Plasma protein binding can be an effective means of improving the pharmacokinetic properties of otherwise short lived molecules. Using peptide phage display, we identified a series of peptides having the core sequence DICLPRWGCLW that specifically bind serum albumin from multiple species with high affinity. These peptides bind to albumin with 1:1 stoichiometry at a site distinct from known small molecule binding sites. Using surface plasmon resonance, the dissociation equilibrium constant of peptide SA21 (Ac-RLIEDICLPRWGCLWE-DEDDNH2) was determined to be 266 ± 8, 320 ± 22, and 467 ± 47 nM for rat, rabbit, and human albumin, respectively. SA21 has an unusually long half-life of 2.3 h when injected by intravenous bolus into rabbits. A related sequence, fused to the anti-tissue factor Fab of D3H44 (Presta, L., Sims, P., Meng, Y. G., Moran, P., Bullens, S., Bunting, S., Schoenfeld, J., Lowe, D., Lai, J., Rancatore, P., Iverson, M., Lim, A., Chisholm, V., Kelley, R. F., Riederer, M., and Kirchhofer, D. (2001) Thromb. Haemost. 85, 379–389), enabled the Fab to bind albumin with similar affinity to that of SA21 while retaining the ability of the Fab to bind tissue factor. This interaction with albumin resulted in reduced in vivo clearance of 25- and 58-fold in mice and rabbits, respectively, when compared with the wild-type D3H44 Fab. The half-life was extended 37-fold to 32.4 h in rabbits and 26-fold to 10.4 h in mice, achieving 25–45% of the albumin half-life in these animals. These half-lives exceed those of a Fab and are comparable with those seen for polyethylene glycol-conjugated Fab molecules, immunoadhesins, and albumin fusions, suggesting a novel and generic method for improving the pharmacokinetic properties of rapidly cleared proteins.

The effectiveness of recombinant protein pharmaceuticals depends heavily on the intrinsic pharmacokinetics of the natural protein. Because the kidney generally filters out molecules below 60 kDa, efforts to reduce clearance have focused on increasing molecular size through protein fusions, glycosylation, or the addition of polyethylene glycol polymers (i.e. PEG). For example, fusions to large long lived proteins such as albumin (1, 2) or the Fc portion of an IgG (3), the introduction of glycosylation sites (4), and conjugation with PEG (5–7) have been used. Through these methods, the in vivo exposure of protein therapeutics has been extended.

Small molecule drugs have long relied on their association with various plasma components to improve their pharmacokinetic properties in vivo; however, a drug associated with plasma protein is usually unavailable for binding to the target although its half-life is extended. Since only the unbound fraction of the small molecule is generally functionally active, a fine balance must be maintained between the concentration of free drug required for efficacy and the frequency at which it must be administered (8).

Albumin (molecular mass ~67 kDa) is the most abundant protein in plasma, present at 50 mg/ml (600 μM), and has a half-life of 19 days in humans (9, 10). Albumin serves to maintain plasma pH, contributes to colloidal blood pressure, functions as carrier of many metabolites and fatty acids, and serves as a major drug transport protein in plasma. There are several major small molecule binding sites in albumin that have been described. Warfarin is known to bind at site I, benzodiazepines and indoles at site II, and cardenolides and biliary acids at site III. In addition, there is an important metal ion binding site.

Noncovalent association with albumin has been shown to extend the half-life of short lived proteins. A recombinant fusion of the albumin binding domain from streptococcal protein G to human complement receptor type I increased its half-life 3-fold to 5 h in rats (11). In addition, fusion to this domain has served to enhance the immunological response directed to peptide antigens (12). In another example, when insulin was acylated with fatty acids to promote association with albumin (13, 14), a protracted effect was observed when injected subcutaneously in rabbits or pigs. Together, these studies demonstrate a linkage between albumin binding and prolonged action.

In this report, peptide phage display was used to develop peptides that selectively bind albumin with high affinity. These peptides bind to albumin from multiple species at a novel site distinct from the known classical binding sites. To test whether association of a short lived protein with albumin could improve cellular homeostatic effects, cell counts, and protein half-life.
its pharmacokinetic properties, one albumin binding peptide was added to a Fab through the use of a simple recombinant fusion that rendered it capable of binding albumin without affecting antigen binding. We demonstrate this approach as a viable route to increasing the half-life of potentially important protein pharmaceuticals.

MATERIALS AND METHODS

Phage Libraries and Selection Conditions—Eight phage libraries expressing random peptide sequences fused to the major coat protein, PBS (15), were pooled into four groups: pool A contained CXGXPX, CXGXXGXX, and CXGXX, where j = 4; pool C contained CX and CXGXX, where j = 4–6; pool D contained CXGXX, where j = 7–10. X represents any of the 20 naturally occurring L-amino acids, and in pools A and B, i + j + k = 18 and [i − k] < 2. Each library has in excess of 10^10 clones.

The phage library pools were suspended in binding buffer (PBS, 1% ovalbumin, 0.05% Tween 20) and sorted against rabbit, rat, or human albumin (Sigma) immobilized directly on Maxisorp plates (Nunc, Roskilde, Denmark) at 10 μg/ml in PBS overnight at 4 °C. Plates were blocked for 1 h at 25 °C using PBS, containing 1% ovalbumin except for round 4, where a Tris-buffered saline-casein blocker (Pierce) was used. Phage were added to a binding buffer for 2 h. Unbound phage were removed by washing with PBS, 0.05% Tween 20, and bound phage were eluted with 500 mM KC1, 10 mM HCl, pH 2.5. Eluted phage were propagated in XL1-Blue cells with VCSM13 helper phage (Stratagene, La Jolla, CA). Enrichment was monitored by titrating the number of phage that bound to an albumin-coated well compared with a well coated with ovalbumin or casein.

Phage Binding Assay—Phage clones (~10^11 phage) were added to Maxisorp plates coated with mouse, rat, rabbit, bovine, rhesus, or human albumin (Sigma) as described above. The microtiter plate was washed with PBS, 0.05% Tween 20, and bound phage were detected following incubation with HRP/anti-M13 conjugate (Amersham Biosciences) in PBS, 0.05% Tween 20. The amount of HRP bound was measured using ABTS/H2O2 substrate (Kirkegard & Perry Laboratories, Gaithersburg, MD) and monitoring the absorbance at 405 nm.

Partial and Complete Randomization on Monovalent Phage—A soft randomized library was designed using an oligonucleotide coding for clone RB but synthesized with a 70:10:10:10 mixture of bases as described (16). Both the soft randomized and fully randomized libraries were sorted against rat, rabbit, and human serum albumin as above.

Peptide Synthesis—Peptides were synthesized by either manual or automated (Milligen 9050) Fmoc (N-(9-fluorenlyl)methoxy carbonyl)-based solid phase synthesis on a 0.25-mmol scale using a PEG-polystyrene resin as described (17). The carboxyl terminal lysine of peptide SA06 was derivatized with N-hydroxysuccinimide-LC-biotin as recommended by the manufacturer (Pierce) and purified by reverse phase high pressure liquid chromatography, yielding SA08b (Ac-QGLIGDICLPRWGCGLWDVK-NH2, where K represents lysine- biotin).

Peptide Competition Assay—Rat, rabbit, or mouse albumin was immobilized directly on Maxisorp plates and blocked as above. Samples, serially diluted in binding buffer were added to the plate, followed immediately by the addition of 10 mM SA08b for 1 h at 25 °C. SA08b has an EC50 of 2 and 4 ng/ml for rat and rabbit albumin, respectively. The microtiter plate was washed with PBS, 0.05% Tween 20, and bound SA08b was detected with streptavidin/HRP (Roche Molecular Bio- sciences) in PBS, 0.05% Tween 20. The amount of HRP bound was measured using ABTS/H2O2 substrate as above.

Affinity Measurements by Surface Plasmon Resonance—The binding affinities between SA peptides and albumin were obtained using a BIACore 3000 (BIACore Inc., Piscataway, NJ). Human, rabbit, and rat albumin were captured on a CM5 chip using amine coupling at ~5000 resonance units. SA peptides at 0, 0.625, 1.25, 2.5, 5, and 10 μM were injected at a flow rate of 20 μl/min for 30 s. The bound peptides were allowed to dissociate for 5 min before matrix regeneration using 10 mM glycine, pH 3. The signal from an injection passing over an uncoupled cell was subtracted from that of an immobilized cell to generate sensograms of the amount of peptide bound as a function of time. The running buffer, PBS containing 0.05% Tween 20, was used for all sample dilutions. BIACore kinetic evaluation software (version 3.1) was used to determine Ka, from the association and dissociation rates using a one-to-one binding model.

Pharmacokinetic Study of SA21 in Rabbits—Three male New Zealand White rabbits were administered an intravenous bolus dose of 2 mg/kg of SA21 in PBS. Eighteen blood samples were collected at serial time points just prior to dosing and from 1 min to 21 days postdosing. Samples were collected in tubes containing sodium citrate as an anti- coagulant and then centrifuged, and the plasma portion was frozen at 70 °C until analysis using an electrospray ionization, LC/MS/MS method.

The mass spectrometer was used an API 4000 (Applied Biosystems/ MDS Sciex, Foster City, CA). The autosampler was a CTC PAL System (Leap Technologies, Chapel Hill, NC) equipped with a cooling stack. The mass spectrometer was a QTrap 4000 in positive ion mode with an electrospray vaporizer. Data was collected using Analyst 1.5.2 software. MDS Sciex, Foster City, CA). The autosampler was a CTC PAL System (Leap Technologies, Chapel Hill, NC) equipped with a cooling stack. The mass spectrometer was a QTrap 4000 in positive ion mode with an electrospray vaporizer. Data was collected using Analyst 1.5.2 software.
with PBS, 0.05% Tween 20, and 50 nM biotinylated TF in binding buffer was added for 1 h. The microtiter plate was washed with PBS, 0.05% Tween 20, and streptavidin-HRP was added. After a final wash, bound HRP was measured as above.

Pharmacokinetics of D3H44 Variants in Rabbits and Mice—Groups of New Zealand White rabbits were given an intravenous bolus of 400–525 µg/kg D3H44 variants (D3H44 Fab, D3H44-L, D3H44-Ls) into the marginal ear vein. Plasma samples were obtained from an arterial catheter placed in the contralateral ear over a 21-day period for analysis by TF ELISA (see below). Individual plasma concentration versus time profiles were considered to have fallen in the linear range of the standard curve was used to calculate MD), and the change in absorbance was monitored at 450 nm. Data were averaged for each treatment group. Differences between groups were determined by analysis of variance, with significance at p < 0.05.

Groups of nine BALB/c mice received a 5.0 mg/kg intravenous bolus of D3H44 Fab or D3H44-L into the tail vein. Plasma samples were obtained by eye bleed from three mice per time point over 2–9 days and assayed for concentration of D3H44 using the TF ELISA. The average plasma concentration was obtained for each time point and fitted to a two-compartment elimination model using WinNonlin version 3.0 (Pharsight, Inc., Mountain View, CA). The pharmacokinetic parameters of clearance, V, steady state volume of distribution, t, AUC, and AUC corrected for actual dose administered (AUC/dose) were obtained by eye bleed from three mice per time point over 2–9 days and the change in absorbance was monitored at 450 nm. Data were averaged for each treatment group. Differences between groups were determined by analysis of variance, with significance at p < 0.05.

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Quantitation of D3H44 Variants—The concentration of D3H44 Fab, D3H44-L, and D3H44-Ls in rabbit plasma was determined using a TF ELISA. Maxisorp plates were coated overnight at 4 °C with 1 µg/ml TF (Genentech, Inc., South San Francisco, CA) in 50 mM sodium carbonate/plasma and the standard curve range was 0.31–5000 nM. Biotinylated TF in binding buffer, pH 9.6. Plates were blocked with 0.5% ovalbumin in PBS, pH 7.4. Diluted antibody standards (0.25–50 ng/ml) and samples (minimum dilution 1:100) in PBS containing 0.5% ovalbumin, 0.05% polysorbate 20, 0.35% NaCl, 5 mM EDTA, 0.25% CHAPS, 0.2% bovine γ-globulins (Sigma), and 1% plasma were added to the plates for 2 h. Antibody bound to the plates was detected with HRP-conjugated goat anti-human Fab’2 antibody (Jackson ImmunoResearch, West Grove, PA). Bound HRP was measured using the substrate 3,3’,5,5’-tetramethyl benzidine/H2O2 (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the change in absorbance was monitored at 450 nm. Data falling in the linear range of the standard curve was used to calculate D3H44 concentrations in the samples. D3H44 Fab and D3H44-L in mouse plasma were assayed in the same enzyme-linked immunosorbent assay except that samples were diluted in buffer without 1% albumin, and the standard curve range was 0.31–40 ng/ml.

RESULTS

Identification and Maturation of Peptides That Bind to Serum Albumin—Naive peptide phage library pools A through D were selected against rat, rabbit, and human albumin. Each pool, with the exception of pool D when human albumin was the target, showed enrichment for each species of albumin. The sequences from the enriched pools revealed in each case that a single clone had taken over the pool. The inferred peptide sequences from these clones are shown in Table I.

Interestingly, albumin has greater than 70% amino acid sequence identity between these species, yet unique peptide sequences originating from within a given phage library pool were identified for each species. The sequence similarity observed between clones HB and HC, RA and RD, and RB and RC, despite their origins from independent phage library pools, suggests the importance of the homologous residues in binding to the respective species of albumin. Individual phage clones were examined using a phage binding assay, a quick qualitative screen to assess species selectivity. Whereas phage clones generally bound only to the albumin for which they were selected, clones HB and HC, selected for binding to human albumin, also bound to rat albumin; and clone RB, selected for binding to rat albumin, bound albumin from all three species (Table I). None of the phage clones bound to structurally unrelated ovalbumin, indicating that the interaction with albumin was specific.

Because of its broad recognition of rat, rabbit, and human albumin, clone RB was chosen for sequence maturation on

| Library Pool | Clones Selected for Binding to Human Serum Albumin | Phage Binding |
|-------------|-------------------------------------------------|---------------|
| HA          | EYRSFDTDPWAKKGDRLPL | +++ | - |
| HB          | RPESFVYKEMETEFSEQ | ++ | - |
| HC          | EMQTPPVWLM | ++ | - |
| BA          | GENVGTFSLMAECKGQVM | +++ | - |
| BB          | MEOFAFGYEGIWECLMHEGK | +++ | - |
| BC          | DLODVTDFWF | +++ | - |
| BD          | KSQSELHMLVLVEGSLF | +++ | - |
| RA          | RNECDLIVVLEMGLEMWAYV | +++ | - |
| RB          | QRYMDPCLPWGLWOLGDGF | +++ | - |
| RC          | GHIQLGIDLPCWGLWOLGDGF | +++ | - |

| Clones Selected for Binding to Rat Serum Albumin | Phage Binding |
|------------------------------------------------|---------------|
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |

TABLE I

Sequences of phage clones selected from polyvalent naive libraries for binding rat, rabbit, or human albumin

The locations of fixed cysteines from the library design are shaded. Sequence identity among clones derived from different phage libraries is boxed. A qualitative assessment of the ability of phage bearing the indicated peptide sequence to bind human (HSA), rabbit (BuSA), or rat (RSA) albumin is indicated.

| Clones Selected for Binding to Rat Serum Albumin | Phage Binding |
|------------------------------------------------|---------------|
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |

TABLE II

Sequences of phage clones selected for binding rat, rabbit, or human albumin following soft randomization of clone RB

Amino acids positions identical in clone RB, the starting sequence used for soft randomization are shaded. A qualitative assessment of the ability of phage bearing the indicated peptide sequence to bind human (HSA), rabbit (BuSA), rat (RSA), bovine (BSA), rhesus (RhSA), and mouse (MSA) albumin is indicated (nd indicates not determined).

| Clones Selected for Binding to Rat Serum Albumin | Phage Binding |
|------------------------------------------------|---------------|
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |
| RB      | QRYMDPCLPWGLWOLGDGF | +++ | + +++ |
| RC      | GHIQLGIDLPCWGLWOLGDGF | +++ | + +++ |

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other species including bovine, rhesus, and mouse albumin (Table II).

Since at any given position, the amino acid present in the parent sequence was designed to appear approximately half the time in these libraries, only fully conserved positions are likely to indicate important structural or contact elements that support albumin binding. A final library that kept these highly selected residues (underlined) constant, XDXCLPXYWGCLWX₄, while allowing all 20 amino acids at the 11 remaining positions allowed a more extensive search of pertinent sequence space. The sequence preferences at each randomized position resulting from selection against rabbit albumin are shown in Fig. 1. A similar profile was observed from sequences selected for binding rat and human albumin (not shown). For each species of albumin, there was a strong preference for Ile at position 7 and Arg at position 11, thus generating a core consensus of DICLPRWGCLW. Additionally, there was a general preference for negatively charged residues (Asp or Glu) at positions flanking this core, particularly on the carboxyl terminus.

Characterization of Albumin Binding Peptides—Several peptides patterned after the sequences selected for albumin binding were synthesized. Their binding to human, rabbit, and rat albumin was assessed by Biacore, and a peptide competition assay was used to assess their affinity for rabbit and mouse albumin (Tables III and IV). The IC₅₀ values obtained for binding to rabbit albumin compared favorably with IC₅₀ values determined by Biacore (Table III). In comparison with rabbit and rat albumin, the peptides bind more weakly to human and more tightly to mouse albumin; however, the rank affinity of a given peptide is generally maintained from species to species. Peptide SA15, representing the consensus for binding rabbit albumin (Fig. 1), had the lowest IC₅₀ value in the peptide binding assay and highest affinity by surface plasmon resonance for rabbit albumin (Table III). A linear peptide, identical to SA06 but with both Cys residues changed to Ala, had an IC₅₀ greater than 50 µM, demonstrating the importance of the disulfide. In addition, the affinity of the peptides for rabbit albumin was assessed by Biacore, and a peptide competition assay was used to assess their affinity for rabbit and mouse albumin (Tables III and IV). The IC₅₀ values obtained for binding to rabbit albumin compared favorably with IC₅₀ values determined by Biacore (Table III).

**Table III**

**Comparison of the peptide competition assay with Biacore for selected peptides**

Selected peptides were tested for binding human (HSA), rabbit (BuSA), rat (RSA) and mouse (MSA) albumin using the peptide competition assay or surface plasmon resonance as indicated and described under "Materials and Methods."

| Peptide | Sequence | Peptide competition assay (IC₅₀, nM) | Surface plasmon resonance (kd, nM) |
|---------|----------|-------------------------------------|-----------------------------------|
|         |          | BuSA      | MSA      | BuSA  | MSA  |
| SA21    | Ac-R L I E D I C L P R W G C L W E D D -NH₂ | 270 ± 110 | 7 ± 2 | 467 ± 47 | 320 ± 22 | 266 ± 8 |
| SA06    | Q R L M E D I C L P R W G C L W E D D F -NH₂ | 130 ± 50 | 6 ± 2 | 803 ± 62 | 143 ± 5 | 229 ± 9 |
| SA08    | Ac-Q G L I G D I C L P R W G C L W G D S V K -NH₂ | 51 ± 11 | 12 ± 2 | 858 ± 59 | 108 ± 5 | 158 ± 3 |
| SA15    | G E W E D I C L P R W G C L W E E D -NH₂ | 13 ± 12 | 5 ± 1 | 878 ± 58 | 65 ± 3 | 150 ± 5 |

**Table IV**

**The effect of peptide length on binding rabbit albumin in the peptide competition assay**

| Peptide | Sequence | IC₅₀ (nM) |
|---------|----------|----------|
| SA20    | Ac-Q R L I E D I C L P R W G C L W E D D F -NH₂ | 260 |
| SA21    | Ac-R L I E D I C L P R W G C L W E D D -NH₂ | 270 ± 110 |
| SA22    | Ac-R L I E D I C L P R W G C L W E D -NH₂ | 430 ± 170 |
| SA29    | Ac-R L I E D I C L P R W G C L W E D -NH₂ | 420 ± 90 |
| SA31    | Ac-R L I E D I C L P R W G C L W -NH₂ | 200 |
| SA33    | Ac-R L I E D I C L P R W G C L -NH₂ | 4310 ± 2770 |
| SA35    | Ac-R L I E D I C L P R W G C L -NH₂ | >250,000 |
| SA23    | Ac- L I E D I C L P R W G C L W E D -NH₂ | 360 ± 140 |
| SA24    | Ac- I E D I C L P R W G C L W E D -NH₂ | 1380 ± 410 |
| SA25    | Ac- E D I C L P R W G C L W E D -NH₂ | 2730 ± 1300 |
| SA26    | Ac- D I C L P R W G C L W E D -NH₂ | 3120 ± 660 |
| SA27    | Ac- I C L P R W G C L W E D -NH₂ | 86,700 ± 21,800 |
| SA28    | Ac- C L P R W G C L W E D -NH₂ | >400,000 |
| SA30    | Ac- I E D I C L P R W G C L W -NH₂ | 1800 ± 590 |
| SA32    | Ac- E D I C L P R W G C L W -NH₂ | 2170 ± 520 |
| SA04    | D I C L P R W G C L W -NH₂ | 8540 ± 4620 |
| SA34    | Ac- D I C L P R W G C L -NH₂ | 28,210 ± 6500 |
| SA19    | D I C L P R W G C L -NH₂ | 24,510 ± 2100 |
| SA18    | I C L P R W G C L W -NH₂ | 124,900 |
| SA36    | Ac- I C L P R W G C -NH₂ | >250,000 |
amin diminished with reduction in the length of the peptides (Table IV). A core of about 10 amino acids (SA34 and SA19, Table IV) having an IC50 of ~25 μM, could be improved 6-fold by the addition of 4 residues to its amino terminus (SA33) or 8.6-fold by the addition of 3 residues to its carboxyl terminus (SA26). The addition of all 7 residues resulted in a 60-fold improvement in the IC50 (SA22), indicating that these additions have an additive effect.

**Characterization of the Albumin Binding Site**—When the binding of RB-B8 or RB-H1 phage to rabbit albumin was monitored over a pH range from 2.9 to 9.0, optimum binding was observed above pH 6.0 for both clones (data not shown). Binding decreased below pH 6.0 until no binding was observed at pH 2.9. A similar pattern was observed for the binding of these clones to human and rat albumin. The similar amino acid preferences and pH profile are consistent with a similar binding environment on each species of albumin.

Since albumin plays an important role as a carrier of many ligands and drugs, we tested whether known albumin ligands might compete with peptide binding. The addition of site I (indomethacin, phenylbutazone, warfarin) or site II (ibuprofen, aspirin, L-tryptophan, dansylsarcosine, diazepam) ligands, a fatty acid (myristic acid), or a metal ion (CuCl2) at concentrations up to 100 μM had no effect on SA08b peptide binding to rat or rabbit albumin in the peptide competition assay (not shown).

We were curious as to whether unrelated clones initially identified for binding to albumin (Table I) might compete with our matured multispecies binding peptides. Whereas RD and BA phage selectively bind only to rat and rabbit albumin, respectively, these clones were clearly blocked by the addition of SA08 (Fig. 2). In contrast, binding of clones HA and HB to human albumin was not blocked by SA08 and thus bind to a different site.

**Pharmacokinetics of SA21**—In vivo, peptides can be rapidly metabolized or eliminated due to glomerular filtration, resulting in a short half-life. We hypothesized that association with albumin would result in a peptide with improved pharmacokinetics and chose to study SA21 because it was stable in citrated rabbit serum in vitro over a 24-h period at 37 °C as monitored by LC/MS/MS. With its high affinity for rabbit albumin (Table III) and the high concentration of albumin in plasma, we calculated that SA21 should remain greater than 99.95% bound to rabbit albumin in vivo.

The pharmacokinetic profile of SA21 in rabbits is shown in Fig. 3 compared with an unrelated control peptide of similar size, 1a (Ac-ALCDNPRIDRWYCQFVEG-NH2) and an engineered variant that binds to albumin, 1m (an amino-terminal naphthalene acyl sulfonamide derivative of 1a) (22). SA21 showed reduced clearance compared with 1a and 1m with a significantly longer half-life of 2.3 h compared with 7.6 and 30 min, respectively (Table V).

**Characterization of Albumin Binding Fab Fusions**—Compared with an IgG, Fab fragments have relatively fast clearance of ~42–72 ml/kg/h in rabbits (23). D3H44 (18) is a humanized antibody that binds human TF and acts as an anticoagulant. To test whether association of the D3H44 Fab with albumin can increase its half-life in vivo, the SA06 sequence was recombinantly fused through a short flexible linker to the carboxyl terminus of the light chain, yielding D3H44-L. D3H44-Ls was also constructed and lacks the disulfide linking the light and heavy chains of the Fab. D3H44-Ls was designed to avoid potential folding problems that may be caused by the addition of a disulfide-bonded peptide. The addition of SA06 provided a simple purification scheme utilizing a TF affinity column followed by an albumin affinity column. Although D3H44-L and D3H44-Ls were judged to be greater than 90% pure following the TF affinity column alone, only 14% of the D3H44-L was bound and retained on the subsequent rabbit albumin affinity column in contrast to 54% of the D3H44-Ls. The higher overall yield obtained with D3H44-Ls suggested improper folding of the SA06 disulfide due to its proximity to the interchain disulfide between the heavy and light chains.

Following both column purification steps, D3H44-L was incubated with TF, rabbit albumin, or both and analyzed by analytical gel filtration along with SDS-PAGE analysis. The shift in the retention time of the eluting fractions was consistent with a 1:1 stoichiometry between D3H44-L and either TF or rabbit albumin and a stoichiometry of 1:1:1 in the presence of both TF and rabbit albumin. For example, the retention time of
the peak that contained rabbit albumin and D3H44-L by SDS-PAGE analysis suggested a molecular mass of 123 kDa compared with the calculated molecular mass of 119 kDa for a 1:1 complex.

The affinity of purified Fab fusions for rabbit albumin and TF were examined in the following assays. First, in comparison with SA06, both D3H44-L and D3H44-Ls had a similar ability to compete for binding to immobilized rabbit albumin. In contrast, D3H44 Fab is unable to bind to rabbit albumin (Fig. 4). Second, D3H44-L and D3H44-Ls not only bind to TF but inhibit its function to the same degree as D3H44 Fab (Fig. 5a). Third, to further investigate whether the binding of rabbit albumin to D3H44-L or D3H44-Ls would preclude binding to TF, an albumin/TF sandwich assay was used (Fig. 6). In this assay, binding to immobilized rabbit albumin was detected with biotinylated TF. The results demonstrate that D3H44-L and D3H44-Ls are able to simultaneously bind albumin and TF, whereas D3H44 Fab is unable to bind albumin and thus does not generate a signal upon the addition of biotinylated TF.

Consistent with these assays, D3H44-L and D3H44-Ls also prolong the prothrombin time assay that measures TF-dependent clotting in human plasma (Fig. 5b). Since D3H44-L and D3H44-Ls bind more tightly to rabbit than to human albumin, they were also tested in a prothrombin time assay using rabbit plasma where clotting was initiated with human TF. Similar results were obtained (not shown). Taken together, D3H44-L and D3H44-Ls have essentially equivalent combined functions of the D3H44 Fab and an albumin binding peptide, and these two functions do not interfere with each other.

Pharmacokinetic Analysis of D3H44 Variants—The pharmacokinetics of D3H44 Fab, D3H44-L, and D3H44-Ls were compared in rabbits (Fig. 7a, Table V). The clearance of D3H44-L and D3H44-Ls decreased 58- and 43-fold, and the half-life increased ~40-fold to 32.4 and 38.3 h, respectively, compared with 0.8 h for D3H44 Fab (Fig. 7a). In mice, D3H44-L had a 25-fold reduction in clearance compared with D3H44 Fab, consistent with the results obtained in the rabbits (Fig. 7b and Table V). D3H44-L had a half-life of 10.4 h, representing a 26-fold increase over the D3H44 Fab.

**TABLE V**

| Rabbit parameter | Units | SA21 | D3H44 Fab | D3H44-L | D3H44-Ls | Mouse parameter | Units | Fab estimate | D3H44-L estimate |
|------------------|-------|------|-----------|--------|---------|----------------|-------|--------------|----------------|
| Dose mg/kg       |       | 2.00 | 0.40      | 0.47   | 0.52    | Dose mg/kg     |       | 5.0          | 5.0            |
| AUC/dose (b-µg/ml)/ (mg/kg) | 77.7  | 4.1  | 14.80     | 3.1    | 840     | 78             | 633   | 157          |                |
| CL ml/h/kg       | 12.9  | 0.7  | 69.9      | 16.2   | 1.20    | 0.11           | 1.64  | 0.37         |                |
| t 1/2 h          | 2.31  | 0.24 | 0.876     | 0.213  | 32.4    | 3.2            | 38.3  | 8.8          |                |
| V 1 ml/kg        | 42.8  | 2.9  | 90.6      | 36.4   | 56.2    | 10.1           | 87.6  | 1.7          |                |
| V ss ml/kg       | 221   | 95   | 113       | 7      | 176     | 11             |       |              |                |

FIG. 4. D3H44-L and D3H44-Ls bind to rabbit albumin with similar affinity to SA06. A comparison of SA06 (○), D3H44-L (●), D3H44-L (△, dashed line), and D3H44 Fab (●) in the peptide competition assay using immobilized rabbit albumin. Lines represent the data fit to a four-parameter equation from which the following IC 50 values were calculated for this representative data set: SA06, 80 nM; D3H44-L, 130 nM; D3H44-Ls, 50 nM; D3H44 Fab, >100 µM.

**DISCUSSION**

A variety of albumin binding peptide phage were identified from the naïve peptide libraries that were screened. During the affinity maturation of clone RB, which gave rise to a series of peptides that recognize albumin from multiple species, the core sequence DIQLPRWGCWL was identified. Although a linear peptide, having both Cys substituted with Ala, demonstrated the importance of the disulfide, none of the matured peptides examined by NMR appeared to be structured in solution.² Interestingly, SA08, a matured sequence derived from libraries patterned after the sequence of the multispecies binding clone RB, inhibited the binding of clones RD and BA, which selectively bound rat and rabbit albumin, respectively. Although they shared no sequence similarity, they may share the same or overlapping binding site(s) on albumin. SA08 does not appear to compete with classical site I or site II albumin binding ligands; nor does it affect binding at fatty acid and metal binding sites. Peptide binding to albumin is also unaffected over a broad pH range from 6 to 9. Apparently, this conserved peptide binding site on albumin is unique and remains unaltered above pH 8, where albumin is known to undergo a conformational change (9). Whether conformational changes in the structure of albumin at low pH or side chain titration contributes to a loss of peptide binding remains to be determined.

Most peptides are rapidly cleared in vivo as a result of metabolism and renal filtration (24). The 2.3-h half-life of SA21 in rabbits is relatively long when compared with other peptides of similar size, presumably due to its association with albumin. The half-life of the coagulation factor VIIa peptide exosite inhibitor 1a, for example, is only 7.6 min in rabbits but can be prolonged 4-fold by the addition of aromatic groups to its amino terminus to induce binding to albumin (22). Whereas both peptides were stable in citrated rabbit serum for 24 h, differences in metabolism in vivo may occur. Alternatively, differences in the affinities of SA21 and 1m for rabbit albumin may explain the superior pharmaco-

² N. Skelton, unpublished result.
kinetic profile observed for SA21 (Fig. 3); the affinity of 1m for rabbit albumin was not reported.

In another example, the in vivo response to insulin was prolonged using insulin derivatives acylated with fatty acids to enable association of the hormone with albumin (14). Unlike these chemically modified peptides, however, the association of SA21 with albumin is achieved through an amino acid peptide fragment.

**Fig. 5.** Effect of D3H44-L and D3H44 Fab on FX Activation and the prothrombin time (PT). a, inhibition of TF:FVIIa-mediated FX activation by D3H44-L (●), D3H44-Ls (△, dashed line), and D3H44 Fab (○). Data from three independent experiments were fit to a four-parameter equation from which the following IC50 values were calculated: D3H44-L, 0.38 nM; D3H44-Ls, 0.33 nM; D3H44 Fab, 0.23 nM. b, the fold prolongation of TF-dependent clotting by D3H44-L (●), D3H44-Ls (△), and D3H44 Fab (○) in the human prothrombin time assay. Uninhibited clotting time was 9.6 s.

**Fig. 6.** D3H44-L can bind tissue factor and albumin simultaneously. The binding of D3H44-L (●), D3H44-Ls (△, dashed line), and D3H44 Fab (○) to immobilized rabbit serum albumin was detected using biotinylated TF followed by streptavidin-HRP.

**Fig. 7.** Pharmacokinetics of D3H44-L and D3H44 Fab in rabbits and mice. a, D3H44-L (●), D3H44-Ls (△, dashed line), and D3H44 Fab (○) were dosed at 0.40–0.52 mg/kg into New Zealand White rabbits (3 rabbits/group). b, D3H44-L (●) and D3H44 Fab (○) were dosed at 5 mg/kg into BALB/c mice (9 mice/group). Samples taken at the indicated times were assayed in a TF ELISA.
sequence that can simply be added to any recombinantly expressed protein.

The ability to rapidly generate antibodies as potential therapeutics has stimulated interest in extending their valence, binding affinity, effector functions and pharmacokinetics through engineering. Their antigen binding domains can be readily presented in numerous formats including Fv, scFv, diabodies, Fab, and Fab′2. These immunoglobulin fragments as well as many other promising protein pharmaceuticals, however, are rapidly cleared from the blood, limiting their potential usefulness (25). To test the possibility of extending the half-life of such molecules, an albumin-binding peptide was recombinantly fused to the carboxyl-terminus of the light chain of the D3H44 Fab directed against human TF (18). The addition of SA06 to D3H44 provided a simple purification scheme utilizing a TF affinity column followed by an albumin affinity column. A higher overall yield was obtained with D3H44-Ls compared with D3H44-L, probably due to the absence of the nearby disulfide between the light and heavy chain of the Fab. The loss of this disulfide had no effect on the ability of D3H44-Ls to bind to TF or albumin but is associated with a 40% increase in drug clearance relative to D3H44-L, as evidenced from the pharmacokinetic study in rabbits (Table V). Rodrigues et al. (26) found that the pharmacokinetics of a Fab′2 lacking the light chain-heavy chain disulfides was not affected and suggested that the stability of a Fab′2 is not dramatically altered in the absence of this disulfide bond. In this study, we observed a difference in the clearance as a result of the increased half-lives of the D3H44-L and D3H44-Ls.

The utility of D3H44-L or D3H44-Ls greatly depends upon the capability of the albumin-bound fraction to bind TF. Both the prothrombin time assay and the albumin/TF sandwich assay indicate D3H44-L and D3H44-Ls can bind to albumin and TF simultaneously. Although not surprising, given that the two binding sites are at different ends of the Fab, the ability of the albumin-bound fraction to remain functional may not be retained when trying to enhance the half-life of other protein-peptide fusions. If an increased half-life comes at the expense of impaired function, a higher dose may be required to provide an efficacious concentration of free drug (8). The fraction of free protein could be increased by using shorter peptides with reduced affinity for albumin (Table IV); however, this is also likely to reduce the half-life.

Besides renal filtration, metabolism is an important parameter affecting half-life. Although clearance is greatly reduced relative to the D3H44 Fab, both SA21 and D3H44-L are cleared faster than one might expect simply based on their calculated free concentration in plasma and the glomerular filtration rate in rabbits (27). The pharmacokinetics of SA21 and D3H44-L are not influenced by binding to any in vivo target other than albumin, since D3H44 does not recognize rabbit TF. Based on these assumptions, metabolism accounts for the vast majority of the clearance for both SA21 and D3H44-L.

Further, the faster clearance and shorter half-life of SA21 compared with D3H44-L is seemingly at odds with their similar affinity for albumin and probably reflects an increased metabolism of the peptide rather than differences in the rate of filtration. Susceptibility of the peptide to metabolic proteolysis while bound to albumin may be shielded upon fusion with the Fab or reduced as a result of the introduction of an amino-terminal fusion. As an example, stabilization of the 10-amino acid peptide hormone, GnRH, which has a half-life of 2–8 min, was achieved by introducing carboxyl-terminal modifications and other stabilizing changes to yield variants with half-lives over 4 h (28). Whereas association with albumin can extend the exposure of molecules in vivo, the stability of these molecules can remain as a limiting feature that governs their half-life.

This study represents the first attempt to improve the half-life of an immunoglobulin fragment without significantly altering the hydrodynamic size of the molecule. Through the use of a simple recombinant fusion, selective tight binding to albumin and a prolonged half-life are achieved. D3H44-L has a half-life of 32.4 h in rabbits or an increase of 37-fold relative to the D3H44-Fab (Table V). This half-life is comparable with that of a D3H44 Fab conjugated to 20,000 or 40,000 PEG and is superior to the half-life of a D3H44 Fab′2 (half-lives of 18, 69, and 8.8 h, respectively). In mice, the 10.4 h half-life for D3H44-L represents a 26-fold improvement. Interestingly, these half-lives correlate with the reported half-life of albumin in these species of 5–6 days in rabbits (29) and 1 day in mice (30). Despite potential metabolism differences and the weaker affinity for human albumin (Table III), the 19-day half-life for human albumin suggests that large improvements in the half-life of a Fab in human are possible.

Moreover, the ability to achieve an increased half-life without a dramatic increase in size may present an advantage when trying to generate tumor targeting and imaging molecules (25, 31, 32). A low molecular weight agent may have an advantage in its ability to diffuse into tissues; however, a sufficient time of exposure is required for adequate absorption. Generally, a small protein such as a scFv can diffuse rapidly into tissues, but the bulk of the material is lost due to extremely fast renal filtration. On the other hand, an IgG remains circulating for several days, providing ample exposure but minimal tumor penetration due to the poor diffusion of such a large protein. A small long lived molecule, such as an albumin binding Fab, could be ideal as an imaging or tumor-targeting agent.

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