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Functionality of the STNV translational enhancer domain correlates with affinity for two wheat germ factors

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ABSTRACT

The satellite tobacco necrosis virus RNA is uncapped and requires a 3′ translational enhancer domain (TED) for translation. Both in the wheat germ extract and in tobacco, TED stimulates *in cis* translation of heterologous, uncapped RNAs. In this study we investigated to what extent translation stimulation by TED depends on binding to wheat germ factors. We show that *in vitro* TED binds at least seven wheat germ proteins. Translation and crosslinking assays, to which TED or TED derivatives with reduced functionality were included as competitor, showed that TED function correlates with binding to a 28 kDa protein (p28). One particular condition of competition revealed that p28 binding is not obligatory for TED function. Under this condition, a 30 kDa protein (p30) binds to TED. Importantly, affinity of p30 correlates with functionality of TED. These results strongly suggest that TED has the capacity to stimulate translation by recruiting the translational machinery either via binding to p28 or via binding to p30.

INTRODUCTION

In eukaryotic cells, different modes of translation initiation occur. An unusual mechanism is used by the uncapped RNAs of satellite tobacco necrosis virus (STNV), tobacco necrosis virus and barley yellow dwarf virus, which activate translation through 3′-UTR sequences (1–4; F.Meulewaeter unpublished results). STNV translation requires the translational enhancer domain (TED), which is located within a 120 nt sequence immediately downstream of the coding region. TED stimulates translation of uncapped RNAs synergistically with the STNV leader both in wheat germ and in tobacco (1–3). Furthermore, it stimulates cap-independent translation autonomously from different positions within the mRNA (5). How TED stimulates recruitment of the translational machinery to the mRNA 5′ end is not known.

The most likely scenario is that TED uses, at least in part, the same machinery as other translation promoting elements like the cap, the poly(A) tail or internal ribosome entry sites. The role of the cap is to facilitate translation initiation via a direct interaction with eukaryotic translation initiation factor (eIF)4E that, together with eIF4A and eIF4G, forms the eIF4F complex. In a subsequent step, eIF4A and eIF4B unwind the secondary structure of the leader to allow binding of the 43S pre-initiation complex (consisting of the 40S ribosomal subunit, Met-tRNA$_i$, eIF2 and eIF3), probably via an interaction of eIF3 with eIF4G. The initiation complex then scans to the first AUG start codon where translation initiates (6,7). In addition to the interaction with eIF4E, the cap may use additional ways of recruiting the translational machinery. Recently it was shown that in a yeast extract the nuclear cap-binding complex interacts with eIF4G and stimulates translation (8). Specifically in plants, two additional cap-binding factors are present, eIFiso4E and the novel cap-binding protein (nCBP), which both stimulate translation *in vitro* (9,10).

Cap-dependent translation is supported by the poly(A) tail (11). The poly(A) tail binds eIF4F, eIF4B and eIF4G either directly (12) or indirectly via the poly(A)-binding protein (13–15). The poly(A) tail is believed to deliver eIF4F and eIF4B to the mRNA 5′ end via a cap-dependent circularisation of the mRNA (16). The poly(A) tail not only stimulates translation synergistically with the cap but also autonomously, at least under non-competitive conditions *in vitro* (17–19). This implies that *in vivo* the poly(A) tail may stimulate translation both in a cap-dependent and in a cap-independent way.

Internal ribosome entry sites (IRES elements) mediate a translation mechanism that acts independently from the cap. IRES elements have been identified in animals both on cellular and on viral RNAs. They may recruit the translational machinery via binding of eIF4G (20) or eIF3 (21), but binding of other proteins is also involved (22–24). eIF4E is not required for translation that is driven by most of the IRES elements (25). This indicates that different translation-stimulating *cis*-elements differ in the way the first contact with the translational machinery is made.

In an analogy to the other translation-promoting *cis*-elements, it is likely that TED stimulates translation through binding of cellular proteins as an initial step in recruiting the translational machinery to the mRNA. In this study we found that binding of at least two wheat germ proteins, one of 28 kDa (p28) and one of 30 kDa (p30) to TED correlates with functionality. The simplest explanation for these observations is that TED can activate translation via binding to either p28 or p30.

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MATERIALS AND METHODS

Plasmid constructions

pFM191A and pFM191B are described by Meulewaeter *et al.* (3,5). pVE192 is a pGEM-3Z-derived vector containing the *cat* coding sequence with an *Nhe*I restriction site immediately downstream of the stop codon (unpublished results). pRvL15 is a pGEM-derived vector containing the complete STNV-2 trailer sequence as described by Danthinne *et al.* (26) (unpublished results).

pZR1 and pZR2 were made by insertion of the *Nde*I–*Nco*I fragment of pFM191B and pFM191A, respectively, between the *Nco*I and *Nde*I sites of the vector pVE192.

pRvLR655TED was constructed in two steps. First, a PCR product containing the STNV-2 trailer sequence was made on pRvL15 with the upstream primer RL26 (5′-CTATGTA-GCTAGCAGGACGCTGAAAGATGCGTA-3′) (*Nhe*I site underlined) and a downstream primer complementary to vector sequences downstream of the STNV-2 trailer sequence. Secondly, the PCR product was digested with *Nhe*I and *Nsi*I (using the *Nsi*I restriction site in the STNV-2 trailer), and this insert was cloned between the *Nhe*I and *Pst*I sites of the pZR1 vector.

pRvLT655TED, pRvLT656TED, pRvLT657TED, pRvLT658TED, pRvLT660TED and pRvLT688TED were made using the same strategy, with the upstream PCR primers RL26, RL30 (5′-CTATGTAGCTAGCGGACGCTGAAAGATGCG-TAG-3′), RL31 (5′-CTATGTAGCTAGCGACGCTGAAAGA-TGCGTAGC-3′), RL28 (5′-CTATGTAGCTAGCACGCTGA-AAGATGCGTAGCT-3′), RL29 (5′-CTATGTAGCTAGCG-CTGAAAGATGCGTAGCTAC-3′) and JonecP2 (5′-CTATG-TAGCTAGCTGCACTTCCTGGTGCAAAGC-3′), respectively (*Nhe*I site underlined), and the PCR products were cloned in the vector pZR2. pRvLR660TED was constructed essentially in the same way as pRvLT660TED, but the PCR product was cloned in pZR1.

pT7N655TED, pT7N656TED, pT7N657TED, pT7N658TED and pT7N660TED were constructed by insertion of an *Nde*I–*Kpn*I fragment of pFM108 (1), in which the overhang of the *Kpn*I site was removed with Klenow enzyme, between the *Nde*I and Klenow-blunted *Nhe*I site of the vectors pRvLT655TED, pRvLT656TED, pRvLT657TED, pRvLT658TED and pRvLT660TED, respectively.

In vitro transcription of unlabelled RNAs

In vitro transcription was performed essentially as described by Meulewaeter *et al.* (5). The templates for the synthesis of the RNAs with the tobacco mosaic virus (TMV) leader, the *cat* coding region and $TED_{(655–753)}$, $TED_{(656–753)}$, $TED_{(657–753)}$, $TED_{(658–753)}$, $TED_{(660–753)}$ or $TED_{(688–753)}$ were synthesised via PCR with Forw18nt (5′-GGGTTTTCCCAGTCACGAC-3′) as upstream primer and RL17 (5′-CTAGTTCAGGACTACTG-TCC-3′) as downstream primer, on the templates pRvLT655-TED, pRvLT656TED, pRvLT657TED, pRvLT658TED, pRvLT660TED and pRvLT688TED, respectively.

The templates for synthesis of $TED_{(655–753)}$, $TED_{(656–753)}$, $TED_{(657–753)}$, $TED_{(658–753)}$ and $TED_{(660–753)}$ were generated using the same strategy on the templates pT7N655TED, pT7N656TED, pT7N657TED, pT7N658TED and pT7N660TED, respectively. The template for synthesis of the RLcat RNA was *Mnl*I-digested pRvLR660TED. The template for synthesis of RNA-R655TED was synthesised via PCR with the primers Forw18nt and RL17 on pRvLR655TED.

In vitro translation

In vitro translation was done essentially as described by Meulewaeter *et al.* (5). Reactions were performed in the presence of [^{35}S]methionine at 25°C. Protein synthesis was quantified using a Storm 820 PhosphorImager (Molecular Dynamics) and ImageQuaNT 5.0 software. Protein accumulation (P) as a function of time (t) was analysed using the mathematical description given by Danthinne *et al.* (1):

$$P(t) = (aR_0/b)[1 - e^{-b(t-T)}] \quad \mathbf{1}$$

in which T corresponds to the time point at which the first translation product is completed, a is the translational efficiency of the mRNA (equivalent to protein molecules synthesised per mRNA molecule per time unit at $t = T$), R_0 is the initial RNA input (at $t = 0$) and b is a constant that is inversely proportional to the functional half-life of the mRNA ($= t_{1/2}$) according to the relation $t_{1/2} = \ln 2/b$ (1). The functional half-life is the time in which the protein accumulation rate halves and measures thus the stability of the mRNA that is actively translated, as opposed to the chemical stability that measures the physical integrity of the transcript. As in these experiments the input of translatable mRNA is equal under all conditions, the product $aR_0 = A$ (equivalent to protein synthesis rate at $t = T$) also reflects the translational efficiency of the mRNA. Equation **1** can also be written as:

$$P(t) = (A \cdot t_{1/2}/\ln 2)[1 - e^{-(\ln 2/t_{1/2})(t-T)}] \quad \mathbf{2}$$

From equation **2** it can be deduced that $P(\infty) = (A \cdot t_{1/2})/\ln 2$, showing that the protein peak level is proportional to both the translational efficiency and the functional half-life of the mRNA. By non-linear regression using equation **2** and the GraphPad Prism 3.0 software, a best fitting curve to the experimental data points was calculated and values for A, $t_{1/2}$ and T were obtained. The translational efficiency as shown in Figure 1C corresponds to A.

UV crosslinking assay

The UV crosslinking assays were performed essentially as described by Kaminski *et al.* (22). TED RNA probes with a high specific activity were synthesised from the same template as the unlabelled $TED_{(655–753)}$ RNA (see above) in an 80 µl reaction [40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 1 mM CTP, 1 mM UTP, 0.1 mM ATP, 0.1 mM GTP, 200 µCi [α-^{32}P]ATP (0.8 µM), 200 µCi [α-^{32}P]GTP (0.8 µM), 280 U T7 RNA polymerase (Amersham Pharmacia)], which results in a specific activity of the TED RNA of 3×10^{18} d.p.m./mol. The RNA was purified over a polyacrylamide gel and incubated in a concentration of 10 fmol/µl with the wheat germ extract (2 µg protein/µl) in a 20 µl reaction (18.8 mM HEPES–KOH pH 7.5, 3.2 mM Tris–Ac pH 7.5, 110 mM KAc, 1 mM $MgAc_2$) during 20 min at room temperature. The reactions were irradiated using a Stratalinker (Stratagene) at an energy level of 2.4 J/cm². The unbound RNA was removed with RNaseA (0.5 µg/µl) and RNaseVI (3.6 U/ml) (Pharmacia) during 1 h at 37°C. Optionally, in addition to RNase, proteinase K was also added (50 µg/ml). The samples were loaded on an SDS–15% polyacrylamide gel and the TED-binding proteins were visualised by autoradiography.

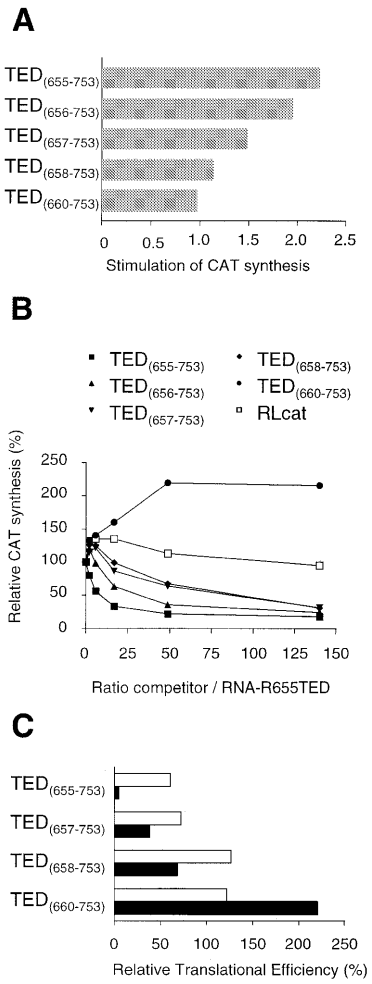

Figure 1. Functionality of TED correlates with its ability to compete for TED-dependent translation *in trans*. (**A**) TED deletion derivatives have a lower functionality. RNAs containing the Ω-fragment of the TMV as leader (5), the *cat* coding region and different TED derivatives were translated in wheat germ. The amount of CAT synthesised from these RNAs was determined after 100 min. The stimulation of CAT synthesis by the different TED derivatives is given as compared to the non-functional $TED_{(688–753)}$ (1). (**B**) *In cis* functionality of TED derivatives correlates with efficiency of competition *in trans*. 0.5 pmol RNA-R655TED, consisting of a 19 nt polylinker sequence (5), *cat* and TED, was translated in a 12.5 µl reaction mixture during 120 min in the presence of 0–70 pmol of different competitor RNAs. The amount of CAT is given as a percentage of the amount in the absence of competitor. (**C**) TED derivatives compete for TED-dependent translation at the level of translational efficiency. RNA-R655TED was translated in wheat germ in the presence of a 5-fold molar excess (open bars) and a 70-fold molar excess (black bars) of different competitor RNAs. The CAT synthesis was determined at seven time points and from the resulting protein accumulation profile the translational efficiency of the TED-containing RNA was determined. The values for the translational efficiency are given as percentages of the values in the absence of competitor.

RESULTS

TED inhibits *in trans* TED-dependent translation

The most likely way in which TED recruits the translational machinery to the mRNA 5′ end is through binding of host factors. In such a scenario, the presence of TED RNA *in trans* would titrate such factor, and thus reduce translation of an uncapped, TED-containing mRNA. To get a first insight into whether this is the case, we translated a TED-containing *cat* RNA in wheat germ in the presence of competitor TED RNA. The TED-containing *cat* RNA (RNA-R655TED) contains a 19 nt random leader (5) fused to the *cat* coding region and the STNV-2 nt 655–753, which have TED activity. RNA containing STNV nt 655–753 [$TED_{(655–753)}$] was used as specific competitor. As a control, a non-specific competitor RNA (RLcat RNA) was used that consists of the 19 nt random leader fused to the first 60 nt of the *cat* coding sequence. This sequence is identical to the first 79 nt of RNA-R655TED and does not stimulate cap-independent translation (5). In an initial set of experiments, we determined whether protein synthesis from the TED-containing *cat* RNA is affected by increasing concentrations of the competitor RNAs.

Figure 1B shows that CAT synthesis was not affected by the presence of the non-specific competitor RLcat RNA. In contrast, CAT protein synthesis was progressively reduced by an increasing concentration of $TED_{(655–753)}$ as competitor, to ~20% at a 50-fold and higher molar excess. This implies that an excess of functional TED *in trans* inhibits TED-dependent translation. These results are thus consistent with the assumption that TED activity requires a direct interaction with wheat germ factors.

TED binds at least seven wheat germ proteins

We next investigated whether TED has affinity for one or more factors in the wheat germ extract. This would give a first insight into candidate proteins with a role in TED-dependent translation and, more importantly, it would provide insight in the extent to which the TED-binding factors may have to compete with each other for binding to TED. We therefore performed a UV-crosslinking assay of ^{32}P-labelled $TED_{(655–753)}$ RNA with the wheat germ protein extract in the absence of any specific or non-specific competitor RNA. This assay was done in the same buffer as used in the wheat germ translation assay, to ensure that the functionally relevant interactions occurred. For the same reason, the ratio of input RNA over protein was the same as in the *in vitro* translation reaction, albeit at 4-fold lower concentrations.

As shown in Figure 2, crosslinking of TED to wheat germ extract resulted in the appearance of about eight radiolabelled products. Products migrating as 25 kDa proteins and smaller were also present without crosslinking, which indicates that only the seven remaining products are likely TED-binding proteins. These seven products were neither visible in the absence of wheat germ, nor after treatment of the samples with proteinase K, which confirms that they are wheat germ proteins. In conclusion, these results show that at least seven proteins in the wheat germ extract bind directly to TED. As it is unlikely that all these proteins have non-overlapping binding sites on TED, this result implies that the TED-binding proteins have to compete with each other for binding to TED.

Mutations in TED that reduce translation stimulation also reduce the capacity to inhibit TED-dependent translation *in trans*

A likely feature of a wheat germ factor with a role in TED-dependent translation would be a specific affinity for the functional TED sequence. To identify such factor, we set up a series of experiments to establish the relationship between TED activity and the ability to interact with the TED-binding

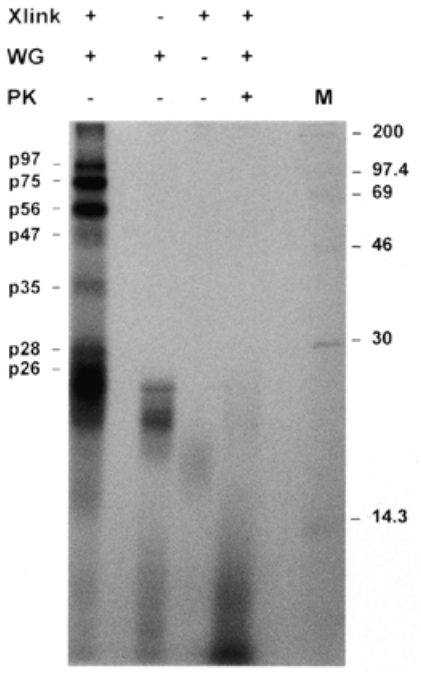

Figure 2. TED crosslinks to at least seven wheat germ proteins. Radiolabelled $TED_{(655–753)}$ RNA (5 fmol/µl) was incubated with or without wheat germ extract (WG). The RNA–protein interactions were crosslinked with UV light (Xlink), except for the no-crosslinking control. The unbound RNA was degraded with RNase A and RNase VI in the absence or presence of proteinase K (PK). The samples were separated on an SDS–polyacrylamide gel. The molecular weight of the marker proteins (M) in kDa is indicated on the right, and the calculated molecular weight of the TED-binding proteins is indicated on the left.

proteins. We opted for a strategy to deplete the extract from some of the TED-binding proteins by adding a surplus of functional TED or of TED deletion derivatives, and to monitor the ability of TED to stimulate translation as well as to interact with wheat germ factors. In the first set of experiments, the feasibility of the approach was tested. We determined the extent to which a deletion series of TED with a known reduction in translation stimulatory activity (Fig. 1A) downregulates TED-dependent translation *in trans*. To this end, RNA-R655TED was translated in wheat germ in the presence of increasing concentrations of competitor $TED_{(655–753)}$, or competitor RNAs with 5′ TED deletions [$TED_{(656–753)}$, $TED_{(657–753)}$, $TED_{(658–753)}$ and $TED_{(660–753)}$].

Figure 1B shows that CAT protein synthesis from the TED-containing RNA was most reduced when functional $TED_{(655–753)}$ was used as competitor. The absence of a single 5′ nucleotide in $TED_{(656–753)}$ reduced the inhibitory effect at low competitor concentrations. The absence of the two and three most 5′ nucleotides, respectively, i.e. $TED_{(657–753)}$ and $TED_{(658–753)}$, further reduced the competitiveness at low concentrations. To be equally effective as the functional $TED_{(655–753)}$ competitor, a 140-fold molar excess of these mutant RNAs over the TED-containing RNA was required. The lack of the five most 5′ nucleotides in $TED_{(660–753)}$ made this RNA unable to act as a competitor for TED-dependent translation at the tested concentrations. Unexpectedly, addition of this competitor resulted reproducibly in a 2-fold increase of CAT synthesis as compared to synthesis in the absence of competitor.

In summary, these data show that the 5′ TED deletion mutants with reduced functionality have a reduced ability to inhibit TED-dependent translation *in trans*, and thus most likely a decreased ability to titrate factors from the wheat germ extract.

TED derivatives *in trans* specifically affect stimulation of translational efficiency by TED

We previously showed that TED stimulates translation by enhancing the translational efficiency of the mRNA (5), i.e. the capacity to initiate translation. If the TED derivatives *in trans* act by competing with TED for binding to a factor required for TED function, they should affect the translational efficiency of the TED-containing RNA, and a dose relationship between availability of these factors and the translational efficiency of the RNA should exist. To validate whether this is the case, CAT protein accumulation profiles from RNA-R655TED translation in wheat germ were determined in the presence of a 5- or 70-fold molar excess of competitor TED RNA or of 5′ TED mutant RNAs. From these profiles the translational efficiency and the functional half-life of RNA-R655TED were determined.

The presence of TED and the TED deletion mutants *in trans* did not significantly affect the functional half-life of the TED-containing RNA (data not shown). In contrast, as shown in Figure 1C, the competitor TED reduced the translational efficiency ~20-fold when present in 70-fold excess. The 5′ deletion series of TED showed a diminishing *trans* effect on translational efficiency when the number of missing 5′ nucleotides increased. Interestingly, the presence of a 70-fold excess of $TED_{(660–753)}$ as competitor stimulated the translational efficiency ~2-fold. This shows that both functional TED and the 5′ mutants *in trans* specifically affect the translational efficiency of the TED-containing mRNA. This finding strongly suggests that TED activates translation via binding to wheat germ factors, and that the 5′ TED deletion mutants have a reduced affinity for such factors. The latter observation makes the 5′ TED mutants suitable substrates for the identification of wheat germ factors with a role in TED-dependent translation.

TED function correlates with affinity for a wheat germ 28 kDa protein albeit not strictly

The experiments presented in Figure 1 indicate that the factors required for TED function have a higher affinity for TED than for the 5′ TED deletion mutants. To identify which of the wheat germ TED-binding proteins have this property, we analysed the extent to which the different proteins are titrated by functional TED and by the 5′ TED deletion mutants. Therefore, ^{32}P-labelled $TED_{(655–753)}$ RNA was UV crosslinked to wheat germ proteins in the presence of increasing concentrations of unlabelled competitor TED or 5′ TED deletion mutant RNAs, or the RLcat RNA that neither stimulates translation *in cis* nor competes for TED-dependent translation *in trans* (see above).

Figure 3 shows that addition of unlabelled functional TED RNA in the UV-crosslinking assay decreased the intensity of all crosslinking products. One crosslinking product, corresponding to a protein of 28 kDa (p28), was titrated more efficiently than the others. At a 5-fold molar excess of unlabelled TED, this product was hardly visible. It was absent

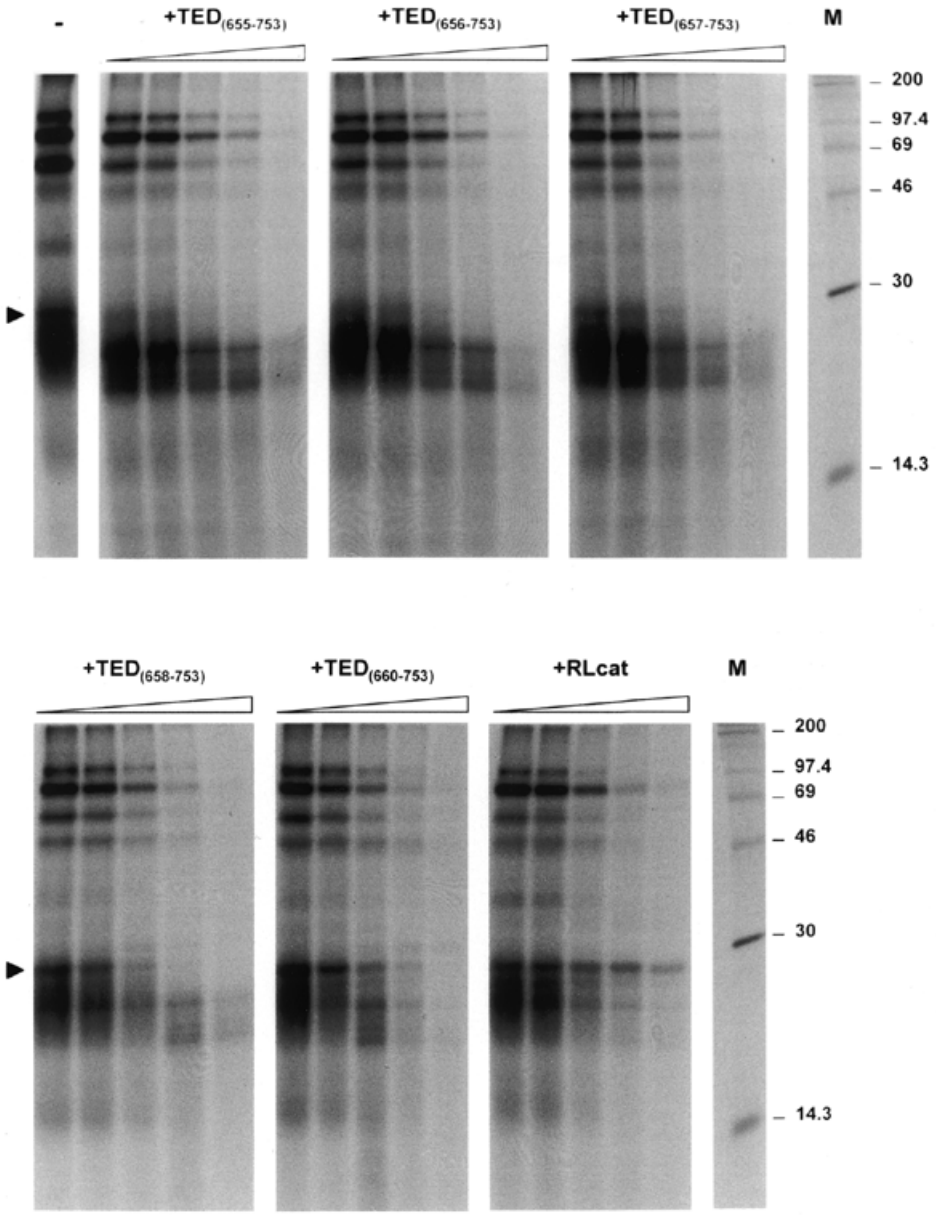

Figure 3. Functional TED specifically binds a 28 kDa protein. UV-crosslinking assay of radiolabelled $TED_{(655–753)}$ RNA with wheat germ without competitor RNA (–), or in the presence of different unlabelled competitor RNAs in a 5-, 14-, 45-, 150- or 500-fold molar excess. The samples were separated on an SDS–polyacrylamide gel. The migration of the 28 kDa TED-binding protein is indicated by an arrowhead. The molecular weight (in kDa) of the marker proteins (M) is indicated on the right.

at a 45-fold excess, whereas most other proteins were still visible. When we added an excess of the RLcat RNA *in trans*, the intensity of almost all migration products decreased to the same extent as in the presence of TED. However, p28 was visible at all concentrations of competitor RLcat RNA. These results show that the 28 kDa TED-binding factor has a higher affinity for the TED RNA than for the RLcat RNA.

When the 5′ TED deletion mutants were added *in trans*, the intensity of all crosslinking products decreased. p28 was the only product that was competed away less efficiently by these mutants than by functional TED. Furthermore, the competition assays with the series of TED 5′ deletion mutants showed that increasing deletions at the 5′ end of TED decreased the efficiency of competition for binding to p28. Consistently, the competitor concentration at which binding of p28 to TED was no longer observable increased with the size of the 5′ deletion of TED. This shows that p28 has a higher affinity for functional TED than for the non-functional TED mutants, and that its affinity decreases with progressive deletions at the 5′ end of TED. When combining the effects of the 5′ deletion mutants *in trans* on TED-dependent translation (Fig. 1B) and TED-protein affinity (Fig. 3), it appears that the ability of TED to promote translation in the presence of competitor correlates with binding of p28 to TED. These results indicate that p28 plays a role in TED-dependent translation.

The results obtained with $TED_{(660–753)}$ in the TED-dependent translation assays and in the crosslink assays do not follow this trend. Figure 3 shows that a 150-fold molar excess of $TED_{(660–753)}$

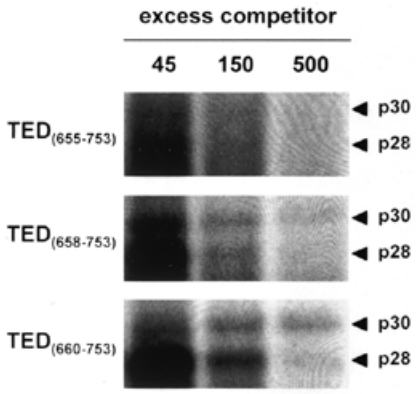

Figure 4. The presence of $TED_{(660–753)}$ *in trans* promotes binding of a 30 kDa protein to TED. UV-crosslinking assay as described in Figure 3. The 28 and 30 kDa TED-binding proteins are indicated on the right.

significantly reduced binding of p28 to TED. However, a 140-fold molar excess of competitor $TED_{(660–753)}$ did not repress TED-dependent translation (Fig. 1B). This implies that under certain conditions, TED may be able to activate translation independently from p28.

Functionality of TED correlates with affinity for a 30 kDa protein

The ability of TED to activate translation in the absence of binding of p28 would suggest an interaction with another wheat germ factor. Longer exposures of the autoradiogram of the crosslinking competition assay (Fig. 4) revealed that at 45- to 500-fold excess of the competitor $TED_{(658–753)}$ and $TED_{(660–753)}$, TED reproducibly crosslinked to a 30 kDa protein (p30). This product was not visible in the presence of any of the other competitors (Fig. 4 and data not shown). These data imply that $TED_{(655–753)}$, $TED_{(656–753)}$ and $TED_{(657–753)}$ have affinity for p30, whereas in the non-functional mutants $TED_{(658–753)}$ and $TED_{(660–753)}$ this affinity is reduced. Affinity for p30 thus correlates with functionality of TED, suggesting a role for p30 in translation. Furthermore, the persistent binding of p30 to TED in the presence of increasing concentrations of $TED_{(660–753)}$ as competitor offers an explanation for the ongoing translation of the TED-containing RNA under high concentrations of this competitor, when p28 is competed away (Fig. 1B). These findings suggest that p30 somehow functionally substitutes for p28.

DISCUSSION

The STNV translational enhancer domain stimulates translation in a cap-independent manner from a position downstream of the coding region. In this study, we investigated whether binding to host-encoded factors is required for TED function. We found that TED function correlates with binding to a 28 kDa protein, which indicates that TED recruits the translational machinery to the mRNA 5′ end through affinity for p28. Furthermore we showed that, in the presence of a specific 5′ TED deletion mutant that titrates p28, TED is still able to stimulate translation. Under these conditions, binding of a protein of 30 kDa to TED was observed.

The most likely explanation for these data is that TED has affinity for both p28 and p30, and that these two proteins compete for a similar binding site. In the presence of both p28 and p30, p28 binds to TED, either because of a higher concentration, and/or because of a higher affinity. This mutually exclusive binding of p28 and p30 can also explain the observation that in the presence of the non-specific RLcat RNA, binding of p30 to TED is absent. The TED derivatives $TED_{(656–753)}$ and $TED_{(657–753)}$ would have affinity for both of these proteins, albeit reduced compared to functional TED, which allows them to titrate both p28 and p30. The $TED_{(658–753)}$ and $TED_{(660–753)}$ RNAs also appear to have affinity for p28, which reduces binding of p28 to TED at high concentrations of these RNAs as competitors. The affinity of these TED-derivatives for p30 seems to be more reduced than for p28, which results in an increase of the amount of free p30 over free p28 under these titration conditions, so that p30 can efficiently compete with p28 for binding to functional TED.

Affinity of both p28 and p30 correlates with functionality of TED, which makes these two proteins likely candidate factors with a role in TED-dependent translation. How p28 and p30 may stimulate translation is not known. In an analogy to what is suggested for the poly(A) tail (12,14), TED could recruit the translational machinery via direct or indirect binding to translation factors. p28 and p30 might therefore be either 'bridging factors' between TED and the translational machinery, or they may be translation factors themselves. Known plant initiation factors with a molecular weight similar to p28 and p30 are the cap-binding proteins eIF4E (26 kDa), eIFiso4E (28 kDa) and nCBP (24 kDa), one of the subunits of eIF3 (28 kDa), and the anti-ribosome association factor eIF6 (25 kDa) (6,9,27). As TED most likely stimulates binding of the 40S ribosomal subunit to the mRNA 5′ end (5), eIF6, which binds the 60S ribosomal subunit, is not a likely candidate factor that stimulates TED-dependent translation. In contrast, TED might have a role in recruitment of the 40S ribosomal subunit to the mRNA 5′ end via eIF3 binding. This may be further supported by the complementarity of TED with the 18S rRNA of the 40S ribosomal subunit (1). Alternatively, p28 and p30 might be the cap-binding factors. This would be consistent with the observation that the cap-binding factor eIFiso4E can interact with TED (28), and that TED lowers the requirement of eIF4F for STNV translation (2). If this were the case, the mechanism of TED-dependent translation would be very similar to that of cap-dependent translation. As p28 appears to be the 'default' TED-binding factor in wheat germ, we have set up a purification protocol to determine its identity and to investigate its role in TED-dependent translation. Although this resulted in pure p28, the amount was insufficient to determine its identity, and further upscaling is still required (data not shown).

From the current data it is unclear under what biological conditions the p30-dependent path becomes a preferred route. Under the conditions that the TED-containing RNAs are translated in wheat germ, virtually all the TED RNA is expected to be occupied by p28. The detected translational output would then be almost completely dependent on the p28–TED interaction, and the capability of TED to stimulate translation via an interaction with p30 would seem superfluous. However, when STNV infects a plant cell, it will be competing with some 300 000 other mRNAs for binding to the cellular proteins. If the plant mRNAs also have affinity to p28, titration of p28 by cellular mRNAs may allow p30 to bind to TED. In this way the STNV RNA could be translated under a wider range of competitive conditions.

The existence of more than one way to recruit the translational machinery is probably not unique to TED, but might also apply to the 5′ cap. First, in yeast extracts it was shown that both eIF4E and the nuclear cap-binding complex stimulate translation via eIF4G binding (8). Secondly, specifically in plants, three cap-binding factors eIF4E, eIFiso4E and nCBP have been described that stimulate translation *in vitro* (9,10). It has been suggested that the plant cell might use the activity of different cap-binding factors as a discriminatory mechanism in selection of the RNAs for translation (27). This is exemplified by the fact that eIFiso4E has a higher affinity for hypermethylated cap structures than eIF4E, and that the nCBP has a higher affinity for the cap than eIF4E and eIFiso4E, but a lower activity in stimulating translation (10). The latter example shows a similarity with the TED-binding factors, as p30 appears to have a lower affinity for TED than p28, but a higher capacity to stimulate translation. The presence of more than one cap-binding protein might also be required for regulation of translation during specific conditions. For example, the levels of eIF4E and eIFiso4E are modulated during development (27), and the pH dependence of eIF4E and eIFiso4E is different (6). Having two routes to initiate TED-dependent translation might be for the same reason. It is likely that a viral RNA is equipped to be minimally dependent on conditions in the host cell and is able to compete efficiently with plant mRNAs for translation under most conditions.

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