Potent Antithrombin Activity and Delayed Clearance From the Circulation Characterize Recombinant Hirudin Genetically Fused to Albumin

By Summer Syed, Philip D. Schuyler, Myron Kulczycky, and William P. Sheffield

In this study we sought to extend the plasma half-life while maintaining the potent antithrombin activity of hirudin. We hypothesized that gene fusion of hirudin to albumin would result in the expression of a slowly cleared hirudin molecule. A hirudin variant 3 (HV3) cDNA was obtained by gene synthesis, while a 1,996-bp full-length rabbit serum albumin (RSA) cDNA was selected from a rabbit liver cDNA library. Expression of the former in COS-1 cells conferred antithrombin activity on media conditioned by the cells, while expression of the latter resulted in the secretion of a 67-kD protein that reacted with mono-specific anti-RSA antibodies. Having shown independent expression of the two proteins, we next expressed two fusion proteins: HV3 linked via its C-terminus to albumin (HLA), and HV3 linked via its N-terminus to albumin (ALH). The former, but not the latter, inhibited both the amidolytic and fibrinogenolytic activities of thrombin. HLA also retained the dye-binding characteristics of RSA, as judged by Affi-Gel Blue chromatography. Highly similar concentrations of either commercial HV1 (40 nmol/L) or HLA (30 nmol/L) were required to halve the initial rate of thrombin reaction with chromogenic substrate S2238, suggesting the retention of high-affinity inhibition of thrombin by the fusion protein. An His-tagged form of HLA was purified by Ni²⁺-chelate affinity and heparin-Sepharose chromatography. The purified, radioiodinated protein was injected into rabbits, and demonstrated a catalytic half-life of 4.60 ± 0.16 days. This represents an extension of hirudin half-life in vivo of greater than two orders of magnitude; gel analysis of HLA[H₆] recovered from rabbits showed that it circulated in intact form. Our results provide a rationale for future testing of the biological effects of HLA, and support our initial hypothesis.

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MATERIALS AND METHODS

Materials

A plasmid encoding human serum albumin, designated p(Alb)3, was generously provided by Dr James Wilson (University of Michigan).
gan Medical Center, Ann Arbor). Recombinant desulfato-hirudin HV1 (CGP 39393), hereafter referred to as commercial HV1, was kindly provided by Dr R.B. Wallis (Ciba-Geigy Pharmaceuticals, Horsham, UK). Human α-thrombin (>3,300 NIH U/mg; >93% active) was the generous gift of Dr John Fenton (New York State Division of Biologics, Albany). Oligonucleotides were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University (Hamilton, Ontario, Canada). All enzymes for the modification of DNA were purchased from Promega (Madison, WI), with the exception of Pfu polymerase (Strategene, La Jolla, CA) and Taq polymerase (Perkin-Elmer, Mississauga, Ontario, Canada). T7 sequencing kits, Sephaglas DNA purification kits, and deoxyribonucleotides were from Pharmacia LKB Biotechnology (Baie d’Urfe, Quebec, Canada). Canadian Life Technologies (Burlington, Ontario, Canada) was the supplier of competent Escherichia coli DH5α cells, Lipofectin reagent, penicillin/streptomycin, and dye-free Dulbecco’s minimal essential medium (DMEM) for cell culture. COS-1 cells were obtained from the American type Culture Collection (Rockville, MD). The radioactive products (35-S)-methionine and (35-S)-cysteine (TransSynSlab) were bought from ICN (Mississauga, Ontario, Canada), as were methionine and cysteine-deficient DMEM; 35-S-dATP and Na234O4 were from Mandel (Toronto, Ontario, Canada). Aflit-Gel Blue (50-100 mesh), and nitrocellulose blotting membranes were purchased from BioRad (Mississauga, Ontario, Canada). Rabbit serum albumin and bovine serum albumin (RSA and BSA) were from Sigma (St Louis, MO). Alkaline phosphatase-conjugated rabbit-antichicken IgG was from Jackson Labs (Bar Harbor, ME). A commercial rabbit liver cDNA library (5-STRETCH; Clontech, Palo Alto, CA) was also purchased. QiaGen (Chatsworth, CA) was the source of plasmid DNA isolation kits and Ni2+-chelate affinity resin. Chromogenic substrate S-2238 (H-D-Phenylalanyl-L-pipecolyl-L-ar- was used to identify a plasmid containing the RSA cDNA in the Genbank (accession number U18344). Construction of expression plasmids. Five expression plasmids were constructed, as described below. (1) A 216-bp Sfi I-EcoRI restriction fragment of pABCD (see above) and a 132-bp fragment of pGEM7zf (+)-rAT-III (52345) were gel-purified and inserted into the EcoRI site of the eukaryotic expression vector pSG5,21 in a three-part ligation. The resulting plasmid, pSGS-HV3, encoded the 32-amino acid secretory signal sequence of rabbit antithrombin, fused to the 66 codon HV3 cDNA assembled by gene synthesis. (2) For expression of recombinant RSA, the 1,996-bp cdNA was released from p(Alb)192 by EcoRI digestion and inserted into the corresponding site of pSG5. Analysis of candidate subclone miniprep DNA was used to identify a plasmid containing the RSA cdNA in the sense (pSG5-RSA) and antisense (pSG5-ASR) orientations with respect to the SV40 promoter of pSG5. (3) To fuse the mature form of RSA to HV3, pSGS-HV3 was used as a template in a standard PCR reaction primed by employing a pSG5-specific primer that bound upstream of the insert (primer 3525, 5'-GTAATACGAC TCATATAGG-3') and primer 4115 (5'-AACCATGGAG CCA- CCTCCGC CACCTCACC TTATGAGGA C-3'). The latter replaces the termination codon of pSG5-HV3 with (Gly)6-Ser- Met. The RSA cDNA was similarly modified, in a reaction primed by the SP6 primer and primer 3951 (5'-CTCATCGGAA CACAT- AAAA GTGAGAT-3'). The resulting plasmid was designated pSG5-ALH (for albumin linked to hirudin). Human serum albumin and bovine serum albumin (RSA and BSA) were from Canadian Life Technologies (Burlington, Ontario, Canada). Five expression plasmid pSG5-RSA was injected subcutaneously into laying hens, and IgG partially purified from yolks obtained from the eggs as described previously.26

Molecular cloning of rabbit serum albumin. A λgt-11 rabbit liver cDNA library was screened by hybridization as previously described12,22 using a 2,200-bp BamHI fragment of pAlb31 containing the entire human albumin cdNA to generate random-primer 3P-labeled probes. The phage clone giving the most intense hybridization signal on a preliminary screen of 2 × 105 plaques was transferred to the EcoRI site of pGEM3zf (+) p(Alb)72. The 1,600-bp cdNA insert of p(Alb)72 was used to rescreen the library, and yielded a clone with a 1,996-bp insert that was transferred to the EcoRI site of pUC19 and the resulting plasmid designated p(Alb)192. This full-length RSA cdNA was sequenced on both strands using the dyeodeoxy process, a method facilitated by the use of p(Alb)192 derivatives with nested deletions.24 The sequence of the RSA cdNA was deposited in Genbank (accession number U18344).

Methods

Gene synthesis of HV3. Four mutually priming oligonucleotides were used to synthesize a hirudin variant 3 (HV3) cdNA [modified from ref 18]: primers A (5'-GCGTCCTGGGCCGCGTTGG TTG-TATGAC TACAAGACT GAGCGGAGC CTGGCACA ACCGAGCCA ACCGACCC ACCGGGAGG AACCATGGAG CCA- CCTCCGC CACCTCACC TTATGAGGA C-3') and D (5'-GGA- TCTCGAC TATGAGCC ATCCAGGG AGTGCGCT CAGGCTC TCTACGAC AGCGGAGC ATGGCTGCT CACCGACCA GCC- CTC ACCCGGAC AGGTGTGCT G-5'). The resulting PCR products, AB (112 bp) and CD (124 bp) were gel-purified, made blunt-ended using the Klenow fragment of DNA polymerase I, and introduced into the EcoRV site of pGEM5zf (+) (Promega) using standard manipulations.20 DNA sequencing showed that all resulting subclones contained some errors, but these misincorporations were fully corrected by additional PCR reactions (eg, a near-perfect match CD subclone was used as the template in a standard PCR reaction primed by an SP6 primer and primer D). Misincorporations at both 5' and 3' ends of AB were combined by similar PCR manipulations involving primers encoding the first 27 bp of primers A and B. A 150-bp Acc I digestion product of the resulting AB clone [in pGEM5zf (+)] was inserted into the Acc I–linearized CD clone [in pGEM7zf (+)]. This final HV3 plasmid was found to be without misincorporations by DNA sequencing, and designated plasmid pABCD. This final construct contained nucleotides 124-138 of a rabbit antithrombin cdNA21 (encoding a portion of the signal sequence) joined in frame to the 66 codons of HV3 and a termination codon.

Expression of recombinant RSA. The 1,996-bp cdNA was released from p(Alb)192 by EcoRI digestion and inserted into the corresponding site of pSG5. Analysis of candidate subclone miniprep DNA was used to identify a plasmid containing the RSA cdNA in the sense (pSG5-RSA) and antisense (pSG5-ASR) orientations with respect to the SV40 promoter of pSG5. (3) To fuse the mature form of RSA to HV3, pSGS-HV3 was used as a template in a standard PCR reaction primed by employing a pSG5-specific primer that bound upstream of the insert (primer 3525, 5'-GTAATACGAC TCATATAGG-3') and primer 4115 (5'-AACCATGGAG CCA- CCTCCGC CACCTCACC TTATGAGGA C-3'). The latter replaces the termination codon of pSG5-HV3 with (Gly)6-Ser-Met. The RSA cdNA was similarly modified, in a reaction primed by the SP6 primer and primer 3951 (5'-CTCATCGGAA CACAT- AAAA GTGAGAT-3'). The resulting plasmid was designated pSG5-ALH (for albumin linked to hirudin). Human serum albumin and bovine serum albumin (RSA and BSA) were from Canadian Life Technologies (Burlington, Ontario, Canada). Five expression plasmid pSG5-RSA was injected subcutaneously into laying hens, and IgG partially purified from yolks obtained from the eggs as described previously.26
Affinity-purified, monospecific antibodies were obtained following negative selection on immobilized BSA, and positive selection on immobilized RSA, and used to develop an ELISA for recombinant products containing RSA. Briefly, affinity-purified chicken anti-RSA antibodies were used as the trapping antibody, and a biotinylated preparation of HLA(H)6 were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and dialyzed against 4.0 L of TBS, and batch-adsorbed to a 2.0-mL vol of Affi-Gel Blue. The resin was washed 5X with TBS and eluted with 2 mol/L NaCl. After dialysis against TBS, the preparation (14 mL) was batch-adsorbed to 2.0 mL of heparin-Sepharose, and the unbound fraction concentrated by ultrafiltration and dialyzed against phosphate-buffered saline. This final preparation contained 12 μg of HLA(H)6, (as judged by BioRad protein assays standardized with BSA) and was free of contaminating protein as judged by SDS-PAGE.

In vivo experiments. Male New Zealand White rabbits (2.8 to 3.2 kg) were injected with 2 × 107 dpm/kg each of 125I-labeled HLA(H)6 purified as described above. At various times after injection, blood samples were drawn from the marginal vein of the other ear into 1/10 volume of acid-citrate-dextrose (ACD) anticoagulant. Plasma samples (0.3 mL) were precipitated by addition of an equal volume of 20% trichloroacetic acid (TCA) and the radioactivity in the resulting pellets was quantified using a gamma counter.

Pharmacokinetic analysis. The clearance data were analyzed using the technique of curve peeling, as described,29 using Lotus 1,2,3 software (Lotus Development Corp, Cambridge, MA). Briefly, the logarithm of the residual radioactivity was plotted against the time after injection in decimal days. The terminal exponential phase of the clearance was curve-fit using linear regression. Subtraction of the equation describing this line from the remaining data yielded a biphasic curve. The curve-peeling process was repeated; after the second curve-peeling, a single linear component remained on the semi-log plot, indicating the need for three terms in the equation, indicative of a three-compartment model.29 Fusion protein clearance, then, is described by the following equation:

\[
y(t) = C_1 e^{-a_1 t} + C_2 e^{-a_2 t} + C_3 e^{-a_3 t}
\]

where \(C_i\) are coefficients, and \(a(n)\) are rate constants. Fractional catabolic rates, half-lives, and distributions were calculated from \(C_i\) and \(a(n)\) values derived from the curve peeling as described by Carlson et al.30,31

General. Previous publications from this laboratory have described routine protocols for SDS-PAGE, fluorography, and autoradiography; concentration of conditioned cell media; transient transfection and metabolic labeling of COS cells; iodination of α-thrombin; and double-stranded DNA sequencing. Sequence management and alignments were performed using GENEPRO (Riverside Scientific, Seattle, WA) software. All protein samples analyzed by SDS-PAGE in this study were reduced with 2-mercaptoethanol before electrophoresis.

RESULTS

Gene Synthesis of HV3 and Expression in COS Cells

To test the concept that clearance of anticoagulant proteins could be modulated via gene fusion to albumin, it was first necessary to obtain independent recombinant expression of each moiety of the future fusion protein. Because any hirudin-albumin fusion protein would be expected to contain 20 disulfide bonds (and an unpaired Cys residue), we chose to express the proteins in COS cells, taking advantage of the optimal folding and disulfide bond formation afforded by passage through the mammalian cell secretory pathway. Accordingly, the hirudin cDNA was obtained via gene synthe-
of the hirudin-albumin fusion proteins in rabbit experimental models, we elected to use RSA as the albumin moiety. Because no RSA cDNA had been described, RSA was cloned by screening a rabbit liver cDNA library in λgt-11, using hybridization to a human serum albumin (HSA) cDNA. The longest clone that was obtained is 1,996 nucleotides long, and is comprised of a 1,824-bp open reading frame, preceded by 51 nucleotides of 5′ untranslated region, and followed by 111 bp of 3′ untranslated sequence that terminates in a polyadenylation signal (TATAAA). Based on the complete identity of the first 24 codons to the HSA pre-pro sequence, the next residue was designated Glu 1. As shown in Fig 2A,

Use of mutually priming, 60- to 70-nucleotide long primers resulted in the gene synthesis of HV3 in two parts. As has been previously noted, the method is relatively error-prone; nevertheless, because in our hands the few misincorporations that were noted clustered at the termini of the products, they were easily repaired in an additional round of PCR. Transfection of COS cells with the resulting expression plasmid, pSG5-HV3, resulted in the appearance in the conditioned media of thrombin inhibitory activity. Addition of this conditioned media, but not that of mock-transfected cells, reduced amidolysis of the chromogenic substrate by thrombin (Fig 1). The bulk of this inhibitory activity was lost after dialysis through 12- to 14-kD molecular weight cut-off (MWCO) membranes (Fig 1), but was retained following dialysis against 6- to 8-kD MWCO dialysis tubing (not shown). In addition, clotting times determined in the presence of HV3 were prolonged from 43 to 52 seconds (see Fig 4B). These results are consistent with the successful production of recombinant HV3 in COS cells and its secretion from the cells in a form capable of inhibiting both thrombin-mediated amidolysis and fibrinogenolysis.

Molecular Cloning and Expression of Rabbit Serum Albumin

Because we planned to investigate the clearance, and eventually the anticoagulant and antithrombotic properties of the hirudin-albumin fusion proteins in rabbit experimental models, we elected to use RSA as the albumin moiety. Because no RSA cDNA had been described, RSA was cloned by screening a rabbit liver cDNA library in λgt-11, using hybridization to a human serum albumin (HSA) cDNA. The longest clone that was obtained is 1,996 nucleotides long, and is comprised of a 1,824-bp open reading frame, preceded by 51 nucleotides of 5′ untranslated region, and followed by 111 bp of 3′ untranslated sequence that terminates in a polyadenylation signal (TATAAA). Based on the complete identity of the first 24 codons to the HSA pre-pro sequence, the next residue was designated Glu 1. As shown in Fig 2A,

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the mature HSA\textsuperscript{31} and RSA proteins share 73% amino acid identity. Like other animal albumins,\textsuperscript{34} RSA is one residue smaller than its human counterpart, and comprises 584 amino acids to give a predicted molecular weight of 66,019. All of its 35 cysteine residues are conserved, as is its single tryptophan.

Insertion of the RSA cDNA into the pSG5 eukaryotic cell expression vector in the sense orientation, followed by transfection of COS cells, resulted in the appearance in the conditioned media of a 67-kD polypeptide that was detected on immunoblots using a monospecific, affinity-purified chicken anti-RSA IgG (Fig 2B, lane Se). This protein comigrated with plasma-derived RSA, and was absent from media conditioned by cells transfected with pSG5 containing RSA in the antisense orientation (Fig 2B, lane As). These results confirm the successful cloning and expression of RSA in COS cells.

**Production of Fusion Proteins ALH and HLA**

As the generation of hirudin fusion proteins had not been previously reported, it was not known a priori whether N-terminal or C-terminal fusion would be more desirable. Accordingly, two fusion proteins were generated: HLA (HV3 linked to albumin), in which RSA was attached to the C-terminus of hirudin; and ALH (albumin linked to HV3), in which RSA was attached to the N-terminus of hirudin. In both cases, a hexaglycine spacer was incorporated into the design of the fusion protein to promote independence of folding of the two parental proteins; in the case of HLA, Gly\textsubscript{6}-Met-Ser separate Glu 66 of HV3 and Glu 1 of RSA (Fig 3A, L1), while in the case of ALH, Met-Val-Gly\textsubscript{6} separate Ala 581 of RSA and Ile 1 of HV3 (Fig 3A, L2). As shown in Fig 3B, transfection of COS cells with expression plasmids encoding these chimeric proteins resulted in the appearance of anti-RSA immunoreactive products with decreased mobility compared with recombinant COS-derived RSA; the mobility of ALH was slightly greater than that of the 74-kD HLA. In repeated transfection experiments, the concentration of ALH secreted into the medium never exceeded 20% of the levels achieved with HLA; moreover, whereas HLA inhibited both the amidolytic and fibrinogenolytic activity of thrombin to an extent similar to that obtained with unfused hirudins (Fig 4), ALH was without detectable effects. Importantly, neither activity of HLA was diminished by dialysis of HLA-containing conditioned media (using 12-kD MWCO), a finding that supports the integrity of the fusion protein in the conditioned media (Fig 3B). Accordingly, ALH was not further investigated, whereas characterization of HLA continued.

**Retention of Parent Protein Characteristics by HLA**

Having shown the retention of thrombin inhibitory characteristics similar to that of unfused hirudin by HLA, we wished to characterize the albumin moiety of the fusion protein in a similar way. Because albumin lacks known enzymatic or enzyme inhibitory activities, we chose to assess the binding of HLA to immobilized Cibacron blue dye (Affi-Gel Blue; BioRad), a well-known property of the serum albumins.\textsuperscript{35} The \textsuperscript{35}S-labeled products of metabolic labeling of COS cells transfected with pSG5, pSG5-RSA, and pSG5-HLA were passed over Affi-Gel Blue mini-columns, washed with TBS, and eluted with either 1.0 or 2.0 mol/L NaCl. Equivalent amounts of each eluate were TCA-precipitated, and detected by SDS-PAGE and autoradiography. As shown in Fig 5, highly similar elution patterns for both proteins were observed; moreover, no ELISA-detectable material was found in either the flow-through or the wash in either case. These results are consistent with the retention of at least some albumin-like properties in the fusion protein HLA.

**Analysis of Thrombin Inhibition and Binding by Fused and Unfused Hirudins**

To compare the ability of HLA and HV1 to inhibit thrombin in a more quantitative manner, kinetic titration experiments were performed. No decrease in the affinity of interaction of the fusion protein with thrombin was detected in experiments in which the concentration of HLA or unfused commercial HV1 required to halve the initial rate of throm-
Purification of His-Tagged HLA

The retention of albumin-like dye-binding characteristics by fusion protein HLA suggested that it might also have acquired the property of slow plasma clearance; its retention of high-affinity thrombin-binding capacity made investigating its clearance worthwhile. To facilitate HLA purification, given the relatively small amounts of protein produced by COS cells (1 to 4 mg/100-mm plate of cells), pSG5-HLA was altered to encode a protein identical to HLA, but with a His6 tag on the C-terminus. After determining that the product of the resulting plasmid, pSG5-HLA(H)6, inhibited thrombin in an indistinguishable manner from HLA lacking the His tag, 30 plates of cells were transfected with the pSG5-HLA(H)6. Their conditioned media was pooled, and HLA(H)6 was purified by Ni2+-chelate affinity chromatography. Examination of material eluted from the nickel column showed that it contained a polypeptide of the expected size (75 kD) and minor quantities of a contaminating protein of 45 kD (Fig 7A, lane 6). This contaminant copurified with the major product on Affi-Gel Blue but, unlike the 75-kD protein, could be removed through its binding to heparin-Sepharose to yield a homogenous preparation (Fig 7A, lanes 6 through 8). The 75-kD species was identified as HLA(H)6, as it reacted both with anti-RSA antibodies and with thrombin, in a blot overlay assay (Fig 7B). Moreover, the purified material inhibited thrombin amidolytic activity in a manner indistinguishable from nontagged HLA. Iodination of this material using the Iodogen method (Fig 8, inset A, HH6) supported the assertion that the material was purified, because no other bands were visible on the autoradiogram.

In Vivo Clearance of Radiolabeled, His-Tagged HLA

The clearance of HLA(H)6 was quantified following injection of three rabbits (weight 2.2 to 2.4 kg) with tracer levels of 125I-labeled HLA(H)6 (32 million cpm protein bound radioactivity each, corresponding to 1.6 mg). The protein-bound radioactivity in blood samples taken over the next 2 weeks was determined, and the residual radioactivity was plotted against time (Fig 8). Even after 2 weeks (336 hours), greater than 2% of the injected material remained in the circulation. Computer-assisted curve peeling revealed three terms in the equation required to describe the clearance curve (see Materials and Methods), suggesting that a three-compartment model was most accurate. The results showed a catabolic half-life of 4.60 ± 0.16 days, a noncirculating vascular half-life of 0.48 ± 0.03 days, and a circulatory half-life of 0.029 ± 0.006 days. Fractional catabolic rates and distributions from these experiments are presented in Table 1, compared with those reported for RSA. Compared with RSA, HLA(H)6 exhibits similar pharmacokinetic behavior, with a somewhat larger distribution into the extravascular space, and a somewhat increased rate of catabolism in the plasma compartment. Compared with catabolic half-life estimates (1/2β values) and fractional catabolic rates for whole-body elimination of hirudin on the order of hours,
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Fig 5. Gel analysis of eluates from Af®-Gel Blue columns. Equivalent amounts of media transfected with pSG5-RSA (RSA), pSG5-HLA (HLA), or pSG5 (CON), and metabolically labeled with 35S-Met and -Cys were chromatographed on separate Af®-Gel Blue minicolumns. Bound material was eluted with the salt concentrations shown below the lanes, and resolved by SDS-PAGE followed by fluorography and autoradiography. Molecular-weight markers (94, 67, 43 kD) are shown at left.

These results suggest that fusion protein HLA acquires the long in vivo residency time of serum albumin.

Two additional experiments were also performed. In the first, an identical dose of 125I-labeled HLA(H)6 as in the full clearance study was used in one rabbit, while another received an equivalent dose of 125I-labeled plasma-derived RSA. Plasma samples were collected over time from both animals, and analyzed on nondenaturing gels (Fig 8, inset A). This mode of electrophoresis allowed separation of the HLA(H)6 from the large, unlabeled albumin band, and showed a clear difference in mobility between labeled fusion protein and labeled RSA in plasma, one that persisted for at least 6 hours. No breakdown products were detected, suggesting that the fusion protein remains intact in vivo. Secondly, we injected the same tracer dose of labeled HLA(H)6 into each of two rabbits, and compared the residual plasma radioactivity after 4 hours with two rabbits injected with an equivalent dose of 125I-labeled commercial HV1; 45% ± 3% of the former, but only 10% ± 4% of the latter remained in the plasma. Urine from both sets of animals was collected, and after 24 hours 76% ± 6% of the HV1-, but only 12% ± 1% of the HLA(H)6-derived radioactivity was found in the urine. These results are consistent with both our more extensive clearance study (Fig 8) and previously published results, and also support the contention that fusion protein HLA(H)6 clears less rapidly than unfused hirudin.

DISCUSSION

The purpose of this investigation was to determine if a genetic fusion of hirudin and albumin could be performed in a way that would yield a novel polypeptide which retained the potent antithrombin activity of hirudin and the slow clearance of serum albumin. COS cell expression was selected over systems in which higher yields are possible, to avoid complicating the characterization of candidate proteins with the necessity to consider de-aggregation or solubilization and refolding protocols. The first priority of this project was to establish functional and separate expression of the two parental proteins to be fused. As shown in Figs 1 and 4, transfection of the synthetic HV3 cDNA into COS cells resulted in the appearance of an antithrombin activity in the conditioned media of comparable affinity to that of commercial HV1 produced in yeast. Similarly, transfection of the full-length RSA cDNA into COS cells resulted in the appearance of recombinant RSA with electrophoretic (Fig 2) and chromogenic (Fig 5) properties indistinguishable from its plasma-derived counterpart.

To maximize the probability of independent folding and independence of action of the two portions of the fusion proteins, a hexaglycine spacer was engineered into both candidate fusion proteins, HLA and ALH. Similar strategies have been successfully used in the generation of heavy- and light-chain Ig fusion proteins.37 HLA, but not ALH, inhibited thrombin-mediated cleavage of both chromogenic and natural substrates (Fig 4). Although it has been reported that even minor perturbations of the amino-terminal region of hirudin interfered with antithrombin activity, our results do not preclude nonspecific explanations of the lack of function of ALH. Examination of the fusion proteins coelectrophoresed on an immunoblot (Fig 3B) suggests that ALH exhibits a faster mobility, indicative either of degradation or misfolding (such as that exhibited by antithrombin Utah in ref 31). The latter explanation is supported by the finding of consistently reduced secretion or synthesis of ALH compared to HLA (not shown). Irrespective of the mechanism(s) involved in the lack of activity of ALH, these results suggested that further characterization of HLA, but not ALH, was warranted.

Both quantitative binding and kinetic experiments showed no large differences in reactivity when the inhibition of thrombin by the fusion protein was compared with its inhibition by unfused hirudin. Kinetic titration experiments (Fig 6) showed near equivalence in terms of the concentration of inhibitor required to reduce the initial rate of thrombin inhibition by twofold (40 nmol/L HV1 v 30 nmol/L HLA). Similarly, in a binding assay in which 125I-labeled thrombin could bind to either immobilized HV1, or soluble HLA, or vice versa, highly similar IC50 values (73 pmol/L for HLA
the case of HLA, this level of injected protein was still present in the circulation after 2 weeks. This represents an increase in catabolic half-life from 0.7 hours\(^{36}\) to \(4.60 \pm 0.16\) days. This value is much closer to that obtained for the clearance of radiolabeled RSA in rabbits (5.55 \(\pm 0.11\) days\(^{39}\) and suggests that via incorporation into HLA, the clearance of HV3 has been made much more like that of RSA than HV1. Gel analysis of HLA recovered from rabbit blood after injection, compared with RSA treated in an identical fashion, renders unlikely the possibility of separation of the hirudin and albumin domains in HLA (Fig 8, inset A). Reduced clearance is also supported by our finding of decreased levels of radioactivity derived from injected labeled proteins in the urine of rabbits treated with HLA, as opposed to unfused hirudin.

Previous efforts to increase the half-life of hirudin in the circulation have included addition of dextran particles and polyethylene glycol chains to the inhibitor, resulting in an increase in \(t_{1/2}\beta\) of approximately one order of magnitude\(^{14,15}\); we have demonstrated in this study that gene fusion increases the \(t_{1/2}\beta\) of hirudin by greater than two orders of magnitude (160-fold). In addition, coupling procedures that use reactive amino groups will, at least in part, modify the N-terminus of hirudin, a region where even minor modifications can

![Graph](image)

**Fig 6.** Kinetic titration of fused and unfused hirudin as an inhibitor of thrombin-mediated amidolysis. The initial velocity of the reaction of thrombin with chromogenic substrate S2238 was determined in the presence of increasing concentrations of either unfused (HV1, top graph) or fused hirudin (HLA, bottom graph). Results shown are from one paired experiment that is representative of a total of three.

\(v\) 95 pmol/L for HV1) were obtained. The observation that HLA was slightly more inhibitory in both sets of experiments than unfused HV1 may derive from the incorporation of HV3 rather than HV1 into the fusion protein, because this hirudin variant exhibits a threefold to fourfold increase in affinity for thrombin compared with HV1.\(^{32}\) These quantitative results confirm the retention of potent antithrombin activity characterized by fusion protein HLA.

These findings, together with the retention of albumin-like dye-binding characteristics by HLA (Fig 5), prompted us to investigate the clearance of HLA in vivo. This experiment was made feasible by the generation of an His-tagged HLA that could be purified in the microgram quantities necessary for radiolabeling. In previous investigations of the clearance of radiolabeled HV1 in rabbits, 2 hours after injection only 2% of the HV1 remained in the circulation\(^{36}\); in

![Graph](image)

**Fig 7.** Purification and characterization of HLA(Hi)\(_6\). (A) A 10% SDS gel stained with Coomassie Blue, on which aliquots of fractions containing HLA(Hi)\(_6\) were electrophoresed at different stages of its preparation. Lane 2, conditioned media from Cos cells transfected with pSG5-HLA(Hi)\(_6\); lane 3, flow-through from Ni-NTA-agarose column; lanes 4 and 5, wash fractions with and without Tween 20; lane 6, peak of eluate from Ni-NTA-agarose column; lane 7, eluate from Affi-Gel Blue; lane 8, flow-through from heparin-Sepharose. Lane 9 contains 1.0 \(\mu\)g of plasma-derived RSA, and lane 1 shows molecular weight markers (same as Fig 3). (B) Additional electrophoresis of aliquots of samples shown in lanes 7 and 9 in (A); the gel was cut in three, and portions stained with Coomassie Blue (Stain), immuno-blotted with anti-RSA as in Fig 3, or blot-overlaid with thrombin (IIa).
Fig 8. Clearance of HLA(H)6 from the rabbit circulation. Rabbits were injected with 125I-HLA(H)6, and the fraction of the protein-bound radioactivity remaining in the circulation was determined over time (n = 3, ±SD). (Inset A) An autoradiogram of a 10% polyacrylamide nondenaturing gel (Native), on which 5 μL of plasma from rabbits injected with HLA (first four lanes) and RSA (last four lanes) have been electrophoresed. (Inset B) An autoradiogram of a 10% SDS gel (SDS) on which aliquots of radiiodinated purified HLA(H)6 (HH6), and purified plasma-derived RSA (RSA) have been electrophoresed.

have negative effects on function.38 Such heterogeneity is also expected to occur in approaches that use chemical cross-linking to form adducts of hirudin with other proteins, eg, streptokinase40 and albumin.41 In neither instance was the fate of these adducts determined in vivo.

Clinical trials comparing hirudin to heparin and other conventional therapies have had mixed results, suggesting potential advantages of hirudin in the prevention of deep-vein thrombosis.42 However, unexpectedly high rates of bleeding complications in the setting of coronary thrombolysis suggest a relatively narrow window of safety.43,44 The availability of hirudins with longer durations of action could be relevant to this problem, in that the ability to use lower, but longer-acting, doses could conceivably expand this window. Irrespective of the future uses of hirudin per se, our results suggest that gene fusion to long-lived plasma proteins is a feasible means to extend the availability of anticoagulant peptides, and provide the impetus to scale-up production of HLA to study its biologic effects in vivo. With respect to this last point, it would clearly have been informative to have shown prolonged effects of HLA, rather than simply its prolonged presence in vivo; however, the minimum dose required for such an effect exceeded our capacity to generate HLA protein in COS-1 cells. We are in the process of full characterization of HLA produced in yeast and anticipate that this expression system will allow us to determine the effectiveness of HLA as an antithrombotic agent in future experiments.

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