Chapter 4
RNase T2 Family: Enzymatic Properties, Functional Diversity, and Evolution of Ancient Ribonucleases

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Contents

Abstract RNase T2 enzymes are transferase-type endoribonucleases that produce oligonucleotides and/or mononucleotides with a terminal $3'$ phosphate via a $2',3'$ cyclic phosphate intermediate. These RNases are found in all eukaryotes and also in bacteria and viruses, where they have a wide range of biological activities. Some have a housekeeping role, degrading rRNA, and mutations affecting this function result in alterations in cellular homeostasis and are associated with brain lesions in vertebrates. Others have a variety of specialized roles including antimicrobial defense, phosphate scavenging, rejection of "self" pollen, and even nitrogen storage. Members of this family have also acquired functions that are independent of their ribonuclease activity. One of these catalysis-independent functions is implicated in the control of cellular growth, and lack of RNASET2 protein in humans is correlated with several classes of tumors. This review will discuss the basic structure, enzymatic properties, and biological roles of this ancient RNase family.

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4.1 Introduction

Prokaryotic and eukaryotic cells possess a large number of ribonucleases (RNases) that participate in many cellular functions, from DNA replication, to control of gene expression, to defense against microorganisms. Among those, a set of RNases are secreted or localized inside cellular structures associated with the secretory pathway, the vacuole, or lysosomes; thus, these enzymes are found in a space not normally associated with the presence of RNA. These RNases belong to a superfamily of enzymes that catalyze RNA cleavage via a $2'$-$3'$-cyclic phosphate intermediate at the $3'$-terminus of the resulting oligo- or mononucleotide products.

Historically, $2'$-$3'$-cyclizing RNases have been classified into three groups according to mass, base specificity, pH preference, and origin. According to Irie (1999), these correspond to the RNase T1, RNase A, and RNase T2 families. The RNase T1 family includes alkaline RNases with a molecular mass ~12 kDa and pH optima between 7 and 8 that are found in fungi and bacteria (Irie 1999; Deshpande and Shankar 2002). The vertebrate-specific RNase A family (discussed in Chaps. 1, 2, and 3) comprises proteins with a molecular mass between 13 and 14 kDa and either an alkaline (7–8) or weakly acidic (6.5–7) pH preference. Finally, the RNase T2 family includes RNases with an average molecular mass around 25 kDa that were originally categorized as acid RNases (Irie 1999). However, the RNase T2 family is very diverse, with members present in almost all eukaryotic genomes and many bacterial and even viral genomes. As will be discussed here, these proteins have a wide size range and broad pH preferences ranging from extreme acidic to very basic. Currently, the best criterion to classify a protein as a member of the RNase T2 family is the presence in its primary sequence of specific amino acid motifs associated with the RNase active site that are conserved in every T2 protein (Irie 1999).

Studies on RNase T2 enzymes began almost a century ago, when Noguchi described the presence of nucleic-acid degrading enzymes in Takadiastase, an enzyme mixture prepared from the fungus *Aspergillus oryzae* (Noguchi 1924). Later, Sato and Egami (1957) purified RNase T1 and RNase T2 from this mixture and showed that they had different biochemical properties. Since then, many fungal, plant, and animal RNase T2 enzymes have been purified, cloned, and characterized. More recently, X-ray crystallographic analyses and mutagenesis studies allowed the identification of the catalysis mechanism and substrate specificity determinants, providing a very good understanding of the enzymological properties of the family.

On the other hand, data on the biological roles of the RNase T2 family lagged well behind the large accumulation of biochemical data, in spite of the apparent importance of these enzymes that are conserved in almost all organisms. The discovery that proteins associated with self-incompatibility in several plants had RNase activity and were part of the RNase T2 family opened the door to a series of studies on the function of these enzymes. Since then, the spectrum of biological activities for this family of enzymes has increased exponentially. Some RNase T2

enzymes are involved in a housekeeping role, turning over ribosomal RNA to maintain cellular homeostasis, while others have acquired specialized functions as defense against microorganisms, determinants of pathogenicity, phosphate scavenging, and even nitrogen storage. Moreover, these proteins have gained functions that are independent of their catalytic activity. These novel functions seem to be important to control cell proliferation and have a significant impact in tumorigenesis.

## 4.2 Protein Properties and Overall Structure

RNase T2 proteins have apparent molecular weights that fall in the range of 19 to ~97 kDa, with the majority between 20 and 40 kDa (Deshpande and Shankar 2002). In spite of this variability, protein and gene sequence analyses determined that most enzymes have polypeptide chains corresponding to ~20–30 kDa. In most cases, the size range is determined by glycosylation.

Early analyses of RNase T2 showed that enzyme preparations were heterogeneous in molecular weight and separated into six fractions on gel filtrations. Amino acid and carbohydrate analyses revealed that in each of these fractions, the protein moiety corresponded to RNase T2 and the heterogeneities were due to the carbohydrate content, mainly galactose (Kanaya and Uchida 1981). Detailed analysis of the glycan moieties present on S-RNases from tobacco and wild tomato (both from the Solanaceae family) found that these proteins are N-glycosylated. The complex patterns containing glucose, mannose, and glucosamine varied largely among alleles of the S-RNase locus, even in a single species (Oxley et al. 1996; Parry et al. 1998). Similar analyses of S-RNase 4 from *Pyrus pyrifolia* (Rosaceae family) identified additional sugar chains, including xylose, fucose, N-acetylglucosamine, and chitobiose (Ishimizu et al. 1999). Effective use of N-Glycosidase F to remove sugar moieties from plant, animal, viral, and fungal enzymes indicated that N-glycosylation is a common modification of proteins in this family (Inada et al. 1991; MacIntosh et al. 2001; Langedijk et al. 2002; Campomenosi et al. 2006; Hillwig et al. 2011). In addition, O-glycosylation has been found in a few RNases. RNase Le37, from the fungus *Lentinus edodes*, has a C-terminal extension rich in Ser and Thr residues that are O-glycosylated, in addition to the common N-glycosylation observed on Asn residues (Inokuchi et al. 2000). A similar pattern of O-glycosylation is possibly present in another fungal protein with a C-terminal extension, RNase Irp1 (Watanabe et al. 1995).

In addition to the C-terminal extension observed for the proteins RNase Le37 and RNase Irp1 from Basidiomycetes fungi, other enzymes with C-terminal extensions are Rny1 from yeast (MacIntosh et al. 2001), and E^{rns} from classical swine fever virus [CSFV (Langedijk 2002)]; although in the latter cases, no O-glycosylation has been observed. In addition, E^{rns} is the only RNase T2 enzyme to exist as a homodimer. In this case, the holoenzyme is a disulfide-linked

homodimer of ~90 kDa, with approximately half of the molecular weight being contributed by the sugar moieties (Schneider et al. 1993; Langedijk et al. 2002).

Crystal structures are available for *Nicotiana alata* and *Pyrus pyrifolia* S-RNases (Ida et al. 2001; Matsuura et al. 2001), wound-inducible RNases NW and NT from *Nicotiana glutinosa* leaves (Kawano et al. 2002, 2006), RNase LE from cultured tomato cells (Tanaka et al. 2000), RNase MC1 from bitter gourd (Nakagawa et al. 1999), trichomaglin from root tubers of *Trichosanthes lepiniate* (Gan et al. 2004), RNase Rh from the filamentous fungus *R. niveus* (Kurihara et al. 1996), and EcRNase I from *Escherichia coli* (Rodriguez et al. 2008). A conserved overall structure has been found for bacterial, fungal, and plant RNase T2 proteins (Fig. 4.1), despite the low sequence conservation between prokaryotic and eukaryotic enzymes [bacterial EcRNase I shows less than 35% sequence identity with plant RNase T2 family members and less than 15% sequence identity with fungal and animal members (Rodriguez et al. 2008)]. Although sequence identity is low, crystallographic analyses showed that all have a core of hydrophobic residues in similar positions (Deshpande and Shankar 2002; Rodriguez et al. 2008). In addition, the conserved structure of all characterized RNases consists of a four-stranded antiparallel β-sheet (strands β1, β2, β4, and β5), a small two-stranded antiparallel
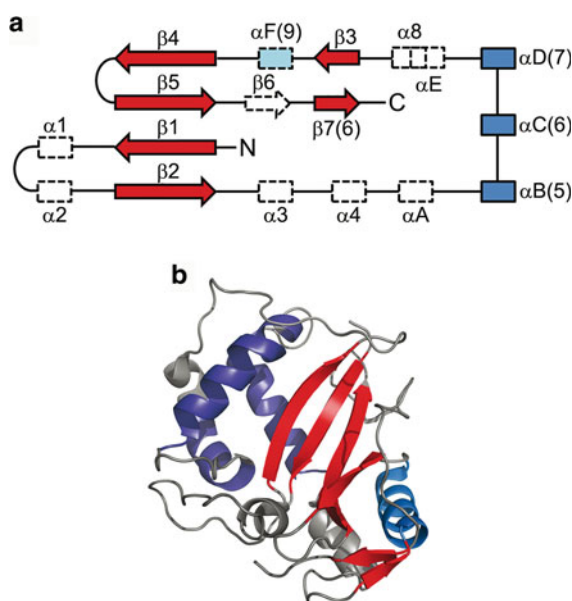
Fig. 4.1 Overall structure of RNase T2 proteins. (**a**) Topology diagram of RNase T2 proteins showing structural elements. Elements conserved in all members of the family are shown in *red* (β-sheets, *arrows*) and *dark blue* (α-helixes, *rectangles*). *Light blue* indicates partial conservation. Other elements are not conserved in all proteins. Nomenclature follows that of RNase Rh and RNase LE, with the nomenclature used for EcRNase I in parentheses (figure modified from Rodriguez et al. 2008). (**b**) Ribbon diagram of RNase LE (PDB ID: 1DIX), using the same color coding as in (**a**)

β-sheet (strands β3 and β7), and three α-helices (αB, αC, and αD) that are absolutely conserved. Another α-helix (αF) is conserved but with relative position and orientation more variable (Rodriguez et al. 2008). Other structural motifs are found in individual proteins, with different degrees of conservation (Fig. 4.1). Multiple alignments of available RNase T2 sequences showed very high conservation only for the amino acids belonging to the active site of these enzymes (Irie 1999; Rodriguez et al. 2008; MacIntosh et al. 2010), which reside mainly in strands β2 and β5 and helix αC (Kurihara et al. 1996; Tanaka et al. 2000; Rodriguez et al. 2008).

All RNase T2 proteins have several highly conserved Cys residues, which form a variable number of disulfide bridges that stabilize the protein structure in an active conformation. The position of C-C bridges was determined by chemical methods (Kawata et al. 1988; Ishimizu et al. 1995), mass spectrometry (Oxley and Bacic 1996; Langedijk et al. 2002), and X-ray crystallography (Kurihara et al. 1996; Tanaka et al. 2000; Rodriguez et al. 2008). Two C-C bridges are conserved in all RNase T2 enzymes (Irie 1999). Two others are generally conserved in the plant/ animal subgroup, and the total number of C-C bridges varies between four in the self-incompatibility S_{F11}-RNase from *N. alata* (Ida et al. 2001) to seven in trichomaglin (Gan et al. 2004). Similarly, three bridges different from those found in plants and animals are commonly conserved in fungal enzymes in addition to the two conserved in all RNase T2 proteins (Kurihara et al. 1996; Irie 1999). RNase I from *E. coli* has a total of four C-C bridges (Rodriguez et al. 2008), with the two non-conserved bridges positioned close in space to those found in eukaryotic enzymes. E^{rns}, an envelope glycoprotein from CSFV and other pestiviruses and member of the RNase T2 family (Schneider et al. 1993; Hulst et al. 1994), has four C-C bridges, and one of these bridges is an unusual vicinal disulfide bridge between cysteines 68 and 69 (Langedijk et al. 2002). Since a C-C bridge between adjacent cysteine residues cannot have a long-range structural role, it was proposed that this bridge has another function, perhaps contributing to the formation of homodimers (Langedijk et al. 2002), as E^{rns} is the only RNase T2 enzyme that has been described to exist as a dimer (Konig et al. 1995; Langedijk et al. 2002).

4.3 Enzymatic Activity

Enzymes from the RNase T2 family are transferase type endoribonucleases that produce oligonucleotides and/or mononucleotides with a terminal 3′ phosphate via a 2′,3′ cyclic phosphate intermediate (Irie 1999). These enzymes do not have strict base specificity, although some can have base preferences that vary from enzyme to enzyme [an extensive review of individual enzyme preferences was published by Deshpande and Shankar (2002)].

The reaction mechanism was studied mostly using fungal enzymes, but since amino acid residues implicated in catalysis and the geometry of the active site are absolutely conserved in all RNase T2 enzymes so far characterized, it is safe to

assume that the same reaction mechanism is employed by all enzymes in the family. Chemical modification experiments of fungal and plant enzymes showed that two histidine residues, His46 and His109 (using RNase Rh numbering), are essential for RNase activity (Kawata et al. 1990; Parry et al. 1997). These findings are consistent with the observation that all RNase T2 enzymes have two regions of almost absolute amino acid conservation. These regions correspond to the conserved active site (CAS) of the enzyme and are named CAS I and CAS II, with the consensus sequences F/WTL/IHGLWP and FWXHEWXKHGTC respectively (Irie 1999). The only His residue in CAS I corresponds to His49, and the second His in CAS II corresponds to His109 in RNase Rh.

The residues corresponding to CAS I and CAS II are found in the conserved strand β2 and helix αC in all the crystal structures of RNase T2 proteins, with residues His46, Trp49, His 104, Glu105, Lys 108, and His 109 (RNase Rh numbering) forming the active site of the enzyme (Fig. 4.2a) (Kurihara et al. 1996; Tanaka et al. 2000). Based on enzyme properties and the geometry of the active site, a two-step (transphosphorylation and hydrolysis) general acid–base catalysis mechanism (Fig. 4.2a) was proposed (Irie 1999; Tanaka et al. 2000). In the first step, His46 acts as the general acid and His109 as the general base to generate the 2′,3′ cyclic phosphate intermediate. His46, with a higher pK_a than His109 (Kawata et al. 1990; Ohgi et al. 1992), interacts with the 5′ oxygen of the scissile phosphodiester bond and donates a proton to the released nucleotide. His109, as the general catalyst, removes the hydrogen of the 2′-OH of the ribose moiety. In the second step, the role of the two histidines is reversed; His46 acts as a general base and His109 as a general acid. Unprotonated His46 activates water, resulting in an activated hydroxyl group that attacks the P-O group of the cyclic phosphate. The proton is donated by His109, now acting as general acid.

This mechanism is also confirmed by site-directed mutagenesis studies. Mutations in either His residue (H46F or H109F) resulted in virtually inactive enzymes, with less than 0.02% of WT RNase Rh activity (Ohgi et al. 1992). Similarly, mutations in the His residue equivalent to His109 in a *Petunia inflata* *S*-RNase resulted in an enzyme with no detectable activity (Huang et al. 1994) and loss of a histidine residue of *S*-locus ribonuclease equivalent to His46 was associated with loss of RNase activity in *Lycopersicon peruvianum* (Royo et al. 1994). Analysis of the crystal structure of RNase MC1 from bitter gourd seeds indicated that the putative catalytic residues of RNase MC1 can be easily superimposed with the catalytic residues of RNase Rh (Nakagawa et al. 1999), and site-directed mutagenesis confirmed the role of His46 in catalysis (Numata et al. 2000). However, mutations in the His residue corresponding to His109 from Rh resulted in a RNase MC1 mutant with about 20% of the WT activity, suggesting that in RNase MC1, other residues could replace this His role but less efficiently (Numata et al. 2000).

Other conserved amino acid residues have also been implicated in catalysis. Trp49 mutants showed between 84% and 99% reduction in activity compared with WT, depending on the substitution and substrate used (Ohgi et al. 1996b, 1997b), and crystal structures indicated that its indole ring forms a hydrogen bond with the
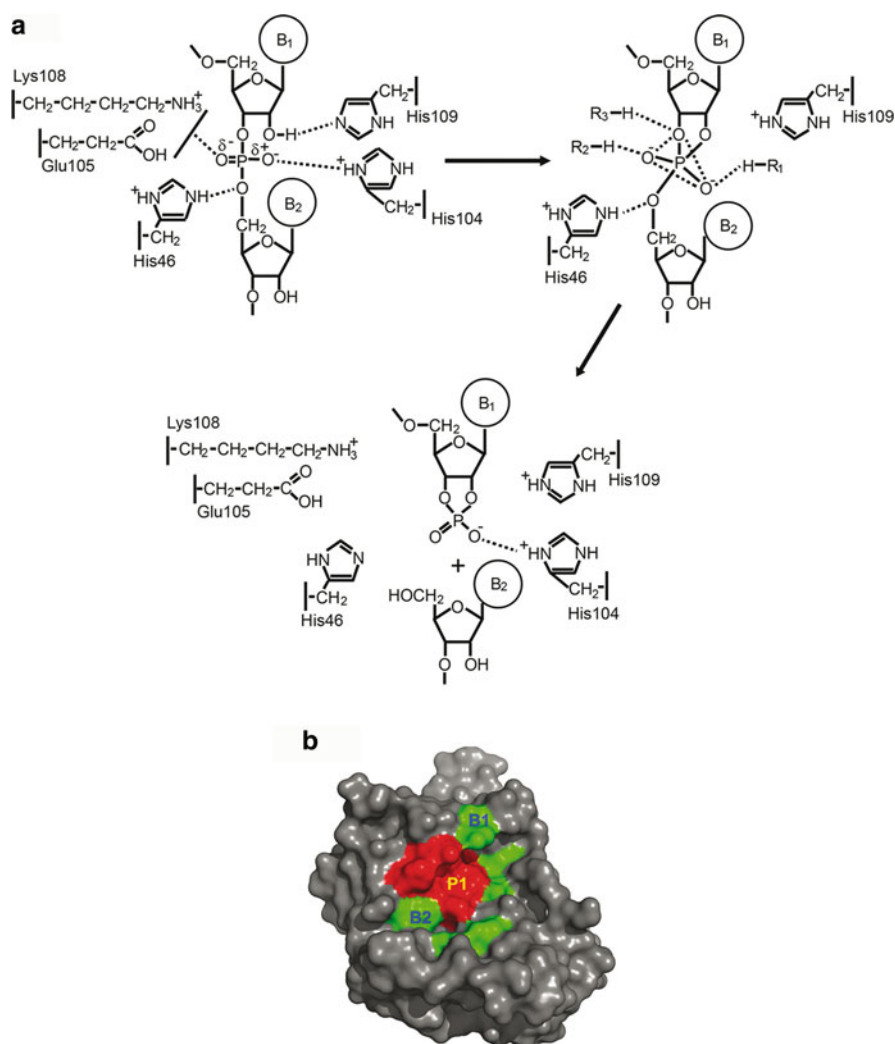
Fig. 4.2 Active site, substrate binding and mechanism of catalysis of RNase T2 enzymes. (**a**) First step of the proposed mechanism of catalysis of RNase T2 enzymes. In this step (transphosphorylation), His46 acts as general acid and His109 as general base. The other amino acid residues contribute to the reaction by stabilizing the pentacovalent phosphate intermediate or binding the substrate. In the second step (hydrolysis, not shown), the role of the two histidines is reversed, His46 acts as general base and His109 as general acid. Residue numbers refer to the RNase Rh sequence (modified from Irie 1999). (**b**) Space filling model of RNase LE showing the P1 (*red*), B1 (*green*), and B2 (*green*) sites

carboxyl group of Glu105 and has a partial stacking interaction with the imidazole ring of His109 (Kurihara et al. 1996; Tanaka et al. 2000). Thus, it was proposed that one of the roles of Trp49 is to fix the side-chains of active site residues

(Tanaka et al. 2000). Glu105 seems to be catalytically crucial and probably operates to polarize the P=O bond or to stabilize the pentacovalent intermediate (Tanaka et al. 2000). Glu105 mutants were strongly inactivated; however, while substitution for alanine resulted in a enzyme with only 0.07% of the WT activity, glutamine or aspartate at that position resulted in enzyme with about 1% of the WT activity(Ohgi et al. 1993), suggesting that Q and D can provide some catalytic function albeit with lower efficiency. His104 also seems to be critical for catalysis, as a H104F mutant only maintained about 1% of the WT activity (Ohgi et al. 1992). Since the K_m for several substrates increased for this His104 mutant, H104 was considered to be a binding site for the negatively charged phosphate group of the substrate (Ohgi et al. 1992; Tanaka et al. 2000). It is important to note that many RNase T2 enzymes have natural substitutions at positions corresponding to His104 and Glu105. Many plant S-RNases contain a glutamine in the position corresponding to Glu105 and a histidine is almost never found at the position corresponding to His104 [see for example Richman et al. 1997; Vieira et al. 2008]. The absence of these residues may account for the low specific activity of many S-RNases (Ida et al. 2001). In most fish RNase T2 enzymes, the position corresponding to His104 is occupied by a tyrosine or a series of polar or charged amino acids, suggesting that these enzymes may also have lower specific activity (Suzuki et al. 2005; Hillwig et al. 2009). This finding led Suzuki et al. (2005) to propose that His104 may also be important for stabilization of the pentacovalent intermediate that would result in higher specific activity. In E. coli RNase I, this histidine is also replaced by tyrosine (Meador and Kennell 1990).

Another residue important for substrate binding to the active site is Lys108. Crystal structures showed that the side-chain of this residue is directed toward the phosphate group (Tanaka et al. 2000), and site-directed mutagenesis experiments indicated that positively charged residues at this position maintain relatively high specific activity, and that residues with hydroxyl groups that can donate protons via hydrogen bonding are preferred (Ohgi et al. 1995, 1996a). Naturally occurring substitutions at this position also confirm this idea; a few fungal and plant RNases present threonine and arginine in place of this conserved lysine (Tanaka et al. 2000).

By analogy to the nomenclature used to define the subsites of RNase A (Richards and Wyckoff 1971), the active site described above has been named "P1 site" (Fig. 4.2b) and contains the catalytic residues and the residues involved in binding to the substrate's phosphate group. Also following this analogy, two other binding sites can be defined. These sites, "B1" and "B2", correspond to hydrophobic pockets on either side of the P1 site and constitute the binding sites for the bases of the nucleotides on each side of the scissile phosphodiester bond (Fig. 4.2b).

RNase T2 enzymes are base-nonspecific, but individual enzymes show unique base preferences, as shown by the release of mononucleotides from RNA, the rates of hydrolysis of homopolynucleotides, and the rates of hydrolysis of dinucleotide phosphates (Irie 1999; Deshpande and Shankar 2002). Some RNases are classified as adenylic acid preferential (RNase Rh), guanylic acid preferential (RNase LE and RNase NW), or uridylic acid preferential (RNase MC1 and RNase CL1),

while others show comparable rate of hydrolysis at any base position (RNase NT and EcRNase I).

Base preference is determined by the residues that form the B1 and B2 sites. The crystal structure of RNase Rh in complex with 2′-AMP showed that the adenine base forms hydrogen bonds with an aspartate and stacking interactions with a tyrosine and a tryptophan residues in the B1 site (Irie 1999). The tryptophan residue corresponds to Trp49; thus, this amino acid has dual functions, participating in the catalytic reaction and in substrate recognition. A D51N RNase Rh mutant showed markedly decreased enzymatic activity toward ApU but not toward UpU, and the substitution of Asp51 by Asn caused the enzyme to become more guanine nucleotide-preferential, suggesting that Asp51 is important for base recognition (Ohgi et al. 1993). Other mutations in the Asp, Tyr, and Trp positions also resulted in enzymes with altered preferences with respect to the 5′ position composition of dinucleoside phosphate substrates (Ohgi et al. 1996b, c, 1997b).

The B2 site of RNase Rh also presents some amino acids that are highly conserved among RNase T2 enzymes, such as Phe101 and Pro92. In the B2 site of crystals of the RNase Rh/d(ApC) complex, the cytosine base is stacked with the side-chain of Phe101 (Irie 1999). Site-directed mutagenesis of Phe101 (Ohgi et al. 2000) suggested that the side-chain of this amino acid interacts with the B2 base probably by π/π or CH/π interactions, and that these contacts are important to maintain enzymatic activity. These mutations also affected the substrate preference of the B2 site. The same effects on base preference were also observed in site-directed mutagenesis experiments that altered two other B2 site residues, Ser 93 and Gln32 (Ohgi et al. 2003; Sanda et al. 2005). Although these experiments targeted residues that are part of the B2 site, small effects on the base preference of the B1 site were also observed. There is not yet an explanation for these observations, but it has been speculated that indirect conformational changes are responsible for this effect (Sanda et al. 2005).

In plant RNases, the Trp and Tyr residues of the B1 site are also conserved (Tanaka et al. 2000; Kawano et al. 2002; Kawano et al. 2006), and may form stacking interactions with the B1 base as reported for RNase Rh. In tomato RNase LE, the position corresponding to Asp51 is occupied by Asn, and RNase LE shows guanine preference. Mutation of this residue to Asp changes the substrate preference to adenylic as in RNase Rh (Ohgi et al. 1997a), suggesting that the base recognition at the B1 site is similar in plant and fungal enzymes. However, in RNase MC1, which has a strong preference for uracil in the 3′ position of the substrate, but does not discriminate based on the composition of the substrate at the 5′ position (Irie et al. 1993), the B1 site is less conserved. The presence of Ser instead of Tyr in this position results in the lack of a hydrophobic pocket and probably explains the low selectivity in the B1 site (Nakagawa et al. 1999). Similar changes observed in S-RNases (Ida et al. 2001; Matsuura et al. 2001), which result in single-sided stacking, could also explain the lack of selectivity of these enzymes at this position (Matsuura et al. 2001).

In *E. coli* RNase I, the B1 site is quite different from the one described in plants and fungi. In this nonselective RNase, only the Trp residue is conserved, and forms

a one-sided stacking with the base. All other interactions, including van der Waals contacts and hydrogen bonds, are unique to E. coli and its closer relatives (Rodriguez et al. 2008). In addition, three water molecules at the bottom of the B1 cleft provide all the hydrogen bonds required for binding to the base (except one involving the side-chain of a B1 residue). Thus, this nonspecific binding site uses a series of water bridges to allow recognition of different bases in combination with base stacking to achieve broad specificity while maintaining good affinity (Rodriguez et al. 2008). This mechanism is different from the binding mechanism observed in the B1 site of other nonselective RNases like RNase NT from tobacco, which uses a series of different hydrogen bonds directly between protein and base (Kawano et al. 2006).

Most mononucleotide complexes of plant RNases show the B2 site occupied. In most cases, a Phe residue, corresponding to Phe101 of RNase Rh, is conserved and participates in stacking interactions with the base, as described for the fungal B2 site (Suzuki et al. 2000; Kawano et al. 2002, 2006). Additional stacking interactions and an extensive network of hydrogen bonds between the base and side-chains of conserved and variable residues located in the B2 site provide additional contacts responsible for base preference. Mutation of amino acids involved in these hydrogen bonds resulted in enzymes with altered specificity (Numata et al. 2001, 2003). In some cases, the size of the B2 site also contributes to base preference. For example, mutation of an Asp in the B2 site of uridylic-preferential RNase MC1 to either Thr or Ser resulted in an enlarged site able to accept a guanine (Numata et al. 2003), and the large size of the B2 site of RNase NT has been proposed as one of the determinants of the purine preference of this enzyme (Kawano et al. 2006).

The amino acids forming the B2 site of RNase I are well conserved among bacteria sequences but are different to those in plants and animal. However, stacking interactions (including a Phe residue) and an extensive hydrogen bond network are also observed in this site. Additionally, a water molecule bridging a B2 amino acid side-chain and a base group has also been observed (Rodriguez et al. 2008).

The bacterial enzyme is the only one that has been crystallized with an oligonucleotide as a substrate mimic [the decadeoxynucleotide d(CGCGATCGCG)]. The structures of EcRNase I in its free and ligand-bound state are very similar, without any significant difference in backbone conformation, indicating a lock-and-key type of binding (Rodriguez et al. 2008).

RNase T2 enzymes are generally regarded as having an acidic pH preference, between pH 4 and 5.5 (Irie 1999; Deshpande and Shankar 2002; Luhtala and Parker 2010). This is certainly the case of the fungal RNases first identified and characterized: RNase T2 optimum pH is 4.5 (Uchida 1966), and RNase Rh prefers pH 5.0 (Tomoyeda et al. 1969); and this is also true for a number of RNase T2 enzymes found in fungi and plants [summarized by Deshpande and Shankar (2002)], and also in animals (Inokuchi et al. 1997; Kusano et al. 1998; Suzuki et al. 2005; Campomenosi et al. 2006; Hillwig et al. 2009). However, many other RNase T2 enzymes have a near-neutral or basic pH preferences. For example, bacterial RNase I has a pH preference around 8.0 (Spahr and Hollingworth 1961),

and plant *S*-RNases also prefer basic pH (McClure et al. 1989; Singh et al. 1991; Deshpande and Shankar 2002). Other RNases are more active near neutral pH, including viral E^{rns} (Schneider et al. 1993), Arabidopsis RNS2 (Hillwig et al. 2011), and yeast RNY1 (G.C. MacIntosh, unpublished). The structural bases of these differences are unknown.

4.4 Biological Roles and Evolution

Members of the RNase T2 family are found in almost all groups of living organisms including virus, bacteria, fungi, plants, and animals (Irie 1999; Hillwig et al. 2009; MacIntosh et al. 2010), although they are not found in the *Archaea* (Condon and Putzer 2002). This family is particularly well conserved in eukaryotes; at least one gene has been found in every eukaryotic genome so far sequenced, with the exception of trypanosomes (Garcia-Silva et al. 2010; G.C. MacIntosh, unpublished) suggesting that RNase T2 enzymes perform an important biological role that has been conserved throughout evolution.

Phylogenetic analyses of plant and animal RNase T2 proteins showed that this family has been very successful in plants, where it has undergone extensive expansion accompanied by high rates of gene duplication and gene loss, resulting in variable numbers of genes in different species (MacIntosh et al. 2010). On the other hand, RNase T2 has been maintained as a single copy gene in most animal species (Hillwig et al. 2009; R. Bailey and G.C. MacIntosh, unpublished).

Evolutionary and expression analyses have found that plant RNase T2 genes can be classified in three classes (Igic and Kohn 2001; Steinbachs and Holsinger 2002; Roalson and McCubbin 2003; MacIntosh et al. 2010). Class I genes show tissue specificity and are generally regulated by stress. Gene duplication and deactivation occurring differentially among lineages [gene sorting (Zhang et al. 2000)] resulted in high diversification of Class I genes, possibly accompanied by the acquisition of novel functions after these duplication events (MacIntosh et al. 2010). Class III genes may have originated from a Class I gene, or at least they seem to share a common ancestor (Steinbachs and Holsinger 2002; Roalson and McCubbin 2003; MacIntosh et al. 2010). Class III genes correspond to the *S*-RNases and potential precursors of these enzymes (Igic and Kohn 2001; Steinbachs and Holsinger 2002; MacIntosh et al. 2010). *S*-RNases have a flower-specific role.

In contrast to Classes I and III, Class II enzymes seem to have conserved more ancestral characteristics, and genes in this class are conserved in all plant species analyzed, with generally one Class II gene in each genome and an evolutionary pattern that follows organismal phylogenies. Most Class II genes are constitutively expressed (MacIntosh et al. 2010; Köthke and Köck 2011). These evolutionary and expression characteristics suggested that plant Class II RNase T2 enzymes may have a housekeeping role (MacIntosh et al. 2010).

Animal RNase T2 genes have not undergone extensive duplication events or diversification. Most animal genomes so far analyzed have only one RNase T2

gene. A study on the phylogenetic relationships of RNase T2 proteins from deuterostomes showed that only one copy is present in each genome, with the exception of bony fishes that have two genes corresponding to this family (Hillwig et al. 2009), with one of the copies forming a fish-specific clade and the other belonging to the clade conserved in all chordates. Expression analyses showed the fish RNase in the conserved clade is expressed in all tissues and all developmental stages (Hillwig et al. 2009), and similar expression patterns have been reported for human RNASET2 (Henneke et al. 2009). These findings suggested that animal RNase T2 enzymes may be the counterpart of plant Class II RNases and also perform a housekeeping role (Hillwig et al. 2009) that could be conserved in plants and animals.

4.4.1 *Cytotoxic Ribonucleases, Defense Responses, and Self-Incompatibility*

While studies on RNase T2 enzymology go back more than half a century, little information on the biological role of these proteins, with a few exceptions, was known until recently. The first enzymes of this family with a definitive assigned function were the *S*-RNases. *S*-RNases are the pistil component of the mechanism of gametophytic self-incompatibility in three plant families (Solanaceae, Rosaceae, and Plantaginaceae). Self-incompatibility (S) prevents self-pollination, and in these families is determined by two components, the *S*-RNase in pistils and the *S*-locus F-box (SLF) protein expressed in pollen, that are genetically linked in the highly polymorphic *S*-locus. The different variants of the *S*-locus that occur in each organism are commonly known as haplotypes. If the haplotype of pollen, which is haploid, matches one of the two haplotypes of the diploid pistil, the pollen is recognized as self-pollen and pollen tube growth is inhibited. On the other hand, pollen that carries a haplotype different from the haplotypes of the pistil is recognized as nonself pollen and the pollen tube is allowed to grow through the style, resulting in fertilization of the ovule (reviewed by Kumar and McClure 2010; Meng et al. 2010).

The pistil *S*-locus gene was originally cloned from *Nicotiana alata*, and described as a secreted glycoprotein (Anderson et al. 1986). Later, its homology to fungal RNase T2 enzymes was recognized, and its RNase activity was confirmed (McClure et al. 1989). Transgenic petunia plants expressing *S*-RNases with single amino acid mutations in the active site, which produced inactive enzymes, were used to demonstrate that the RNase activity is essential for rejection of self-pollen (Huang et al. 1994), and these experiments were confirmed by the identification of naturally occurring wild tomato mutants that had changes in the active site of *S*-RNases that resulted in lack of activity and loss of self-incompatibility (Royo et al. 1994). It has been shown that *S*-RNases are secreted from cells of the stigma, style, and ovary into the extracellular matrix that guides the pollen tube to the ovule

(Cornish et al. 1987; Anderson et al. 1989), and they enter the pollen tubes, both in compatible and incompatible interactions (Luu et al. 2000; Goldraij et al. 2006). However, specific degradation of ribosomal RNA in pollen tubes occurs only during the incompatible interaction (McClure et al. 1990). Thus, it was hypothesized that the pollen *S*-locus component would inhibit *S*-RNase active during compatible interactions (Golz et al. 2001; Kao and Tsukamoto 2004).

The identification of the *S*-pollen component proved more difficult; only recently, it was found that the product of the pollen *S*-locus is an F-box protein (*SLF*) that putatively is part of the multi-subunit E3 ubiquitin ligase complex (Qiao et al. 2004a, b; Sijacic et al. 2004). All current models for the mechanism of self-incompatibility agree that the *S*-RNase acts as a cytotoxin that inhibits pollen tube growth during incompatible interactions by degradation of rRNA in pollen. Specificity of this reaction is given by interaction of *S*-RNase with SFL. This interaction may result in the degradation of *S*-RNase in compatible but not in incompatible interactions, or in differential release of *S*-RNase from an intracellular compartment into the cytoplasm, depending on the model (Hua et al. 2008; Kumar and McClure 2010; Meng et al. 2010).

Although the three plant families in which self-incompatibility is based on cytotoxic *S*-RNases are not closely related phylogenetically, most evidence suggests that this mechanism evolved only once and was the ancestral state in the majority of dicots (Igic and Kohn 2001; Steinbachs and Holsinger 2002; Vieira et al. 2008). The cytotoxic activity of *S*-RNases and their expression in flowers led to the hypothesis that gametophytic self-incompatibility evolved through the recruitment of an ancient flower ribonuclease involved in defense mechanisms against pathogens for the use in defense against "invasion" by self-pollen tubes (Lee et al. 1992; Hiscock et al. 1996; Nasrallah 2005). Plant RNase T2 proteins that do not participate in self-incompatibility are frequently called S-like RNases. Many of these S-like RNases have been, in fact, associated with defense responses.

Most evidence for a defensive role in plants comes from gene expression analyses. For example, the expression of *RNase NE*, a S-like RNase from *Nicotiana tabacum*, is induced in response to the oomycete pathogen *Phytophthora parasitica* (Galiana et al. 1997). A highly similar gene from *N. tabacum*, *RNase Nk1*, is induced by cucumber mosaic virus infections (Ohno and Ehara 2005), and expression of *RNase NW* is induced in *Nicotiana glutinosa* plants infected with tobacco mosaic virus (Kurata et al. 2002). Microarray analyses indicated that several S-like RNase genes from rice are induced in response to infections by the bacterium *Xanthomonas oryzae* and the fungus *Magnaporthe grisea* (MacIntosh et al. 2010). These expression patterns suggested that RNase T2 enzymes could have an antimicrobial effect; however, only for RNase NE, this activity has been demonstrated. Addition of purified RNase NE inhibited hyphal growth from *P. parasitica* zoospores and from *Fusarium oxysporum* conidia in vitro, and infiltration of RNase NE into the extracellular space of tobacco leaves inhibited the development of *P. parasitica* in vivo (Hugot et al. 2002). This antimicrobial effect of RNase NE is dependent on the ribonuclease activity of the protein, since a protein with a point mutation in one of the active site His residues failed to inhibit hyphal growth in vitro (Hugot et al. 2002).

This defense role supports the hypothesis that *S*-RNases evolved from an antimicrobial RNase. Even now, *S*-RNases may maintain a defense role in addition to their role in self-incompatibility. Petunia *S*-RNases are expressed in nectaries, the organ that produces floral nectar, together with other RNase T2 enzymes with intermediate characteristics between *S*- and S-like RNases (Hillwig et al. 2010a, b). Moreover, a proteomics analysis identified *S*-RNases as bona fide nectar proteins (Hillwig et al. 2010a). In nectar, these enzymes may also have an antimicrobial role to inhibit the growth of fungi and bacteria that would be favored in this rich medium. In fact, petunia nectar has a strong antibacterial activity (Hillwig et al. 2010b).

Many S-like RNases are also induced in response to insect feeding and mechanical wounding, including Arabidopsis RNS1, tobacco RNase NW and RNase Nk1, tomato RNase LE, zinnia ZnRNaseII, and several rice and soybean RNase T2 genes (Ye and Droste 1996; Kariu et al. 1998; LeBrasseur et al. 2002; Gross et al. 2004; Bodenhausen and Reymond 2007; Hillwig et al. 2008; MacIntosh et al. 2010). Although the role of S-like RNases in the response to insects and wounding is not clear, it has been proposed that they could be antimicrobial enzymes that inhibit colonization by fungal, bacterial, and viral pathogens that use the wound as an entry site, or they could participate in phosphate remobilization during the healing process (LeBrasseur et al. 2002; Ohno and Ehara 2005). The extracellular nature of some of these RNases is consistent with an antimicrobial role (Jost et al. 1991; Bariola et al. 1999; Hugot et al. 2002).

In addition to plant S-like RNases and *S*-RNases, other RNase T2 proteins have cytotoxic effects on different cells; however, in some cases, the mechanisms of action underlying cytotoxicity, and even the need for RNase activity, are not clear. Viral E^{rns} acts as a virulence factor for pestiviruses. This protein is secreted from CSFV-infected cells (Rumenapf et al. 1993), and it displays cytotoxic effects against lymphocytes in cell cultures through induction of an apoptotic process (Bruschke et al. 1997); moreover, this effect is specific since E^{rns} is not cytotoxic to epithelial cells. However, immunosuppression associated with virus persistence in the animal host seems to rely both on RNase activity of E^{rns} and other protein features unrelated to its activity (Meyers et al. 1999; von Freyburg et al. 2004; Xia et al. 2007; Magkouras et al. 2008; Sainz et al. 2008; Tews et al. 2009). Omega-1, a glycoprotein secreted by eggs of the parasitic helminth *Schistosoma mansoni*, is an active RNase T2 enzyme (Fitzsimmons et al. 2005). This protein is the main elicitor of a potent CD4 T helper (Th) cell response that is necessary for parasite eggs to cross the endothelium and migrate to the intestinal lumen, from where they can exit the body to continue their lifecycle (Pearce 2005; Everts et al. 2009; Steinfelder et al. 2009). In immunosuppressed mice, omega-1 has a cytotoxic effect on hepatocytes, and it has been speculated that RNase activity is necessary for this effect (Dunne et al. 1991; Fitzsimmons et al. 2005). However, the ability of omega-1 to promote Th2 lymphocyte differentiation is independent of its RNase activity (Everts et al. 2009; Steinfelder et al. 2009).

Rny1, the only RNase T2 found in *Saccharomyces cerevisiae* (MacIntosh et al. 2001), is also involved in cytotoxic responses. Rny1 is localized to the vacuole and

also secreted to the growth medium (MacIntosh et al. 2001; Thompson and Parker 2009); however, during oxidative stress, it is released from the vacuole to the cytoplasm, where it can cleave tRNA and rRNA (Thompson and Parker 2009), a function that clearly relies on its RNase activity. In addition, Rny1 is able to promote cell death through a mechanism that is independent of its catalytic activity (Thompson and Parker 2009).

4.4.2 Phosphate Scavenging and a Housekeeping Role in rRNA Recycling

The original discovery of RNase T2 as a secreted RNase from fungi suggested that it participates in scavenging of phosphate from RNA for nutritional purposes (Deshpande and Shankar 2002). Later, many plant RNases were found to be induced in response to phosphate starvation and during cell death processes when RNA is recycled, reinforcing this idea. The expression of two tomato RNases, RNase LE, and RNase LX, is induced when cultivated tomato cells or seedlings are grown in Pi-deficient media (Jost et al. 1991; Loffler et al. 1993; Kock et al. 1998, 2006). Two RNase T2 genes from Arabidopsis, *RNS1*, and *RNS2*, are also induced by Pi-starvation (Taylor et al. 1993; Bariola et al. 1994). The induction of RNase T2 genes as part of a phosphate scavenging system seems conserved in all plants. Other dicot genes regulated by Pi-starvation include tobacco *RNase NE* (Dodds et al. 1996), *AhSL28* from Antirrhinum (Liang et al. 2002), and *RNase PD2* from almond. Pi-starvation-regulated genes in monocots include *OsRNS5*, *OsRNS7*, and *OsRNS8* from rice (MacIntosh et al. 2010), and *WRN2* and *WRN3* from wheat (Chang et al. 2005).

Analysis of Arabidopsis seedlings grown on plates in which the only source of phosphate was RNA showed a strong increase in RNS1 activity and a weaker increase in RNS2 activity, and indicated that plants can use external RNA as a source of phosphate through the induction of a scavenging system (Chen et al. 2000). The presence of RNase T2 enzymes in the digestive liquid of the carnivorous plants *Drosera adelae* (Okabe et al. 2005) and *Nepenthes ventricosa* (Stephenson and Hogan 2006) also confirms a role in nutrition through phosphate scavenging for this family of RNases.

While some of the enzymes induced by Pi-starvation are extracellular and could be released to the medium, such as RNase LE and RNS1 (Jost et al. 1991; Bariola et al. 1999), some intracellular proteins, such as RNS2 and RNase LX (Loffler et al. 1993; Hillwig et al. 2011), are also induced by this stress, suggesting that internal pools of RNA are also subjected to scavenging. In addition, the expression of *RNS2* and *RNase LX* increases during senescence (Taylor et al. 1993; Lers et al. 1998; Lehmann et al. 2001), a regulation also observed for *AhSL28* and for *VRN1* from the green alga *Volvox carteri* (Shimizu et al. 2001; Liang et al. 2002). Moreover, *ZRNaseI* is expressed in the late stage of in vitro tracheary element differentiation

in zinnia (Ye and Droste 1996) and *RNase LX* expression has been observed in xylem differentiation (Lehmann et al. 2001). These observations indicated that the corresponding proteins could be involved in recycling of phosphate during processes involving cell death. Antisense suppression of *RNase LX* expression resulted in delayed senescence and leaf abscission, suggesting that RNase LX may not only recycle phosphate during senescence and abscission, but could also participate in the control of these processes (Lers et al. 2006). Even more intriguing is the fact that some of the rice RNases induced by phosphate starvation (*OsRNS5*, *OsRNS7*) have accumulated mutations in the active site that most likely render the proteins inactive (MacIntosh et al. 2010). The function of these proteins is yet unknown.

A scavenging role has also been proposed for non-plant RNase T2 enzymes. The edible mushroom *Pholiota nameko* grows well in phosphate-deficient conditions, and this characteristic was attributed to its ability to secrete RNases, including one RNase T2 enzyme, to the medium under Pi-limiting conditions (Tasaki et al. 2004). The human parasite *Entamoeba histolytica* is a nucleo-base auxotroph that needs to obtain purines and pyrimidines from its host. This parasite constitutively secretes two RNase T2 enzymes during axenic culture, and it was hypothesized that these proteins are utilized to scavenge nucleotides to support this organism's growth (McGugan et al. 2007).

The proposed housekeeping role of RNase T2 enzymes is also related to phosphate and/or nucleotide recycling. Although the Arabidopsis *RNS2* gene is upregulated by Pi-starvation and during senescence, it is normally expressed at high levels in all tissues and developmental stages (Hillwig et al. 2011). Phylogenetic analysis determined that RNS2 belongs to the Class II category (Igic and Kohn 2001; MacIntosh et al. 2010), which suggested that it could have a housekeeping function. Fluorescently tagged protein was used to determine that RNS2 localizes to the endoplasmic reticulum and the vacuole (Hillwig et al. 2011). Mutants lacking RNS2 activity accumulate RNA intracellularly, mainly in the vacuole, and half-life analysis showed that rRNA decays more slowly in these mutants. Moreover, expression of RNS2 is necessary for maintenance of cellular homeostasis, since the *rns2* mutants showed constitutive autophagy. Based on these results, it was suggested that the housekeeping role of RNase T2 enzymes is to participate in the degradation of ribosomes not only when plants are under nutritional stress but also under normal growth conditions (Hillwig et al. 2011). Cells lacking RNS2 activity may sense a nutritional imbalance that triggers autophagy to compensate for the lack of normal rRNA degradation.

This housekeeping role seems to be conserved in all eukaryotes. The zebrafish genome contains two RNase T2 genes, with only one, *RNASET2*, conserved in other vertebrates (Hillwig et al. 2009). The RNASET2 protein of zebrafish was also found in the endoplasmic reticulum and lysosomes (Haud et al. 2011). Similarly, the homologous human protein, also RNASET2, is found in lysosomal fractions (Campomenosi et al. 2006). Mutant zebrafish that lack RNASET2 activity had enlarged lysosomes that accumulated rRNA in brain cells, and RNASET2 depletion in HEK 293 cells resulted in increased lysosome biogenesis (Haud et al. 2011). Moreover, magnetic resonance microimaging of mutant zebrafish revealed white

matter lesions that may be the results of altered lysosomal function, suggesting that lack of RNASET2 also affects cellular homeostasis in this system (Haud et al. 2011). *RNASET2* mutation in humans is linked with a leukoencephalopathy characterized by cortical cysts and multifocal white matter lesions (Henneke et al. 2009), similar to those found in zebrafish; these findings indicate that RNASET2 function is conserved in fish and humans. In these cases, it was also proposed that RNASET2 functions in normal rRNA decay (Haud et al. 2011).

Analysis of mutant yeast cells lacking Rny1 activity also detected deficiencies in rRNA cleavage, although in this case, the effect was only observed when cells were subjected to oxidative stress, when a portion of this protein is released from the vacuole to the cytoplasm (Thompson and Parker 2009). Whether Rny1 participates in rRNA decay under normal conditions is not known.

4.4.3 Catalysis-Independent Functions and Cancer

Several lines of evidence point to a role for RNASET2 as a regulator of cell growth that can affect tumor progression in humans. The *RNASET2* gene maps to a region of chromosome 6 that is frequently deleted in a variety of cancer cells (Trubia et al. 1997). In an extensive survey, no mutations were found in the *RNASET2* gene in ovarian tumor tissues, but its expression was significantly reduced in 30% of primary ovarian tumors and in 75% of ovarian tumor cell lines (Acquati et al. 2001, 2005). Similarly, reduction of *RNASET2* expression was found in lymphomas and melanomas (Steinemann et al. 2003; Monti et al. 2008). Cancer cell lines transfected with *RNASET2* showed a reduction in clonogenicity, and reduced development of tumors and metastatic potential of cell lines in nude mice models was observed (Acquati et al. 2001, 2005; Smirnoff et al. 2006). While the effect of RNASET2 on growth of cancer cell cultures in vitro is controversial (Liu et al. 2002), a consistent effect as tumor antagonizing/malignancy suppressor has been found for this protein in vivo (Acquati et al. 2011).

Remarkably, the tumor suppression function of RNASET2 is independent of its catalytic activity. Versions of the *RNASET2* gene, in which the sequence encoding the active site histidines were mutated, were also effective in reducing tumorigenesis and metastasis (Acquati et al. 2005); and similar results were obtained with heat inactivated protein (Smirnoff et al. 2006). Moreover, the antitumorigenic, antiangiogenic, and antimetastatic effects of ACTIBIND, an RNase T2 protein isolated from *Aspergillus niger*, are also independent of its catalytic activity (Roiz et al. 2006; Schwartz et al. 2007). It has been shown that ACTIBIND is internalized by tumor cells (Schwartz et al. 2007). Both RNASET2 and ACTIBIND are able to bind actin, and ACTIBIND can disrupt the internal actin network and reduce cell motility (Roiz et al. 2006; Smirnoff et al. 2006).

The catalysis-independent mechanism of action of these proteins in tumor suppression is not understood. In addition to binding the actin network, internalized ACTIBIND can be found localized in the nucleus, where it may act as a competitive

inhibitor of angiogenin (Schwartz et al. 2007). Inhibition of angiogenin effects have also been reported for RNASET2 (Smirnoff et al. 2006); and it is known that in addition to its lysosomal localization, RNASET2 is secreted from cells to the extracellular space, where it could then find its target cells (Acquati et al. 2005). Overexpression of RNASET2 in SV40-immortalized fibroblasts resulted in reduction of colony-forming efficiency and growth rate accompanied by reduction in the expression of transcripts involved in Akt signaling, cell cycle control, and cell proliferation (Liu et al. 2010). Finally, RNASET2's control of ovarian tumorigenesis seems to be the result of modification of the cellular microenvironment and the induction of immunocompetent cells of the monocyte/macrophage lineage (Acquati et al. 2011). Thus, it is possible that the tumor suppression effect is exerted by these proteins at several cellular levels.

In addition to these antitumor roles, other catalysis-independent roles for RNase T2 proteins have been reported or hypothesized. RNase activity is not necessary for modulation of cell viability in yeast by Rny1 during oxidative stress responses (Thompson and Parker 2009). Some of the immunosuppressive effects of viral E^{rns} might also be independent of RNase activity (Luhtala and Parker 2010); it has been hypothesized that an antimicrobial role of plant RNases may rely in part on properties of RNase T2 proteins separate from their catalytic activity (MacIntosh et al. 2010). In fact, several highly expressed plant members of the RNase T2 family have accumulated mutation in the active site resulting in inactive proteins and expression of these proteins is regulated by a variety of biotic and abiotic stress conditions (Gausing 2000; Chang et al. 2003; Wei et al. 2006; MacIntosh et al. 2010). The biological role of these plant inactive RNase T2 proteins is unknown. Inactive RNases are also used as storage proteins in plants; for example, an inactive RNase T2 protein is the main vegetative storage protein of resting rhizomes of *Calystegia sepium* (Van Damme et al. 2000). In this case, RNase activity may be dispensable since the protein is only used as a nitrogen reservoir.

4.5 Conclusion

The presence of the RNase T2 family in prokaryotes and eukaryotes and the almost absolute conservation in the former tells us that these ancient proteins have important functions. The last few years have brought advances in our understanding of this family. However, many questions remain to be answer to comprehend the mechanisms in which RNase T2 enzymes participate. Results from zebrafish and Arabidopsis link RNase T2 enzymes to ribosomal RNA decay; but the mechanism that determines when and why rRNA is targeted for degradation and how this substrate comes together with the enzyme needs to be investigated. The same is true for the effects of human RNASET2 and ACTIBIND on cell proliferation and tumor suppression. How do these proteins enter their target cells? What are their intracellular targets? How do they regulate those targets? Uncovering the properties of these proteins that allow them to regulate cell growth independently of their

catalytic activity will have an important impact on future antitumorigenic therapies. It will be also important to investigate whether plant proteins that have lost their enzymatic activity have maintained a function as regulators of cell growth. The diversification of the RNase T2 family in plants also presents unique opportunities to learn more about the acquisition of new protein functions after gene duplication events. These are just a few of the questions that should keep us engaged in the exploration of this ancient and fascinating protein family.

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