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Improving Protein Pharmacokinetics by Engineering Erythrocyte Affinity

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Abstract: Poor pharmacokinetic profiles are often the underlying reason for the failure of novel protein drugs to reach clinical translation. Current passive half-life improvement methods focus on increasing the apparent hydrodynamic radius of the drug. We sought to develop an active method to increase the circulation half-life of proteins by binding to erythrocytes in blood. Screening a naive phage-displayed peptide library against whole mouse erythrocytes yielded a 12 amino acid peptide (ERY1) that binds the erythrocyte surface with high specificity. ERY1-displaying phage bind mouse and rat erythrocytes 95-fold higher than wild-type phage and exhibit negligible binding to mouse leukocytes, as determined by flow cytometry. Affinity experiments with soluble peptide revealed the extracellular domain of glycophorin-A as the membrane protein ligand. When expressed as an N-terminal fusion to maltose-binding protein and administered intravenously, the erythrocyte-binding variant exhibits a 3.2- to 6.3-fold increase in circulation half-life, 2.15-fold decrease in clearance, and 1.67-fold increase in bioavailability as compared to the wild-type protein. The peptide fails to induce ERY1-reactive immunoglobulin production, furthering the potential of the concept in therapeutic design, although this sequence does not bind human erythrocytes. We conclude that engineering erythrocyte affinity into proteins effectively increases their circulation half-life, thereby offering a solution to improve pharmacokinetic profiles of the numerous therapeutic protein drugs in clinical development.

Keywords: Erythrocyte; pharmacokinetics; cell-binding peptide; phage display; glycophorin

Introduction

The clinical success of therapeutic proteins in treating disease is a hallmark of modern medical care. Of the over 160 protein drugs currently on the market, many still lack optimal delivery and dosing schemes for minimal patient intervention and safety.[1] Of particular importance are the pharmacokinetic parameters of the drug that dictate its duration in circulation and thus its capacity to affect target cells and tissues. Platform technologies that decrease dosage while increasing efficacy of protein drugs may decrease care costs and safety risks, as well as enable clinical translation of therapeutic proteins currently in development.

Current methods to increase protein drug circulation have focused on increasing the apparent hydrodynamic radius of the molecule. These range from chemical conjugation to polyethylene glycol (PEG)[2] or other water-soluble polymers,[3,4] recombinant fusions to serum proteins such as albumin,[5−7] and tandem dimer formulations.[8−10] Though effective in many cases in increasing circulation half-life, especially as the hydrodynamic radius of the graft or fusion increases,[4] these methods offer challenges in manufacturing and maintenance of biological effector function. Heterogeneities in

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conjugation reactions can cause complex product mixtures with varying biological activities, due mostly to the utilization of site-unspecific chemistries. Extensive biochemical characterization often follows precise purification methods to retain a homogeneous therapeutic product.[11−13] Furthermore, attachment of large moieties, such as branched PEGs, to reactive zones of proteins can lead to decreased receptor affinity.[2] Site-specific and reaction-free, recombinant additions to protein drugs need no added purification. Although able to yield remarkable extensions in circulation lifetimes,[14] large protein fusions may complicate expression, as well as interfere with correct folding and disulfide-bond formation of the protein drug to its active form.

Other work has shown that enabling the therapeutic protein to bind to blood components allows for increased circulation of the drug.[15−17] The addition of affinity-matured serum albumin-binding peptides to antibody fragments increased their circulation time 24-fold in mice.[16] Though effective, this method is complicated by the dynamics of albumin recycle by the neonatal Fc receptor (FcRn) and the use of cysteine-constrained cyclic peptides for functionality. Recombinant attachment of albumin-binding antibody domains to therapeutic proteins have shown similar success,[17] yet their large size (11−13 kDa) could pose biophysical hindrances for correct active conformation of the therapeutic.

We sought to develop an active erythrocyte-binding method whereby a short linear peptide fusion increases the circulation time of the protein drug by imparting an affinity to red blood cells. Such a method has a conceptual advantage that the effective size is increased only after entry into the bloodstream, allowing for ease in formulation and faster diffusion through the interstitium into circulation. Such a methodology would lack drawbacks associated with chemical conjugation techniques, be simple to express and manufacture, and bind to a high concentration blood component. The erythrocyte has been previously considered as a target for nanoparticle binding in an *ex vivo* context.[18] Through a novel screening method, peptides were discovered that aided 0.22 μm sphere attachment to erythrocytes, yet no ligand identification nor *in vivo* validation was conducted. Present in blood at around 5×10^6 cells/μL, erythrocytes offer opportune carrier characteristics in that they do not endocytose cell-surface bound components and they exhibit a long circulation life, much longer than the dissociation time scales we would likely discover.[19] We screened a phage-displayed library against whole mouse erythrocytes to discover a novel peptide that binds to the cell surface with high affinity and specificity. In implementing the discovered linear 12-residue peptide fused to the N-terminus of maltose-binding protein (MBP), we demonstrate that erythrocyte binding effectively improves protein drug pharmacokinetic parameters.

Experimental Section

Materials. A complete list of materials used is provided in the Supporting Information.

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Selection of Peptide Ligands toward Mouse Erythrocytes. In each round of screening, 10^{11} input phage were incubated with mouse erythrocytes in PBS with 50 mg/mL BSA (PBSA-50). After 1 h at 37 °C, unbound phage were removed by centrifugation in Percoll at 1500 g for 15 min. A subsequent dissociation step was carried out in PBSA-50 in order to remove low-affinity binding phage. Dissociation duration and temperature were increased in later rounds of screening to increase stringency of the selection process. In round 1, phage binding was followed by a 2 min dissociation step at room temperature prior to washing and elution. In round 2, phage binding was followed by a 10 min dissociation at 37 °C. In rounds 3 and 4, two separate and sequential dissociation steps were conducted at 37 °C: 10 min followed by 15 min in round 3, and 10 min followed by 30 min in round 4. Erythrocyte-associated phage were eluted with 0.2 M glycine, pH 2.2 for 10 min, and the solution was neutralized with 0.15 volumes of 1 M Tris, pH 9.1. Phage amplification and titering were conducted as per the NEB protocol.

Flow Cytometry. To determine phage binding, approximately 10^{10} phage particles were used to label 5×10^5 cells in PBSA-50 in a total volume of 200 μL for 1 h at 37 °C. Following a 4 min centrifugation at 200*g*, cells were resuspended in 200 μL of PBSA-5 and anti-phage-PE was added at a 1:20 dilution for 1 h at room temperature. After a final spin/wash cycle as above, cells were resuspended in PBSA-5 and analyzed on a flow cytometer.

Microscopy. For microscopy staining, approximately 10^{10} phage particles were used to label 5×10^5 cells in PBSA-50 in a total volume of 200 μL for 1 h at 37 °C. Following a 4 min centrifugation at 200 g, cells were resuspended in 200 μL of PBSA-5 and anti-phage-PE was added at a 1:20 dilution for 1 h at room temperature. After a final spin/wash cycle as above, cells were resuspended in 10 μL of PBSA-5, hard set mounted, and imaged on a Leica fluorescence microscope. For confocal imaging, erythrocytes were labeled with phage as described above, followed by a 1 h incubation with rabbit anti-fd bacteriophage antibody (1:200) and goat anti-GYPA (1:200) simultaneously in PBSA-5. Cells were incubated with the appropriate AlexaFluor labeled secondary antibody (1:200) and hard set mounted. Standard image analysis was conducted in ImageJ and IMARIS.

Peptide Synthesis and Purification. The ERY1 (H_2N-WMVLPWLPGTLDGGSGCRG-$CONH_2$) and mismatch (H_2N-PLLTVGMDLWPWGGSGCRG-$CONH_2$) peptides were synthesized using standard solid-phase f-moc chemistry using TGR resin on an automated liquid handler. The peptide was cleaved from the resin for 3 h in 95% trifluoroacetic acid, 2.5% ethanedithiol, 2.5% water, and precipitated in ice-cold diethyl ether. Purification was conducted on a Waters preparative HPLC-MS using a C18 reverse phase column.

Peptide−Biotin Functionalization and Cross-Linking. The ERY1 and mismatch peptide were conjugated to Mts-Atf-biotin as suggested by the manufacturer. In brief, peptides were solubilized in PBS/DMF and reacted with 1.05 equiv of Mts-Atf-biotin overnight at 4 °C. Following clarification of the reaction by centrifugation, biotinylated peptide was incubated with erythrocytes in PBSA-50 for 1 h at 37 °C, cells were washed twice in fresh PBS, and were UV irradiated at 365 nm for 8 min at room temperature. Cells were lysed by sonication, and the lysate was purified using streptavidin-coated magnetic beads.

Cloning and Expression of MBP Constructs. Clonal replicative form M13KE DNA was extracted using a standard plasmid isolation kit. The resultant plasmid was digested with *Acc*651 and *Eag*I to obtain the gIII fusion gene and then ligated into the same sites in pMAL-pIII, yielding the plasmid herein termed pMAL-ERY1. Sequence verified clones were expressed in BL21 *Escherichia coli*. In brief, midlog BL21 cultures were induced with IPTG to a final concentration of 0.3 mM for 3 h at 37 °C. An osmotic shock treatment with 20 mM Tris, 20% sucrose, 2 mM EDTA for 10 min, followed by a second treatment in 5 mM $MgSO_4$ for 15 min at 4 °C, allowed for the periplasmically expressed MBP fusion to be isolated from the cell debris. Purification of the fusion protein was conducted on amylose Sepharose and analyzed for purity by SDS−PAGE.

MBP Variant Pharmacokinetic Analysis. The Swiss Vaud Veterinary Office previously approved all animal procedures. While under anesthesia with ketamine/xylasine, the tail was warmed in 42 °C water and 150 μg of protein was injected in a 100 μL volume directly into the tail vein of 10−12 week old female BALB/c mice. Care was taken to ensure mice were kept at 37 °C while under anesthesia. Blood was drawn by a small scalpel incision on the base of the tail, diluted 10-fold in PBSA-5, 10 mM EDTA, and stored at −20 °C until further analysis. Blood samples were analyzed for MBP concentration by sandwich ELISA. In brief, monoclonal mouse anti-MBP was used as the capture antibody, polyclonal rabbit anti-MBP as the primary antibody, and goat anti-rabbit-HRP as the secondary antibody. The data were analyzed in Prism4 using standard pharmacokinetic compartmental analysis.[20,21] Bioavailability was calculated as the ratio between the area under the curve (AUC) of the blood MBP concentration vs time graph following intravenous administration (Figure 6A) and the AUC following subcutaneous administration (Figure 6B).

Results

Rather than screen against a purified erythrocyte cell-surface protein, we screened against whole erythrocytes to discover affinity partners. Applying 4 rounds of selection against whole erythrocytes substantially enriched the library toward higher-binding phage clones, as illustrated by flow cytometry (Figure 1A). Through the use of density gradient centrifugation and extensive washing, meticulous care was taken to minimize the number of unbound phage escaping round elimination. Furthermore, selection was halted and

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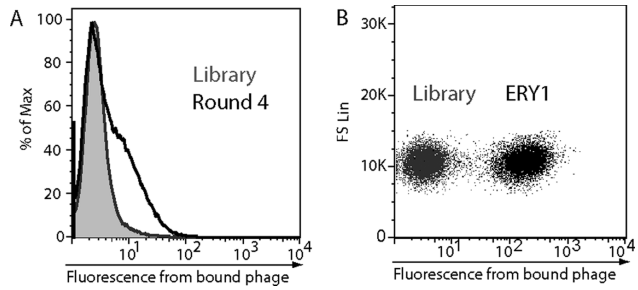

Figure 1. Flow cytometric analysis of erythrocyte binding of phage from round 4 of the selection (A), and the best clone from round 4, clone ERY1, displaying the sequence WMVLPWLPGTLD (B).

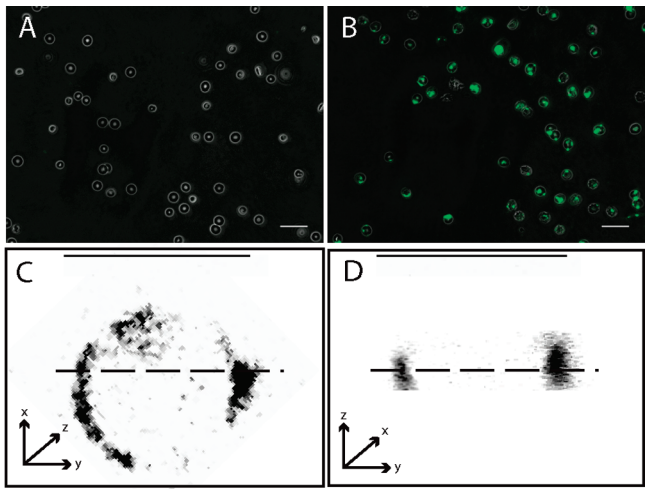

Figure 2. Microscopy of erythrocytes labeled with either library phage (A) or ERY1 phage (B). ERY1 phage specifically localize to the surface of the cell membrane at the equatorial periphery and do not penetrate to the cytoplasm (C, D); scale bar = 20 μm in A, B, 5 μm in C, D.

clones were analyzed early in the screening process so as to prohibit highly infective phage clones from dominating the population. The entire screening process was performed in the presence of a high concentration of serum albumin (50 mg/mL) and at 37 °C to reduce nonspecific binding events and, perhaps more importantly, select for peptides with favorable binding characteristics in blood serum. Clonal analysis after round 4 revealed one phage clone displaying a high-affinity peptide, WMVLPWLPGTLD (herein termed ERY1), toward the mouse erythrocyte cell surface (Figure 1B). When similarity searched using the BLAST algorithm in UniProt, no relevant protein sequence homology was identified toward the full peptide.

Microscopy confirmed that the ERY1 phage binds the erythrocyte cell surface without altering cell morphology and without cytoplasmic translocation. Fluorescence and phase contrast images reiterated the erythrocyte-binding capacity of ERY1 phage (Figure 2B) relative to the nonselected library (Figure 2A). High-resolution confocal imaging revealed that ERY1 phage bind preferentially to the equatorial periphery of the cell surface (Figure 2C and Figure 2D), and that

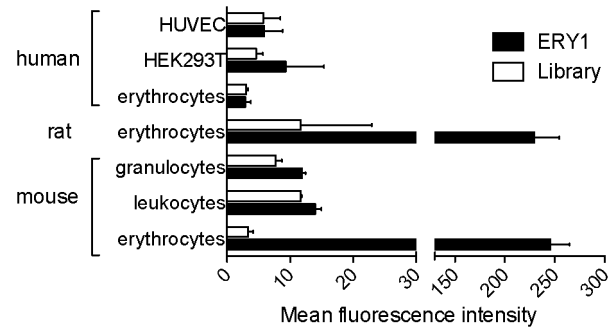

Figure 3. Flow cytometric analysis of ERY1 phage binding to a panel of cell types. Binding to mouse and rat but not human erythrocytes was observed. Values reported as mean ± SD, $n = 2$.

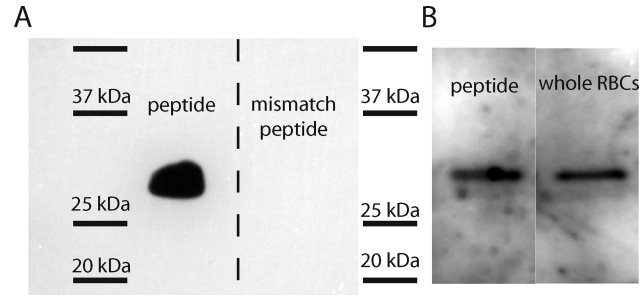

Figure 4. Affinity pull-down of erythrocytes photo-cross-linked with biotinylated peptide shows a unique binding protein at ca. 28 kDa by streptavidin Western blot (A), the identity of which is confirmed by anti-glycophorin-A Western blotting (B).

binding was homogeneous among erythrocytes (Figure 2B, as well as Figure 1B).

Flow cytometric screening of a panel of interspecies cell lines demonstrated the ERY1 phage was specific for mouse and rat erythrocytes, with no measurable binding to mouse leukocytes or human cells (Figure 3). These data suggested that the specific membrane protein acting as the ERY1 ligand was found solely in erythroid cells, and not in myeloid or lymphoid cell lineages. Furthermore, this validated our screening method of using freshly isolated blood with little prior purification other than centrifugation for a target. Though erythrocyte specificity negated numerous potential membrane protein targets as the ERY1 ligand, the result failed to identify a single protein responsible for the interaction.

To search for the molecular target for the ERY1 peptide, we employed affinity pull-down techniques using a biotinylated soluble peptide; this method revealed glycophorin-A (GYPA) as the ERY1 ligand on the erythrocyte membrane. When whole erythrocytes were incubated with ERY1 peptide functionalized with biotin and a photoactivatable cross-linker, a single 28 kDa protein was conjugated with the peptide−biotin complex, as detected by a streptavidin Western blot (Figure 4A, Figure 2A in the Supporting Information). The reaction lysate was extensively washed and purified using streptavidin magnetic beads to ensure no unlabeled proteins from the erythrocyte lysate remained. As expected, the mismatch peptide failed to conjugate

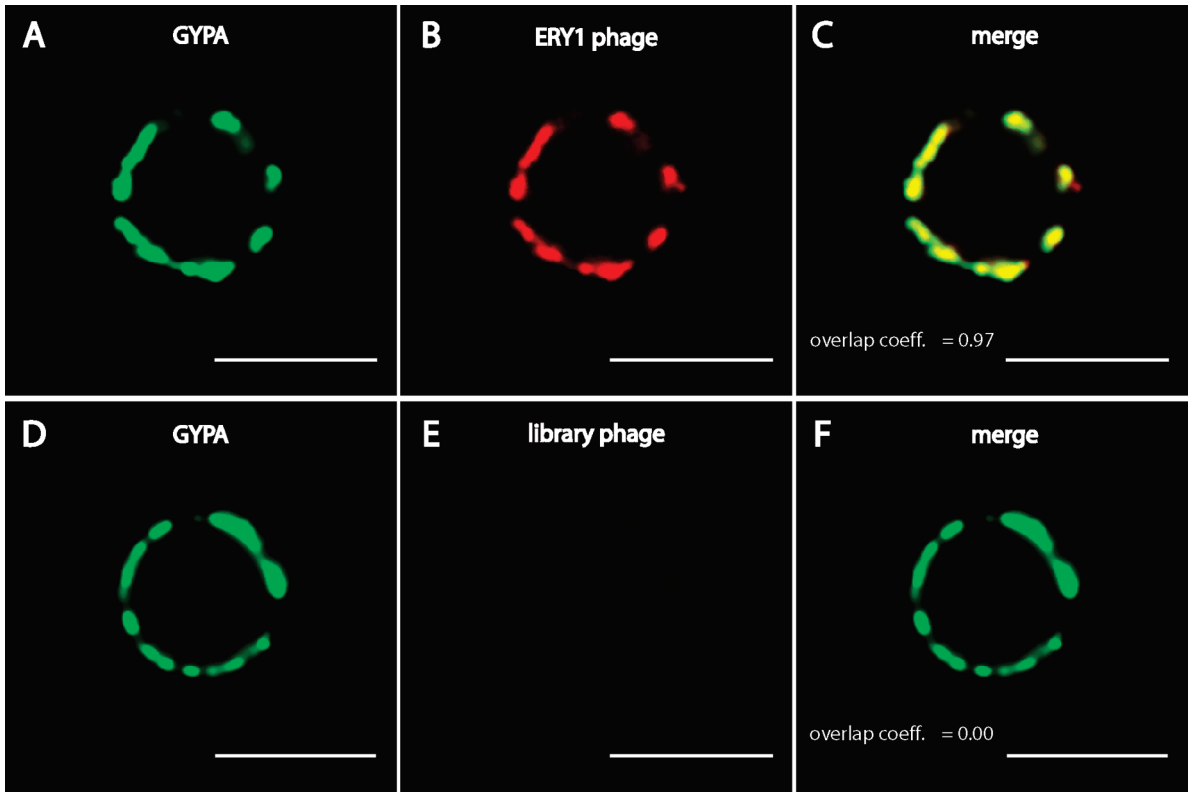

Figure 5. High resolution confocal microscopy on ERY1 and library phage labeled erythrocytes. In ERY1 labeled erythrocytes, colocalization (C) of the anti-phage signal (B) with anti-glycophorin-A staining (A) supports ERY1 binding GYPA, confirming the molecular observations reported in Figure 4. In library phage labeled erythrocytes, no phage staining is seen (E), which obviates any colocalization (F) with anti-glycophorin-A staining (D). Thresholded overlap coefficients are reported for ERY1 (0.97) and library phage (0.00).

to any erythrocyte proteins. The mismatch peptide, PLLTVG-MDLWPW, was designed to contain the same amino acid residues as ERY1, and to match its hydrophobicity topography. Evidence of the apparent size of the interacting protein suggested several smaller, single pass membrane proteins as likely ligands, namely, the glycophorins. Anti-GYPA Western blotting of the same purified samples from the cross-linking reaction confirmed that the candidate biotinlyated protein was indeed GYPA (Figure 4B, Figure 2B in the Supporting Information).

Co-localization of ERY1 phage with GYPA was analyzed by high-resolution confocal microscopy (Figure 5). GYPA is naturally expressed and presented as part of a complex composed of several membrane and cytoskeletal proteins.[22] This is visually evident in GYPA staining, whereby non-uniform labeling was seen at the cell equatorial periphery (Figure 5A). Labeling with ERY1 phage produced extremely similar staining topographies (Figure 5B). A high overlap coefficient of 0.97 was calculated in colocalization analysis, corroborating the conclusion that ERY1 phage and anti-GYPA bind to the same protein (Figure 5C). GYPA clustering was also witnessed in erythrocytes labeled with library phage (Figure 5D), yet no phage binding and thus no colocalization were evident (Figure 5E, Figure 5F).

Biochemical characterization of ERY1-dependent erythrocyte binding was conducted by similar flow cytometric binding assays following various enzymatic treatments of cells (Figure 1 in the Supporting Information). Treatment of erythrocytes with neuraminidase, trypsin, and chymotrypsin markedly increased the binding capacity of ERY1 phage to the cell surface, suggesting sialic acid and extraneous membrane protein removal increased accessibility of the peptide to the binding epitope of GYPA.

To characterize the effect of the ERY1 peptide upon the pharmacokinetics of a protein, we expressed the model protein MBP as an N-terminal fusion with the ERY1 peptide (ERY1-MBP). Upon intra- and extravascular administration, the ERY1-MBP variant exhibited extended circulation relative to the wild-type protein (Figure 6A and Figure 6B). Blood samples at time points taken immediately following injection confirmed that initial concentrations, and thus the dose, were identical in both formulations. Beginning 4 h after intravenous injection, ERY1-MBP was cleared from circulation at a statistically significantly slower rate than the nonbinding wild-type MBP. Following subcutaneous injection, similar trends of heightened blood concentrations of ERY1-MBP were seen sustained throughout the experimental duration.

ERY1-MBP demonstrated a 3.28-fold (for a single-compartment model) to 6.39-fold (for a two-compartment

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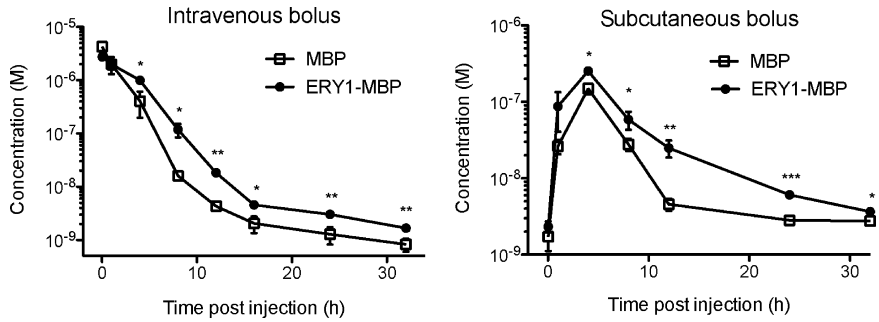

Figure 6. Blood concentration profile of MBP variants following intravenous (A) and subcutaneous (B) administration. Values reported as mean ± SD, $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, as per Student's two-tailed t test.

Table 1. Pharmacokinetic Parameters of Wild-Type MBP and ERY1-MBP[a]

	$t_{1/2}$ (h)	$t^{\alpha}_{1/2}$ (h)	$t^{\beta}_{1/2}$ (h)	Cl (h^{-1})	bioavailability (%)
MBP	0.92 ± 0.17	0.41 ± 0.10	1.11 ± 0.18	0.62 ± 0.10	10.1
ERY1-MBP	3.02 ± 0.57	2.62 ± 0.83	3.17 ± 0.51	0.29 ± 0.03	16.9
fold change	3.28	6.39	2.86	−2.14	1.67
P value	0.01	0.03	0.01	0.02	0.07

[a] Values reported as mean ± SD, $n = 4$, P value as per Student's two-tailed t-test.

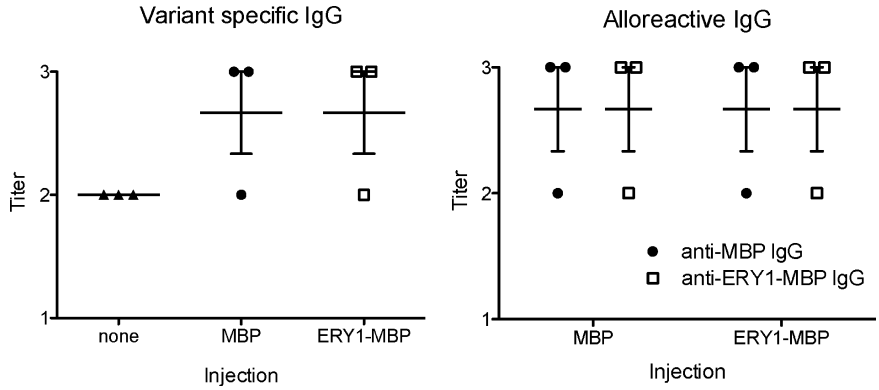

Figure 7. Antibody titers of mice 21 days following injection with MBP variants. Both MBP variants induce slight IgG induction (A), which are alloreactive toward both MBP versions (B), revealing the lack of antibodies raised toward the ERY1 peptide domain in the ERY1-MBP construct.

model) increase in serum half-life, 2.14-fold decrease in clearance, and 1.67-fold increase in bioavailability compared to the wild-type MBP (Table 1). Analyzing concentrations using a standard one-compartment pharmacokinetic model yielded a half-life of 0.92 and 3.02 h for the wild-type and ERY1 variants, respectively. The data were also accurately fit to a two-compartment model ($R^2 \geq 0.98$) to obtain α and β half-lives of 0.41 and 1.11 h, and 2.62 and 3.17 h, for the wild-type and ERY1 variants, respectively.

To explore the potential immunogenicity of the erythrocyte-binding peptide, we examined the humoral immune response in mice administered with ERY1-MBP or wild-type MBP. The ERY1 peptide failed to induce immunoglobulin G (IgG) antibodies reactive toward its own sequence epitope. Variant-specific IgG titers were seen elevated in mice 21 days following subcutaneous injection of either MBP variant (Figure 7A), yet the same IgGs were found to bind to both variants of MBP (Figure 7B). Thus, the murine immunological response to the fusion protein seems focused on the MBP domain and not the fused ERY1 peptide domain.

Discussion

The goal of this study was to develop a recombinant method to improve the pharmacokinetic profiles of protein drugs by imparting a transient affinity to a long-circulating blood component. Enhanced and confined circulation of the drug in the blood compartment would decrease doses necessary for functionality and therefore potentially decrease chances of systemic toxicity. A simple recombinant peptide tag would furthermore reduce the complexity currently associated with chemical conjugation and large protein fusion techniques, which may lead to decreased pharmacodynamic function and increased manufacturing costs.

Phage display was effectively applied in screening against ligands on whole cells to discover a novel erythrocyte-binding peptide. The ERY1 peptide is unique in that it does not show relevant sequence homology to any known proteins in UniProt, nor to previously discovered erythrocyte binding peptides.[18] Though the target sample was molecularly heterogeneous in nature, the selection process converged toward peptides binding to a major membrane protein component, namely, GYPA.

Conceivably, library enrichment could have progressed toward ligands against many other membrane proteins found on the erythrocyte, yet this was not observed. By virtue of the stoichiometry of the erythrocyte membrane proteins, GYPA represents a majority of the solvent accessible polypeptide components present on the cell surface.[23,24] The larger epitope landscape of GYPA seemingly endowed itself with higher peptide interaction possibilities. Interestingly, as a key receptor for *Plasmodium* parasites,[25−27] the glycophorins may inherently contain specific reactive zones attractive for potential foreign polypeptide interactions.

The cellular specificity of the ERY1 peptide toward mouse and rat erythrocytes was both a vital early indicator of the ERY1 membrane epitope and a promising metric of therapeutic potential. Sequence homology between the major erythrocyte membrane proteins in mice and humans is relatively low, yet higher in those of the rat. Furthermore, all attempts to enzymatically degrade the ERY1 epitope on mouse erythrocytes failed (Figure 1 in the Supporting Information), hinting that the epitope was highly glycosylated and thus protected from enzymatic cleavage. Taken together, these data guided our search for the identity of the ERY1-binding membrane protein toward the glycophorins, and the ERY1 ligand was validated as GYPA.

We have demonstrated that erythrocyte binding is an effective method to increase the circulation half-life of a model protein, both after intravenous and after subcutaneous administration. Fusion of MBP with the 12 residue linear ERY1 peptide increased its half-life approximately 3- to 6-fold in mice. Our results closely resemble previous protein engineering work performed on the Fc regions of therapeutic antibodies, where a 10-fold increase in affinity of the antibody toward the FcRn receptor caused a 2.4- to 4-fold increase in serum half-life.[28−30] This methodology was demonstrated to increase the *in vivo* efficacy of bevacizumab (Avastin) and cetuximab (Erbitux) in mice.[31] Given the positive correlation between extension of half-life and clinical efficacy of antibody drugs, our simple erythrocyte affinity tag technology holds high potential value in improving such therapeutics. Clearly, further work to find a ligand binding to human erythrocytes is needed.

Furthermore, the immunological stealth of the ERY1 peptide, as determined by a lack of epitope specific IgG production, bodes well for the safety of the technique, at least for the murine-binding ERY1 sequence. In general, hydrophobicity and hydrogen bonding of anchor residues positively correlate with potential recognition by the major histocompatibility complex II (MHC II),[32,33] which are biochemical characteristics found in the ERY1 peptide. Contrary to this prediction, the ERY1 peptide when fused to MBP did not induce any observable humoral immune response.

We conclude that engineering protein therapeutics with short erythrocyte-binding peptide moieties positively impacts their *in vivo* pharmacokinetic parameters. Our study implemented a novel 12-residue GYPA binding peptide discovered by phage display to increase the half-life of a model protein by 3.28- to 6.39-fold. Though this improvement is not as large as with some current PEGylation and large protein fusion techniques, the recombinant nature in which functionality is imparted, as well as the minor biophysical alteration of the molecule involved, promote our technology's application in smart drug design. As evidenced by the success of anti-albumin antibody fusions,[15,17] we postulate that tags displaying higher affinity than ERY1 would further improve the half-life extension of our technology. The concept of an increase in effective size of the fusion protein only after entry into the bloodstream offers interesting potential.

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Supporting Information Available: A complete list of all reagents used, as well as detailed description of enzymatic treatments of erythrocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

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