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Chimeric ribonuclease as a source of human adapter protein for targeted drug delivery

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Assembled modular complexes for targeted drug delivery can be based on strong non-covalent interactions between a cargo module containing an adapter protein and a docking tag fused to a targeting protein. We have recently constructed a completely humanized adapter/docking tag system based on interactions between 15 amino acid (Hu-tag) and 110 amino acid (HuS) fragments of human ribonuclease I (RNase I). Although recombinant HuS can be expressed and refolded into a functionally active form, the purification procedure is cumbersome and expensive, and more importantly, it yields a significant proportion of improperly folded proteins. Here we describe engineering, high-yield expression, and purification of a chimeric bovine/human RNase (BH-RNase) comprising 1–29 N-terminal amino acids of bovine ribonuclease A and 30–127 amino acids of human RNase I. Unlike RNase I, the chimeric BH-RNase can be cleaved by either subtilisin or proteinase K between A20 and S21, providing a functionally active HuS. The HuS obtained from chimeric BH-RNase differs from wild-type HuS by an N24T substitution; therefore, we have reverted this substitution by mutating N24 to T24 in BH-RNase. This BH-RNase mutant can also be cleaved by subtilisin or proteinase K yielding wild-type HuS. The affinity of HuS obtained from BH-RNase to Hu-tag is approximately five times higher than that for recombinant HuS, reflecting a higher percentage of properly folded proteins.

Keywords: adapter protein/chimeric RNase/delivery complexes/targeting drugs

Introduction

Lack of efficient technology for loading drugs onto targeting proteins hinders advances in targeted drug delivery (Dubowchik and Walker, 1999). Most loading technologies are based on random chemical conjugation of a 'cargo' (such as drugs, drug carriers or adapters for drug carriers) directly to targeting proteins. Chemical modifications of targeting proteins damage their ability to bind to cellular targets, require expensive custom development and yield heterogeneous preparations.

We have recently proposed a novel strategy for modular assembly of targeting complexes for drug delivery (Backer *et al*., 2002a). This technology avoids chemical modification of targeting proteins by using a standardized docking system that includes two modules: a 'docking' tag fused to a targeting protein and a 'payload' module containing an adapter protein for binding to the docking tag. Standardized payload modules are pre-made by linking a cargo to an adapter (Figure 1).

A proof-of-principle for this strategy was obtained with a docking system based on interactions between two fragments of bovine ribonuclease A (RNase A), an N-terminal 1–15 amino acid peptide (S-peptide) and a C-terminal 21–124 amino acid fragment (S-protein) serving as an adapter (Backer *et al*., 2002a). Association of these fragments reconstitutes RNase activity and interactions between these fragments are being used for affinity purification and detection of recombinant proteins fused to S-peptide (Kim and Raines, 1993). Since fragments of bovine RNase A are potentially immunogenic in humans, as RNase A is immunogenic in mice (Matousek *et al*., 2002), we have recently developed a docking system based on interactions between similar fragments of human pancreatic ribonuclease I (RNase I), that we named Hu-peptide and HuS [(Backer *et al*., 2003); see Sorrentino and Libonati (Sorrentino and Libonati, 1997), for a review on human RNases]. It should be noted that attempts to express a native 21–127 amino acid RNase I fragment in *Escherichia coli* were not successful, and the only variant that was synthesized in *E.coli* was an $^{18–127}$HuS(P19A, S20A) with the P19A and S20A amino acid substitutions. Recombinant $^{18–127}$HuS(P19A, S20A) was recovered from inclusion bodies and refolded into a functionally active conformation in the presence of synthetic Hu-peptide. After removal of Hu-peptide, purified $^{18–127}$HuS(P19A, S20A) retained the ability to bind Hu-peptide and Hu-tagged fusion proteins with restoration of ribonuclease activity. Attempts to refold this protein or, for that matter, recombinant bovine S-protein, into functionally active conformations without the corresponding peptides were not successful (Backer *et al*., 2002b, 2003).

This is a somewhat cumbersome and expensive strategy for preparing the quantities of HuS that are necessary for preclinical and early clinical studies. Here we describe a strategy for making native 21–127 amino acid HuS using an engineered chimeric bovine/human RNase (BH-RNase) as a starting material. This strategy is based on a procedure developed for preparation of S-protein via subtilisin cleavage of RNase A

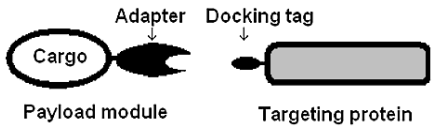

Fig. 1. Modular assembly of targeting complexes for drug delivery. The docking tag is genetically fused to a recombinant targeting protein, e.g. single-chain antibody, growth factor. A cargo (a drug or a drug carrier such as a liposome or a polymer) is linked to an adapter protein. The resulting standardized 'payload module' is docked to any targeting protein armed with a docking tag [based on Backer *et al*. (Backer *et al*., 2002a)].

between A20 and S21 (Richards and Vithayathil, 1959). Unfortunately, limited proteolytic cleavage of RNase I does not release HuS, presumably because of incompatibility of the hinge loop 15–25 with the subtilisin active center (Gupta *et al*., 1999; Pous *et al*., 2001). To reconstruct a cleavage site in this loop, we have constructed BH-RNase containing 1–29 amino acids of RNase A and 30–127 amino acids of RNase I. The 21–29 region of RNase A differs from a corresponding region of RNase I by the presence of N24 instead of T24. In order to reconstruct wild-type RNase I sequence starting from S21, we have also constructed a mutant BH-RNase (N24T). We found that both chimeric RNases can be expressed at a high level, refolded into an enzymatically active conformation, and cleaved by either subtilisin or proteinase K between A20 and S21. The affinity of HuS obtained from BH-RNase to Hu-tagged proteins is ~5-fold higher than that of recombinant $^{18–127}$HuS(P19A, S20A), reflecting a higher proportion of functionally active protein.

Materials and methods

Construction of chimeric RNase

The pET-HP plasmid encoding 1–127 amino acids of human RNase I (el-Joubary *et al*., 1999) was a generous gift from Dr G.D'Alessio (Napoli Federico II University, Naples, Italy). The ORF of an 18–125 fragment of human RNase I with the T24N amino acid substitution was amplified by PCR using a 5′-ATGTCCGCTGCCAGCAGCTCCAACTACTGCAACCAG-ATGATGCGTCG-3′ sense primer (mutation underlined) and an antisense primer, 5′-CTATTCAACACACGCGTCGAA-ATGAACCGG-3′. The PCR product was cloned in the pETBlue-1 using a Perfectly Blunt Cloning Kit (Novagen), and the T24N mutation was confirmed by sequencing. Then, 10 bovine RNase A codons were introduced by PCR using a sense primer, 5′-TTTGAGCGGCAGCACATGGACTCCAGCAC-TTCCGCTGCCAGCAGCTCC-3′. An antisense primer, 5′-TCAAGAGTCTTCAACAGACGCGTCG-3′, introduced co-dons for D126, S127 and a stop-codon. The PCR product was cloned in the pETBlue-1 and the resulting construct was confirmed by sequencing. Seven more bovine codons were introduced in the sequence by amplifying it with a sense primer, 5′-ATGAAGGAAACTGCAGCAGCCAAGTTT-GAGCGGCAGCACATGGACTCC-3′ and the above anti-sense primer. The PCR product was cloned in the pETBlue-1 and confirmed by sequencing. The $^{1–29}$B/$^{30–127}$H-RNase coding sequence was amplified by PCR using a sense primer, 5′-CACAAGCATATGAAGGAAACTGCAGCAGCC-AAG-3′ (*Nde*I site underlined) and an antisense primer, 5′-TACGGTACCTCAAGAGTCTTCAACAGACGCGTCG-3′ (*Kpn*I site underlined), and cloned in *Nde*I–*Kpn*I sites of the pET29a(+) bacterial expression vector (Novagen). The N24T amino acid substitution was introduced in the pET29/$^{1–29}$B/$^{30–127}$H-RNase plasmid DNA by site-directed mutagenesis using a Gene-Tailor Site Directed Mutagenesis Kit (Invitrogen), a sense primer, 5′-TCCGCTGCCAGCAGC-TCCACCTACTGCAACCAG-3′ (mutation underlined) and an antisense primer, 5′-GGAGCTGCTGGCAGCGGA-AGTGCTGGAG-3′. The N24T mutation was confirmed by sequencing.

Purification of HuS

Both $^{1–29}$B/$^{30–127}$H-RNase (BH-RNase) and $^{1–29}$B/$^{30–127}$H-N24T-RNase (BH-N24T) were expressed in BL21(DE3) *E.coli* (Novagen), refolded from inclusion bodies, and purified by ion-exchange chromatography on a HiTrap SP-Fast Flow column (Amersham) and RP-HPLC on C8 as described for recombinant RNase I (Backer *et al*., 2003). To exchange buffer, RP-HPLC purified proteins were loaded on a HiTrap SP-Fast Flow column equilibrated with a buffer containing 20 mM NaOAc pH 6.5 and eluted with a buffer containing 20 mM NaOAc, 0.6 M NaCl, pH 6.5. BH-RNase and its mutant were digested with either subtilisin or proteinase K (Sigma) at a protein to protease ratio of 100:1 (w/w) at room temperature or at 4°C. Bovine peptide was removed from the digestion mixture by RP-HPLC on a C18 column as described (Backer *et al*., 2002b, 2003). After buffer exchange on a HiTrap SP-Fast Flow column performed as described above, HuS-T24N and wild-type HuS were purified by affinity chromatography on a Hu-peptide column. The column was prepared by coupling of a CA-extended Hu-peptide (**CA**-KESRAKKFQRQHMDS synthesized by Genemed Synthesis, Inc., South San Francisco, CA) to Activated Thiol Sepharose 4B (Amersham) according to the manufacturer's protocol. Before coupling, **CA**-Hu-peptide was treated with 100 mM TCEP for 30 min at room temperature, purified on an RP-HPLC C18 column, and transferred into conjugation buffer via buffer exchange on a HiTrap SP-Fast Flow column. Bound HuS was eluted from the affinity column with 0.2 M citric acid. After buffer exchange on a HiTrap SP-Fast Flow column (as described above), proteins were aliquoted and stored at –70°C.

HuS/Hu-tag binding assay

The equilibrium dissociation constant (K_D) values for HuS-T24N/Hu–peptide complexes were determined as described (Backer, 2002b, 2003). Briefly, HuS-T24N at final concentrations of 0.1, 0.2 or 0.3 nM was mixed with varying amounts of Hu-tagged vascular endothelial growth factor [Hu-VEGF, expressed as described in Backer *et al*. (Backer *et al*., 2002a)] in a buffer containing 20 mM Tris–HCl, 100 mM NaCl, pH 7.5, 0.1 mg/ml poly C and incubated for 5 min at room temperature. The activities of the reconstituted ribonucleases were measured by the absorbance at 280 nm of the reaction mixtures cleared from TCA-precipitated material. One optical unit of TCA-soluble material released from poly C incubated with reconstituted ribonuclease was defined as one relative unit of ribonuclease activity. Calculations of the K_D values were performed assuming that the initial rate of the hydrolysis is proportional to the concentration of reconstituted ribonuclease. DYNAFIT software was used for the global fitting with numeric iteration and calculation of the K_D values (Kuzmic, 1996).

Results

Construction and expression of BH-RNase

Bovine RNase A is cleaved by subtilisin between A20 and S21 yielding functionally active S-protein (Richards and Vithayathil, 1959). In contrast, limited proteolytic cleavage of human RNase I does not release HuS, presumably because of incompatibility of the hinge loop 15–25 with the subtilisin active center, most likely due to a conformational change induced by P19 (Gupta *et al*., 1999; Pous *et al*., 2001). Indeed, direct comparison of the bovine and human hinge loops 15–25 backbones (Figure 2A, shown in green and red respectively) revealed significant differences between the two RNases.

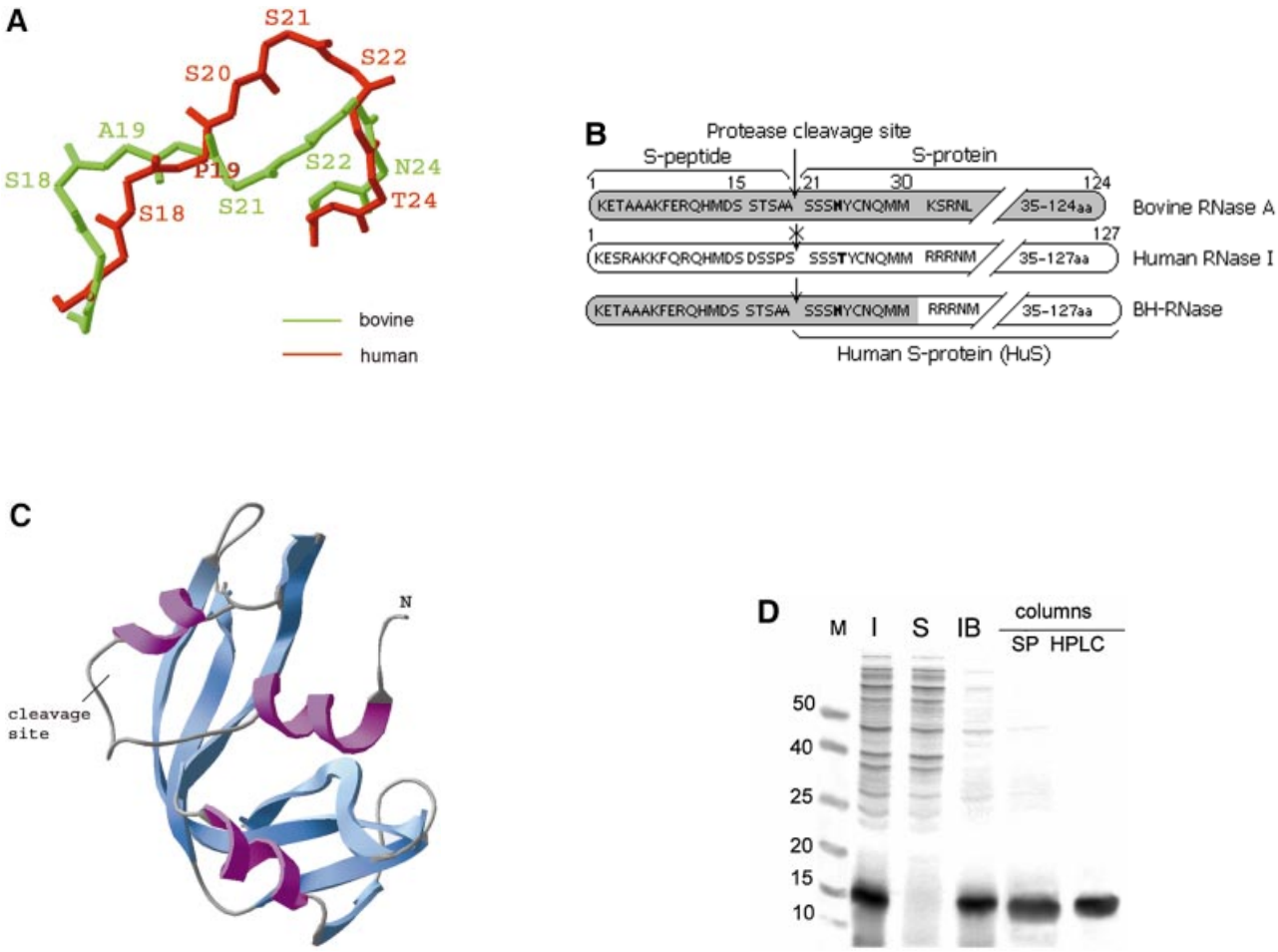

Fig. 2. Construction and expression of BH-RNase. (**A**) The backbone structures of the bovine (green) and human (red) hinge loops 16–25 superimposed by SWISS-PDB viewer (Guex and Peitsch, 1997). (**B**) Construction of BH-RNase. The subtilisin cleavage site is indicated. Position 24, a single difference between RNase A and RNase I in the 20–30 amino acid region, is shown in bold. (**C**) Putative tertiary structure of BH-RNase. The amino acids 1–29 were taken from bovine RNase A (PDB accession No. 1AQP) and appended to amino acids 30–127 of human RNase I (PDB accession No. 1E21). The model was minimized and drawn with SWISS-PDB viewer. α-Helices are in purple and β-sheets are blue ribbons. The expected proteolytic cleavage site between A20 and S21 is indicated. (**D**) BH-RNase purification. Samples saved at different purification stages were analyzed by SDS–PAGE on 17.5% gel followed by SafeBlue staining (Invitrogen). Lane M, molecular weight markers; lane I, induced bacterial lysate; lane S, soluble part of bacterial lysate; lane IB, inclusion bodies; lanes SP and HPLC, samples after purification on SP and HPLC columns, respectively.

In order to develop an RNase-based source of HuS, we have constructed a chimeric BH-RNase containing a 1–29 amino acid fragment of RNase A and a 30–127 amino acid fragment of RNase I (Figure 2B and C). We reasoned that a preferential cleavage of the peptide bond between A20 and S21 by subtilisin might take place in BH-RNase. The 21–29 region of RNase A differs from a corresponding region of RNase I by the presence of N24 instead of T24. In order to reconstruct the wild-type RNase I sequence starting from S21, we have also constructed a mutant BH-N24T.

Both BH-RNase and BH-N24T were expressed in *E.coli* strain BL21(DE3), recovered from inclusion bodies, refolded by a two-step dialysis, and purified via ion-exchange and reverse-phase chromatography as described previously for recombinant RNase I (Backer *et al*., 2003). Purified BH-RNase and BH-N24T had typical yields of 60–80 mg/l, and were found to be >98% pure judging by SDS–PAGE (Figure 2D, for BH-RNase) and HPLC (data not shown). Ribonuclease activities of BH-RNase and BH-N24T were indistinguishable, both were ~2-fold lower than that of recombinant RNase I (prepared as described in Backer *et al*., 2003), and varied from isolation to isolation within 20% (data not shown).

BH-RNase cleavage

Initially, limited proteolytic digestion of BH-RNase with subtilisin was performed at the BH-RNase/protease ratio of 100:1 (w/w) at room temperature. A BH-RNase fragment with an electrophoretic mobility corresponding to that of HuS was transiently accumulated within 1 h under these conditions (Figure 3A, top). However, significant quantities of BH-RNase were degraded by 30–60 min of incubation. Lowering the incubation temperature to 4°C significantly reduced the reaction rate and enhanced the selective proteolysis of the A20–S21 peptide bond, yielding more HuS(T24N) (Figure 3A, bottom).

To further increase the yield of HuS(T24N), we have tested proteinase K that was shown to cleave the A20–S21 bond of RNase A more efficiently then subtilisin (Markert *et al*., 2001).

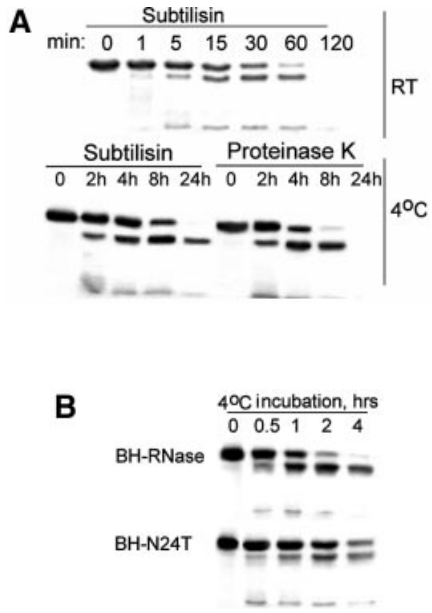

Fig. 3. Subtilisin and proteinase K digestion of BH-RNase. (**A**) BH-RNase was cleaved with either subtilisin or proteinase K. (**B**) BH-RNase and its N24T mutant were cleaved with proteinase K. The BH-RNase/protease ratio was 100:1 (w/w). Samples saved at various incubation times were analyzed by SDS–PAGE on 17.5% gels followed by SafeBlue staining.

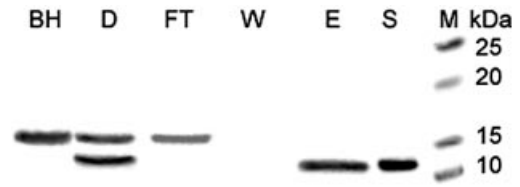

Fig. 4. HuS-T24N affinity purification. BH-RNase was digested with proteinase K [100:1 (w/w) ratio] in 0.1 M Tris–HCl pH 8.0 at 4°C for 4 h. S-peptide was removed by RP-HPLC. After a buffer exchange on a HiTrap SP-Fast Flow column (Amersham), the digestion mixture was loaded on the affinity Hu-peptide containing column. Samples were analyzed by SDS–PAGE on a 17.5% gel followed by SafeBlue staining. Lane BH, undigested BH-RNase; lane D, digestion mixture; lane FT, flow-through fraction; lane W, wash; lane E, elution with 0.2 M citric acid; lane S, HuS protein standard; lane M, molecular weight markers.

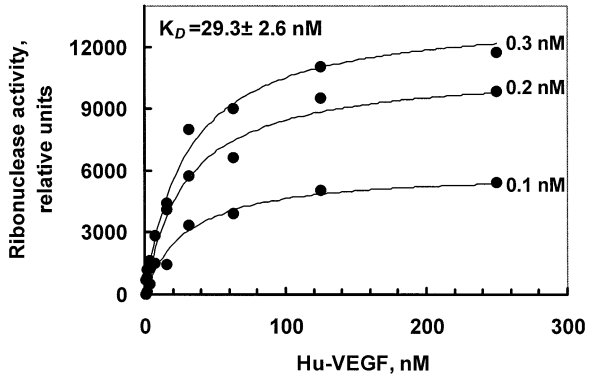

Fig. 5. Affinity of HuS/Hu-VEGF interactions. Reconstituted RNase activity in mixtures of HuS(N24T) and Hu-VEGF was tested and the K_D value was calculated as described (Backer *et al*., 2002b, 2003).

Indeed, we found that limited proteolysis of BH-RNase with proteinase K yielded more HuS(N24T) than subtilisin after a 4 h treatment under the same conditions (Figure 3A, bottom). Interestingly, the BH-N24T mutant that yields wild-type HuS was more resistant to proteinase K than BH-RNase (Figure 3B).

HuS purification

Short peptides released from BH-RNase and BH-N24T during proteolysis were removed from digestion mixtures by RP-HPLC on a C8 column as described for separation of human S-peptide and recombinant $^{18–127}$HuS(P19A, S20A) (Backer *et al*., 2003). In order to separate HuS fragments from undigested ribonucleases we used an affinity column that was constructed by coupling of a CA-extended Hu-peptide (**CA**-KESRAKKFQRQHMDS). After passing reaction mixtures through this column, undigested RNase and minor fragments were found in flow-through fractions, whereas HuS fragments bound to the column were eluted with 0.2 M citric acid (Figure 4, for BH-RNase cleavage). Q-TOF ES MS confirmed the identity of HuS(T24N) (MW 12 146) as a main product obtained from BH-RNase. In addition, 5–10% of purified protein resulted from the cleavage at the S21–S22 bond, as was reported for RNase A (Neumann and Hofsteenge, 1994; Mendez *et al*., 2000).

HuS activity

To characterize the affinity of purified HuS(T24N) to Hu-peptide, we used VEGF fused to Hu-tag [Hu-VEGF, expressed as described in Backer *et al*. (Backer *et al*., 2003)]. Reconstituted ribonuclease activity in HuS/Hu-VEGF mixtures was measured under conditions of equilibrium between free and bound HuS(T24N) as described (Backer *et al*., 2002b, 2003). To derive equilibrium dissociation constant (K_D) values for these complexes, the experimental data were analyzed with DYNAFIT software for the global fitting with numeric iteration and calculation of the K_D values (Kuzmic, 1996) under an assumption that the initial rate of the hydrolysis is proportional to the concentration of reconstituted ribonuclease. We found that the affinity of HuS(T24N) to Hu-VEGF was characterized by a K_D value of 29.3 ± 2.6 nM (Figure 5). These values are approximately five times lower that that obtained for complexes between recombinant $^{18–127}$HuS(P19A, S20A) and Hu-VEGF under the same experimental conditions (K_D of 162 ± 16 nM; Backer *et al*., 2003). To explain this difference, we have tested what proportion of $^{18–127}$HuS(P19A, S20A) binds to the Hu-peptide affinity column and found that only 20–30% of this protein was retained on the column (data not shown). The latter result supports the notion that the difference in K_D values is due to significant heterogeneity of refolded recombinant $^{18–127}$HuS(P19A, S20A) as compared with HuS preparations.

Discussion

Here we report engineering of the first chimeric BH-RNase that combines 29 N-terminal amino acid residues of bovine RNase A fused to 97 C-terminal residues of human RNase I (Figure 2). The choice of N-terminus from bovine RNase A is based on the classic work by Richards and Vithayathil (Richards and Vithayathil, 1959) who found that limited proteolysis of RNase A with subtilisin yields two complimentary fragments known as S-peptide and S-protein [for a review of RNase A, see Raines (Raines, 1998)]. Neither S-peptide nor S-protein alone has enzymatic activity, but reconstitute active ribo-

nuclease upon forming a complex known as RNase S. Subsequent studies established that subtilisin and proteinase K cleave RNase A preferentially between A20 and S21, with minor products formed upon cleavage of nearby peptide bonds (Rauber *et al*., 1978; Neumann and Hofsteenge, 1994; Mendez *et al*., 2000; Markert *et al*., 2001). The sensitivity of the hinge loop 15–25 to proteolytic cleavage varies between closely related mammalian pancreatic RNases, reflecting significant sequence diversity in this particular region [for a discussion see Gupta *et al*. (Gupta *et al*., 1999) and Figure 2 for comparison between RNase A and RNase I]. For example, hinge regions of rat pancreatic RNase (Gupta *et al*., 1999) and human RNase I (Pous *et al*., 2001) do not fit into the catalytic center of subtilisin.

We found that BH-RNase and its N24T mutant are cleaved by both subtilisin and proteinase K at the A20–S21 bond, yielding functionally active HuS fragments. The conditions of cleavage and affinity purification on the S-peptide column are optimized to achieve a 40–50% yield of HuS fragments from chimeric RNases (Figures 3 and 4). Importantly, HuS displays an ~5-fold higher affinity to Hu-tagged proteins than $^{18–127}$HuS(P19A, S20A) [Figure 5 and Backer *et al*. (Backer *et al*., 2003)]. This change might be a result of more homogeneous preparations of HuS that arise upon proteolytic cleavage of chimeric RNase. Indeed, surface plasmon resonance experiments and affinity chromatography on the S-peptide column suggest that refolded $^{18–127}$HuS(P19A, S20A) exists in several conformations with different affinities to Hu-peptide (Backer *et al*., 2003).

We found that the BH-N24T mutant appeared to be more resistant to proteolytic cleavage by subtilisin and proteinase K (Figure 3). This result is somewhat surprising, since molecular modeling indicates that an amino acid in position 24 is not involved in direct interactions with a catalytic triad in subtilisin or proteinase K (data not shown). Unfortunately, this resistance did not lead to a higher yield of wild-type HuS. Nevertheless, since wild-type HuS might be more advantageous in minimizing the risk of an adverse immunological response in potential clinical applications of the adapter/docking tag system, experiments to optimize its production are in progress.

Taken together, our data indicate that chimeric BH-RNase is a superior source of an adapter protein for assembled complexes for targeted drug delivery. Our recent experiments with tumor radionuclide imaging using techetium-99m labeled HuS/Hu-VEGF complexes suggest important new applications for this system (F.Blankenberg *et al*., manuscript in preparation).

References

Backer,M.V., Aloise,R., Przekop,K., Stoletov,K. and Backer,J.M. (2002a) *Bioconjug. Chem.*, **13**, 462–467.

Backer,M.,Gaynutdinov,T., Aloise,R., Przekop,K. and Backer,J. (2002b) *Protein Expr. Purif.*, **26**, 455–461.

Backer,M., Gaynutdinov,T.I., Gorshkova,I.I., Crouch,R.J., Hu,T., Aloise,R., Arab,M., Przekop,K. and Backer,J. (2003) *J. Control. Release*, **89**, 499–511.

Dubowchik,G.M. and Walker,M.A. (1999) *Pharmacol. Ther.*, **83**, 67–123.

el-Joubary,A. *et al.* (1999) *J. Biomol. NMR*, **15**, 265–266.

Guex,N. and Peitsch,M.C. (1997) *Electrophoresis*, **18**, 2714–2723.

Gupta,V., Muyldermans,S., Wyns,L. and Salunke,D.M. (1999) *Proteins*, **35**, 1–12.

Kim,J.S. and Raines,R.T. (1993) *Protein Sci.*, **2**, 348–356.

Kuzmic,P. (1996) *Anal. Biochem.*, **237**, 260–273.

Markert,Y., Koditz,J., Mansfeld,J., Arnold,U. and Ulbrich-Hofmann,R. (2001) *Protein Eng.*, **14**, 791–796.

Matousek,J., Pouckova,P., Soucek,J. and Skvor,J. (2002) *J. Control. Release*, **82**, 29–37.

Mendez,T.J., Johnson,J.V. and Richardson,D.E. (2000) *Anal. Biochem.*, **279**, 114–118.

Neumann,U. and Hofsteenge,J. (1994) *Protein Sci.*, **3**, 248–256.

Pous,J. *et al.* (2001) *Acta Crystallogr. D Biol. Crystallogr.*, **57**, 498–505.

Raines,R.T. (1998) *Chem. Rev.*, **98**, 1045–1065.

Rauber,N.R., Jany,K.D. and Pfleiderer,G. (1978) *Z. Naturforsch. C*, **33**, 660–663.

Richards,F.M. and Vithayathil,P.J. (1959) *J. Biol. Chem.*, **234**, 1459–1465.

Sorrentino,S. and Libonati,M. (1997) *FEBS Lett.*, **404**, 1–5.

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