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Review

Tobacco Etch Virus protease: A shortcut across biotechnologies

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ABSTRACT

About thirty years ago, studies on the RNA genome of Tobacco Etch Virus revealed the presence of an efficient and specific protease, called Tobacco Etch Virus protease (TEVp), that was part of the Nuclear Inclusion a (NIa) enzyme. TEVp is an efficient and specific protease of 27 kDa that has become a valuable biotechnological tool. Nowadays TEVp is a unique endopeptidase largely exploited in biotechnology from industrial applications to *in vitro* and *in vivo* cellular studies. A number of TEVp mutants with different rate of cleavage, stability and specificity have been reported. Similarly, a panel of different target cleavage sites, derived from the canonical ENLYFQ-G/S site, has been established. In this review we describe these aspects of TEVp and some of its multiple applications. A particular focus is on the use and molecular biology of TEVp in living cells and organisms.

Contents

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1. Introduction

Proteases with stringent specificity for the target sequence represent excellent tools for the specific removal of tags and undesired sequences or to achieve precise processing on defined polypeptides. Several different enzymes of mammalian origin have been used for these purposes, such as the coagulation Factor Xa, enterokinases and α-thrombin. However, in some cases they were reported not to be highly specific, having off-targets effects. Viral proteases instead, have evolved in most cases to allow virus replication by precise processing of precursor polyproteins and have thus been used as targets for anti-viral drugs. Some of them are characterised by having stringent sequence specificity and efficiency, such as the human rhinovirus 3C protease, the Potyvirus proteases from the Tobacco Vein Mottling Virus (TVMV) and the Tobacco Etch Virus (TEV) (Blommel and Fox, 2007). Therefore, viral proteases have been widely exploited as biotechnological tools (Waugh, 2011). TEVp was taken in high consideration for these purposes because of its good activity rate and its sequence specificity, which was found to be much more stringent than that of other proteases generally used. In fact to our knowledge, TEVp has never been reported to cleave fusion proteins at unintended locations. Despite its high specificity, TEVp can tolerate a wide variety of residues in position P1′ (the first amino acid downstream the cleavage site) of the substrate, making it the best protease for endoproteolytic removal of affinity tags (Waugh, 2011). In addition, TEVp is the only protease that combines several useful features for biotechnological applications, from easy and economical production to availability of open source vectors and mutants, making it suitable for several different *in vitro* and *in vivo* applications.

2. The tobacco etch virus protease

2.1. Origin

TEV is a member of the Potyviridae family responsible for infections of many different plant species of Solanaceae including *N. tabacum* (Revers and Garcia, 2015). The TEV genome is a 9500 nucleotides-long single stranded RNA of positive polarity that encodes a 3054 amino acids-long polyprotein, which is co-translationally processed in infected cells into 10 mature products by three viral proteases (Adams et al., 2005; Allison et al., 1986). One of the most important TEV proteases is the Nuclear Inclusion protein a (NIa) (Carrington and Dougherty, 1987a), which processes most of the polyprotein (Fig. 1A and C) (Carrington and Dougherty, 1988; Carrington et al., 1988; Dougherty et al., 1988). NIa originates itself from the polyprotein as a 49 kDa precursor that is eventually cleaved into the N-terminal 21 kDa genome-linked protein VPg and the 27 kDa protease catalytic subunit TEVp, which can be detected during the late stages of plant infections (Carrington and Dougherty, 1987b; Dougherty and Parks, 1991).

TEVp belongs to the family of C4 peptidases and is structured as a two-domain antiparallel β-barrel fold, typical of trypsin-like proteases (Nunn et al., 2005; Phan et al., 2002) (Fig. 1B). In fact, TEVp shares homology to serine-proteases despite the presence of a cysteine instead of serine in the catalytic core, which comprises residues His46, Asp81 and Cys151. Consistently, mutation of Cys151 into Ala abolishes protease activity (Parks et al., 1995).

2.2. Specificity

The TEVp specific recognition motif (TEVp cleavage Site, TS) is only 7 amino acids-long: EXXYXQ-S/G (where X can be any amino acid) and proteolysis takes place between residues Gln and Ser or Gly (Dougherty et al., 1989a) (Fig. 1C). Despite an apparent low specificity, many studies have demonstrated that within the EXXYXQ-S/G cleavage site not all residues are equally tolerated. According to crystal structures, experimentally confirmed, residues in positions P6, P4, P3, P2, P1, and P1′ of the substrate directly interact with the enzyme substrate-binding pocket (Phan et al., 2002). Only residue P5 is exposed to the solvent and can tolerate almost any possible amino acid without consequences on enzyme effi-

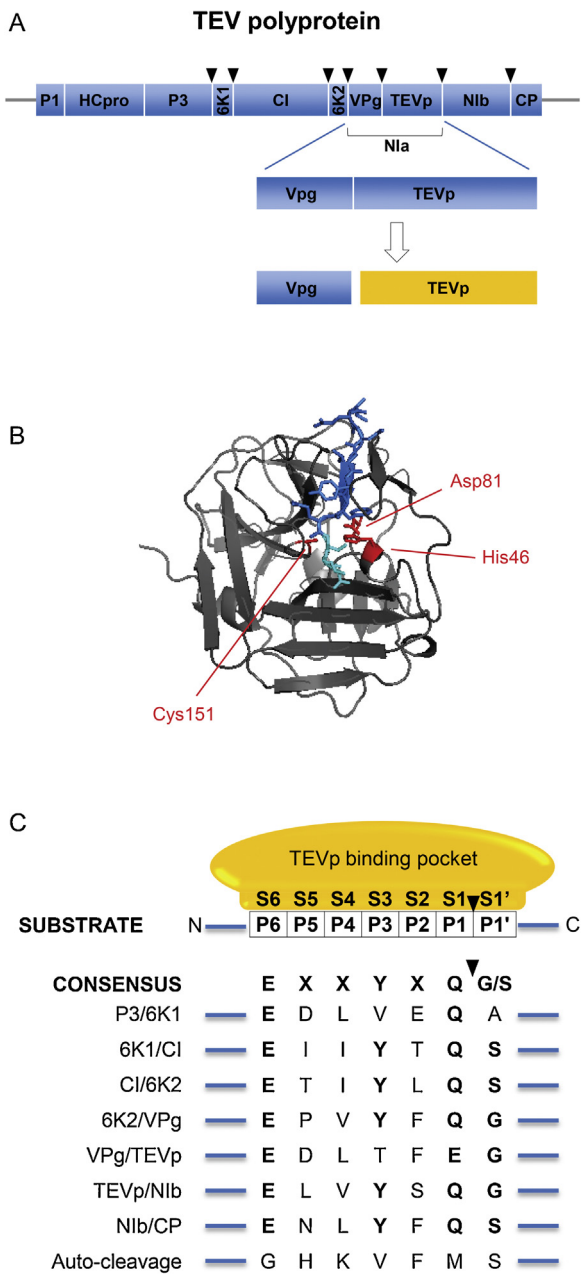

Fig. 1. The Tobacco etch virus protease. (A) TEV polyprotein processed by TEVp at the indicated sites (filled arrowhead). The NIa 49 kDa precursor is finally cleaved into VPg and TEVp. P1, S30 serine protease; HCpro, Helper Component for aphid transmission C6 cysteine protease; P3, protein P3; 6K1 and 2, 6 kDa proteins 1 and 2; CI, cylindrical inclusion protein; VPg, viral protein genome-linked, NIa and NIb: nuclear inclusion proteins a and b; CP, coat protein. (B) TEVp crystal structure, with key residues of the catalytic site shown in red. The substrate is represented with the TS amino acids P6-P1 in blue and peptide from P1′ to the C-terminus end in light blue (adapted from pdb file 1LVM, Phan et al., 2002). (C) Schematic illustration of residues on the TEVp substrate recognition pocket (S) and on the substrate cleavage site (P). The table reports the cleavage site sequences processed by the protease on the TEV polyprotein. Arrowhead, site of cleavage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

ciency (Dougherty et al., 1989a). TEVp has a strong preference for substrates having Gln in position P1, Phe in P2, Tyr in P3 and Leu in P4. Cys and Ile in position P2 and Ile in position P4 are also tolerated (Dougherty et al., 1989a; Phan et al., 2002; Tözsér et al., 2005; Yi et al., 2013). Thus, despite many possible TS combinations tested, the optimum recognition site is ENLYFQ-S/G, which corresponds to the cleavage site between protein fragments NIb and CP on the natural TEV polyprotein (Boulware et al., 2010; Kostallas et al., 2011) (Fig. 1C). Other small aliphatic residues, like Ala, Cys and Met in position P1′ are also well tolerated and efficiently processed, while the presence of Pro completely prevents cleavage (Dougherty et al., 1989a; Kapust et al., 2002b). This tolerance is due to the S1′ pocket of TEVp, which is shallow and allows partial exposure of the P1′ residue side chain to the solvent (Phan et al., 2002). Recently, a TEVp mutant (Arg203Gly and truncated at amino acid 234) named TEV2, was described to have a further relaxed recognition of substrate position P1′, but still unable to process Pro in this position (Renicke et al., 2013). Interestingly, Pro in position P2′, but not other residues, inhibits TEVp activity (Dougherty et al., 1989a). These findings imply that, despite having different kinetic rates, almost every protein fused downstream a TS can be released by the appropriate TEVp, leaving the desired amino acid at the N-terminus. This avoids unwanted N-terminal modifications, and allows to control stability of the released protein according to the N-rule pathway, a noteworthy advantage for the use of TEVp. In fact, two other very specific viral proteases, the human rhinovirus 3C protease and the Potyvirus proteases from the TVMV do not share this characteristic. The human rhinovirus 3C protease requires the Gly-Pro dipeptide immediately after the cleavage site, while the TVMV protease does not have a relaxed specificity on P1′ position, likely due to a smaller and rounder S1′ pocket compared to TEVp (Sun et al., 2010; Waugh, 2011).

TEVp mutants with a changed specificity for Gln in P1 have also been selected. In particular, Asp148 mutated to positively charged residues Arg or Lys was preferentially found in TEVp variants with specificity for Glu in P1 position, while selection for His on P1 favoured mutations with small amino acids (Ala, Ser or Cys) or Pro (Yi et al., 2013). More recently, a novel TEVp mutant selected by directed evolution, which had Asn171 mutated in Asp, showed higher tolerance for Thr and Pro in position P6 (Carrico et al., 2016).

Several proteases are sensible to post-translational modifications of their protein substrates (He et al., 2015). Similarly, a TS containing chemical modifications may not be processed by TEVp. For instance, phosphorylation of Tyr in position P3 was shown to down regulate TEVp catalysis, with the possibility of controlling and monitoring its enzymatic activity through the presence/absence of phosphatases and of a fluorophore-quencher reporter system (He et al., 2015).

2.3. Self-cleavage

The 27 kDa TEVp contains a self-cleavage motif (GHKVFM-S) in its C-terminal portion between residues 213–219, cleaved at Met218 (Kapust et al., 2001; Parks et al., 1995) that presents little homology to the canonical TS.

Apparently conflicting results have been reported on the remaining activity of the self-cleaved enzyme (Nunn et al., 2005; Parks et al., 1995). Loss of activity following self-cleavage was shown to be the consequence of inhibition of the catalytic activity by the cleaved C-terminal end peptide (amino acids 219–242), which remains tightly bound to the protease substrate pocket through residues 235–242 (Nunn et al., 2005), and not to a deficiency of the shorter TEVp polypeptide. Consistently, TEVp truncated at amino acid 218 is active both *in vitro* (Nunn et al., 2005) and in living cells (Cesaratto et al., 2015), although with reduced affinity for the substrate and lower performance (Cesaratto et al., 2015; Nunn et al., 2005).

Many efforts have been made to understand the mechanism of self-cleavage and to reduce this activity introducing point mutations in the internal cleavage motif, without affecting the overall proteolytic activity (Kapust et al., 2001; Lucast et al., 2001; Nunn et al., 2005). Initial attempts were based on mutants at P1′ position of the self-cleavage site. Several amino acids such as Asn (Lucast et al., 2001), Asp, Glu, Lys, Val and Pro (Kapust et al., 2001) substituting Ser219 were reported. Mutants with Val, Asn or Pro were shown to be more resistant than the wild type. However, only variants with Val and Asn maintain satisfying enzymatic efficiency, while mutant Ser219Pro is much less active. An explanation provided by molecular dynamics simulations showed that Val in position 219 enhances flexibility of the catalytic core and formation of a C-terminal helix that avoids self-cleavage and improves the enzymatic activity (Wang et al., 2013). Other mutations within the self-cleavage motif, like Val216Asp (Nunn et al., 2005) and Phe217Lys (Kapust et al., 2001) cause drastic reduction of enzymatic activity.

A valid and more efficient alternative to this strategy was mutation of the C-terminal residues SELVYSQ (236–242). When mutated into NEGGGLE self-cleavage was prevented, as originally observed by the large increase in the yield of *E. coli* produced recombinant TEVp purified through a C-terminal His6-tag (Wu et al., 2009), without decrease of enzymatic activity. This was also true when expressed in mammalian cells (Cesaratto et al., 2015). Similarly, a TEVp truncated version named TEV$^+$, which had the 235–242 C-terminal sequence removed was found to perform better than wild type TEVp in yeast (Taxis et al., 2009). A shorter TEVp truncated at amino acid 224 was recently described to have the same processivity of TEV$^+$ (Renicke et al., 2013).

2.4. Solubility

Early studies on TEVp were carried out with protein purified from infected cells and later with the recombinant enzyme produced in *Escherichia coli* strains. The first TEVp purification protocols allowed yields of around 1 mg/L of active enzyme (Carrington et al., 1988; Parks et al., 1995). In fact, industrial exploitation of TEVp was initially limited by its low solubility following bacterial expression as it forms insoluble aggregates. Different approaches were then implemented to solve this problem: from the use of different *E. coli* strains, such as DH5α, Rosetta (DE$_3$) pLysS, BL21 and Krx (strain Krx allowed higher expression levels) to control of the temperature growth (TEVp is prone to aggregate at higher temperatures) (Fang et al., 2007; Kapust and Waugh, 1999; Kapust et al., 2002a, 2001; Miladi et al., 2011; Van Den Berg et al., 2006; Wu et al., 2009). TEVp was also obtained following refolding of the material recovered from inclusion bodies (Blommel and Fox, 2007; Lucast et al., 2001) and through the widely used approach of fusing it to solubility tags such as poly-histidine or poly-arginine tags (Kapust and Waugh, 1999; Kapust et al., 2001, 2002b), Maltose-binding protein (MBP) and glutathione S-transferase (GST) (Blommel and Fox, 2007; Kapust and Waugh, 1999; Sun et al., 2012), Thioredoxin (TRX) (Kapust and Waugh, 1999), Streptag II (Miladi et al., 2011), hyperacidic protein fusion partners (Zou et al., 2008), SUMO (Zou et al., 2008), the N-utilization substance (NusA) (Zou et al., 2008), superfolded GFP (Wu et al., 2009) or chaperone proteins (Fang et al., 2007). Combining two or more of these variables it is now possible to obtain high scale production of soluble active and highly pure TEVp of up to 300–400 mg/L from bacterial cultures (Blommel and Fox, 2007; Wu et al., 2009).

TEVp aggregation was investigated by selecting mutants through the directed evolution approach, consisting in random mutagenesis of the TEVp sequence based on error prone PCR and

gene shuffling (Van Den Berg et al., 2006). A variant (named TEV_{SH}) harbouring mutations Thr17Ser, Asn68Asp, Asn177Val was shown to be the most soluble one without compromising enzymatic activity (Fang et al., 2013; Wei et al., 2012).

Finally, a TEVp mutant missing the C-terminal amino acids 238-242 (LVYSQ), which are part of the disordered 15 amino acids long C-terminal peptide (Nunn et al., 2005; Phan et al., 2002) was found to be active and more soluble in bacteria than other C-terminal isoform of the enzyme (Blommel and Fox, 2007). Similarly, substitution of the canonical amino acids 238-242 with –NEGGGLE- (Wu et al., 2009) also confers more solubility in *E. coli*. These results suggest an involvement of TEVp C-terminus in the determining protein solubility.

3. TEVp *in vitro* enzymology

TEVp was shown to have high activity rate *in vitro*. However, K_m and k_{cat} values (indicatively K_m 0.061 mM, κ_{cat} 0.16 s^{-1}, κ_{cat}/K_m 2.62 mM^{-1} s^{-1} (Kapust et al., 2001)) were shown to vary depending on the mutant considered as well as on the substrate used (Blommel and Fox, 2007; Fang et al., 2013; Kapust et al., 2001; Nunn et al., 2005; Parks et al., 1995; Wu et al., 2009). Nevertheless, the interest on the TEVp remarkable activity and specificity is connected to its application to remove affinity tags used to purify proteins of interest from crude extracts or to enhance protein solubility (Melcher, 2000; Sun et al., 2012). The most used *in vitro* reaction buffer is 50 mM Tris-HCl 0.5 mM EDTA, 1 mM DTT, pH 8.0. Because of Cys151 in its catalytic site TEVp is resistant to many common serine-protease inhibitors, like phenylmethylsulfonyl fluoride (PMSF) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mM), Tosyl-L-lysyl-chloromethane hydrochloride (TLCK, 1 mM), Bestatin (1 mg/ml), pepstatin A (1 mM), EDTA (1 mM), and E-64 (3 mg/ml). On the other hand, it is highly sensitive (>1 mM) to alkylating agents that react with cysteines, like N-Ethylmaleimide or Iodoacetamide, but also to 5 mM Zn^{2+}, as this cation interacts with Cys and His (Dougherty et al., 1989a,b).

A wide range of enzyme/substrate concentrations have been used for TEVp (from 1/100 to 1/10) without off-target cleavage activities (Eisenmesser et al., 2000; Miladi et al., 2011).

TEVp have similar activity at pH values between 6 and 9 (Parks et al., 1995) and was reported to be maximally active at 30 °C, although it retains more than 50% activity at room temperature (20 °C) and about 10% at 4 °C (Nallamsetty et al., 2004). A drop in activity is reported at temperatures above 30 °C *in vitro* (Nallamsetty et al., 2004). A mutant known as $TEVp^{2M}$ (Leu56Val and Ser135Gly) has enhanced stability at temperatures higher than 40 °C (Fang et al., 2013).

Although TEVp activity is maximal in salt-free buffers, it is often used in reactions containing 0.2 M NaCl (Eisenmesser et al., 2000; Nallamsetty et al., 2004), and well tolerates NaCl concentrations from 0.8 M (Raran-Kurussi et al., 2013) up to 2 M (Parks et al., 1995). More important, TEVp was shown to be active in several buffers used for affinity purifications maintaining relative activities of about 73%, 82%, 58% and 69% in purification buffers for, respectively, poly-histidine tag (50 mM Na_2HPO_4, 0.3 M NaCl, 300 mM imidazole, pH 8.0), MBP (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4, 10 mM maltose), GST (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and Strep tag (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, pH 8.0) (Sun et al., 2012). A recently reported mutant known as $TEVp^{5M}$ (Fang et al., 2013; Wei et al., 2012) harbouring a combination of mutations of $TEVp^{2M}$ (Cabrita et al., 2007) and TEV_{SH} (Van Den Berg et al., 2006) was highly efficient for on-column cleavage on different type of resins (Zhu et al., 2016).

TEVp and its mutants have been reported to tolerate with limited loss of activity molar concentrations of denaturing agents, such as guanidine hydrochloride (2 M), urea (2 M), β-mercaptoethanol (0.7 M), and up to 1% SDS (Cabrita et al., 2007; Fang et al., 2013; Sun et al., 2012). In addition, TEVp is not sensitive to EGTA (10 mM), ethylene glycol (0.17 M), Triton X-100 (2%), Tween-20 (2%), NP-40 (2%) or CHAPS (1%) (Sun et al., 2012). DTT can be substituted by 0.3 mM triscarboxyethylphosphine (TCEP), 2 mM reduced glutathione (Eisenmesser et al., 2000) or other reducing agents, even though their presence is not strictly required (Sun et al., 2012).

TEVp has been used for removal of affinity tags from membrane proteins that require appropriate detergents and in some cases at high concentrations. Cleavage activity was sometimes reduced in such conditions, while in others it was reported to have no effect, independently of the detergent chemical family (Lundbäck et al., 2008; Mohanty et al., 2003; Vergis and Wiener, 2011). Diverse activity was found with three different protein substrates in the presence of the same detergent (Triton X-100) (Lundbäck et al., 2008), suggesting that accessibility of the cleavage site represents a key factor. In fact, TEVp requires a well-exposed TS and relatively far from highly folded domains as it has to be positioned within the catalytic pocket (Sun et al., 2012; Tropea et al., 2009).

TEVp was used in tandem affinity purification (TAP) of properly tagged proteins in complex with associated proteins for mass spectrometry (Andrès et al., 2011; Knuesel, 2003; Puig et al., 2001; Rigaut et al., 1999). The TAP tag was formed by protein-A separated by a TS from the calmodulin-binding peptide (CBP) and applied for purification of protein complexes from yeast and mammalian cells.

TEVp was shown to be functionally active (up to 70% in comparison to the soluble one) when immobilised to resins through cysteines (thiolsulfinate (TSI)-agarose) or lysines (glutaraldehyde (G)-agarose), which can then be stored and reutilised multiple times (Puhl et al., 2009). A more efficient oriented immobilisation of a streptag II-TEVp Ser219Val variant (Miladi et al., 2012) was later reported to retain 80% activity and 51% after nine cycles.

4. TEVp *in vivo*: expression and applications

A number of TEVp *in vivo* applications have been developed and implemented in bacteria, yeast, plant and mammalian cells. They were often achieved by clever ways to control TEVp activity *in vivo*. Accurate regulation of TEVp expression by use of conditional promoters (heat shock, temperature, galactose, light inducible promoters) or precise control of TEVp activation at the post-translational level (development of split-TEVp or control of TEVp localization) are some examples. However, as described below, a tight control of TEVp expression is only required for particular applications rather than to avoid side effects of TEVp expression *in vivo*.

4.1. TEVp expression in bacteria

TEVp expression in the bacterial cytoplasm required some adaptation, but was found to be not toxic. It is mainly used to verify "cleavability" of TS in a fusion protein before large scale *in vitro* digestions. It is a powerful tool to improve exogenous protein production in bacteria (Eisenmesser et al., 2000) and their proper folding (Shih et al., 2014, 2005). A tetracycline-inducible TEVp expression in *E. coli* was used in a system defined as Controlled Intracellular Processing (CIP) to improve yields of recombinant proteins after accumulation as fusion proteins with solubility tags. In this way, solubility of proteins was improved and fusion tags were cleaved *in vivo* before purification (Kapust and Waugh, 2000).

TEVp was used in bacteria that had been modified by TS insertion, *via* mini transposons, into different target proteins for several

applications, such as topological studies of membrane proteins, protein inactivation, insertion mutagenesis experiments, and protein tagging (Ehrmann et al., 1997).

An inducible TEVp was recently used to modulate gene expression in *E. coli* (Copeland et al., 2016) targeting bacterial virulence factors called transcription activator-like effectors (TALEs). TALEs are *trans*-acting regulators commonly used to repress transcription of certain bacterial genes, thanks to their flexibility in targeting specific DNA sequences (Copeland et al., 2014). TALEs engineered with multiple TS were degraded upon induction of TEVp expression, thus removing their repressor activity. Considering the rapid spread of TALEs, it appears as a promising system to regulate gene expression and not limited only to bacteria.

4.2. TEVp expression in eukaryotes

Ectopic expression of TEVp *in vivo* was found to be non-toxic, not only in bacteria, but also in plant cells and yeast (Faber et al., 2001; Henrichs et al., 2005; Taxis et al., 2009) and complex organisms, such as *Drosophila* and *Xenopus* (Pauli et al., 2008; Wawersik et al., 2005). In *Drosophila*, ubiquitous or tissue specific expression of TEVp did not show notable phenotypes (Harder et al., 2008; Pauli et al., 2008) and in HeLa cells, micro-injection of TEVp had no discernible effect on viability or cell proliferation (Satoh and Warren, 2008). Similarly, TEVp cytosolic overexpression in various cell lines (HeLa, HEK-293, PC12, U2OS, COS-7 and COS1) or in primary cell cultures (neurons, astrocytes) did not cause any morphological change nor compromised cell division despite robust expression (Cesaratto et al., 2015; Chen et al., 2010; Wehr et al., 2006). These findings highlight the restricted cleavage specificity of TEVp compared to other proteases, which display toxic phenotypes because of their low specificity that allows proteolysis of endogenous substrates (Hwang et al., 2015; Jenny et al., 2003; Xiao et al., 2007).

4.3. In vivo targeting to specific cellular compartments

In its natural context of viral infection of plant cells, TEVp accumulates mainly in the nucleus before being released from the NIa precursor. Instead, if expressed alone, it localises largely in the cytoplasm (Ceriani et al., 1998). TEVp localisation to specific subcellular compartments in eukaryotic cells such as the nucleus, mitochondria, peroxisome and Endoplasmic Reticulum (ER) requires modification with appropriate targeting signal sequences (Fig. 2). All these topologically targeted variants expanded the possible *in vivo* biotechnological applications of TEVp, from basic cellular studies to the creation of synthetic networks.

4.3.1. ER targeting

TEVp expression in the ER required several specific adaptations. If targeted to the ER lumen of mammalian cells, through the N-terminal fusion of a leader signal peptide, TEVp becomes N-glycosylated (consensus sequence Asn-X-Ser/Thr, where X can be any amino acid except Pro) in four different positions distributed in various regions of the enzyme structure (Asn23, Asn52, Asn68, and Asn171). Two of them (Asn23 and 171), inactivate the enzyme. A TEVp variant (named secTEVp-QSG) bearing a signal peptide (sec) at the N-terminus and mutations on two N-glycosylation sites (Asn23Gln, Thr173Gly) and on an exposed Cys (Cys130Ser), was rationally designed to be active in the ER lumen (Cesaratto et al., 2015). This mutant was successfully used to discriminate topology of membrane proteins within the secretory pathway and to produce a fully-functional secreted immunoglobulin (IgG) from a single mRNA encoding the light (L) and heavy (H) chains separated by a short TS linker (Cesaratto et al., 2015).

In yeasts, TEVp variants were selected through functional screening to have a higher affinity for a TS with a Glu or His at P1 and to be functionally active in the ER lumen (Yi et al., 2013). Interestingly, the TEVp Ser219Pro variant was reported to be active even though its glycosylation state was not investigated. A fourfold-more efficient TEVp mutant, called TEV-Fast, contained two other substitutions in addition to Ser219Val: Gly79Glu and Thr173Ala, the latter abolishing glycosylation of Asn171. Furthermore, most of the TEVp variants selected showed a hot spot of mutations precisely in the same glycosylation site (mainly Asn171 into Ser or Asp or Thr173 into Ala or Gln) strongly suggesting that impaired N-glycosylation of Asn171 represents an important factor for TEVp activity in the ER also in yeasts.

In mammalian cells, despite its targeting to the ER lumen, wild type TEVp was retained in the ER and not secreted, while a single mutation (Asn23Gln) allowed its secretion (unpublished observations and (Cesaratto et al., 2015)). Of note, a fraction of the wild type TEVp and mutants targeted to the ER undergo spontaneous ER to cytosol retro-translocation, most likely because of engagement into the ER-associated degradation (ERAD) pathway. As a result, ER-targeted TEVp variants were all found to be also active on substrates located in the cytosolic compartment (Cesaratto et al., 2015).

4.3.2. Nuclear targeting

TEVp localises to the nuclear compartment by the addition of one or more nuclear localization signals (NLS). An engineered TEVp with the SV40 NLS at both, the N- and C-termini, was used for studies on chromatin (Uhlmann et al., 2000; Xiao et al., 2007). A similar system was adapted for nuclear expression in neurons (Pauli et al., 2008; Pesiridis et al., 2011) to analyse cohesin function in living *Drosophila* (Pauli et al., 2008). An elegant example of an optochemically controlled NLS was reported (Gautier et al., 2010) and more recently applied on TEVp (Engelke et al., 2014). TEVp nuclear localisation was induced by a short illumination pulse of cells expressing a site specific, genetically encoded photocaged lysine within an NLS signal positioned at the TEVp N-terminus. Illumination decaged the single chemically modified Lys (NPMK, nitropiperonylmethyloxycarbonyl lysine), causing NLS activation and nuclear TEVp localisation, thus allowing spatiotemporal control of nuclear proteins engineered with a TS.

4.3.3. Mitochondrial and peroxisomal targeting

The use of the Su9-ATPase mitochondrial targeting sequence (MTS) added at the TEVp N-terminus allowed targeting exclusively to the mitochondria in yeast (Yogev et al., 2007). This TEVp variant was used to demonstrate that the mitochondria protein fumarase is co-translationally translocated into mitochondria. A fumarase harbouring N- or C-terminal TS was analysed in cells expressing the mitochondria targeted TEVp which, in contrast to the cytosolic TEVp, was capable of cleavage thus allowing to determine the translation-coupled import kinetics *in vivo*.

Efficient delivery of an active TEVp to the peroxisomal compartment in yeast was achieved by addition of the peroxisomal targeting sequence PTS1-type (SKL) at the C-terminus and used to determine topology of the membrane-bound peroxisomal proteins peroxins Pex3p and Pex10p, despite the fact that import into peroxisome is post-translational (Faber et al., 2001). This system required expression titration and temporal separation between protein substrates and TEVp to avoid premature cleavage events during transportation to peroxisomes. It was established that C-termini of both peroxins localise on the cytosolic side of the peroxisomal membrane and then proposed to use the TEVp-based assay to investigate peroxisome biogenesis.

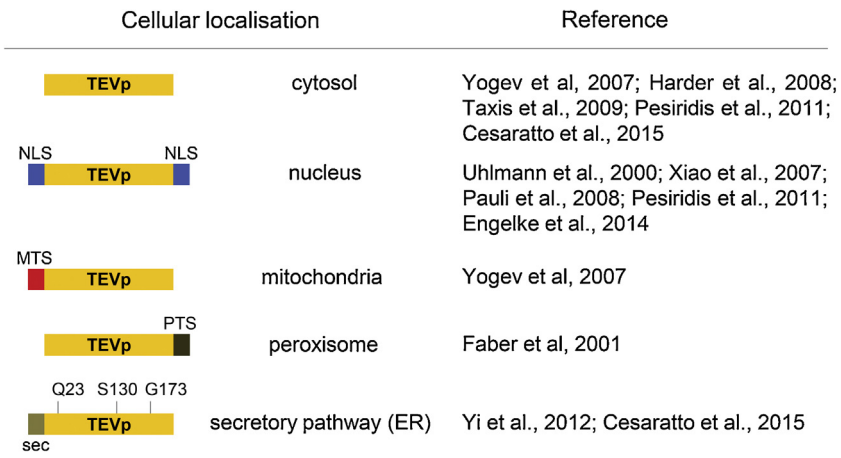

Fig. 2. Subcellular localisation of TEVp variants in eukaryotic cells. Scheme of constructs used for targeting TEVp expression into the indicated subcellular compartments and relevant references. NLS, nuclear localisation signal; MTS mitochondrial targeting signal; PTS, peroxisomal targeting signal; sec, leader signal peptide.

4.4. Stoichiometric expression of multiple proteins

TEVp has been often used *in vivo* to facilitate stoichiometric expression of multiples genes in bacteria (Chen et al., 2010), yeast (Ghiaci et al., 2014), plants (Majer et al., 2015) and mammalian cells (Cesaratto et al., 2015). Using this strategy it is possible to achieve expression of protein subunits at stoichiometric equal levels and at the same physical location. For instance, the equimolar expression of three subunits of diol dehydratase complex in the cytosol of yeasts (Ghiaci et al., 2014) and of the two chains of IgG in the ER of mammalian cells (Cesaratto et al., 2015). Different proteins can be packaged in a single polyprotein together with TEVp, mimicking the successful processing of precursor polypeptide during TEV infection (Chen et al., 2010). An alternative system was described to work in *N. tabacum* plants. A TEV-derived viral vector bearing a cassette with cDNAs of multiple proteins separated by the TS allowed the translation of equimolar amounts of up to three proteins (Majer et al., 2015).

This strategy leaves on the protein substrate the 6 amino acids-long ENLYFQ tag at the C-terminus of the upstream protein and the minimal N-terminal Gly (or Ser) in the downstream one. Other methods developed to express multiple proteins from a single mRNA in eukaryotic cells have been developed, although with some limitations. For instance, the internal ribosomal entry sites usually requires long sequences and IRES-mediated expression does not produce proteins in stoichiometric proportions, as the upstream polypeptide is preferentially expressed (Chen et al., 2009; Mizuguchi et al., 2000).

Similarly, the picornavirus-derived self-processing peptide 2A has been largely used to express multiple proteins from a single open reading frame in mammalian cells. This peptide is a relative short sequence of 17–20 amino acids that causes the release of the upstream polypeptide when its sequence is translated, although translation of the second one continues downstream of 2A sequence. In this way 2A remains fused to the C-terminus of the upstream protein, while the one downstream is only modified with a Pro at the N-terminus (Donnelly et al., 2001; Piperno et al., 2015; Principe et al., 2015). In addition, peptide 2A does not work with bacterial ribosomes (Donnelly et al., 1997) and its efficiency can vary depending on neighbour amino acids (de Felipe et al., 2010, 2006).

Peptide 2A could also cause, depending on the upstream protein, lack of processing resulting in an unresolved fusion of both proteins (Minskaia and Ryan, 2013) and has a variable bias towards the expression of the first protein, thus preventing 1:1 stoichiometric expression of protein subunits (L. Sasset, G. Petris, OR Burrone, unpublished observations). TEVp stoichiometry, instead is 1:1. Expression of a construct encoding a polyprotein containing the ER-targeted TEVp, followed by the L and H chains of an IgG separated by TS, was more efficient in producing and secreting the fully assembled IgG than other constructs based on 2A or in combinations of 2A and TS (Fig. 3). TEVp stoichiometry can be modified, for instance to 1:2 by duplicating one of the subunits in the polyprotein precursor (Chen et al., 2010). As TEVp cleavage is quite fast, it is also possible to envisage that fine-tuning of stoichiometry can be obtained by tagging one of the subunits with stabilizing or destabilizing domains (*i.e.* degron/PEST signals) (Taxis et al., 2009).

4.5. A split-TEVp complementation assay

As other proteins, also TEVp can be split in two non-functional parts that restore proper enzymatic activity upon their association (complementation). This system, defined as protein complementation assay, or PCA, can be used to monitor or induce protein-protein interactions (PPIs), as recently reviewed (Wehr and Rossner, 2015). Two different split-TEVp PCAs were initially reported (Wehr et al., 2006). One consisted of N-TEVp(1–118)/C-TEVp(119–242) and the other of N-TEVp(1–70)/C-TEVp(61–242). In both cases, lower activity was displayed following association (30–40%) compared to full-length TEVp. The rational of the N-TEVp(1–118)/C-TEVp(119–242) PCA is based on reconstitution of the two domains triggered by interaction of fusion partners engineered at their N-termini (Fig. 4A,D). Consequently, the restored TEVp activity can be monitored by direct or indirect reporter systems: proteolytic activation of luciferase (Wehr et al., 2008, 2006), fluorescent proteins and FRET (Wehr et al., 2008; Williams et al., 2009) or transcriptional activation of a reporter gene (Capdevila-Nortes et al., 2012; Djannatian et al., 2011; Wehr et al., 2012, 2008, 2006). Interestingly, this technique facilitated analysis of the more challenging PPIs based on transient phosphorylations (Wehr et al., 2008) of G protein-coupled membrane receptors (GPCRs) activation (Djannatian et al., 2011; Wehr and Rossner, 2015) and cell-cell interactions (Wehr et al., 2008).

Thanks to its sensitivity and flexibility, the split-TEVp PCA was applied in a genome wide high-throughput RNA interference-mediated screening in *Drosophila*. The assay was adapted to monitor activation of the Hippo pathway: split-TEVp domains complementation occurs upon Yki/14.3.3 interaction, which is induced by Hippo activation. Two salt-inducible kinases (Sik 2 and 3) that act as negative modulators in *Drosophila* Hippo signalling pathway were thus identified (Wehr et al., 2012).

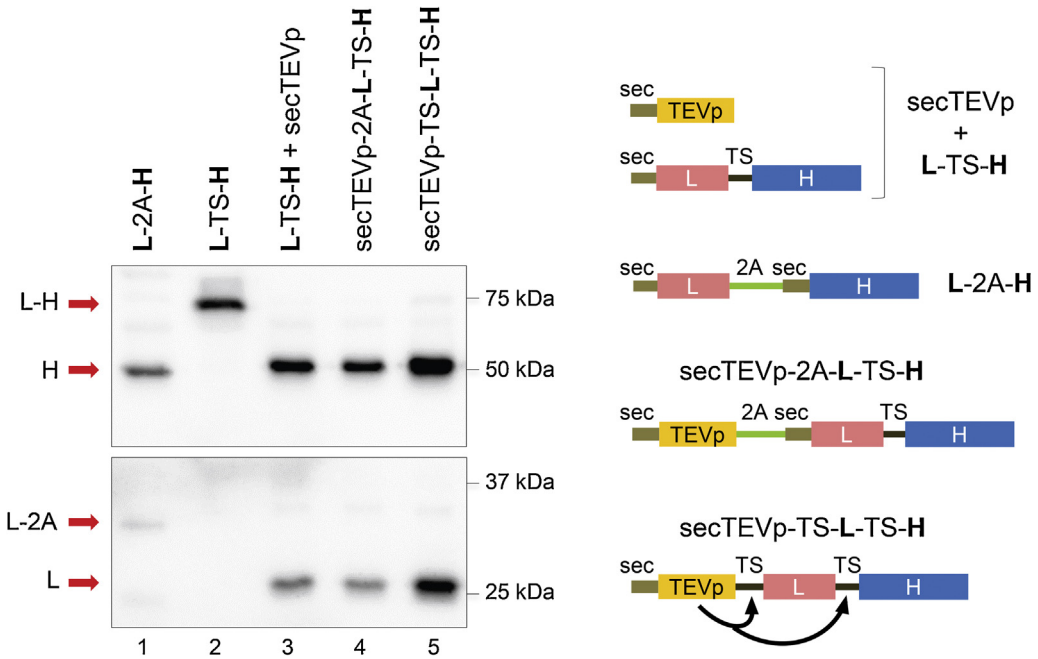

Fig. 3. Use of TEVp for *in vivo* polyprotein processing. Expression of a full-length IgG from constructs encoding L and H chains. Improved expression was obtained from a single linear construct encoding polypeptides secTEVp-QSG active in the ER lumen, (Cesaratto et al., 2015), L and H separated by TEVp cleavage sites TS. A single signal peptide (sec) is needed for translocation into the ER. 2A, Foot and Mouth disease virus derived peptide that allows independent translation of the two L and H polypeptides form a single mRNA.

The use of a ligand-inducible PCA is more suitable to detect soluble PPIs and to reduce signal background. An inducible split-TEVp PCA was described with the N-TEVp and C-TEVp moieties engineered, respectively, at the C-terminus of the rapamycin receptor FKBP (FK506 binding protein) and of its counterpart FRB (the rapamicyn-binding domain of mTOR kinase). Upon rapamycin addition the high affinity association of FKBP and FRB induced complementation of the two TEVp split domains (Fig. 4B) (Gray et al., 2010; Morgan et al., 2015; Williams et al., 2009).

An improved version with reduced leaky proteolytic activity in the absence of rapamycin uses a 23 amino acids shorter C-TEVp fragment (119-219). This system was applied to study the roles and interactions of caspases-3, -6 and -7 (Gray et al., 2010) during cell apoptosis and the caspase-activated DNase CAD (Morgan et al., 2015).

A different PCA based on N-TEVp(1–118)/C-TEVp(119–242) or N-TEVp(1–206)/C-TEVp(119–242) induced by rapamycin and the conditional protein splicing (CPS) was reported in yeast (Fig. 4C) (Sonntag and Mootz, 2011). CPS in based on inteins, that are domains that remove themselves from a precursor protein catalysing the formation of a peptide bond between the two flanking domains. As TEVp, the intein domain can be split into two fragments. The N-TEVp and C-TEVp domains were engineered, respectively, with the N- and C-terminal fragments of a split-intein in the rapamycin-inducible platform. Rapamycin-induced complementation of intein resulted in the covalent stabilisation of the split-TEVp, generating a soluble and more stable (wild type like) TEVp.

The many advantages of using a TEVp PCA, such as its sensitivity, specificity and flexibility, render this methodology highly adjustable to different systems in living cells and organisms.

4.6. Other in vivo TEVp applications

An alternative TEVp-based assay (termed Tango) was developed to study membrane receptor activation (Barnea et al., 2008), with a TS engineered between a membrane receptor and a transcription factor. TEVp, fused to a protein interactor that is recruited upon ligand activation of the receptor, releases the transcription factor that induces expression of a reporter gene. This assay was developed for diverse classes of receptors, such as GPCRs, receptor tyrosine kinases, and steroid hormone receptors. Of note, the TS used had a reduced affinity for TEVp (Leu in position P1′) to enhance specificity of cleavage only upon TEVp recruitment. However, in the specific case of the GPCRs the split-TEVp complementation assay was more sensitive than full-lenght TEVp (Djannatian et al., 2011). The Tango assay was later extended to the study of cytosolic PPIs in the *de novo* purine biosynthesis pathway (Deng et al., 2012).

TEVp expression in living organisms can be used to interfere with protein complexes or functions. A TEVp-based strategy to induce degradation of a specific protein was first described in yeast (Taxis and Knop, 2012; Taxis et al., 2009). The galactose-inducible TEVp cleavage induced N-degron de-protection and consequently protein degradation by the proteasome. This flexible and fast system was applied to the study of cell cycle regulators (Sasabe et al., 2014), inhibition of 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) (Vanella et al., 2016) and inhibition of endocytosis and trafficking to the vacuole (Rodríguez-Limas et al., 2015). A similar application was described in living *Drosophila* flies (Pauli et al., 2008) to explore the role of cohesin complex ring in mitotic and non-mitotic neurons. An inducible TEVp was directed against one of the cohesin ring components, Rad21, engineered with a TS. TEVp cleavage compromised Rad21 function. A distinctive feature of this study was that the direct deactivation of a protein by TEVp cleavage takes place on a much faster time scale than gene deletion or RNA interference. A tetracycline-inducible TEVp was used to study the role of C-terminal fragments (CTFs) of the ribonucleoprotein TDP-43 (Pesiridis et al., 2011), which are found in cytoplasmic inclusions, together with full-lenght TDP-43, in neurons of some detrimental diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD-TDP). The introduction of TS in TDP-43 allowed monitoring of CTFs formation and aggregation after induction of soluble or nuclear TEVp variants.

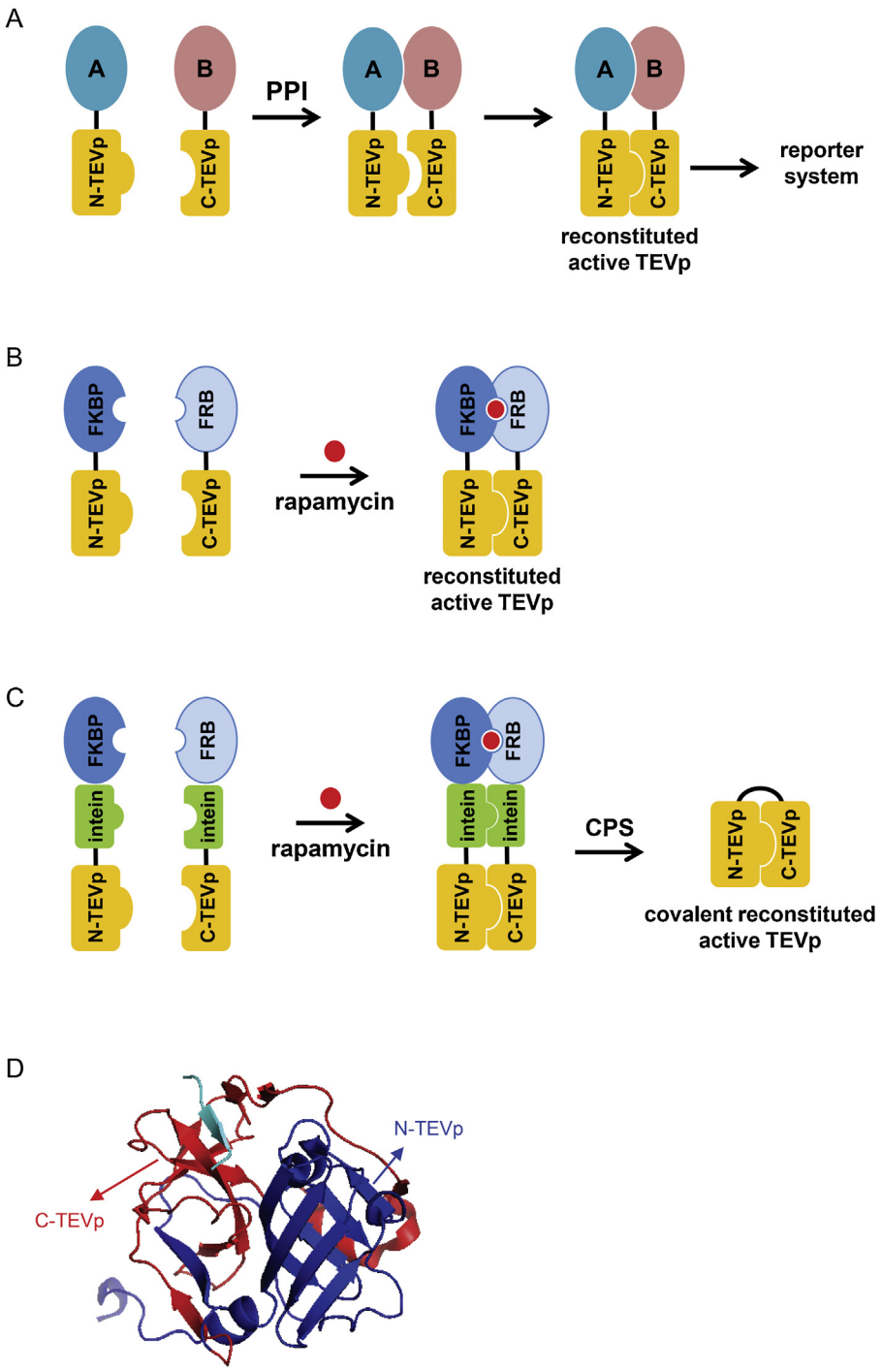

Fig. 4. Split-TEVp complementation assay. (A) Schematic representation of TEVp PCA for the study of protein-protein interactions (PPI). (B) Rapamycin-inducible split-TEVp PCA. (C) Rapamycin-inducible CPS/split-TEVp assay. (D) Crystal structure of reconstituted TEVp; blue, amino acids 1–118 (N-TEVp); red, i 119-219 (C-TEVp). In light blue the substrate peptide (adapted from pdb file 1LVB, Phan et al., 2002). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TEVp can be used as well to reactivate a protein function: for example, specific transcription factors can be introduced in a desired cell type to induce pluripotency (iPS technique), an approach proposed for cell replacement therapies. Delivery of such transcription factors can be achieved by fusing them to a protein transduction domains (PTD) as an alternative to viral transduction. As this protein fusions were less active, a cytosolic TEVp was then used to specifically remove PTDs inside cells, fully restoring their transcriptional activity (Konno et al., 2011).

5. Conclusion

TEVp is the best studied and the most widely used peptidase for *in vitro* and *in vivo* biotechnological applications. TEVp has high specificity *in vitro* and *in vivo*, with lack of toxicity from bacteria to complex organisms. It tolerates different residues in P1′ position of its cleavage recognition site and can be engineered and targeted to different subcellular compartments. It is easy and economically convenient to produce for *in vitro* applications and available from

several expression vectors from open sources. All these features make TEVp a unique tool in life sciences and a shortcut to address several different biotechnological challenges.

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