Improving Protein Pharmacokinetics by Genetic Fusion to Simple Amino Acid Sequences*

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The role of primary amino acid sequences in protein pharmacokinetics, an issue of relevance in both basic knowledge and biotechnology, was addressed here using as a starting point two repetitive antigens from the hemoflagellate Trypanosoma cruzi that are known to stabilize their associated proteins in the bloodstream. A major drawback to their pharmacological application is that these repetitive sequences are highly immunogenic, being therefore the deletion of this characteristic desirable. Based on sequence homology and epitope mapping analyses, an artificial repetitive sequence (PSTAD) was engineered. This motif was tested by genetic fusion to the C terminus of both the trypanosomal trans-sialidase and the rat tyrosine aminotransferase and found to produce a 4.5- to 6-fold increase in the half-life of the associated protein while displaying significantly lower immunogenicity. Residues involved in the stabilizing properties of the novel peptide were mapped by a site-directed mutagenesis approach, allowing us to successfully identify another two motifs. Searching databases for sequences displaying some homology, embedded in proline frameworks and associated to shed virulence factors from unrelated microorganisms, resulted in the identification of four other protein extensions. Remarkably, three of them (from Streptococcus pneumoniae, Actinomyces viscosus, and Escherichia coli) revealed similar pharmacokinetic features, suggesting therefore an analogous evolutionarily acquired mechanism to ensure the biodistribution of their corresponding proteins. Our findings indicate that the insertion of defined motifs into a proline-rich framework constitutes a suitable alternative to construct a chimeric protein with extended half-life in blood.

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Despite its relevance, little is known about the structural requirements that are to be filled by a given protein to remain in blood. This applies even for a long lived blood protein such as the albumin, where detailed knowledge of its structure and function has already been gained (1). The mean residence time in the bloodstream is distinctive for each particular protein and seems to rely on a combination of several mechanisms including proteolytic degradation, hepatic uptake, endothelium permeability, renal excretion, and immunogenic reactions (2). In addition, post-translational modifications such as site-specific carbohydrate attachment (3, 4), sialylation degree (5), and multimerization (6) may modulate this phenomenon. The presence of endogenous receptors for certain molecules also regulates their mean residence time in blood (7–10). Although scarce evidence is at hand, the existence of linear amino acid motifs involved in the bloodstream half-life of proteins might be postulated (11, 12).

Understanding the structural requirements that govern the pharmacokinetics of proteins is not only a relevant basic science issue; the need to maintain substances in circulation within their biologic activity ranges is one of the main objectives in therapeutic and diagnostic applications (13). In this regard, several strategies have been described, including mutagenesis, covalent conjugation of molecules to polymers (14–18), encapsulation in liposomes or viral envelopes, and physical entrapment in particles (2). Other approaches rely on genetic and chemical techniques to create chimeras between a desired protein and a normal constituent of the mammalian plasma that would eventually act as a carrier (19–21). This strategy resembles that displayed by several pathogen-derived molecules that recognize and remain attached to circulating proteins such as immunoglobulins, albumin, fibrinogen, or fibrinogen through the evolutionary acquisition of specific binding domains (22–24).

Trypanosoma cruzi, the protozoan parasite causative of the Chagas’ disease, displays an alternative strategy to improve the pharmacokinetic properties of its trans-sialidase (TS1), a bloodstream-borne virulence factor (25). The presence of a repetitive domain termed shed acute phase antigen (SAPA) on the TS C terminus, although not involved in catalysis (26), raises its half-life in blood (12, 27). This enhanced serum persistence allows the enzyme to be systemically distributed, thus ensuring the interaction with its target tissues during infection (28, 29). Another repetitive shed antigen from T. cruzi known

* The abbreviations used are: TS, trans-sialidase; GST, glutathione S-transferase from Schistosoma japonicum; HRM, hybrid repetitive motif; PBS, phosphate-buffered saline; SAPA, shed acute phase antigen; TAT, tyrosine aminotransferase from rat liver; β2, elimination half-life; TS-HRM, TS with 21 associated HRM repeats; TS-SAPA, TS with 13 associated SAPA repeats; TS-3R, TS with three associated SAPA repeats.
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As Tc13 (30), when genetically fused to TS stabilizes the enzyme in blood to a similar extent as SAPA (12). Therefore, both amino acid sequences provide a simple and valuable tool for the elucidation of peptidic motifs underlying the maintenance of proteins in the circulation. Unfortunately, both SAPA and Tc13 are highly immunogenic, being two of the major antibody targets during the acute phase of T. cruzi infection (31). Molecular dissection of both features (i.e., immunogenicity and stabilization capacity) is therefore highly desirable.

In the present work, we took advantage of sequence alignments and B-cell epitope mapping information from both T. cruzi antigens to design a novel artificial repetitive motif (termed the hybrid repetitive motif (HRM)). As SAPA and Tc13, this motif carries the ability to stabilize reporter proteins in the circulation but displays significantly lower immunogenicity. Critical amino acid residues involved in the pharmacokinetics properties of HRM were identified by a site-directed mutagenesis approach. Results achieved were extended to peptidic motifs present in shed proteins from other pathogenic microorganisms, suggesting an analogous strategy to disseminate their virulence factors inside the vertebrate host.

**EXPERIMENTAL PROCEDURES**

**Mice**—C57Bl/6J and BALB/cJ 60–90-day-old female mice bred in our facilities were indistinctly used. All assays were approved by the ethical committee of our institute.

**Recombinant Proteins**—The minimal DNA unit coding for two 5-a-minotyrosyl repeats of the HRM molecule was generated by annealing the oligonucleotides 5′-pGATTTCACTACGGGACCCAGAATCT-3′ (where p means 5′ phosphorylated oligonucleotide) to its complementary sequence. Oligonucleotides (250 pmol each) were mixed, boiled for 5 min, and incubated overnight at 16 °C with 1,000 units of T4 DNA ligase and 75 μM ATP. Five units of Espl restrictionendonuclease were included in the ligation mixture to release tail-to-tail ligated products. DNA-modifying enzymes were from New England Biolabs (Beverly, MA), unless otherwise stated. Fragments showing maximal extent of polymerization were purified from agarose gels and subjected to a second round of ligation as described above. DNA fragments showing the desired molecular mass were purified, incubated for 1 h at 72 °C with T4p polymerase in the presence of ATPG 0.4 μM, and subsequently cloned into the pGEM T-easy vector (both from Promega (Madison, WI)). The accuracy and the actual number of repeats were determined by DNA sequencing. To obtain the TS-HRM fusion protein, this insert was subcloned into the EcoRI site located at the 3′ end of the TS gene in the pTsac plasmid (12) constructed in pTrcHisA vector (Invitrogen). The HRM-encoding fragment was also subcloned into the HRM encoding fragment was also subcloned into the pET24a (Stratagene, La Jolla, CA) and amplified by PCR (primers 5′-ACCCGGACCCAGAATCT-3′ and 5′-CGATTTCACTACGGGACCCAGAATCT-3′). After XhoI/KpnI digestion sites (underlined in the sequences), the amplicon was inserted upstream of the EcoRI site of pTrcHisA (Invitrogen). The degenerate repetitive sequence of the sialidase from Actinomyces viscosus strain DSM43798 (32) (GenBank™ X62276/L06898) was obtained by KpnI/ScaI digestion of a plasmid containing the whole nanH gene, a kind generous of Drs. R. Schauer and P. Roggentin. The fragment was ligated to EcoRI adaptors constructed with the oligonucleotide pairs 5′-GATTCTATACGAGCT-3′/5′-GCGTTATAG-3′ and 5′-ATTCTAACGTGGTACG-3′/5′-GCGTTAGGCCG-3′ inserted into the pTsac as above. The C terminus of the sialidase A encoded by the nanH gene from Streptococcus pneumoniae (33) (GenBank™ number Q59959) was amplified from genomic DNA using the primers 5′-GAATGAGTAACGAAAGA-3′ and 5′-TATAGCTGCGATGTT-3′. The PCR product was cloned into the pGEM T-easy vector, released by EcoRI digestion, and subcloned into the pTsac. The same strategy was employed to clone the hydrophilic extension of a TS-like gene from Trypanosoma cruzi (34) (GenBank™ number AY249142) (primers 5′-ATCTTTCCGTGTTGAAAC-3′/5′-TCAATCTGTTCCGATTTAGG-3′) and the C terminus of the EsfP protein from the enteropathogenic Escherichia coli O145:NM strain (35, 36) (GenBank™ number AY243566) (primers 5′-TTACCGCCGTGCAGG-3′/5′-TTACCCCTTGTCATGG-3′). All constructs were subjected to DNA sequencing, and the molecular properties of the encoded proteins were predicted with the aid of the LaserGene software (DNASTAR Inc., Madison, WI).

**Expression, Purification, and in Vitro Evaluation of Recombinant Proteins**—TS and TAT recombinant proteins were expressed in E. coli XL1-Blue (Stratagene) and purified to homogeneity, as judged by Coomassie Blue-stained SDS-PAGE, by immobilized metal affinity chromatography through Ni²⁺-charged HiTrap chelating columns using the His tag located at their N termini provided by their respective cloning vectors, followed by ion exchange chromatography through MonoQ columns (both from Amersham Biosciences).

**GST**—GST-SAPA (12), and GST-HRM proteins were purified by affinity chromatography through GSTrap columns (Amersham Biosciences) following manufacturers’ guidelines.

**Pharmacokinetics Studies**—Mice were intravenously injected with 3 μg (30–40 pmol) of the indicated TS recombinant protein contained in 0.2 ml of PBS by the retro-ocular sinus. Blood samples were taken from the tail at different intervals as required by each experimental design. Sera were assayed for remnant TS activity by measuring the transferance of the sialyl residue from sialyl-lactose (Sigma) to 125I-lactose (Amersham Biosciences) as described (37). Results were calculated in cpm/μl of plasma as enzyme source. Specific TS activity, thermal inactivation at 37 °C, and proteolytic degradation in normal mouse plasma (12, 27) rendered similar values for every chimeric TS generated (data not shown).

TAT and TAT-HRM proteins were labeled with 125I (PerkinElmer Life Sciences) using IODO-GEN-coated tubes (Pierce), and unincorporated 125I was removed by Hi-Trap Desalting columns (Amersham Biosciences). About 1.5 × 10⁶ cpm (specific activities: TAT, 145,000 cpm/μg, TAT-HRM, 130,000 cpm/μg) were given to the sera collected at different times postinjection were resolved by SDS-PAGE, and labeled proteins were quantified by 1D Image Analysis software (Eastman Kodak Co.) after autoradiography.

In all cases, results were expressed relative to values found at the 3-min postinjection time that was taken as 100%. The intravascular half-life values (t of the elimination phase, t½) were determined from the second part of the decay curve by least squares regression using the slope of a curve fitted to the data points (16, 38).

**Animal Immunization and Antibody Evaluation**—Mice were administered with four intravenous doses (10 μg/each) of recombinant TS proteins every 10 days by the retro-ocular route. One week after the last dose, the reactivity of sera to the SAPA or the HRM motif was evaluated by enzyme-linked immunosorobent assay. Briefly, poly styrene microplates (Maxisorp, NUNC a/s, Roskilde, Denmark) were coated with GST-SAPA or GST-HRM (100 ng/well, about 1.6 pmol of GST-SAPA, or 2.3 pmol of GST-HRM) in PBS overnight. Wells coated with GST were used as negative controls. The amount of GST fusion proteins in each plate was tested assaying three wells/plate with proper dilutions (100 dilutions (100 μl/well) were seeded and incubated for 1 h. Reaction at 37 °C, and then serum serial dilution of an anti-GST rabbit serum. Plates were blocked with 5% nonfat milk in PBS for 2 h at room temperature, and then serial serum (100 μl/well) were seeded and incubated for 1 h. Reactivity was revealed by the addition of peroxidase-conjugated secondary antibodies (Sigma) followed by 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Amer sham BioSciences). Plates were blocked with 5% nonfat milk in PBS for 2 h at room temperature, and then serial serum 100 μl/well were seeded and incubated for 1 h. Reaction was revealed by the addition of peroxidase-conjugated secondary antibodies (Sigma) followed by 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Amer sham BioSciences). Sera obtained from naive animals and assayed in the same plate were taken as negative controls.

**SPOT Assays**—B-cell epitope mapping of antigen Tc13 was achieved by SPOT assay as described (39). Briefly, filters containing peptide
The HRM Motif Extends the Bloodstream Half-life of Genetically Fused Proteins—The use of TS as a reporter protein in pharmacokinetic assays was previously validated (12). It also provides some experimental advantages such as its easy enzymatic activity measurement and the absence of specific inhibitors in normal mammalian serum (41). Therefore, 21 HRM units were inserted in frame at the C terminus of TS (TS-HRM; Fig. 2A). This addendum is roughly equivalent in mass to nine SAPA repeats, thus slightly above the threshold of eight SAPA units determined to improve the TS permanence in the circulation (12). TS-HRM protein was injected into mice by the intravenous route, and remnant TS activity was monitored in blood samples collected at different times postinjection. As controls, three different TS proteins previously characterized (12) were used (see Fig. 2A). All of these proteins exhibit similar specific TS activity when assayed in vitro (data not shown) and were thus administered in equimolar amounts (35–40 pmol/mouse). As shown in Fig. 2B, TS-HRM and TS-SAPA displayed similar half-lives (t½ of 40 and 39 h, respectively) and can be detected in the bloodstream up to 3 days postinjection. In contrast, TS without any C-terminal extension was absent from circulation, and only 10% of the input of the TS harboring three SAPA repeats (TS-3R) remained after 24 h. In a separate set of experiments, a TS with only seven HRM-associated motifs, roughly equivalent in mass to three SAPA units, was also tested, displaying a similar half-life to that estimated for the TS-3R molecule (t½ of 24 versus 18 h, respectively), thus suggesting that HRM and SAPA motifs share similar pharmacokinetic characteristics.

To assay whether the effect of the HRM motif can be extended to proteins other than TS, the rat liver TAT was chosen as a mammalian protein model. The purified recombinant TAT and TAT-HRM proteins were 125I-labeled and administered intravenously. From the plotting of the autoradiographic signals derived from SDS-PAGE (Fig. 2C), the half-life of the TAT-HRM was extended about 6 times when compared with that of TAT (from 1.3 to 7.9 h). Findings displayed in Fig. 2 indicate that the HRM motif abridges all the molecular features involved in the stabilization properties of both SAPA and Tc13 parental sequences.

The HRM Motif Displays Lower Immunogenicity than SAPA—To evaluate the immunogenic properties of the HRM repeat, mice were intravenously administered with four doses of either TS-HRM, TS-3R, or TS-SAPA (10 μg each, Fig. 2A) and the specific antibody response directed against the repetitive domains was assayed by enzyme-linked immunosorbent assay. Although intravenous injection often leads to poor immune responses, high titers of SAPA directed antibodies were detected in TS-SAPA-immunized animals (Fig. 3). Furthermore, the TS-3R protein that contains only three SAPA-repeats and is rapidly cleared from the circulation (Fig. 2B) elicits a similar humoral response as TS-SAPA (Fig. 3). In contrast, the HRM motif displays a significantly reduced immunogenicity although it harbors 21 repetitive units. Reactivity of sera from TS-HRM-immunized animals remained under the cut-off value at the end of the immunization schedule (Fig. 3). Overall, selected amino acid replacements introduced to generate the HRM molecule significantly impair its immunogenic properties, probably by disruption of both SAPA and Tc13 B-cell epitopes (see above and Fig. 1). Therefore, the designed HRM repetitive motif retains the ability to lengthen the permanence of associated proteins in blood while exhibiting a remarkable reduction in its immunogenic properties.
activity. Data are expressed as the mean ± S.D. (n = 3 animals). The asterisks denote significant differences (p < 0.05) from TS-SAPA. Data for normal mouse serum are presented as single columns, since undistinguishable reactivities were recorded against GST, GST-SAPA, and GST-HRM molecules.

Since the repetitive antigens used herein are embedded in a proline framework (Fig. 1A), we first tested the effect of a longer interproline distance in the pharmacokinetics of the HRM molecule. A repeat unit with prolines spaced by 10 residues was built by replacing prolines for glycine in alternating repeats (PSTADGSTAD, termed HRM-P). As shown in Fig. 4B, the TS-HRM-P fusion protein renders a similar bloodstream half-life value as compared with the parental TS-HRM molecule (tβ = 38 and 40 h, respectively). The second relevant feature displayed by both SAPA and HRM is a biased amino acid composition toward negatively charged residues (Figs. 1A and 2A). Therefore, we replaced the aspartate residue of HRM either for asparagine (PSTAN, termed HRM-N) or lysine (PSTAK, termed HRM-K). The TS-HRM-N recombinant protein displayed the same in vivo performance (tβ = 39 h) as the TS-HRM, indicating that this modification imposes minor constraints on its stabilization properties (Fig. 4B). Conversely, replacement of aspartate by lysine significantly drops the mean residence time of the TS-HRM-K molecule in blood (tβ = 11 h; Fig. 4C). This value is similar to that recorded for the TS without a C termi- nus extension and then even below that of the TS-3R molecule (tβ of 9 and 18 h, respectively) (Fig. 4).

Repetitive Units Present in Secreted Virulence Factors from Other Pathogens Also Stabilize TS in Blood—The acquisition of SAPA and Tc13 extensions might represent a novel strategy to disseminate TS and TS-like molecules in the bloodstream. Hence, it is conceivable that other pathogens unrelated to T. cruzi might have developed a similar mechanism to improve the biodistribution of some of their encoded virulence factors. To test this hypothesis, we searched the data bases for sequences displaying the following features: (a) a certain degree of similarity to SAPA and/or Tc13; (b) a proline-rich hydrophilic framework accounting for their extended secondary structure and maximum solvent exposure; and (c) association to shed virulence factors from pathogenic microorganisms. Four sequences matching these criteria were identified and cloned at the TS C terminus to be tested in our pharmacokinetic assays. These sequences span the degenerate repetitive units present at the C termini of the sialidases from A. viscosus (32, 42) and S. pneumoniae (33), the hydrophilic C-terminal extension of the closely related TS from the fish parasite T. carassii (34), and

Mapping of Residues Involved in HRM Stabilization Properties—Critical amino acid residues involved in the HRM-mediated blood stability were then searched by site-directed mutagenesis. For this purpose, several constructs derived from the HRM sequence were designed and cloned at the TS C terminus. Recombinant proteins having similar mass addenda to TS-HRM were assayed (for molecular properties, see Fig. 4A).
the proline-rich region of the EspF protein from enteropathogenic *E. coli* (35, 36). The complete sequence of these domains together with the predicted molecular properties of the ensuing chimeric TS proteins is indicated in Fig. 5A. The putative alignment of these sequences with SAPA and/or HRM repetitive units is also displayed. As shown in Fig. 5B, both *A. viscosus*- and *S. pneumoniae*-derived sequences increase the persistence of TS in blood to the same extent as SAPA. It is noteworthy that the homologies detected between both molecules and the *T. cruzi* SAPA repeat are restricted to the PST-PAD motif, which explains the improved pharmacokinetics of the latter molecule (see Figs. 1 and 2). The sequence from *E. coli* EspF displays an intermediate but significant effect on the stabilization of TS \((t_{1/2}/H9252 = 24\) h), whereas the extension of *T. carassii* does not show any effect \((t_{1/2}/H9252 = 13\) h).

**DISCUSSION**

The rapid clarification from the bloodstream constitutes a major drawback for many otherwise promising protein pharmaceuticals (17, 43, 44). To overcome this problem, covalent coupling of drugs or proteins to polyethylene glycol has been extensively applied (13). Although polymer conjugation to hormones and enzymes may lead to a reduction in their biological/ enzymatic activities (45–47). Furthermore, chemical coupling methods usually result in a mixture of heterogeneous molecules displaying different *in vivo* performances (2, 45–47). Genetic techniques constitute an interesting alternative, since this method allows the allocation of the desired sequences far from the active site of the target molecule. However, current approaches involve fusion to whole proteins or to entire protein domains that might produce negative effects in the recombinant protein activity as well (48).

A novel approach based on repetitive sequences able *per se* to retain proteins in the circulation was explored here. Two *T. cruzi* repetitive antigens (SAPA and Tc13) able to extend the life span of associated proteins in blood have been previously identified (12). Taking into account both structural and antigenic information of these antigens (Fig. 1) (39), an artificial repetitive unit termed HRM was designed. Pharmacokinetic studies showed that the genetic fusion to HRM produced a 4.5–6-fold increase in the half-life in blood of the associated protein (Fig. 2). Whereas the rapid protein distribution phase from the bloodstream to the extravascular space (termed the \(t_{1/2}/H9251\) phase) seems not to be substantially modified, most of the improvement achieved by HRM is due to a large increase in the \(t_{1/2}/H9252\) phase that corresponds to the intravascular elimination of the injected proteins. As previously described for SAPA and Tc13 (12), the HRM extension does not modify the specific activity of the associated TS, probably due to its flexible secondary structure made up of hydrophilic residues interspaced by prolines. More important, the immunogenicity of HRM was significantly reduced as compared with SAPA, overcoming one of the major hurdles for the biotechnological applications of the parental sequences (Fig. 3).

The involvement of the net charge of the HRM molecule in its *in vivo* function was also addressed here. Replacement of aspartate by asparagine did not alter the functionality of the repeat (TS-HRM-N; Fig. 4B), contrasting with the remarkable effect observed when the residue was replaced by lysine (TS-
HHRM-K; Fig. 4C). This phenomenon might be ascribed to the positive charge added and is consistent with the fact that positively charged proteins are rapidly depleted from blood mainly due to the negative charges present in the endothelium, liver, and the renal glomerule (49). In view of these results, one striking feature is the presence of one lysine per repeat in the original Tc13 sequence (Fig. 1A). The positive charge provided by this residue, however, seems neutralized in the Tc13 unit by the high metabolic rate of blood proteins in mice (55). Here we present evidence indicating that peptidic motifs allowing permanence in blood of the associated proteins can also be embedded in this structure. To search for the requirements concerning the proline spacing, a construction where the prolines were located at 10 residues distance was designed (HRM-P). This repeat retained the ability to extend the half-life of TS in blood (Fig. 4B), showing that this distance between proline residues works properly for this functionality. It should be emphasized, however, that neither the presence of the proline-rich framework nor the overall negative charge of the repetitive sequence guarantees the stabilization phenomenon described here, since molecules combining both attributes such as the intracellular T. cruzi antigens Tc1, Tc30, and Tc36 (12) and the hydrophilic extension of T. carassii TS (Fig. 5) did not significantly extend the TS half-life.

Taken together, our results point out that sequences enriched in hydrophilic and negatively charged residues and inserted in a proline-rich framework constitute likely candidates to create chimeric proteins with extended half-life in blood. In support of this hypothesis, the extensions from the sialidases of A. viscosus and S. pneumoniae that display these features were able to extend the TS permanence in blood although they slightly resemble the SAPA, Tc13, or HRM motifs (Fig. 5). By another hand, the assayed E. coli EspF-derived domain that partially address these requirements have an intermediate effect; meanwhile, the negative charged extension from the TS of T. carassii not having any homology did not work at all. In addition, these findings suggest that the acquisition of an extended domain to improve the pharmacokinetic performance of the virulence factors might constitute a convergent strategy displayed by several pathogens.

The approach followed here can be proposed as a viable route to increase the half-life of proteins in blood through the use of recombinant fusion proteins that can be readily expressed in microbial systems with the potential for scaling up production. Besides, the high metabolic rate of blood proteins in mice (55)
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suggests an even better performance of these repeats in humans or cattle.

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REFERENCES