The cytopathic action of ribonucleases (RNases) requires the interaction of the enzyme with the cellular membrane, its internalization, translocation to the cytosol, and the degradation of ribonucleic acid. The interplay of these processes as well as the role of the thermodynamic and proteolytic stability, the catalytic activity, and the evasion from the intracellular ribonuclease inhibitor (RI) has not yet been fully elucidated. As cytosolic internalization is indispensable for the cytotoxicity of extracellular ribonucleases, we investigated the extent of cytosolic internalization of a cytotoxic, RI-evasive RNase A variant (G88R-RNase A) and of various similarly cytotoxic but RI-sensitive RNase A tandem enzyme variants in comparison to the internalization of the non-cytotoxic and RI-sensitive RNase A. After incubation of K-562 cells with the RNase A variants for 36 h, the internalized amount of RNases was analyzed by rapid cell disruption followed by subcellular fractionation and semiquantitative immunoblotting. The data indicate that an enhanced cellular uptake and an increased entry of the RNases into the cytosol can outweigh the abolishment of catalytic activity by RI. As all RNase A variants proved to be resistant to the proteases present in the different subcellular fractions for more than 100 h, our results suggest that the cytotoxic potency of RNases is determined by an efficient internalization into the cytosol.

The ribonucleolytic activity of ribonucleases (RNases) provides the potential to use these enzymes as therapeutics for tumor treatment. Particularly, members of the RNase A and RNase T1 superfamilies have shown promising cytotoxicity to cancer cells (1–3). Among these enzymes, OnconaseTM (Alfacell Corp., Bloomfield, NJ), an RNase from the Northern Leopard frog, is farthest along the clinical trials (4). To overcome the disadvantage of the renal toxicity of Onconase and a possible immunogenicity of non-mammalian RNases, RNase A or human pancreatic RNase 1 evolved as targets for the development of antitumor agents (5). Because RNases from mammalian sources are silenced due to the tight binding by the intracellular inhibitor protein (RI) and, thus, are not cytotoxic (6), various strategies have been mapped out to accomplish cytotoxicity. Inspired by the cytotoxicity of the amphibian Onconase (5, 7, 8) and the dimeric bovine seminal RNase (9), tremendous efforts have been made to create RNase derivatives that evade RI binding. While the generation of chimeras (10–13), the use of chemical modifications (14–17), or site-directed mutagenesis (5, 6, 18–21) proved to be strategies of rather varying success, we developed RNase A tandem enzymes (22) in which two RNase A molecules are coupled covalently by a peptide linker. Due to steric hindrance, a complete binding by RI should be prevented and in contrast to bovine seminal RNase, the RNase A entities of the tandem constructs cannot dissociate, which would result in a subsequent binding by RI. In fact, the RNase A tandem constructs have been found to be cytotoxic, with IC50 values between 70 and 13 μM (22).

However, to unfold their cytotoxicity in the correct place, the RNases first have to reach the cytosol of the target cells by endocytosis (23) except for RNases from *Rana catesbeiana* and *Rana japonica*, which cause cell death by binding to the cell surface followed by agglutination of the cells (1). To meet the target RNA, the RNases must be released from the endosomes into the cytosol. In contrast to Onconase, which enters the cytosol from the endosome recycling compartment (24), mammalian RNases are translocated into the cytosol from the endosomal/lysosomal pathway (21). In the endosomes (as well as in the lysosomes) and in the cytosol the RNases have to resist the attack by proteases. Furthermore, as a consequence of an estimated cytosolic concentration of RI of 1 μM (6), RNases with a dissociation constant (Kd) of the RNase-RI complex of <10−6 M such as RNase A should be inactivated rapidly by endogenous RI when they reach the cytosol. Thus, it has been suggested that only RNases that can evade RI binding exert cytotoxicity. While in many cases the relation between RI evasion (characterized by Kd) and cytotoxic potency (i.e., IC50 value) obviously holds true (6), derivatives of RNases that have been engineered to be cytotoxic still proved to be inhibited by RI (11, 21, 25). Recently, De Lorenzo et al. (27) could show for an antibody-RNase 1 fusion protein, which is inactivated by RI but is nevertheless strongly cytotoxic (IC50 = 50 nM) (26), that the amount of fusion protein that reaches the cytosol readily neutralizes the endogenous RI. Domain-swapped RNase A species, which are cytotoxic (28, 29), were found to be bound by RI with Kd values comparable with the complex of RNase A and RI (25). Similarly, the RNase A tandem constructs (22) were completely inhibited by RI but
nevertheless proved to be considerably cytotoxic. Consequently, RI evasion cannot be the reason for the cytotoxic effect exerted by the domain-swapped RNase A dimers and the RNase A tandem enzymes. A more avid endocytosis as a consequence of an improved interaction of these cationic proteins with the polyanionic cell surface was proposed as the basis for the cytotoxicity (28, 29). This proposal is supported by the fact that the cationization of RNase A and RNase 1 rendered these non-cytotoxic proteins cytotoxic by improved interaction with the cellular surface (15).

We have analyzed the cellular uptake and subcellular localization of various RI-sensitive but cytotoxic RNase A tandem enzyme variants that differ in length and amino acid composition of the linker sequence in comparison with the RI-sensitive, non-cytotoxic RNase A and the RI-escape, cytotoxic variant G88R-RNase A. The data indicate that endocytosis efficiency is a crucial factor for the magnitude of cytotoxicity of RNase A variants. Obviously, the effect of RI on RI-sensitive RNase A tandem enzymes is abolished if enough of the RNase A molecules reach the cytosol to neutralize the intracellular RI (2). In contrast, proteolytic stability proved to be not limiting for the cytotoxic potency of RNase A variants, as all used cytotoxic RNase A variants and the non-cytotoxic RNase A were stable under the applied conditions for more than 50 h.

EXPERIMENTAL PROCEDURES

Proteins and Chemicals—RNase A from Sigma was purified on a SOURCE S FPLC column (Amersham Biosciences). Growth media for bacterial cultures were from Difco Laboratories (Detroit, MI). *Escherichia coli* strains XL 1 Blue and BL21(DE3) were from Stratagene. RPMI 1640, fetal bovine serum, bovine serum albumin (BSA), enzyme substrates, penicillin, and streptomycin were from Invitrogen; milk powder, bovine serum albumin (BSA), enzyme substrates, Nonidet P-40, and E 64 were from Sigma, and protease inhibitor mixture Complete™ was from Roche Applied Science. Polyclonal anti-RNase A antibodies were a gift from H. Younus, Aligarh Muslim University, India. Polyclonal anti-cathepsin B antibodies were from Biomol GmbH (Hamburg, Germany); monoclonal anti-β-actin antibodies were from Sigma. All other chemicals were of purest grade commercially available.

Expression, Renaturation, and Purification of RNase A Variants—The experimental procedure for expression and renaturation was performed as described previously (22). The proteins were purified on a SOURCE S column (Amersham Biosciences) (50 mM Tris-HCl, pH 7.5, with a linear gradient of 0–500 mM NaCl).

Cells—K-562 cells, which are from a human erythroleukemia cell line, were maintained at 37 °C in a humidified atmosphere containing CO₂ (5% v/v). Culture medium was RPMI 1640 medium supplemented with fetal bovine serum (10% v/v), penicillin (100 units ml⁻¹), and streptomycin (100 μg ml⁻¹). The cells were grown in T7 cell culture bottles in a volume of 100 ml to densities of 0.5–1 × 10⁶ cells ml⁻¹.

Differential Fractionation—Differential fractionation was carried out according to Schröter et al. (30) with minor modifications. All procedures were carried out at 4 °C or on ice. 10 mM Tris acetate buffer, pH 7.0, containing 250 mM sucrose was used as fractionation buffer throughout the procedure. About 1 × 10⁹ cells were harvested by centrifugation at 2,000 × g for 10 min and washed three times. The cell pellet was resuspended in 0.3 ml of buffer and homogenized by ultrasonic treatment (5 pulses of 5 J) (Vibra Cell; Bioblock Scientific, Lyon). The cell homogenate was then centrifuged at 8,000 × g for 10 min to remove debris, intact cells, plasma membranes, and nuclei (30). The supernatant was centrifuged at 100,000 × g for 6 min to isolate mitochondria, endosomes, and lysosomes (fractions E and L) in the pellet. The supernatant was again centrifuged at 130,000 × g for 60 min to remove microsomes (fraction M). The final supernatant contained highly purified cytosol (fraction C). All pellets were washed with fractionation buffer and centrifuged again. The supernatants of the washing steps were discarded.

Separation of Endosomes and Lysosomes—Lysosomes were isolated from the combined fractions E and L by a 20-min hypotonic lysis according to Bohley et al. (31) using an 18-fold volume of distilled water over the pellet. After another centrifugation step (100,000 × g for 8 min), the content of the lysosomes was in the supernatant (fraction L) whereas mitochondria and endosomes were in the pellet (fraction E). The pellet was washed with fractionation buffer and centrifuged again. The supernatant of the washing step was discarded.

Characterization of the Subcellular Fractions—Lysosomes were characterized by the activity of N-acetyl-β-D-gluco- somaminidase (β-AGA) determined by a fluorometric assay according to Schmid et al. (32). The activity of β-AGA was determined in 95 μl of 0.1 M sodium citrate buffer, pH 5.0, 1% (v/v) Nonidet P-40, 0.8 mM 4-methylumbelliferon-N-acetyl-β-D-glucosaminid and 5 μl of the subcellular fraction. Reactions were incubated in a microtiter plate at 25 °C. Fluorescence emission at 460 nm was followed upon excitation at 360 nm on a POLARstar Galaxy Microplate Reader (BMG Labtech, Offenburg, Germany) for 60 min. The amount of the released product methylumbelliferon was determined from the initial slope using a calibration curve of methylumbelliferon ranging from 10 to 400 μM. For calibration, methylumbelliferon was dissolved (10 mM in 0.5 M sodium glycine buffer, pH 10.4, 0.5 M NaCl) and diluted with 0.1 M sodium citrate buffer, pH 5.0, containing 0.02% BSA.

In addition, lysosomes were characterized by determination of the total activity of the cathepsins B, L, and S (catBLS). Activity of catBLS was determined fluorometrically (33) in 95 μl of 0.1 M sodium citrate buffer, pH 5.0, containing 4 mM diithiothreitol, 0.1% BSA, 4 mM EDTA, and 6 μM aprotinin using 0.5 mM benzoylcarbonyl-phenylalanyl-arginine-7-amido-4-methylcoumarin as substrate and 5 μl of the subcellular fraction. Reactions were incubated at 37 °C for 10 min. Activity of catBLS was inhibited by addition of E-64 (final concentration 10 μM) according to Schmid et al. (34). Fluorescence emission at 460 nm was determined on a POLARstar Galaxy Microplate Reader upon excitation at 360 nm after diluting the reaction mixtures 200-fold with 0.1 M sodium citrate buffer, pH 5.0. 7-Amino-4-methylcoumarin was used for calibration between 2 and 20 μM.

The cytosol was characterized by the activity of lactate dehydrogenase according to Storrie and Madden (35). The activity of lactate dehydrogenase was determined in a mixture of 150 μl of 50 mM KH₂PO₄, pH 7.5, containing 0.31 mM sodium pyru-
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The reaction was started by addition of 5 μl of subcellular fraction. The decrease of absorbance was followed continuously in a 1 × 0.2-cm cuvette on a UV-visible spectrometer (Ultraspec 3000; Pharmacia Biotech) at 340 nm for 1 min. Lactate dehydrogenase activity was estimated by the use of the extinction coefficient for NADH of 6,300 M⁻¹ cm⁻¹.

**Immunoblotting**—10 ml of K-562 cells (density 0.5–1 × 10⁶ cells ml⁻¹) were incubated for 36 h with 100 μM RNase A entities (100 μM monomeric RNase A variant or 50 μM RNase A tandem enzyme, respectively). As a control, 10 ml of K-562 cells were incubated with phosphate-buffered saline to detect cross-reactivity of the antibodies. After harvesting, extracts of subcellular fractions were prepared and characterized as described above. Additionally, samples of the centrifuged cell homogenate (without debris, intact cells, plasma membranes, and nuclei) were used to quantify the total internalization (T). The same amount of protein for each subcellular fraction (300 ng for total internalization, 60 ng for fraction E, 25 ng for fraction L, and 250 ng for fraction C) was then applied to SDS-PAGE (see below), and proteins were blotted onto an Amersham Biosciences Hybond²⁺ nitrocellulose membrane (GE Healthcare) (60 min at 1 mA cm⁻²). After treatment with milk powder (2.5%, w/v, in Tris-buffered saline, containing 1% v/v Tween), the corresponding parts of the membranes were incubated with polyclonal anti-RNase A (34 μg ml⁻¹) and anti-rabbit IgG peroxidase antibodies, with monoclonal anti-FITC antibodies, and proteins were blotted onto an Amersham Biosciences Hybond²⁺ nitrocellulose membrane and visualized as described above. β-actin was used as internal standard.

**SDS-PAGE and Determination of the Rate Constants of Proteolysis (kₓ)—** Electrophoresis was carried out on a Midget Electrophoresis Unit (Hoefer, San Francisco, CA) according to Laemmli (36) using 5 and 15% (w/v) acrylamide for stacking and separating gels. The gels were stained with Coomassie Brilliant Blue G250. After destaining, the gels were evaluated using a CD 60 densitometer (Desaga, Heidelberg, Germany) at 595 nm. Values of kₓ were calculated from the decrease in the peak areas of the band of intact protein as a function of time of proteolysis, which followed pseudo-first-order kinetics. The determination of kₓ was performed in triplicate.

**RESULTS**

As RNase A variants differ remarkably in their cytotoxic potency as well as in their capability to evade RI binding, we compared the following variants with respect to cellular uptake and proteolytic stability in order to contribute to the clarification of the contradictory statements on the crucial factors for the cytotoxicity of RNases: RNase A, which is RI-sensitive (kₜ = 44 fM) (37) and non-cytotoxic, G88R-RNase A, which is RI-escape (kₜ = 0.4 nm) (6) and cytotoxic (IC₅₀ = 7 μM) (6), and the RNase A tandem enzymes with the linker sequences GP₃G, GP₃G, GP₃G, SGSGSG, and SGRSGRSG, which are RI-sensitive but cytotoxic (IC₅₀ = 70, 20, 22, 40, and 13 μM, respectively) (22). All RNase A variants are of similar stability and activity.

**Cell Homogenization and Characterization of the Subcellular Fractions**—The harvested cells were homogenized by ultrasonic treatment using 5 pulses of 5 J as more rigorous homogenization conditions increased the portion of destroyed organelles. Subcellular fractions E, L, and M obtained by differential fractionation (Fig. 1) as described under “Experimental Procedures” were analyzed for the lysosomal marker enzymes β-AGA and catBLS as well as for the cytosolic marker enzyme lactate dehydrogenase.

As can be seen in Fig. 2, the highest activity of the lysosomal marker enzyme was in fraction L (10.9 ± 2.1 units mg⁻¹ corre-
Internalization of RNase A Variants

Proteolysis of RNase A Variants by the Cytosolic and Lysosomal Subcellular Fractions and in Intact K-562 Cells—As internalized RNases face attack by the proteases in the lysosomes as well as in the cytosol, thereby abolishing their catalytic activity, we analyzed the proteolytic susceptibility of the RNase A variants in fractions C and L as well as the proteolytic degradation of an internalized RNase A variant in intact K-562 cells. As exemplified in Fig. 3 for G88R-RNase A and the GP-G-RNase A tandem enzyme, no protein degradation was detectable for all RNase A variants. All these enzymes were stable in the presence of fractions C and L for more than 100 h (Fig. 3). Moreover, the RNase A tandem enzymes were also stable for 54 h in vivo when internalized into intact K-562 cells as shown for the GP-G-RNase A tandem enzyme (Fig. 4). No proteolysis products were detectable by immunoblotting. On the contrary, proteolytic degradation of BSA as a control by fraction C and fraction L was highly reproducible with $k_p$ values of $(4.0 \pm 0.2) \times 10^{-2}$ h$^{-1}$ and $(3.5 \pm 0.3) \times 10^{-2}$ h$^{-1}$, respectively.

Detection of RNase A Variants in the Subcellular Fractions—To quantify the uptake of the RNase A variants into the cells, K-562 cells were incubated in the presence of the respective RNase A variants for 36 h, harvested, and disrupted by ultrasonic treatment. Subcellular fractions were separated by differential fractionation as described under “Experimental Procedures.” Relative amounts of RNase A variants were determined by semiquantitative immunoblotting. As can be seen in Fig. 5, RNase A is clearly detectable only in fraction T. While the GP-G-, GP$_4$G-, GP$_5$G-, and SGSSGSS-RNase A tandem enzymes are enriched in this fraction ~5-fold over RNase A (Fig. 5), the SGRSGRSG-RNase A tandem enzyme, which is the most cytotoxic RNase A tandem enzyme (I$_{50}$ = 13 μM) (22), is enriched more than 14-fold. In addition, G88R-RNase A, which shows I$_{50}$ values in a similar range (6), is enriched ~10-fold. In fractions C, E, and L no RNase A is detectable (Fig. 5), whereas the bands representing G88R-RNase A and the tandem enzymes are clearly visible. In fractions C and E, the SGRSGRS-RNase A tandem enzyme again is most abundant, followed by the SGSSGSS-, GP$_3$G-, GP$_4$G-, and GP$_5$G-RNase A tandem enzymes, but G88R-RNase A is accumulated less. In contrast, in fraction L there are only minor differences in the responding to 78% of the total β-AGA activity). In fraction E, β-AGA activity was $2.2 \pm 1.1$ units mg$^{-1}$ whereas the activity of β-AGA in fractions C and M was marginal ($0.5 \pm 0.2$ and $0.4 \pm 0.1$ units mg$^{-1}$, respectively). As expected, the highest value of catBLS activity was also found in fraction L with $20.2 \pm 3.9$ units mg$^{-1}$; little cathepsin activity was found in endosomes ($0.9 \pm 0.1$ units mg$^{-1}$). The catBLS activity in fraction C and fraction M was negligible with values of $0.1 \pm 0.05$ units mg$^{-1}$ each. In contrast, the cytosolic marker, lactate dehydrogenase activity, was exclusively found in the cytosol ($112.9 \pm 3.6$ units mg$^{-1}$). These data prove that almost no organelles were disrupted by our cell homogenization method.

Subcellular Fractions and in Intact K-562 Cells—As internalized RNases face attack by the proteases in the lysosomes as well as in the cytosol, thereby abolishing their catalytic activity, we analyzed the proteolytic susceptibility of the RNase A variants in fractions C and L as well as the proteolytic degradation of an internalized RNase A variant in intact K-562 cells. As exemplified in Fig. 3 for G88R-RNase A and the GP-G-RNase A tandem enzyme, no protein degradation was detectable for all RNase A variants. All these enzymes were stable in the presence of fractions C and L for more than 100 h (Fig. 3). Moreover, the RNase A tandem enzymes were also stable for 54 h in vivo when internalized into intact K-562 cells as shown for the GP-G-RNase A tandem enzyme (Fig. 4). No proteolysis products were detectable by immunoblotting. On the contrary, proteolytic degradation of BSA as a control by fraction C and fraction L was highly reproducible with $k_p$ values of $(4.0 \pm 0.2) \times 10^{-2}$ h$^{-1}$ and $(3.5 \pm 0.3) \times 10^{-2}$ h$^{-1}$, respectively.

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the mean graphs show the intensity of the RNase A variants corrected for the intensity of the respective standards. Data give the mean ± S.D., n = 4.

**DISCUSSION**

**Contradictions in the Reason for the Cytotoxicity of RNases—**
The cytotoxic potency of RNase-based drugs is determined by their ability to interact with the cellular membrane, the extent of endocytosis, their ability to reach the cytosol, their resistance toward proteolytic degradation in the endosomes/lysosomes and in the cytosol, their capability to evade RI binding, and by their ribonucleolytic activity. As expected, in general the better an RNase evades RI binding the higher is its cytotoxicity (6, 20). However, there are numerous exceptions as RI-sensitive RNase variants were found to be cytotoxic (11, 21, 22, 25, 27) and RI-escape variants were found to be not cytotoxic (21). Moreover, microinjected RNase A was cytotoxic (38) and non-cytotoxic RNases were found to remain non-cytotoxic even if RI had been silenced by small interfering RNA (39). Consequently, there have to be other factors than RI evasion that likewise affect cytotoxic potency. As microinjected RNase A (40), RNases conjugated to delivery molecules (11, 26, 41–44), and modified RNases with an increased net charge (15, 16, 45, 46) proved to be cytotoxic, the efficiency of internalization was suggested to limit the cytotoxicity of RNase constructs. In fact, when RNase 1, which is not cytotoxic, was fused to a delivery moiety it became strongly cytotoxic due to a dramatically improved conveyance to the cytosol where it proved to overcome the level of endogenous RI (27).

By virtue of their basic pl values, RNases of the RNase A superfamily bind to the negatively charged surface of cells (23). As cells should be anxious to prevent foreign RNases from entering the cell, no RNase receptor is expected to be found but RNases enter cells by endocytosis (23). To exert their cytotoxicity, the RNases have to be released from the endosomes into the cytosol. Here, for the first time differential fractionation of K-562 cells, which had been treated with RNase A variants, in combination with semiquantitative immunoblotting was exploited to analyze the content of the RNases in the subcellular fractions after endocytosis.

**Subcellular Quantification of RNase A Variants Suggests Endocytosis as Main Determinant of Cytotoxicity—**As deduced from the activity of the respective marker enzymes (Fig. 2), ultrasonic cell disruption followed by ultracentrifugation and hypotonic lysis of the lysosomes yields a sufficient separation into cytosolic, endosomal, lysosomal, and microsomal fractions of K-562 cells. To evaluate the importance of endocytosis over other feasible factors for the cytotoxicity we chose RI-sensitive, non-cytotoxic RNase A, the RI-evasive, cytotoxic mutant enzyme G88R-RNase A, and various RI-sensitive RNase A tandem variants but nevertheless cytotoxic RNase A tandem enzymes.

After incubation of K-562 cells with the RNases, RNase A was poorly detectable in fraction T but no longer traceable in fractions C, E, and L (Fig. 5). This finding is in line with the lack of cytotoxicity of RNase A. Haigis and Raines (23), however, had reported that fluorescein-labeled RNases are endocytosed into acid vesicles such as endosomes and lysosomes from where they have to be released into the cytosol to exert their cytotoxicity. As RNase A is RI-sensitive in vitro (5, 6), the lack of cytotoxicity was attributed to an inactivation in the cytosol. However, as elaborated above, other studies have pointed to a limitation of the cytotoxicity by the efficiency of internalization (11, 15, 40, 45). Interestingly, for the cytotoxic variants G88R-RNase A and Onconase an enhanced binding to the cell surface can be seen (23); an improved interaction with the cell surface as reason for the cytotoxicity has also been proposed for domain-swapped RNase A multimers (25) and RNase A tandem enzymes (22). Our quantitative results unambiguously show that the cytotoxic variant G88R-RNase A accumulates in the cytosol (Fig. 5) where it might exert cytotoxicity by evading RI binding. All studied RNase A tandem enzymes were found in fraction T as well as in fractions C, E, and L to a significantly higher extent than RNase A (Fig. 5), indicating a considerably improved endocytotic uptake into K-562 cells. Interestingly, the SGRSGRSG-RNase A tandem enzyme, which is the most cytotoxic variant (IC_{50} = 13 μM) (22), is the most abundant of all enzymes in both the cytosol and the endosomes. Binding to RI in the cytosol, however, diminishes its cytotoxicity, thus becoming slightly less potent than G88R-RNase A, which is released worse from the endosomes into the cytosol (Fig. 5) but evades RI binding (6). Obviously, however, G88R-RNase A is routed more efficiently to the lysosomes than the RNase A tandem enzymes.

**RNase A Variants Resist Proteolytic Attack in the Subcellular Fractions—**As the RNases face the attack by both lysosomal and cytosolic proteases, we investigated their stability toward extracts of those subcellular compartments. In contrast to BSA,
which was properly degraded, RNase A, G88R-RNase A, and all RNase A tandem enzyme variants were found to be resistant toward proteolytic attack in both the cytosolic and the lysosomal fractions (Fig. 3). Neff et al. (47) determined a half-life of 10–40 h for 125I-labeled BSA microinjected into fibroblasts, which corresponds well to the half-lives of ~17 and 20 h for BSA in fractions C and L, respectively, as found here.

Using the same technique as for BSA, Neff et al. (47) determined a half-life of 55–95 h for RNase A in fibroblasts, and McElligott et al. (48) showed that microinjected and endocytosed RNase A were both mainly degraded in the lysosomes. Although the proteolytic resistance of the RNase A variants in the cytosol is in accordance with those reports, our data on the proteolytic resistance of RNase A and the RNase A variants in the lysosomal fraction clearly differ from the above-mentioned reports. A loss of proteolytic activity of the cytosolic and lysosomal fractions can be ruled out as BSA is steadily degraded. McElligott et al. (48) showed that microinjected and endocyto-

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