ORIGINAL ARTICLE

Engineering a serum-resistant and thermostable vesicular stomatitis virus G glycoprotein for pseudotyping retroviral and lentiviral vectors

B-Y Hwang and DV Schaffer

Vesicular stomatitis virus G glycoprotein (VSV-G) is the most widely used envelope protein for retroviral and lentiviral vector pseudotyping; however, serum inactivation of VSV-G pseudotyped vectors is a significant challenge for *in vivo* gene delivery. To address this problem, we conducted directed evolution of VSV-G to increase its resistance to human serum neutralization. After six selection cycles, numerous common mutations were present. On the basis of their location within VSV-G, we analyzed whether substitutions in several surface exposed residues could endow viral vectors with higher resistance to serum. S162T, T230N and T368A mutations enhanced serum resistance, and additionally K66T, T368A and E380K substitutions increased the thermostability of VSV-G pseudotyped retroviral vectors, an advantageous byproduct of the selection strategy. Analysis of a number of combined mutants revealed that VSV-G harboring T230N + T368A or K66T + S162T + T230N + T368A mutations exhibited both higher *in vitro* resistance to human serum and higher thermostability, as well as enhanced resistance to rabbit and mouse serum. Finally, lentiviral vectors pseudotyped with these variants were more resistant to human serum in a murine model. These serum-resistant and thermostable VSV-G variants may aid the application of retroviral and lentiviral vectors to gene therapy.

INTRODUCTION

Since the first human gene therapy trial in 1989,[1] there has been remarkable progress in both understanding the genetic basis of human disease and developing improved gene delivery vector systems to treat them.[2–4] For the latter, clinical gene delivery to date has focused primarily on viral vehicles owing to their evolved ability to efficiently transport genetic information to into cells. However, viral vectors still face a number of challenges including immunogenencity, targeted delivery, safe genome integration and vector production.[5–9]

Pseudotyping—the replacement of a virus' attachment protein with that of a different virus—has enabled progress in addressing several of these concerns.[10–15] For example, vesicular stomatitis virus G glycoprotein (VSV-G) is the most widely used glycoprotein for retroviral and lentiviral vector pseudotyping, as it offers several advantages including effective delivery to a broad range of cell types, enhanced vector stability and increased infectious titer.[16,17] That said, VSV-G is cytotoxic to producer cells,[18] though the use of tetracycline-regulating promoters has alleviated this problem and facilitated the generation of stable cell lines expressing VSV-G.[19] As an additional problem, however, serum inactivation of VSV-G pseudotyped viral vectors impedes their *in vivo* use.[20] This inactivation is mediated by proteins of the complement cascade. In general, complement activation can occur via classical, alternative, and lectin pathways, and these systems are tightly regulated by the complement regulatory proteins.[21] However, the precise mechanism of VSV-G inactivation and the protein regions involved are not known.

Several approaches have been explored to overcome serum inactivation. Although known inhibitors of complement improve vector survival in serum *in vitro*,[22] systemic delivery of complement inhibitors can be accompanied by toxicity.[23] Incorporating complement regulatory proteins directly into a virus—including human decay-accelerating factor/CD55 and/or membrane cofactor protein/CD46—enhanced the resistance of the virus to human serum inactivation.[24–26] However, the efficiency of complement regulatory proteins incorporation into virions varied with the types of virus and producer cell, and the direct fusion of complement regulatory proteins to viral proteins resulted in low titers. As a result, only systemic use of modified adenovirus has been reported.[27] As another approach, chemical 'shielding' of VSV-G via bioconjugation of polyethylene glycol or polyethylenimine enhanced the serum resistance of lentiviral vectors,[28,29] though vector titer was reduced, and a chemical modification adds an additional step in vector production for clinical development. Alternatively, using different, serum-resistant glycoproteins—such as feline endogenous virus envelope protein RD114 or cocal virus envelope[30,31]—for pseudotyping could pose a solution. However, RD114 pseudotyping resulted in lower titers (∼100-fold) compared with the VSV-G pseudotyping, and its use *in vivo* has thus been somewhat limited to date.[32,33] Furthermore, the cocal envelope has not been tested *in vivo* to date, in contrast to the considerable *in vivo* characterization of VSV-G. Finally, utilizing packaging cell lines, in which alpha-galactosyl-transferase genes are disrupted, can generate vector lacking galactosyl-α(1,3)galactose epitopes, and thereby reduce

Chemical and Biomolecular Engineering, Bioengineering and the Helen Wills Neuroscience Institute, University of California, Berkeley, CA, USA. Correspondence: Professor DV Schaffer, Chemical and Biomolecular Engineering, Bioengineering and the Helen Wills Neuroscience Institute, University of California, 176 Stanley Hall, Berkeley, CA 94720-3220, USA.
E-mail: schaffer@berkeley.edu

sensitivity to human complement, through this approach has not been broadly explored.[25]

Directed evolution has recently been developed and implemented to improve numerous properties of viral vectors, and this approach can be effective even in the absence of a mechanistic understanding of challenges facing a vector system. In particular, multiple studies have applied molecular evolution to improve vector stability, pseudotyping efficiency, transduction efficiency, resistance to antibodies, genomic integration selectivity and other properties.[34–39] Here, we explore whether directed evolution of the VSV-G envelope may enable pseudotyped viral vectors to resist neutralization by human serum. Through a combination of evolution and site-directed mutagenesis, we created VSV-G variants that are both resistant to a panel of human and animal sera and are thermostable. Furthermore, variants exhibited enhanced gene delivery in the presence of human serum in vivo.

RESULTS

Library construction and selection

VSV-G libraries were constructed by error-prone PCR at two different mutation rates: a 2×10^6 mutant library with a low error rate of 1–4 nucleotide mutations per VSV-G sequence and a 2×10^5 mutant library with 3–8 nucleotide mutations per sequence, as quantified by sequencing of randomly chosen clones. For selection, these libraries were inserted into a retroviral vector plasmid that was used to package a library of vector particles, where each harbored a vector genome encoding the VSV-G variant incorporated in the envelope of that particle. This requires that ~1 plasmid carrying a VSV-G variant be transfected into a producer cell during packaging. For initial optimization of this process, Human Embryonic Kidney (HEK) 293T cells were transfected with 62.5 ng—2 μg of the retroviral vector pCLPIT GFP followed by flow cytometry analysis (Supplementary Data Figure S1). Upon transfection with 62.5 ng of pCLPIT GFP, ~15% of cells expressed GFP, suggesting that these conditions may introduce on average <1 plasmid per cell and could thus produce the desired virion library. Therefore, the two CLPIT VSV-G libraries were packaged separately using these conditions, and the resulting vectors were combined for selection.

To develop a strategy for selecting VSV-G variants resistant to serum neutralization, retroviral vector pseudotyped with wild-type VSV-G was diluted fivefold in a mixture of human serum from 18 donors and incubated at 37 °C (Supplementary Data Figure S2). Vector infectivity progressively decreased with longer incubations, such that only ~5% of the vector remained infective after 6 h. The VSV-G library was thus selected to evade human serum inactivation by incubating the virions with serum at 37 °C for 6 h. 293T cells were then infected with the treated viral vector library (at a multiplicity of infection<0.1 to prevent multiple infections of a single cell). Following the selection with 1 μg ml⁻¹ of puromycin, the selected viral pool was rescued via transfection of the pCMV gag/pol helper plasmid.

This process was repeated for six selection steps, and VSV-G variants were then recovered from 293T cell genomic DNA via PCR and inserted into the plasmid pcDNA IVS to generate helper plasmids for vector production. DNA sequencing revealed that while there were no duplicates among 36 randomly chosen VSV-G clones, common mutations were present (Figure 1a; Supplementary Data Figure S3). The positions of these 'hot spot' mutations were analyzed within the structure of the prefusion form of VSV-G (Figure 1b),[40] which indicated that Lys66, Ser162, Asp208, Ser212, Lys216, Thr230, Thr368 and Glu380 are located on the protein surface. In addition, Thr344 is located in the core region, Ile98 and Phe125 are located on the leg region that points toward the viral membrane and Phe470 is located on the transmembrane domain of VSV-G. The latter, nonsolvent exposed

residues (Thr344, Ile98, Phe125 and Phe470) would appear unable to interact with components of human serum, and the former set of mutations was instead investigated.

### Functional analysis of individual VSV-G mutations

As there were no dominant individual clones following selection, we analyzed the roles of the common mutations in potential VSV-G serum resistance. Site-directed mutagenesis was used to introduce the individual, identified point mutations into the surface-exposed positions of wild-type VSV-G (Figure 1), and GFP-expressing retroviral vectors were packaged. We found that with the exception of S162T, the genomic titers (G-titers) of mutant vectors were equivalent to wild-type vector (Figure 2a), suggesting that VSV-G can tolerate mutations that emerged from this library selection without the loss of assembly. The mutant infectious titers (I-titers) were similar to wild type, with the exceptions that VSV-G E380K exhibited a 40% increase, and K66T and T368A showed a 70% decrease, respectively, in infectivity (Figure 2b). We investigated the relative ratio of I-titer to G-titer of all mutant variants (Figure 2c and Supplementary Data Figure S4C). Vector containing K66T, S162T, T230N or T368A VSV-G showed somewhat lower I-titer/G-titer ratios compared with the wild type, suggesting that these mutations slightly decreased the infectivities of the viral vectors. We also investigated the relative ratio of VSV-G expression level, as obtained with a VSV-G Enzyme-linked immunosorbent assay (ELISA), to G-titer (Figure 2c), and did not observe any significant differences compared with wild-type VSV-G.

Human serum inactivation of variant VSV-G retroviral vectors

The human serum inactivation of retroviral vectors bearing wild type and single mutant VSV-G variants was examined. As noninfectious of empty particles could conceivably affect a neutralization assay, we used the results of a VSV-G ELISA assay (Supplementary Data Figure S4C) to equalize the amount of VSV-G added to each sample. The resulting ELISA-normalized viral vectors levels were incubated with the mixture of human serum from 18 donors. Following serum incubation at 37 °C for 1 h, titers were quantitated and reported as the percentage of remaining titer compared with that of control samples incubated at 37 °C for 1 h with phosphate-buffered saline (PBS) (pH 7.4) (Figure 3a) or heat inactivated (56 °C, 30 min) human sera (HIHS) (Supplementary Data Figure S5A). Viral vectors were not inactivated in HIHS, suggesting that the inactivation may be caused by complement (Supplementary Data Figure S5B), and the results of these two normalization were thus very comparable. In particular, individual VSV-G mutants S162T ($P<0.05$), T230N ($P<0.05$) or T368A ($P<0.05$) showed significantly increased human serum resistance compared with wild-type VSV-G (Figure 3a).

Incubation in serum at 37 °C may select not only for serum resistance but serendipitously also for thermostability, so we further examined the results for the vectors before and after incubation for 1 h in PBS (Figure 3b). Mutants carrying K66T, T368A or E380K interestingly showed higher thermostability compared with wild-type VSV-G ($P<0.05$). Because of the combined effects of serum resistance and thermal stability, a mutant carrying T368A showed >1.6-fold higher infectivity ($P<0.01$) compared with wild-type VSV-G upon incubation in human serum at 37 °C for 1 h (Figure 3c). On the basis of these data, the S162T, T230N and T368A mutations were deemed beneficial for serum resistance.

Combining mutations that individually contribute to a given property can further enhance that property.[41,42] We therefore, generated a number of double and triple VSV-G mutants from individual mutations shown to confer serum resistance. All VSV-G mutants analyzed showed significantly higher resistance ($P<0.01$ or 0.05) to human serum (gray bars in Figure 4a). Furthermore, all such mutants showed similar thermal stability compared with wild-type VSV-G (striped bars in Figure 4a). For comparison, we
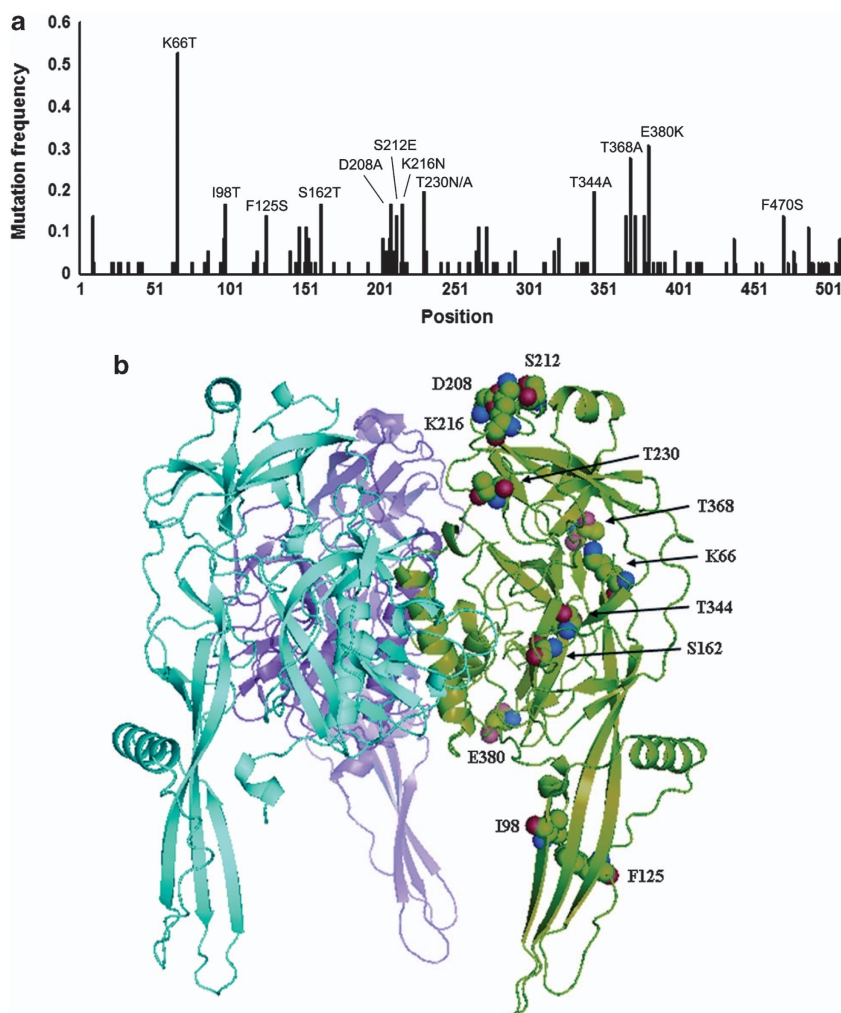
Figure 1. Common mutations following directed evolution of VSV-G. (**a**) The frequency of mutation at each amino acid residue of VSV-G among 36 randomly chosen VSV-G clones after five or six selection steps. (**b**) The location of each apparent 'hot spot mutation' in the crystal structure of the prefusion form of VSV-G (PDB ID: 2J6J). Figure was made using PyMol (http://www.pymol.org). Each monomer of VSV-G was colored in green, purple and sky blue, respectively. Green, blue and red balls represent carbon, nitrogen and oxygen atoms, respectively.

also prepared retroviral vector pseudotyped with an RD114 envelope, which has previously been reported to have higher serum resistance than wild-type VSV-G,[33,43] as another control. As reported, RD114 showed increased resistance to human serum compared with wild-type VSV-G, though again this envelope cannot package vector to the high titers needed for *in vivo* use.[33] Collectively, the T230N + T368A mutant showed the highest residual infectivity ($\sim$68%) compared with the residual infectivity of parental VSV-G ($\sim$37%) and similar residual infectivity ($\sim$68%) of RD114 after incubation in human serum at 37 °C for 1 h.

K66T, T368A and E380K mutations increased VSV-G thermal stability (Figure 3), and we thus investigated the effects of adding these mutations to the promising serum-resistant variants. Several variants containing K66T or K66T + E380K substitutions showed similar or slightly higher thermal stability compared with wild-type VSV-G, and several mutants (K66T + S162T + T230N + T368A, K66T + T368A + E380K and K66T + S162T + T230N + E380K) also showed statistically higher serum resistance ($P < 0.05$) (Figures 4b and c).

Human serum inactivation of variant VSV-G lentiviral vectors
Although the envelope compositions of retroviral and lentiviral vectors are likely similar, we also analyzed the ability of VSV-G

variants to confer serum resistance to lentivirus. On the basis of results with retroviral vectors, we analyzed the top five VSV-G variants (S162T + T230N, S162T + T368A, T230N + T368A, K66T + T368A + E380K and K66T + S162T + T230N + T368A) showing higher serum resistance for retrovirus. Equal amounts of packaged lentivirus (7×10^4 GFP TU) were diluted fivefold in human serum, or PBS (pH 7.4) as a control, and incubated at 37 °C for 1 h. Vector pseudotyped with several mutant VSV-G showed a greater resistance to human serum compared with wild-type VSV-G pseudotyped lentiviral vector (Figure 5). Importantly, mutants T230N + T368A or K66T + S162T + T230N + T368A had higher combined thermostability and serum resistance than wild-type VSV-G.

Vector inactivation by animal serum
VSV-G pseudotyped viral vectors can be inactivated by sera from mouse, rat and guinea pig, which can impact the interpretation of animal studies.[25,44] The relative sensitivity of VSV-G pseudotyped retroviral or lentiviral vectors to several animal sera was thus tested (Figures 6a and b, respectively). S162T + T230N, T230N + T368A or K66T + S162T + T230N + T368A VSV-G showed statistically higher resistance to mouse and rabbit sera for both retroviral and lentiviral vectors.
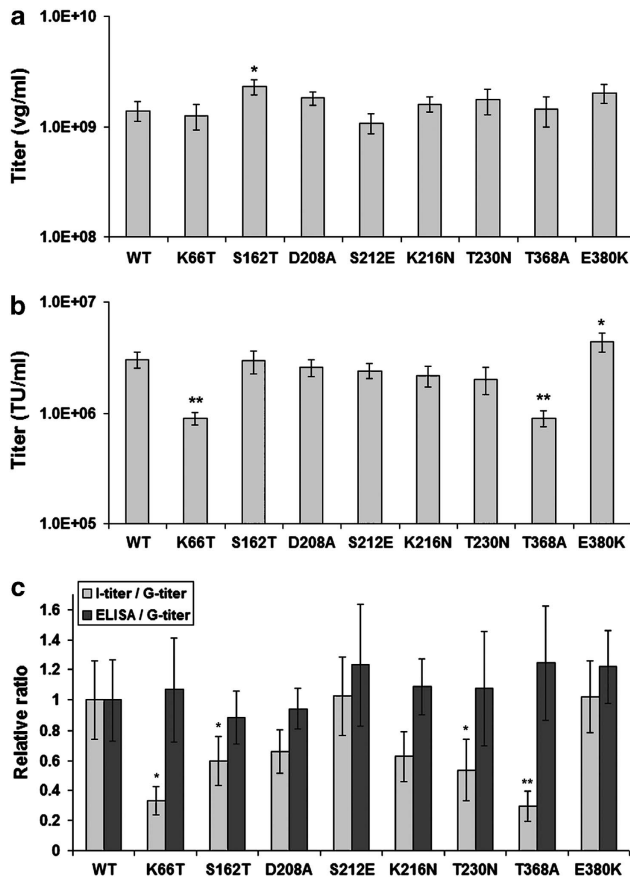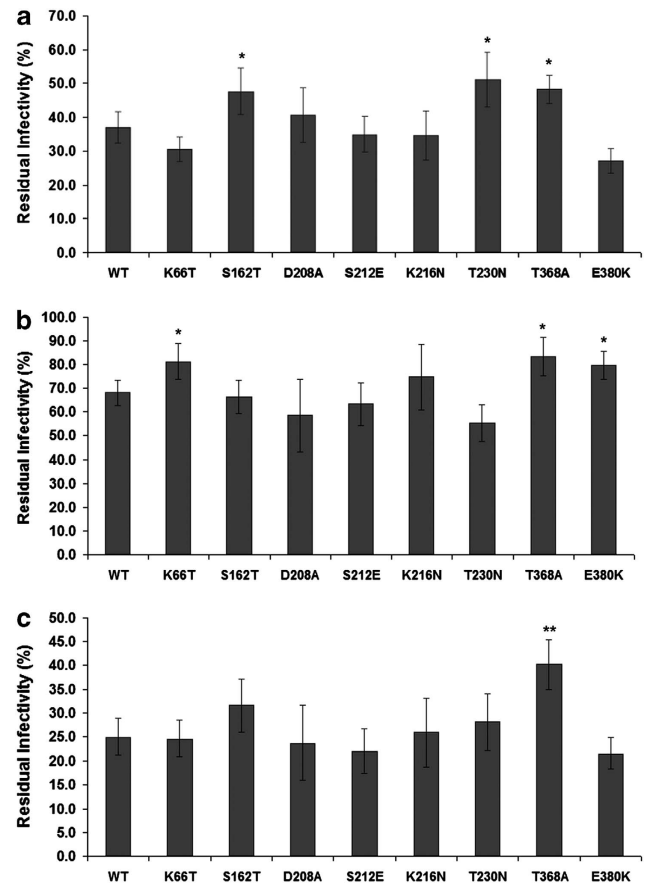
Figure 2. Genomic and infectious titers of VSV-G chimeric retroviral vectors. Murine retroviral vector was packaged with the pCLPIT GFP vector plasmid, pCMV gag-pol and pcDNA IVS VSV-G helper plasmid containing individual VSV-G variants. (**a**) Vector genomic titers (G-titers) were measured by real-time quantitative PCR. (**b**) Transduction efficiencies on 293T cells were determined by flow cytometry analysis of retroviral vector mediated GFP expression. (**c**) Relative ratios of infectious titers (I-titers) to G-titers (gray bars) and VSV-G ELISA data to G-titers (black bars) of the retroviral vectors were calculated with the ratio of wild-type VSV-G pseudotyped retroviral vector as 1, respectively. Error bars denote s.d. ($n = 3$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with infectivity of wild-type VSV-G, as determined using an one-way analysis of variance (ANOVA).

Figure 3. Human serum resistance and thermostability of retroviral vectors pseudotyped with VSV-G mutants. The amounts of viral vectors were normalized based on VSV-G ELISA assay. (**a**) Human serum neutralization was quantified by measuring vector titers after incubation with human serum at 37 °C for 1 h, relative to those after incubation with PBS at 37 °C for 1 h. (**b**) Thermal effects were determined by quantifying relative titers after incubation with PBS at 37 °C for 1 h compared with those without incubation at 37 °C. (**c**) Human serum neutralization and thermal effects were determined by calculation of relative titers after incubation with human serum at 37 °C for 1 h compared with those without incubation at 37 °C. Error bars denote s.d. ($n = 4$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with infectivity of wild-type VSV-G, as determined using an one-way ANOVA.

In vitro transduction of several cell lines

Although the infectivities of 293T cells were comparable with wild-type VSV-G in the absence of human serum (Figure 2), we characterized transduction of several other cell lines to determine whether the mutations affected infectivity (Figure 7). Although the infectivities of the variants were somewhat lower for several cell types, the trends in infectivity for all lines was similar between wild-type and mutant VSV-G. Interestingly, the mutant carrying K66T + S162T + T230N + T368A showed statistically higher transduction efficiency to HeLa cells compared with wild type ($P < 0.01$).

*In vivo* analysis of human serum resistance

The ability of two promising variants—which we now term mutant 1 (T230N + T368A) and mutant 2 (K66T + S162T + T230N + T368A)—to resist human serum neutralization in a murine model was analyzed. For the *in vivo* neutralization assays, vector could be added to 100% human serum; however, human serum

administration *in vivo* results in an ~ 10-fold dilution of the serum into circulation. To tune the level of vector that should be administered to observe neutralization, a preliminary study with wild-type VSV-G was conducted, and the interval between serum and lentiviral vector administration was also varied. BALB/c mice were injected with 200 μl of human serum, or PBS as a control, into the tail vein. One, 5, or 24 h later, lentiviral vector encoding firefly luciferase was administered. For an 8×10^{10} vector genome administration, after two weeks expression was observed in the liver, the primary site of lentiviral vector transduction.[45] Also, expression levels were lower for vector administered 1 or 5 h, but not 24 h, after infection of human serum, compared with the PBS control (data not shown).

To assess neutralization of the VSV-G mutants, equal levels of lentiviral vector (8×10^{10} vector genomes) were injected via the tail vein 1 h after the administration of 200 μl of human serum or PBS. After 2 weeks, the mice were killed, and luciferase expression levels were analyzed in the liver. The presence of human serum reduced luciferase expression mediated by wild-type VSV-G to
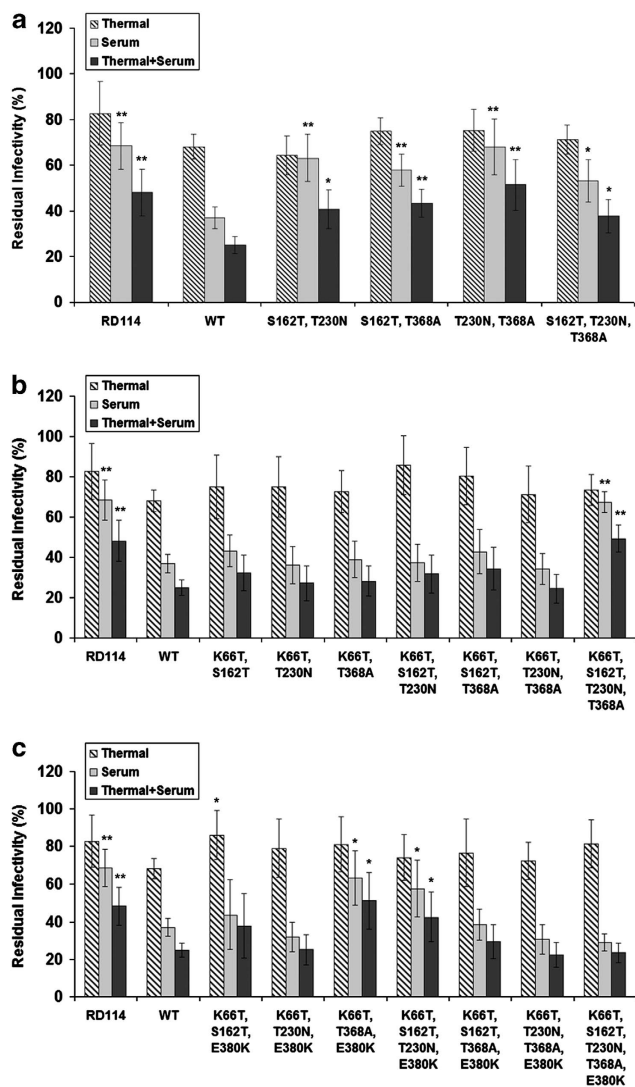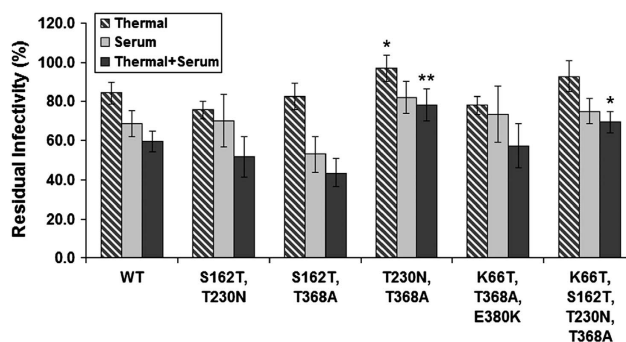
Figure 4. Human serum neutralization and thermostability of retroviral vectors pseudotyped with VSV-G variants that combine several 'hot spot mutations'. VSV-G mutants with combined beneficial mutations were generated by site-directed mutagenesis. The amounts of viral vectors were normalized based on VSV-G ELISA assay. Thermal effects, human serum neutralization and combined serum neutralization and thermal effects were determined by quantifying relative titers after incubation with PBS at 37 °C for 1 h compared with those without incubation at 37 °C, after incubation with human serum at 37 °C for 1 h compared with those after incubation with PBS at 37 °C for 1 h, and after incubation with human serum at 37 °C for 1 h compared with those without incubation at 37 °C, respectively. (a) Variants contained 'hot spot mutations' for human serum resistance. (b) K66T and (c) E380K were added to enhance the thermal stability of retroviral vectors. Error bars denote s.d. ($n = 4$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with the wild-type VSV-G, as determined using an one-way ANOVA.

only 22.1% of PBS-injected controls in the liver. The human serum reduced mutants 1 and 2 to only 60% of PBS controls, levels that were nearly threefold higher than wild-type VSV-G under the same conditions (Figure 8).

DISCUSSION

Because of their broad tropism and high stability, VSV-G pseudotyped retroviral and lentiviral vectors are promising


Figure 5. Human serum neutralization and thermostability of lentiviral vectors pseudotyped with VSV-G variants. A standard GFP encoding lentiviral vector was packaged with five VSV-G mutants that appeared promising in the retroviral results. Five VSV-G variants (S162T + T230N, S162T + T368A, T230N + T368A, K66T + T368A + E380K and K66T + S162T + T230N + T368A) showing higher resistance and thermal stability for retroviral vector packaging were selected. Thermal effects, human serum neutralization and combined serum neutralization and thermal effects were determined by quantifying relative titers after incubation with PBS at 37 °C for 1 h compared with those without incubation at 37 °C, after incubation with human serum at 37 °C for 1 h compared with those after incubation with PBS at 37 °C for 1 h, and after incubation with human serum at 37 °C for 1 h compared with those without incubation at 37 °C, respectively. Error bars denote s.d. ($n = 4$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with the wild-type VSV-G, as determined using an one-way ANOVA.

vehicles for gene transfer gene in vitro and in vivo. However, while VSV-G pseudotyped vectors have been increasingly explored for ex vivo gene delivery in clinical trials,[46,47] serum neutralization of the VSV-G pseudotyped viral vectors is a significant obstacle for direct in vivo administration. In this study, we conducted directed evolution of VSV-G to create serum-resistant VSV-G mutants. After six selection steps, followed by functional analysis of common mutations, we identified several mutations (S162T, T230N and T368A) that enhanced VSV-G serum resistance. Furthermore, as incubation at 37 °C was inherent to the selection, we also identified several adventitious mutations (K66T, T368A and E380K) that apparently increased VSV-G thermostability. Upon combining these two classes of mutations, retroviral and lentiviral vectors pseudotyped with two resulting VSV-G variants (T230N + T368A and K66T + S162T + T230N + T368A) showed higher resistance to human and animal sera, as well as increased thermostability compared with wild-type VSV-G. While adeno-associated virus has been evolved to resist antibody neutralization,[38,48,49] to our knowledge this is the first effort to harness iterative random mutagenesis and selection to endow retroviral or lentiviral vectors with the capacity to resist serum inactivation.

VSV-G pseudotyped viral vectors can be inactivated in serum by complement,[20,30] a major element of the innate immune response that in general functions via classical, alternative and lectin pathways.[21] All pathways can mediate virus opsonization, virolysis and anaphylatoxin and chemotaxin production. However, the particular mechanisms by which complement inactivates VSV-G presenting virions are unknown. The mutation sites (Ser162, Thr230 and Thr368) we identified do not lie within several known antibody binding epitopes,[50] indicating that these residues may lie in previously uncharacterized epitopes, or that VSV-G may interact with complement via mechanisms independent of antibody neutralization. Despite the lack of mechanistic information underlying a given gene delivery problem, directed evolution can still be used to create enhanced variants, which in
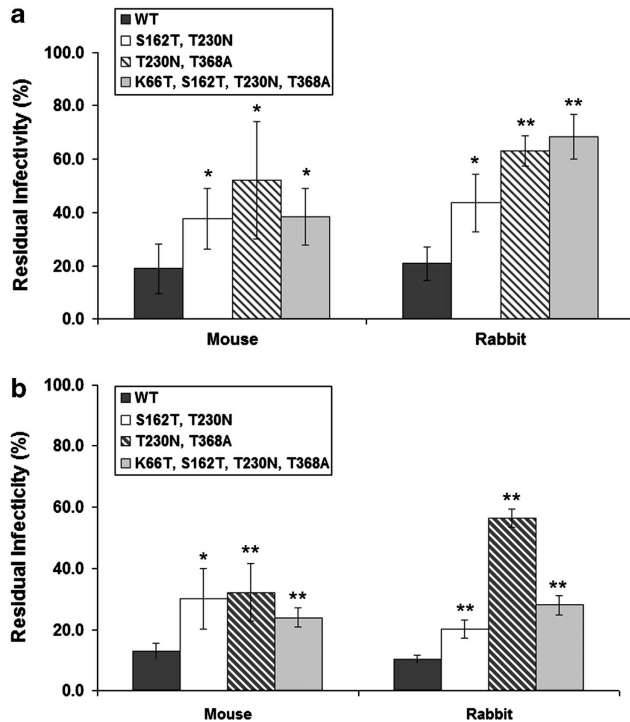
Figure 6. Aminal serum neutralization of retroviral and lentiviral vectors pseudotyped with VSV-G variants. For three variants (S162T + T230N, T230N + T368A and K66T + S162T + T230N + T368A) and wild-type VSV-G, neutralization by animal sera was examined. (**a**) Retroviral vectors and (**b**) lentiviral vectors were diluted fivefold in animal sera and incubated at 37 °C for 1 h. Serum inactivation was determined by quantifying relative titers after incubation with human serum at 37 °C for 1 h compared with those after incubation with PBS at 37 °C for 1 h. Error bars denote s.d. ($n = 4$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with the wild-type VSV-G, as determined using an one-way ANOVA.
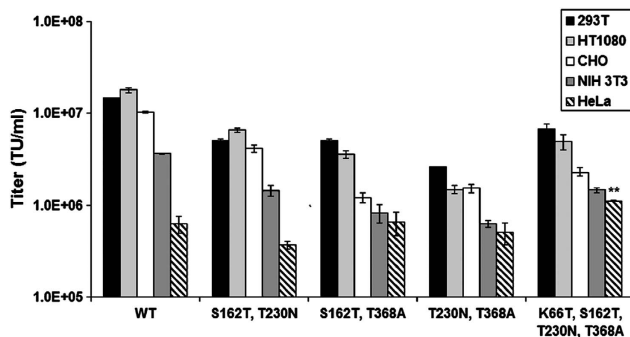
Figure 7. *In vitro* transduction of multiple cell lines with retroviral vectors pseudotyped with VSV-G variants. Retroviral vectors expressing GFP were used to transduce a panel of cell lines: HEK 293T, HT1080 (human fibrosarcoma cell line), CHO K1, NIH 3T3 (mouse embryonic fibroblast cell line), and HeLa cells to assess the transduction profile of the novel VSV-G variants. Error bars denote s.d. ($n = 3$). ** indicates statistical differences of $P < 0.01$, as determined using an one-way ANOVA.

this case could aid future efforts to investigate mechanisms of retroviral or lentiviral vector inactivation by complement.

As a byproduct of the evolution, mutations that enhanced the thermal stability of VSV-G were also identified, which can potentially enhance vector production and reduce the dosage of
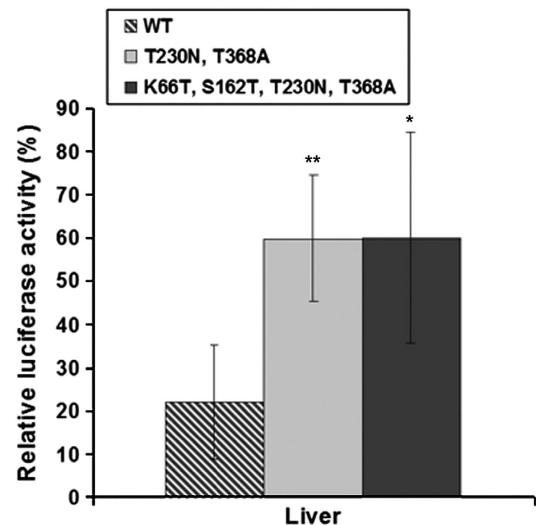
Figure 8. Human serum neutralization *in vivo*. For two variants (T230N + T368A and K66T + S162T + T230N + T368A) and wild type VSV-G, lentiviral vectors encoding luciferase were administered via tail vein injection to female BALB/c mice one hour after human serum or PBS introduction. After two weeks, levels of luciferase activity were determined and normalized to total protein for each sample analyzed. Relative luciferase expression in liver was determined by quantifying relative enzyme activity from human serum-primed mice relative to activity from naïve mice. Error bars denote s.d. ($n = 4$). * and ** indicate statistical differences of $P < 0.05$ and $P < 0.01$, respectively, compared with wild type VSV-G, as determined using an one-way ANOVA.

administration. The extracellular half-life of retroviral vectors lies between 3.5 and 8 h at 37 °C,[35,51,52] and our measurement ($t_{1/2} = \sim 3$ h) is close to this range. Therefore, our incubation of the library for 6 h in human serum at 37 °C also selected for enhanced thermostability. An increased protein thermal stability can be caused by several factors such as disulfide bond formation, hydrophobic interactions or change of electrostatic interactions of the surface.[53] The E380K mutation in VSV-G may eliminate a like-charge repulsion with Asp381 and create opposite-charge attraction on the protein surface to confer thermostability to the protein. By comparison, potential mechanisms for K66T and T368A thermostabilization are not as readily apparent. At any rate, combining these mutations with others that conferred serum resistance resulted in vectors with both the properties. In particular, mutants 1 and 2 showed a twofold increase in half-life at 37 °C ($t_{1/2} = \sim 6$ h). These improvements of thermal stability may have also contributed slightly to a greater stability to vector concentration by ultracentrifugation (data not shown).

Cocal-pseudotyped lentiviral vectors may have potential for *in vivo* gene therapy, due to their high titers, broad tropism, stability and reported increased resistance to human serum compared with VSV-G pseudotyped vectors.[31] However, unlike VSV-G, their *in vivo* use has not yet been reported. RD114-pseudotped vectors also exhibit resistance to human serum. Green *et al.* [54] conducted immunostaining for RD114 receptors, which were present in the colon, testis, bone marrow, skeletal muscle and skin epithelia. However, the receptors are absent in the lung, thyroid and artery, suggesting that the application of RD114-pseudotyped vectors to treat some diseases may be limited. By comparison, VSV-G pseudotyped lentiviral vectors have potential for broad tropism.[55]

In this study, we evolved VSV-G and successfully created variants with higher resistance to human, rabbit and mouse sera *in vitro* and human serum *in vivo*. This work therefore further establishes the power of directed evolution to improve viral

vectors, and these results may enhance the utility of retroviral and lentiviral vectors to treat human disease. In addition, VSV itself has emerged as a promising candidate in the field of oncolytic virus therapy. Therefore, incorporating a VSV variant encoding a human serum-resistant and thermostable VSV-G may enhance the therapeutic potential of VSV for treating human cancer.

MATERIALS AND METHODS

Library construction and selection

Random mutagenesis libraries were generated by standard error-prone PCR of a VSV-G template using VSV-G fwd and VSV-G rev as primers (see Supplementary Table S1 for all primer sequences). The amplified PCR fragments were digested with Sfi I and Xho I, and the resulting fragments were ligated into the corresponding sites of the retroviral vector pCLPIT, which contains a puromycin resistance gene and places VSV-G under a tetracycline responsive promoter.

HEK 293T cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (Invitrogen, Calrsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen) at $37\,^{\circ}C$ and 5% CO_2. The VSV-G library was packaged into retroviral vectors via calcium phosphate transfection of 62.5 ng of pCLPIT VSV-G Lib, 6 μg of pCMV gag-pol, and 14 μg of pBluescript II SK (Stratagene, Santa Clara, CA, USA) in an $\sim 80\%$ confluent 10 cm dish of HEK 293T cells. The library vector supernatant was twice harvested, 2 and 3 days post-transfection, and then concentrated by ultracentrifugation. For selection, 18 individual human serum samples were obtained from Innovative Research, Inc. (Southfield, MI, USA). The viral vector library was diluted fivefold in the pooled human serum and incubated for 6 h at $37\,^{\circ}C$. These viral vectors were used to infect 293T cells at a multiplicity of infection of < 0.1. The infected cells were selected and enriched using 1 μg ml^{-1} of puromycin for 2 or 3 weeks. Packaging-competent virus variants were rescued through transfection of pCMV gag/pol into the expanded 293T cells and concentrated by ultracentrifugation for the next round of screening.

Sequence analysis and site-directed mutagenesis

After five and six rounds of the selection process, genomic DNA of the infected 293T cells was extracted using the QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). For sequencing of individual VSV-G clones, the VSV-G genes were amplified from this genomic DNA by PCR (using VSV-G IVS fwd and VSV-G IVS rev primers). The resulting PCR fragments were digested with Eco RI, and the fragments were ligated into the corresponding site of pcDNA IVS.[56] Randomly chosen clones were sequenced (with IVS seq fwd, IVS seq rev and VSV-G seq in primers).

Site-directed mutagenesis was conducted on the plasmid pcDNA IVS VSV-G using the QuickChange PCR-based technique, with Pfu polymerase and primers described in Supplementary Table S1. The amplified PCR products were treated with Dpn I and propagated into Escherichia coli DH10B. Mutations were verified by DNA sequencing.

Viral production

The individual VSV-G mutant retroviral vectors were packaged with 10 μg of pCLPIT GFP, 6 μg of pCMV gag-pol and 4 μg of pcDNA IVS VSV-G (or pcDNA IVS VSV-G mut) in an $\sim 80\%$ confluent 10 cm dish of HEK 293T cells. RD114 envelope pseudotyped retroviral vector was packaged with pcDNA IVS RD114, which was obtained by the substitution of VSV-G gene by RD114 gene from pLTR-RD114A (Addgene, Cambridge, MA, USA). The culture medium was changed 12 h post-transfection, and the viral supernatant was collected both 24 and 48 h later and concentrated by ultracentrifugation. To determine the titers of GFP-expressing vectors, 4×10^5 293T cells were infected with at least three different volumes of vector supernatant or concentrate. The cells were assayed for GFP expression by flow cytometry 3 days after infection. Vector genomic titers were measured by real-time quantitative PCR using the iCycler iQ Real Time Detection System (Bio-Rad, Hercules, CA, USA) and SYBR Green I (Invitrogen) with primers 5'-ATTGACTGAGTCGCCCGG-3' (forward) and 5'-AGCGAGACCACAAGTCGGAT-3' (reverse). Individual VSV-G mutant lentiviral vectors were packaged with 10 μg of lentiviral transfer vector HIV-CSCG, 5 μg of pMDLg/pRRE, 1.5 μg of pRSV Rev and 3.5 μg of pcDNA IVS VSV-G (or pcDNA IVS VSV-G mut), and a process similar to retroviral vector production. Lentiviral vectors harboring firefly luciferase were packaged with 10 μg of Fluc, 5 μg of pMDLg/pRRE, 1.5 μg of pRSV Rev and 3.5 μg of pcDNA IVS VSV-G (or pcDNA IVS VSV-G mut).[57]

Serum inactivation assay

In addition to the 18 human serum samples were obtained from Innovative Research, Inc. (Southfield, MI, USA), mouse and rabbit serum were purchased from Sigma (St Louis, MO, USA). Serum inactivation experiments were carried out as described previously with some modifications.[58] Briefly, 20 μl of viral vector normalized based on VSV-G ELISA data was diluted fivefold in human sera, heat-inactivated human sera ($56\,^{\circ}C$, 30 min), or PBS (pH 7.4) as a control. Each sample was incubated for 1 h at $37\,^{\circ}C$. All samples were then serially diluted and titered on 293T cells. Gene transfer was evaluated by flow cytometer for GFP expression 3 days after infection. To determine the change in titer after exposure to serum, the percentage of GFP-expressing cells for vector incubation in the serum was divided by the percentage of GFP-expressing cells for vector in PBS and reported as the percentage of titer recovery. To investigate thermal stability of the viral vectors, samples in PBS without incubation at $37\,^{\circ}C$ was used as a control.

ELISA assay

To evaluate the expression level of VSV-G of purified viral vectors, the binding of viral vector populations to a VSV-G polyclonal antibody was monitored by ELISA.[59] MaxiSorp (Nunc) 96-well plates were coated at $4\,^{\circ}C$ overnight with 50 μl of viral vectors diluted in carbonate coating buffer (100 mM, pH 9.6) and washed three times with PBS (pH 7.4). The wells were blocked by adding 200 μl of PBS with 5% nonfat dry milk at room temperature for 2 h and washed twice with PBS. Hundred microlitres of biotin conjugated anti-VSV-G tag antibody (1:3000) (Abcam, Cambridge, MA, USA) in carbonate coating buffer was added. After incubation overnight at $4\,^{\circ}C$, the plates were washed four times with PBS and 100 μl of streptavidin-alkaline phosphatase (1:1000) (Invitrogen) was added and incubated for 2 h at room temperature. After four times washing with PBS, p-nitrophenly-phosphate substrate solution (Invitrogen) was added. Plates were read to determine the OD_{405} after 30 min incubation at room temperature.

Transduction analysis in vitro

To determine the relative transduction efficiencies the selected mutants compared with parental wild-type VSV-G pseudotyped retroviral vectors, HEK 293T, CHO K1, NIH 3T3 (mouse embryonic fibroblast cell line), HeLa and HT1080 cells were plated at a density of 4.0×10^5 cells per well 24 h before infection. Cells were infected with wild-type or VSV-G variants pseudotyped retroviral vectors. The fraction of GFP-positive cells was assessed 72 h post infection using a Beckman-Coulter Cytomics FC-500 flow cytometer.

In vivo human serum inactivation

Eight-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA; $n = 4$) were used for all experiments. Two hundred microlitres of pooled human serum, or PBS as a control, was administered into the tail vein with a 30-gauge needle. One hour after human serum or PBS introduction, recombinant lentiviral vectors carrying complementary DNA encoding firefly luciferase under the control of human ubiquitin promoter was injected into the tail vein. Two weeks after virus injection, animals were transcardially perfused with PBS, and liver was harvested and frozen. Frozen tissue samples were homogenized in $1 \times$ reporter lysis buffer (Promega, Madison, WI, USA) and clarified by centrifugation for 10 min at $10\,000\,g$. Luciferase reporter activities were determined as previously described,[60] and the luciferase signal was normalized to total protein content as determined by a bicinchoninic acid assay (Pierce, Rockford, IL, USA). Animal protocols were approved by the University of California, Berkeley Animal Care and Use Committee and conducted in accordance with the National Institutes of Health guidelines.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)