within the native virus where it is found as a structural protein estimated to be present at 600 to 1000 copies per virion (Roller & Roizman, 1992). From several independent studies, we concluded that, on introduction into the cell by the infecting virus, US11 protein first enters the nucleus together with the viral DNA before passing back into the cytoplasm where it exists as heterogeneous polymers (Diaz et al., 1992; Massé et al., 1990; Puvion-Dutilleul, 1987; Roller & Roizman, 1992). Based upon these considerations, and because US11 protein is an RNA-binding protein, we proposed some years ago that US11 protein could be involved in the nucleo-cytoplasmic transport of specific mRNA (Diaz et al., 1993). Moreover, the striking similarities displayed by US11 protein with the retroviral regulatory proteins Rex and Rev of human T-lymphotropic virus type I (HTLV-I) and human immunodeficiency virus type I (HIV-1), respectively, led us to postulate that this very basic phospho-protein, which concentrates in the nucleoli of infected cells, could intervene post-transcriptionally in the life cycle of these complex retroviruses. Indeed, we have demonstrated that US11 protein can substitute for Rex and, under certain conditions, for Rev, in transactivating expression of the retroviral genes encoding envelope glycoproteins (Diaz et al., 1996). US11 protein binds specifically to the Rex responsive element (XRE) located in the 3′ untranslated region of HTLV-I envelope mRNA (Diaz et al., 1996). However, the RNA-binding characteristics of US11 protein have not yet been determined and no direct comparison has been made between US11 protein-binding affinity for XRE and that for UL34 RNA.

Rex and Rev post-transcriptionally regulate the replication cycles of HTLV-I and HIV-1, respectively (Cullen, 1992). These transactivator proteins are required for the nucleo-cytoplasmic transport of unspliced gag–pol and singly spliced env transcripts. Rex and Rev interact directly with their cis-acting RNA target sequences, designated respectively XRE and the Rev-responsive element (RRE), before driving into the cytoplasm the incompletely spliced mRNA encoding the structural proteins (Fischer et al., 1994). Therefore, these Rex and Rev complex transactivating functions involve multiple specific interactions, encompassing protein–RNA and protein–protein interactions, for providing intracellular movements of the protein–RNA complexes. This is achieved by distinct domains structurally organized within the proteins, each playing a defined role. Nuclear localization signals allow the newly synthesized proteins to migrate to the nucleus where they are able to interact with RNA in a sequence-specific manner via the RNA-binding domains (Cochrane et al., 1990; Siomi et al., 1988). Multimerization domains lead to the recruitment of additional Rex or Rev molecules, and effector domains direct the target RNA to the nuclear pore through binding to nucleoporin-like factors (Bogerd & Greene, 1993; Bogerd et al., 1996; Hope et al., 1992; Malim et al., 1989; Meyer et al., 1996).

Since US11 protein can substitute for Rex and Rev in transactivating envelope glycoprotein gene expression, and because it is also able to bind XRE and RRE, we hypothesized that US11 protein should be organized into at least two functionally distinct domains: an RNA-binding domain allowing sequence-specific interaction with responsive elements within UL34 and human retrovirus mRNA, and an effector domain which might be responsible for nucleo-cytoplasmic transport of the target RNA. To verify this hypothesis, several deletions were made within US11 protein, and the function of the deleted proteins tested: first, for RNA-binding using both HTLV-I XRE and the US11 responsive element located within UL34 mRNA as target sequences; and second, for transactivation of HTLV-I env gene expression through a functional test allowing detection of envelope glycoprotein production (Diaz et al., 1996). The results of this study clearly allow us to delineate two distinct domains within US11 protein. The N-terminal region of US11 protein is necessary for transactivation of HTLV-I env gene expression, and is therefore the so-called effector domain of the protein. Moreover, a deleted form of US11 protein lacking the effector domain acts as a trans-dominant negative mutant. The C-terminal half of the protein is sufficient to mediate RNA-binding with high affinity and specificity. Binding affinities of US11 protein for the XRE and for UL34 RNA are very similar. These effector and RNA-binding domains exhibit primary and secondary structures completely different from those of other known RNA-binding proteins displaying such functions. In particular, the RNA-binding domain is characterized by 20 repeats of the XPR sequence. Secondary structure predictions of this new RNA-binding motif reveal a possible polyproline type II helix organization.

Methods

Cell culture and transfections. HeLa cells were grown as monolayers in Eagle’s minimum essential medium supplemented with 5% heat-inactivated foetal calf serum. Cells were transfected by the calcium phosphate precipitation procedure as previously described (Greco et al., 1994).

Construction and control of recombinant fusion proteins. Construction of the recombinant fusion proteins was achieved by cloning different parts of the US11 coding sequence (HSV-1 strain KOS) in-frame to the glutathione S-transferase (GST) coding sequence of pGEX-2T (Pharmacia). DNA fragments containing portions of the US11 coding sequence were obtained by PCR amplification using pG-3 (Simonin et al., 1981), as a template plasmid. The deduced amino acid sequence of US11 protein from the KOS strain is almost identical to that of strain 17 (McGeoch et al., 1985), the main difference being the two SPREPR repeats located at the C terminus, as previously described (Simonin et al., 1995). US11 protein from the KOS strain is 149 amino acids long, while US11 proteins from strains 17 and
MP are 161 amino acids long. Five expression vectors were obtained which contained the GST coding sequence followed by either the entire US11 coding sequence (pGEX-2) or by parts of the US11 coding sequence (pGEX-5, pGEX-6, pGEX-11 and pGEX-15). Fusion proteins were purified from Escherichia coli DH5α (Gibco BRL) transformed with the different plasmids and stimulated with IPTG for 3 h as previously described (Diaz et al., 1993). Fusion protein synthesized from pGEX-2 was named GST-US11 and contained the 149 amino acids of US11 (see Fig. 2a). For mutant fusion proteins, the letter ‘m’ was added to GST-US11 followed by the respective numbers of the pGEX vectors from which the proteins were synthesized. GST-US11-m15 and GST-US11-m11 contained the first 40 and 84 amino acids of US11, respectively; GST-US11-m5 and GST-US11-m6 contained the last 109 and 62 amino acids of US11.

Western blot, syncytia counting and immuno-fluorescence analyses. After transfection of HeLa cells with pCMV-US11 in the presence or absence of pCMV-US11(Δ1–40), Western blot analysis of the different forms of US11 protein was carried out following SDS–PAGE (Laemmli, 1970) as previously described (Diaz et al., 1993). Syncytia formation was visualized 48 h after transfection of 1.5 × 10⁵ HeLa cells with 1 µg pHTLV-env, 1 µg pcTAX (Rimsky et al., 1988) and 0.5 µg pCMV-US11 together with different amounts of pCMV-US11(Δ1–40). These co-transfections were carried out with an identical amount of total plasmid (3 µg) containing the CMV immediate early promoter, by supplementation with pCMV (Diaz et al., 1996). The number of syncytia containing at least five nuclei was scored under an inverted microscope at low magnification (× 200) after May–Grünwald–Giemsa staining. For immuno-fluorescence, 10⁵ HeLa cells were transfected with 1.8 µg pCMV-US11(Δ1–40). At 48 h after transfection, cells were fixed with paraformaldehyde and permeabilized with Triton X-100. The deleted form of US11 protein was localized with anti-US11 rabbit antibody diluted 50-fold (Diaz et al., 1993) and fluorescein isothiocyanate-labelled second antibody.

Protein–RNA binding analysis. A fragment of the HTLV-I genome (Seiki et al., 1983) located between nucleotides 8601 and 8876 and containing the CDNA encoding the entire XRE was obtained by PCR amplification from pTAX-LTR (Rimsky et al., 1989) and cloned into pBluescript II KS+. A part of the UL34 gene of HSV-1 (strain KOS) located between the EcoRI and SmaI endonuclease restriction sites of psg-3 (Goldin et al., 1981) was subcloned into pBS. This region of the UL34 gene encompasses the binding site of US11 protein (Roller & Reizman, 1991). RNA containing the XRE or the US11 binding site of UL34 was synthesized from the pBS-derived plasmids using T3 RNA polymerase and [α-³²P]UTP (Promega) as previously described (Diaz et al., 1996). As a control, an RNA (pBS RNA) was synthesized from pBS itself, after digestion with Poulil, using T3 RNA polymerase and [α-³²P]UTP. [³²P]-labelled RNA was resuspended in 50 mM NaCl containing BSA 0.25 mg/ml, denatured at 80 °C for 5 min, then renatured at 37 °C for 20 min. For each gel retardation assay, the fusion proteins at a given concentration were incubated with 10 µg yeast tRNA (Boehringer) at 4 °C for 10 min. Then, 2 × 10⁶ c.p.m. (about 0.5 ng) of the internally labelled RNA was added to the samples and the incubation extended for a further 10 min at 4 °C. Reaction mixtures were directly submitted to electrophoresis using a 4% polyacrylamide gel in standard TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) containing 4% glycerol. Electrophoresis was performed at 4 °C for 450 V·h. Gels were fixed for 10 min in 7.5% acetic acid–30% methanol, dried and submitted to autoradiography for 30 min. Measurement of the radioactivity contained in the different bands was performed on the dried gels by [³²P]quantification using a Storage Phosphor Imaging System (GS-525, Bio-Rad).

Molecular modelling of US11 protein C-terminal domain. Computer models of the US11 C-terminal domain comprising the last 76 residues were generated using the Biosym Technologies modules INSIGHT II, BIOPOLYMER and DISCOVER (version 230), run on a Silicon Graphics Indigo Elan workstation. Appropriate φ and ψ angles were assigned to each residue of the US11 polypeptide according to its predicted secondary structure, using both the Chou–Fasman and GOR algorithms. Thus the two predicted configurations of the polypeptide, a 3₁₀-helix and a polyproline type II helix, were built. The resulting structures were first refined by an energy minimization procedure consisting of 500 steps of steepest descent followed by 1000 cycles of conjugate gradients to a maximum derivative of 0.1 kcal/A/mol, using the consistent valence forcefield, and then subjected to an extensive cycle of molecular dynamics.

Results

Binding of US11 protein to the XRE

Characteristics of US11 protein binding to the XRE and to UL34 RNA were determined and compared by gel retardation assay. To this aim, US11 protein was purified as a recombinant GST-US11 fusion protein and incubated with [³²P]-labelled XRE or UL34 RNA synthesized in vitro; [³²P]-labelled pBS RNA provided a negative control. The amount of RNA was kept constant and incubation with GST-US11 protein was carried out with increasing amounts of the fusion protein, in the presence of a 3 × 10⁻⁵-fold excess of non-specific competitor RNA. RNA–protein complexes were then separated from free RNA by electrophoresis through non-denaturing polyacrylamide gels and revealed by autoradiography (Fig. 1). Binding of GST-US11 protein to UL34 RNA was not significantly modified by varying the concentrations of MgCl₂ (from 1 to 6 mM) and KCl (from 12.5 to 200 mM), or by the presence of 100 mM NaCl, whereas binding was slightly increased when the temperature was raised from 4 to 42 °C (data not shown). GST-US11 protein bound to the XRE and UL34 RNA with about the same affinity, complete binding being achieved at 570 to 1140 nM protein (Fig. 1a, b). By contrast, binding of GST-US11 to the control pBS RNA was very weak and insufficient to provide electrophoretic retardation of the probe, even at high protein concentration (Fig. 1b).

To evaluate the binding affinity of GST-US11 for the three RNAs, dissociation constants (Kd) of the RNA–protein complexes were calculated. Radioactivity in free RNA and in GST-US11–RNA complexes was measured on dried gels for each GST-US11 concentration (Fig. 1c). The Kd was defined as the concentration of GST-US11 protein required to bind 50% of the RNA probe. GST-US11 interacted strongly with both XRE and UL34 RNA with Kd ranging between 100 and 130 nM. By contrast, the Kd of the GST-US11–pBS RNA complex was about 1500 nM. In addition, the US11 concentration-dependent electrophoretic mobility suggested a possible in vitro oligomerization of GST-US11 on the probe. Such oligomerization of GST-US11 molecules bound to specific target

Downloaded from www.microbiologyresearch.org by
IP: 54.70.40.11
On: Fri, 11 Jan 2019 09:20:46
RNA ruled out a possible involvement of the GST part of the fusion protein in the oligomerization process, GST alone being only able to dimerize (Wen et al., 1995).

**Identification of the US11 protein domain mediating the specific binding to the XRE**

We next determined whether a single and continuous discrete domain of US11 protein was responsible for the high affinity and specific binding to the XRE and to UL34 RNA (Fig. 2). To this aim, recombinant fusion mutant proteins containing parts of US11 fused in-frame with GST were constructed and purified (Fig. 2a, b). GST-US11-m6 contained the C-terminal half of US11 characterized by 20 XPR repeats. GST-US11-m11 contained the N-terminal region of US11, encompassing the first methionine residue up to the beginning of the XPR repeats. In addition, because it was shown previously that US11(Δ1–40) protein lacking the first 40 amino acids was unable to transactivate HTLV-I envelope gene expression (Diaz et al., 1996), an equivalent of this deleted protein was constructed (GST-US11-m5) together with the complementary GST-US11 fusion protein containing only the first 40 amino
acids of US11 (GST-US11-m15). When analysed by SDS-PAGE after purification, all these different forms of GST-US11 protein gave a single band of similar intensity when stained with Coomassie brilliant blue (Fig. 2b). Binding affinities of the different recombinant proteins to the XRE and UL34 RNA were then determined by gel retardation assay as before (Fig. 2c). As expected, GST-US11 interacted with the XRE and UL34 RNA. The deleted GST-US11-m5 and GST-US11-m6 also bound to the XRE and UL34 RNA, giving rise to protein–RNA complexes of distinct electrophoretic mobilities. Conversely, GST, GST-US11-m15 and GST-US11-m11 were not able to bind the XRE or UL34 RNA. As already shown, GST-Rex did not bind to UL34 RNA but only to the XRE (Diaz et al., 1996). To evaluate the binding affinity of GST-US11-m6, deleted protein–RNA complexes was performed with constant levels of 32P-labelled RNA (lanes 1 to 4), UL34 RNA (lanes 5 to 8) or pBS RNA (lanes 9 to 12), as in Fig. 1, and increasing concentrations of GST-US11-m6. Reaction mixtures were separated by electrophoresis through a non-denaturing polyacrylamide gel. After fixation and drying, the gel was submitted to autoradiography for 30 min. As a control, 32P-labelled RNA was incubated without protein (lanes 1, 5 and 9). (b) Gel retardation assay of GST-US11–XRE and GST-US11–m5–XRE complexes. 32P-labelled XRE (0.3 ng) was incubated with either GST-US11 (114 to 1140 nM, lanes 2 to 8) or GST-US11-m5 (125 to 1254 nM, lanes 10 to 16). The fragment of US11 present in GST-US11-m5 is identical to that expressed by pCMV-US11(A1–40). Reaction mixtures were analysed as in (a). As a control, 32P-labelled RNA was incubated without protein (lanes 1 and 9).

![Fig. 3. Specific binding of GST-US11-m5 and GST-US11-m6 deleted proteins to RNA. (a) Gel retardation assay of GST-US11-m6 deleted protein–RNA complexes was performed with constant levels of 32P-labelled RNA (lanes 1 to 4), UL34 RNA (lanes 5 to 8) or pBS RNA (lanes 9 to 12), as in Fig. 1, and increasing concentrations of GST-US11-m6. Reaction mixtures were separated by electrophoresis through a non-denaturing polyacrylamide gel. After fixation and drying, the gel was submitted to autoradiography for 30 min. As a control, 32P-labelled RNA was incubated without protein (lanes 1, 5 and 9). (b) Gel retardation assay of GST-US11–XRE and GST-US11–m5–XRE complexes. 32P-labelled XRE (0.3 ng) was incubated with either GST-US11 (114 to 1140 nM, lanes 2 to 8) or GST-US11-m5 (125 to 1254 nM, lanes 10 to 16). The fragment of US11 present in GST-US11-m5 is identical to that expressed by pCMV-US11(A1–40). Reaction mixtures were analysed as in (a). As a control, 32P-labelled RNA was incubated without protein (lanes 1 and 9).](image-url)

protein interacted strongly and very similarly with both the XRE and UL34 RNA, but very weakly with pBS RNA (Fig. 3a). This behaviour, similar to that observed for the wild-type GST-US11 protein, strongly suggested that the domain of US11 containing the 20 XPR repeats was the part of the molecule mediating high affinity and specificity in RNA-binding.

Analysis of the RNA-binding properties of GST-US11-m5 mutant protein, which corresponded to the non-functional US11(A1–40) deleted protein, revealed that the binding efficiency of this protein was different from that of GST-US11 wild-type protein. Indeed, the electrophoretic mobility of GST-US11-m5 complexed to either the XRE or UL34 RNA was always lower than that of the wild-type complexed to the same probes. Moreover, the amount of residual unbound RNA was lower when incubated with the same amount of the deleted protein compared with the wild-type protein (Fig. 2c, lanes 5 and 6 and lanes 13 and 14). To examine whether these differences were significant, we performed gel retardation assays with a constant level of the XRE and increasing amounts of the GST-US11-m5 deleted protein and of the GST-US11 wild-type protein as control. These analyses (Fig. 3b) showed that the binding affinity for the XRE was higher for the deleted protein than for GST-US11 wild-type protein, the Kd being about 30% lower. Under our conditions, 456 nM GST-US11 was required to bind approximately the same amount of XRE as 356 nM GST-US11-m5 (Fig. 3b, lanes 5 and 12). Again, the mobility of the GST-US11-m5–XRE complex was lower than that of the GST-US11–XRE complex at high protein concentration, and higher at low protein concentration (Fig. 3b). These results suggested that multimerization ability was superior for the deleted GST-US11 than for the wild-type GST-US11 protein and not the consequence of the deleted protein adopting a less compact conformation than the wild-type.

**Computer modelling of the RNA-binding domain of US11**

The sequence of the US11 RNA-binding domain was remarkable since it was built with a highly repeated tripeptide motif (XPR) in which the presence of proline was indicative of a turn-like structure as the basic component, and excluded the formation of α-helix or β-strand segments from the outset. Two main types of secondary structure were able to accommodate such repeated turn motifs, the right-handed 3_{10}-helix and the poly-α-proline type II helix. Both had the ability to give rise to structural regions significantly longer than the ones defined by canonical α-helices or β-strands and, as such, they pertained more to the supersecondary level of folding than to the mere secondary one. Secondary structure prediction performed on the last 76 residues of the C-terminal domain pointed to the sole presence of consecutive turns. In consequence we explored the two aforementioned structures, a right-handed 3_{10}-helix...
Fig. 4. Computer modelling of US11 RNA-binding domain (residues 85 to 161 of US11 protein strain 17 containing 24 XPR repeats). Two potential structures were explored, a $3_{10}$-helix (left) and a polyproline helix (right). Proline rings are displayed in red, arginine side-chains in indigo. The side-chains corresponding to the third position of the repeat tripeptide motif XPR have been coloured in yellow for Glu and Asp, turquoise for Thr and Ser, magenta for Val and Ala. The polypeptide backbone appears as a white-coloured ribbon. Hydrogen atoms have been omitted for the sake of clarity.

and an $\alpha$ poly-$\ell$-proline type II helix, as potential structures for the US11 C-terminal domain (Fig. 4). In each case, the characteristic standard $\Phi$ and $\Psi$ angles were input at the beginning and the resulting structures were submitted to an extensive cycle of minimization and molecular dynamics to take into account the modulation by the actual polypeptide sequence. Energy minimization and molecular dynamic calculations indicated greater stability for the poly-$\ell$-proline type II helix configuration, which also appeared more compact. The C-terminal half of US11 protein encompassing the RNA-binding domain might therefore exhibit a polyproline type II helix conformation, stabilized by a proline at every third residue (for a review, see Williamson, 1994).

Identification of a trans-dominant inhibiting activity for US11(Δ1–40) protein

In view of the fact that the US11(Δ1–40) protein had lost its ability to transactivate expression of HTLV-I envelope glycoprotein gene expression (Diaz et al., 1996), and because it was still able bind to the XRE and to oligomerize in vitro, we tested the possibility that the deleted form of US11 protein could act as a trans-dominant negative mutant. First of all, synthesis of either US11 or US11(Δ1–40) protein alone was verified in HeLa cells transfected with increasing amounts of either pCMV-US11 or pCMV-US11(Δ1–40). The level of US11(Δ1–40) protein accumulation after transfection of
HeLa cells (10^5) were transfected with pCMV-US11(Δ1–40). At 48 h after transfection, the deleted form of US11 protein was detected by indirect immuno-fluorescence using an inverted microscope at low magnification (x 100).

Sub-cellular distribution of US11(Δ1–40) non-functional protein. HeLa cells (10^5) were transfected with pCMV-US11(Δ1–40). At 48 h after transfection, the deleted form of US11 protein was detected by indirect immuno-fluorescence using an inverted microscope at low magnification (x 100).

To assess the trans-dominant negative effect of US11(Δ1–40) protein, we used the previously described US11-dependent syncytia formation assay (Diaz et al., 1996). HeLa cells were co-transfected with a constant amount of Tax-directed pHTLV-env and pCMV-US11, together with increasing amounts of pCMV-US11(Δ1–40). The number of syncytia containing at least five nuclei was scored 48 h after transfection (Fig. 6a). As expected, co-expression of US11 together with env and tax led to the formation of syncytia. However, co-expression of pCMV-US11(Δ1–40) inhibited very strongly the appearance of multinucleated cells. To verify whether this inhibition was due to a true trans-dominant negative effect of US11(Δ1–40) on US11 protein, or to an impairment of US11 synthesis by US11(Δ1–40), the amount of US11 and US11(Δ1–40) was verified by Western blot analysis (Fig. 6b). The amount of transfected plasmids containing the CMV promoter was kept constant for each transfection. Increasing the amount of pCMV-US11(Δ1–40) from 0.5 to 2 µg, while keeping constant pCMV-US11 at 0.5 µg, did not significantly modify the accumulation of either US11 or US11(Δ1–40) protein. In addition, cellular toxicity of US11(Δ1–40) was ruled out because expression of a control CAT reporter plasmid was not impaired when co-transfected with pCMV-US11(Δ1–40) (data not shown). All these results suggested very strongly that US11(Δ1–40) indeed acted as a trans-dominant negative protein in inhibiting US11 wild-type protein function.

Discussion

In this study, we have demonstrated that HSV-1 US11 protein interacts with HTLV-1 XRE with high affinity. RNA-binding assays were carried out with recombinant GST-US11 fusion proteins and 32P-labelled target RNA synthesized in vitro, in order to compare the binding affinity of US11 protein for the XRE with that for HSV-1 UL34 RNA. The RNA encoding the essential viral protein UL34 was chosen as a reference among the RNA previously shown to interact with US11 protein because this RNA contains a sequence of about 95 nucleotides required for the specific binding of US11 and because of its physiological relevance in the course of the virus cycle (Roller & Roizman, 1991). The binding affinities of US11 protein for the XRE and for UL34 RNA were evaluated by determination of the apparent Kd of the different RNA–protein complexes in the presence of a high level of non-specific RNA.
In our experimental conditions, binding affinities of US11 protein for the XRE and for UL34 RNA were very similar, with $K_d$ ranging from 100 to 130 nM, and at least 10 times higher than that for the pBS RNA ($K_d \sim 1500$ nM) used as control. This 10-fold difference in apparent $K_d$ between specific US11 protein binding to either XRE or UL34 RNA and non-specific US11 protein binding to other RNA sequences is sufficient to account for the specificity of US11 protein binding. As already emphasized, the binding constant of ribosomal S4 protein to its natural site on the 16S ribosomal RNA is only 5-fold higher than that for non-specific binding between S4 and tRNA. $K_d$ for S4–16S RNA interaction being only 14 mM (Heaphy et al., 1990; Vartikar & Draper, 1989). In addition, the $K_d$ determined here for US11 protein interactions with specific RNAs are in the range of those determined for other RNA-binding proteins. For example, nucleolin, a major nucleolar protein that contains four consensus RNA-binding domains, interacts specifically with nascent pre-rRNA with high affinity and an apparent $K_d$ of 50 to 100 nM (Ghisolfi-Nieto et al., 1996). Furthermore, binding affinity estimated for GST-US11 with the XRE and UL34 RNA is as strong as that evaluated for recombinant GST-Rex and GST-Rev fusion proteins with the XRE and RRE (Bogerd et al., 1992; Malim & Cullen, 1991). The apparent $K_d$ calculated for a recombinant Rev protein with an RRE-containing RNA was found to be even lower, around 1 nM. However, the binding experiments leading to this value were carried out using filter-binding assays with a recombinant Rev protein instead of a fusion protein, and in the absence of non-specific competitor RNA, making $K_d$ values difficult to compare (Burd & Dreyfuss, 1994; Daly et al., 1989).

Database searches, as well as visual inspection of the sequence, did not reveal similarities between US11 protein and any other known RNA-binding proteins (Burd & Dreyfuss, 1994). The C-terminal half of US11 protein being very rich in arginine residues, it seemed highly likely that this region is responsible for the RNA-binding activity. However, although it has already been shown that this region of US11 is probably involved in RNA binding (Roller et al., 1996), it has not been demonstrated whether the XPR repeats alone are necessary and sufficient to bind RNA with high affinity and specificity. Our first mutational analyses demonstrated that the C-terminal half of US11 protein containing the XPR repeats is the only one exhibiting RNA-binding activity. Moreover, these XPR repeats were able to bind the XRE and UL34 RNA with a very similar high affinity. From these results we conclude that US11 protein might display an RNA chaperone activity (Herschlag, 1995) mediated by its C-terminal domain made up of the XPR repeats. One may speculate that US11 protein is involved in the correct processing of the RNA to which it has the ability to bind. US11 protein might contribute to the correct folding of the XRE and to the subsequent nucleo-cytoplasmic export and translation of HTLV-I env mRNA.

Many RNA-binding proteins have modular structures consisting of one or several copies of various RNA-binding domains, often coupled with so-called auxiliary domains. Some of the already identified RNA-binding modules include the RNA recognition motif, the double-stranded RNA-binding domain and the K-homologous domain (for a review, see Burd & Dreyfuss, 1994). The structures of these domains, determined by crystallography and NMR, have revealed common features such as an $\alpha$–$\beta$ topology and the presence of a potential RNA-binding surface made up of aromatic and basic residues. As stated in the Results section, the conformation of the US11 RNA-binding domain is most likely a polyproline type II helix. This raises the question as to whether the US11 C-terminal domain could constitute a novel RNA-binding motif, as already suggested (Roller et al., 1996). It is difficult, at this stage, to anticipate the mechanism by which this type of domain could mediate a specific interaction with an RNA target, and only a few hypotheses can be put forward. Arginine, which is present at every third residue, should generate both positively-charged groups for interaction with RNA and a network of hydrogen-donor groups for more specific interactions. Along this line, the capacity of arginine specifically to recognize guanine (Shimoni & Glusker, 1995) has been shown to play an essential role in HIV-1 Rev–RRE and Tat–TAR interactions (Grate & Wilson, 1997). In addition to imposing the structural constraints leading to the polyproline conformation, proline residues could promote hydrophobic interactions with RNA bases. Finally, the flexibility of the polyproline type II helix is an advantage in ligand-binding activity, be it nucleic acids or other proteins (Williamson, 1994).

The finding that US11($\Delta$1–40) was not only non-functional (Diaz et al., 1996) but also acted as a trans-dominant negative form of US11, although it was able to bind the XRE (this study), demonstrates that the RNA-binding domain of US11 is not sufficient for US11 function. This strongly suggests that the N-terminal region of US11 protein consisting of the first 40 amino acids represents an effector domain which probably interacts with cellular proteins. However, because the steady-state amount of US11($\Delta$1–40) protein present in HeLa cells after transfection was lower than that of the wild-type US11 protein, it was necessary to verify whether the non-functional phenotype of the deleted protein was due to the lack of a functional domain, or reflected only the fact that a certain amount of protein was required to obtain post-transcriptional transactivation activity. We have shown previously that the functional replacement of Rex by US11 protein can be evaluated in HeLa cells, the efficient synthesis and targeting of the envelope glycoprotein being visualized by the formation of multinucleated cells (Diaz et al., 1996). Using this functional assay, we have shown that US11($\Delta$1–40) protein is able to inhibit the function of the wild-type protein. Demonstration of the trans-dominant negative phenotype of US11($\Delta$1–40) protein strongly suggests that US11($\Delta$1–40) protein is non-functional because of the deletion of its N-terminal region which therefore should contain the effector domain of the...
protein. In this functional assay, analysis of the amounts of US11 and US11(Δ1–40) protein revealed that inhibition of US11 function was not due to an impairment of US11 protein synthesis induced by the simultaneous expression of wild-type and mutant US11 genes. Mechanistically, there are several ways to explain the dominant negative effect of the deleted protein. We have demonstrated that US11 protein is able to form large polymers of up to 200 US11 protein molecules (Diaz et al., 1993). This oligomerization was also demonstrated in vitro by gel retardation assay, the XPR repeats alone still being able to oligomerize on the XRE. Therefore, if oligomerization of wild-type US11 protein is necessary for its function, as it is for Rex and Rev, the deleted and the wild-type US11 proteins might form inactive hetero-oligomers. However, because US11(Δ1–40) protein exhibited a higher RNA-binding affinity for the XRE than for the wild-type protein, the deleted protein might also compete with the wild-type protein for binding to the XRE, thus impairing an efficient processing of env mRNA due to the absence of the effector domain. Whether the effector domain of US11 protein contains a nuclear export signal (NES) remains to be determined. If an NES is localized within the first 40 amino acids, it should be a so-called atypical effector domain, since no consensus sequence of the HIV-Rev type could be identified in this region (Kim et al., 1996). However, many hydrophobic residues are concentrated in the N-terminal region of US11 protein, making them potential candidates to be part of such a signal.

This work was supported by Centre National de la Recherche Scientifique (CNRS) and by a grant to J.-J.M. from Agence Nationale de Recherches sur le SIDA (ANRS).

References


Received 20 January 1998; Accepted 10 March 1998