Molecular Engineering of Viral Gene Delivery Vehicles

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Abstract

Viruses can be engineered to efficiently deliver exogenous genes, but their natural gene delivery properties often fail to meet human therapeutic needs. Therefore, engineering viral vectors with new properties, including enhanced targeting abilities and resistance to immune responses, is a growing area of research. This review discusses protein engineering approaches to generate viral vectors with novel gene delivery capabilities. Rational design of viral vectors has yielded successful advances in vitro, and to an extent in vivo. However, there is often insufficient knowledge of viral structure-function relationships to reengineer existing functions or create new capabilities, such as virus-cell interactions, whose molecular basis is distributed throughout the primary sequence of the viral proteins. Therefore, high-throughput library and directed evolution methods offer alternative approaches to engineer viral vectors with desired properties. Parallel and integrated efforts in rational and library-based design promise to aid the translation of engineered viral vectors toward the clinic.

Contents

INTRODUCTION

Over the past two decades, gene therapy has demonstrated strong potential to treat various diseases, such as cystic fibrosis, hemophilia, Alzheimer's disease, and some cancers (1–3). The two primary approaches currently employed to deliver the therapeutic genetic material are synthetic and viral delivery vehicles. Whereas synthetic vectors, usually a polymer-DNA or lipid-DNA complex, offer safety and modularity, they remain too inefficient for most clinical applications. However, viruses have evolved in nature to efficiently deliver their own genetic payload to specific cells, and standard molecular biology methods can be employed to swap therapeutic transgenes in place of some or all of the viral genes. Promising results from numerous in vitro studies and animal disease models have fueled a large number of human clinical trials that utilize viral vectors. Greater than half of all clinical trials to date have employed adenoviral (Ad), retroviral/lentiviral, or adeno-associated viral (AAV) vectors for gene delivery (1). However, both basic research studies and these trials have identified several gene delivery barriers that reduce the therapeutic efficacy of the viral vectors (2, 4, 5).

These biological gene delivery barriers occur in two forms: systemic (e.g., pre-existing immunity) (4, 6–8) or cellular (e.g., binding to the cell surface) (9, 10). Over evolutionary timescales, viruses have evolved ways to circumvent many of these gene delivery barriers; however, most vectors still suffer numerous shortcomings along their delivery pathway. Within a given class or family of viruses, swapping the viral attachment proteins (VAPs) of different members on a common vector scaffold, a process known as pseudotyping, offers the potential to create vectors with new specificities and efficiencies. However, in many cases, there exists no natural variant with the desired gene delivery properties, as many properties of viruses are not optimal for human therapeutic use, not surprisingly because natural evolution did not require some therapeutically desirable properties for viral "survival."

Therefore, engineering novel viral vectors with customized, user-defined gene delivery properties is an exciting and promising area of research. However, viruses are highly complex biological entities, as exemplified by their intricate multimeric protein shells, or capsids, which encompass

Ad: Adenovirus

AAV: Adeno-associated virus

VAP: viral attachment protein

complex genomes that utilize overlapping reading frames and alternative splicing for highly economical use of coding space. This complexity presents many challenges to engineering novel functions without disrupting key properties crucial for efficient viral function. Recently, synthetic biology approaches have successfully assembled smaller functional parts into a variety of complex systems (11, 12), and by analogy, engineering single viral proteins with defined functions offers the potential to create complex viral vector systems with user-defined gene delivery properties. This review outlines the major protein engineering techniques that have been utilized in viral vector engineering, highlights the key features of each method, and discusses the future applications of designed viral vectors. We focus on Ad, retroviral/lentiviral, and AAV vectors, and we refer readers to reviews of other viral vector systems that have also demonstrated some success in gene delivery and vector engineering (2).

Transduction: the entire system of gene delivery and expression

CAR: coxsackievirus B and adenovirus receptor

Serotype: a group of related biological entities, such as viruses, that share a common set of antigens

BACKGROUND

Adenovirus

Ad, a member of the family *Adenoviridae* and genus *Mastadenovirus*, is a nonenveloped virus with a 36-kilobase (kb) double-stranded DNA genome. Several features of Ad make it an attractive candidate as a gene delivery vehicle, including its ability to grow as high-titer recombinant virus, large transgene capacity, and efficient transduction of both dividing and nondividing cells (13–15). Through the use of alternative splicing and bidirectional transcription, the viral genome encodes more than 50 viral proteins, flanked by ~100 base pair (bp) inverted terminal repeats (ITRs) (**Figure 1**) (16). The 70–100 nm diameter icosahedral viral capsid consists of an exterior composed mostly of three proteins, the hexon (240 trimers), fiber (12 monomers), and penton (5 pentamers), as well as other structural proteins involved in capsid stabilization and genome association. The fiber and penton mediate binding to cell surface receptors such as coxsackievirus B and adenovirus receptor (CAR) (17), CD46 (18), and α_v integrins (19). A complete review of Ad biology has recently been presented elsewhere (16).

To date, more than 51 human and many nonhuman serotypes of Ad, which are classified as A–F, have been found to mediate gene delivery to a wide range of tissues, such as the respiratory tract, eye, liver, and urinary tract (16). The majority of studies have utilized Ad5; however, many other variants are now being explored (20, 21). Several generations of replication-deficient Ad vectors have been produced, where each stage involves the deletion of several additional viral genes (22), culminating in a helper-dependent vector that lacks the full viral genome (23). However, several significant limitations of Ad vectors exist, such as lengthy production (22), difficult production for helper-dependent virus, preexisting immunity (6, 7), infection of off-target cells (9), and in particular vector immunogenicity (8).

Adeno-Associated Virus

AAV, a member of the family *Parvoviridae* and genus *Dependovirus*, is a nonpathogenic, nonenveloped virus with a 4.7 kb single-stranded DNA genome. As its name implies, AAV requires the presence of certain proteins from a helper virus, usually a member of the adenovirus or herpesvirus family, to complete its lifecycle. It has not been associated with any human disease, and AAV vectors mediate long-term gene expression in a wide array of dividing and nondividing cells in vivo (4, 24, 25), making AAV an ideal candidate for a gene delivery vector.

The viral genome contains two 145-bp ITRs that flank two viral genes, *rep* and *cap*, which, through use of alternative splicing and start codons, generate seven proteins with partially

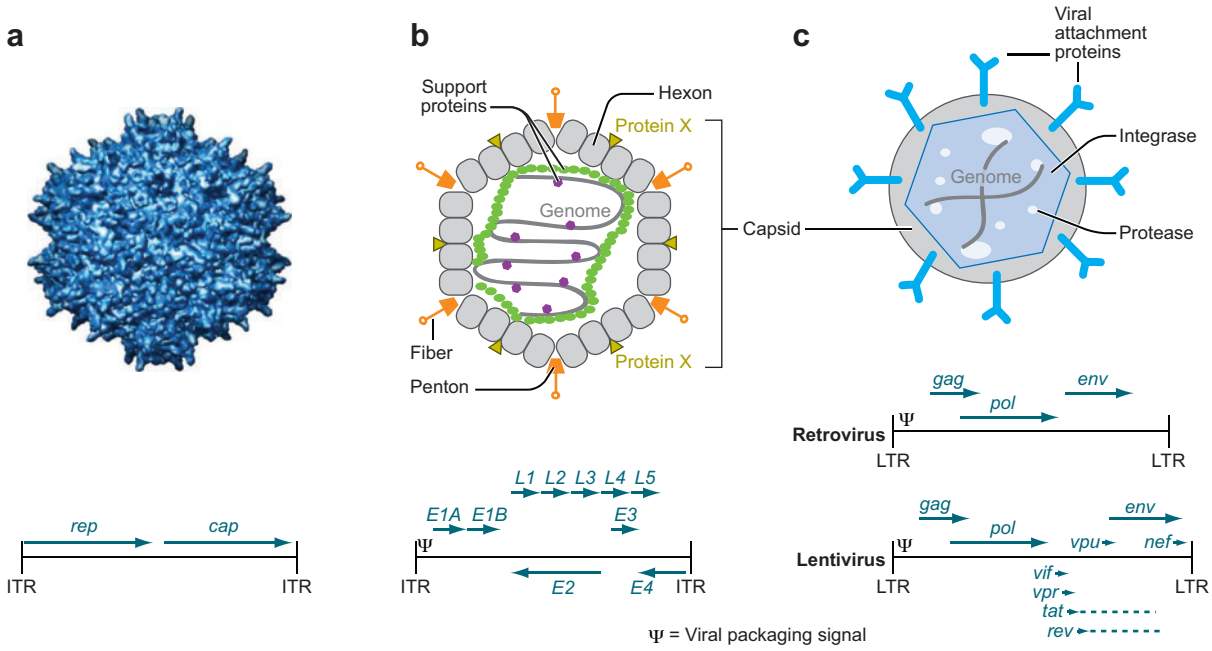

Figure 1

Schematic of the structure of viral particles and organization of the viral genomes. (*a*) Three-dimensional representation of the AAV capsid from VIPER database (**http://viperdb.scripps.edu/**) and schematic of AAV genome. (*b*) Representation of key components of the adenoviral capsid and genome organization. (*c*) Representation of key components of retroviral and lentiviral particles along with the genome organization of each virus.

overlapping sequences (**Figure 1**) (26). The *rep* gene serves as a template for four nonstructural proteins (Rep78, Rep68, Rep52, and Rep40) that possess a broad range of functions in ITR-dependent viral replication, transcriptional regulation, site-specific integration (27), and virion assembly, as reviewed elsewhere (28). The *cap* gene mediates the production of three structural proteins, VP1–3, that assemble at a ratio of ~1:1:18 to form the 60-mer viral capsid of ~25 nm in diameter (28). The capsid determines the gene delivery properties of the virus, including its binding to a variety of cell surface receptors such as heparan sulfate proteoglycan (HSPG) (29), sialic acid (30), fibroblast growth factor receptor (FGFR) (31), and platelet-derived growth factor receptor (PDGFR) (32). The complete AAV biological infection pathway has recently been reviewed in detail (33).

To date, more than 100 different serotypes of AAV have been isolated from both human and nonhuman tissues (34, 35). Most studies to date have focused on AAV serotype 2 (AAV2), but recently several other serotypes, whose sequence variation in the viral capsid confers a broad range of gene delivery properties and options, have shown promising results. Traditionally, the transfection method for AAV production has been time consuming; however, recent advances in the development of AAV packaging cell lines and purification methods such as ion exchange have substantially improved the process and expanded the application of AAV vectors to clinical therapy (36). Finally, several additional limitations to AAV vectors exist, including genome packaging size (37), preexisting immunity (4, 6), poor transduction of some cells (10), and infection of off-target cells (38).

HSPG: heparan sulfate proteoglycan

Retrovirus and Lentivirus

Tropism: cell or tissue delivery range of a virus or vector

MLV: murine leukemia virus

Retroviruses are a family of enveloped viruses with a diploid, 7–12 kb single-stranded, positive sense RNA genome (39). Retroviruses are subdivided into seven groups, including five groups of oncogenic retroviruses, lentiviruses, and spumaviruses (39). Their genomes contain four primary genes: *gag*, which encodes structural proteins such as matrix protein (MA), capsid protein (CA), and nucleocapsid protein (NC); *pro* and *pol*, which encode enzymatic proteins such as protease (PR), reverse transcriptase (RT), and integrase (IN); and *env*, which encodes the surface and transmembrane units of envelope protein (SU and TM, respectively) (**Figure 1**). Retroviruses initiate infection through interaction between their SU and specific cellular receptors, and this interaction is the critical determinant of viral tropism. This binding results in conformational changes of SU and TM, allowing exposure of a hydrophobic fusion peptide in TM. Furthermore, ecotropic Moloney murine leukemia virus (MLV) and avian leucosis virus (ALV) require additional exposure to low pH after endosomal acidification to aid fusion (40, 41). Insertion of the hydrophobic peptide into the cellular membrane subsequently mediates fusion between viral membrane and the cellular plasma membrane or endosome, thereby releasing the core nucleoprotein complex into the cytoplasm. The viral RNA genome is then reverse transcribed into double-stranded DNA, and the resulting duplex is finally integrated into the host chromosome after transport into the host nucleus. This transport can be active, such as lentiviruses that can be imported through nuclear pore complexes in nonmitotic cells, or passive, such as MLV vectors that require cell division to gain access to the nucleus. A complete review of the retrovirus life cycle has recently been presented (39).

Genome integration, which permits stable and prolonged expression of delivered therapeutic genes, makes retroviruses attractive platforms as gene delivery vehicles. Two variants of the well-characterized MLV are frequently used for gene therapy applications: ecotropic MLV, which utilizes the cationic amino acid transporter Rec-1 expressed strictly by murine cells as its receptor, and amphotropic MLV, which uses the phosphate transporter RAM-1 present on various mammalian cells for its receptor (42). In addition, vectors based on HIV-1 and other retroviruses, pseudotyped with various envelope proteins, have been used to mediate gene delivery to both dividing and nondividing cells. Despite advantages such as low immunogenicity and relatively large packaging capacity, retroviral vectors suffer several shortcomings, including low production yields, instability of envelope proteins, and semirandom integration patterns that pose a risk of insertional mutagenesis (5).

RATIONAL DESIGN OF VIRAL VECTORS

Chimeric, Mosaic, and Pseudotyped Particles

While natural viral variants can offer some desirable properties, they can possess several limitations, such as neutralization by host immune responses (4, 6–8), inefficiencies in production and purification (22, 37), poor specificities (9, 38), and poor transduction of therapeutically relevant cells (10, 16). Therefore, protein engineering approaches to generate viral vectors with novel and improved gene delivery properties offer attractive means to address these gene delivery problems. For example, combining functions from two or more viral variants into a single viral vector has the potential to build complex viral vectors from individual viral protein constituents. These methods can entail substitution of a VAP from one serotype with that of another (pseudotyping), assembly of entire viral particles using mixtures of VAPs from different variants (mosaic particles),

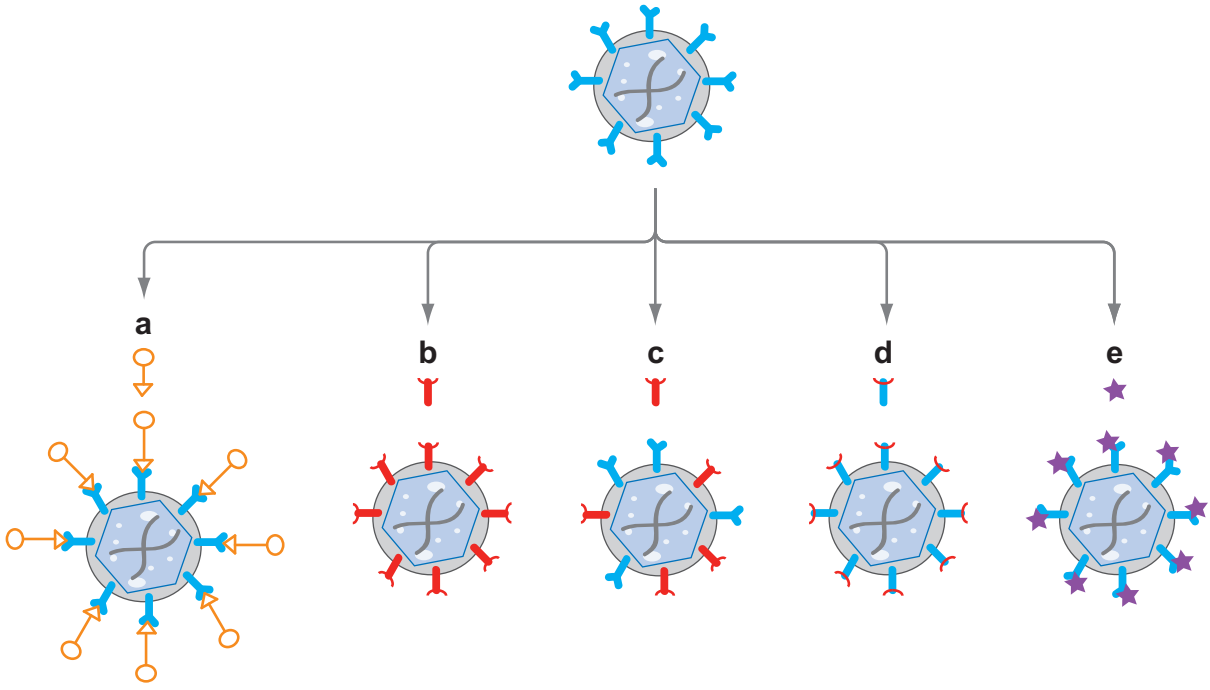

Figure 2

Overview of rational protein engineering strategies for viral vectors. Rational design methods include (*a*) the use of a bispecific adaptor, (*b*) pseudotyping with an alternate capsid or VAP, (*c*,*d*) the generation of mosaic or chimeric particles, and (*e*) genetic engineering of VAP to insert peptide or point mutations.

or swapping smaller VAP domains between serotypes via rational methods or high-throughput recombination techniques (chimeric particles) (**Figure 2**).

Chimeric Ad particles generated by swapping fibers or hexons between Ad5 and other serotypes have exhibited impressive potential to alter viral tropism and enhance vector resistance to preexisting Ad5 antibodies. For example, a recent system was developed to analyze the properties of fibers or hexons from any Ad serotype in the context of the Ad5 capsid and has identified alternate serotype fibers that considerably improve transduction of some cancer and primary cell lines (20). Likewise, swapping of only the hypervariable regions (HVR) of the Ad5 hexon with those of the rare Ad48 serotype was sufficient to evade preexisiting neutralizing antibodies (NAbs) (43). Interestingly, this work also revealed that not all fiber or hexon combinations produce functional Ad chimeras. In studies that tested the modularity of "parts" from distant viral families, chimeric Ad vectors were also constructed with modified fiber-like proteins from T4 bacteriophage (44) and reovirus (45). Furthermore, a modified T4 fibritin fused to single-chain antibodies has been used to retarget Ad vectors to alternate receptors (44), and reovirus σ_1 protein facilitated sialic acid binding and enhanced dendritic cell transduction (45).

Novel AAV vectors have also been generated in multiple ways. The most straightforward approach for AAV, pseudotyping, involves swapping the *cap* gene from one serotype for another. This approach allows for the rapid and modular generation of vectors with the gene delivery properties matching a parent serotype, but methods that combine properties from multiple different serotypes can generate viral vectors with novel functions not found in the natural variants.

HVR: hypervariable region

Chimera: a biological entity, such as a virus, composed of two or more genetic fragments of distinct origin or composition

Because the AAV capsid is composed of 60 copies of VP1, VP2, and VP3, VP monomers from two different AAV serotypes or mutants can be mixed during viral packaging to yield a mosaic AAV capsid that contains a heterogeneous mixture of VP monomers and can thus combine properties from the constituent parents. Two studies have demonstrated the potential of this approach by cotransfecting *cap* genes from different AAV serotypes (AAV1–5) at various stoichiometric ratios (46, 47). The resulting mosaic virions exhibited a range of HSPG and sialic acid–binding properties, in some cases substantially higher than either parent. Furthermore, at some stoichiometric ratios, the mosaic virions displayed novel cell tropisms that differed substantially from either constituent serotype, suggesting the potential for synergistically combining properties of the two parents. This mosaic approach has recently been extended to combine functions of rationally designed AAV mutants (48). As another example, the properties of two AAV1 mutants, containing either a biotin acceptor peptide (BAP) ligand for purification or an arginine-glycine-aspartic acid (RGD) ligand for targeting, were combined to generate mosaics that exhibited enhanced vasculature targeting and could readily be purified by column chromatography (49). In general, however, cotransfection of two *cap* genes likely results in a heterogenous mixture of mosaic virions, some of which may not possess the desired phenotype. Furthermore, the exact copy number of monomer required to confer desirable properties such as enhanced targeting has yet to be determined. Therefore, future efforts to more precisely control the ratios of monomers within such mosaics and more extensive monomer titration studies will enhance the utility of the mosaic capsid approach.

Targeting: the delivery of genetic material to a specific cell type

wt: wild type

VSVG: vesicular stomatitis virus glycoprotein

An alternate strategy to combine properties of different AAV serotypes involves the generation of a single chimeric *cap* gene containing domains from multiple serotypes. For example, through cotransfection of a noninfectious AAV2 mutant genome and an AAV3 *cap* gene, Bowles et al. (50) isolated several chimeras after three rounds of infection and rescue on HeLa cells. This work demonstrated the strong potential of chimeric virus, although a small fraction of full wild-type (wt) AAV3 sequences were recovered, and the number of recombination events was relatively small. In a separate study, domains from the AAV1 *cap* gene were substituted into the analogous regions of the AAV2 *cap* gene to generate a range of chimeras. Most of the resulting variants were functional, and an AAV1 domain that conferred enhanced muscle tropism to AAV2 was identified (51). Recently, we have demonstrated the potential of generating random chimeras with novel gene delivery properties from multiple AAV serotypes using high-efficiency in vitro recombination (discussed in Directed Evolution, below) (J.T. Koerber & D.V. Schaffer, unpublished data).

In the case of retroviruses, pseudotyping is again a straightforward method to alter or improve the properties of vectors (52, 53). As an important example, to extend the tropism of retroviral vectors and to enhance vector stability, envelope proteins from vesicular stomatitis virus (VSVG) have often been used in retroviral and lentiviral vectors (54, 55). VSVG apparently uses ubiquitous lipid-type receptors such as phosphatidylserine (PS), resulting in broad vector tropism to most cells. Alternatively, the lymphocytic choriomeningitis virus (LCMV) envelope protein, which is less cytotoxic than VSVG, increases retroviral vector stability and also enables the development of stable retrovirus packaging cell lines (56).

Systemic injection of retroviral vectors pseudotyped with various envelope proteins results in predominant transduction of the liver and spleen (55). However, upon local injection, the various envelope proteins used for pseudotyping confer drastically different tropism. For example, feline immunodeficiency virus (FIV)-based lentiviral vectors injected into the mouse brain showed preferential transduction of astrocytes and oligodendrocytes when pseudotyped with Ross River virus (RRV) envelope proteins (57), whereas pseudotyping with LCMV envelope proteins led to transduction of neural stem cells or progenitor cells (58). These transduction patterns are different from those of VSVG-pseudotyped lentiviral vectors, which exhibit a strong preference for mature neurons (58).

mAb: monoclonal antibody

Envelope proteins from viruses with unique tropism can enable gene delivery to cell types or tissue regions normally resistant to transduction. For example, VSVG-pseudotyped lentiviral vectors can infect polarized airway epithelial cells via the basolateral but not the apical side. However, because tight junctions block virus access to the basolateral membrane of cells in vivo, transduction efficiency is very low (59). However, lentiviral vectors pseudotyped with envelope proteins from viruses that infect respiratory tissues or cells, including Ebola viruses, respiratory syncytial virus (RSV), fowl plaque viruses, and influenza viruses, can successfully transduce airway epithelial cells from the apical side (60). Likewise, VSVG-pseudotyped lentivirus efficiently transduces muscle locally (61), but lentiviral vectors pseudotyped with rabies envelope proteins undergo retrograde transport to the spinal cord upon intramuscular injection (62).

Interestingly, surface proteins overexpressed by producer cells can be also efficiently incorporated into virion particles during vector production, facilitating novel targeted gene delivery opportunities. For example, retroviral and lentiviral vectors can be pseudotyped with the HIV-1 primary receptors and coreceptors, CD4 and CCR5, CD4 and CXCR4, or hybrid receptors composed of the four extracellular loops of CD4 and CXCR4. The resulting vector could infect cells expressing HIV-1 envelope proteins and cells infected by HIV-1 (63, 64), enabling novel antiviral therapy approaches. Likewise, incorporation of receptors for Rous sarcoma virus and ecotropic MLV into vectors allowed targeted infection of cells expressing the corresponding envelope proteins, although the infectious titers were 10- to 100-fold lower than those of common envelope-pseudotyped retroviral vectors (65).

Finally, as discussed below (Directed Evolution) chimeric VAP libraries coupled with high-throughput selection offers the potential to generate novel solutions to the various gene delivery barriers (66).

Adaptors and Chemical Modification

Bifunctional adaptors, which combine a vector-binding region with a novel functional domain, can endow vector systems with new capabilities (**Figure 2**). The primary advantage of this approach is the ability to change viral properties in a highly modular manner, often without genetic modification of the viral vector.

Direct modification methods of Ad vectors can be grouped into four different areas: chemical conjugation, soluble CAR (sCAR) adaptors, bispecific antibody adaptors, and monoclonal antibody (mAb) linkers. Initial efforts at chemical modification of Ad vectors via PEGylation (polyethylene glycol) of the capsid conferred modest resistance to neutralizing antibodies, but with dramatic losses in viral infectivities due partially to viral aggregation and a strong dependence on precise reaction conditions (67, 68). Further progress has resulted in PEGylated vectors with similar gene delivery properties to unmodified vectors in vivo and reduced innate immune responses to the vector (69). Recently, two studies have expanded the applicability of these PEGylated vectors by conjugating a targeting ligand to the distal termini of PEG moieties on the virus. PEG vectors linked to fibroblast growth factor 2 (FGF2) exhibited tenfold higher transduction of one cancer cell line and enhanced gene expression in an in vivo tumor model (70). In other work, conjugation of an RGD ligand or an anti-E-selection antibody to the PEGylated Ad vectors yielded moderately targeted gene delivery (71). Recently, a platform for site-specific chemical modification of Ad vectors has been developed based on conjugation to a cysteine motif in the surface exposed HI loop of the Ad fiber (72). The potential of this platform for robust, reproducible modification of Ad vectors was demonstrated through detargeting of the Ad vector via PEGylation and subsequent retargeting via site-specific conjugation of transferrin.

Several different bispecific adaptor platforms have been developed for targeting Ad vectors. One such platform involves the fusion of sCAR, which binds the Ad fiber, with a targeting ligand. The initial study, performed with a sCAR-epidermal growth factor (EGF) ligand, reduced binding to CAR^+ cells, and at the optimal adaptor:virus ratio enhanced transduction levels by up to 40-fold on EGF receptor (EGFR)-expressing cells (73). However, too much adaptor actually inhibited binding to the cells, potentially via competition with the virus for EGFR binding, highlighting a potential limitation of this approach. A related platform employed a bispecific adaptor with a mAb recognizing the wt Ad capsid linked to a targeting ligand (74) or mAb (75). Finally, genetic insertion of the Fc binding region of protein A (the ZZ domain) into a truncated fiber (76), the C terminus of the fiber (77), and the HI loop of the Ad fiber (78) has permitted the creation of Ad vectors that bind mAbs and can thereby be targeted to a variety of cell types. For example, a mAb against CD40 enabled this Ad vector variant to transduce primary human dendritic cells at levels 30-fold higher than wtAd5 (77), and use of an anti-EGFR mAb enhanced transduction by up to 77-fold on some primary cell lines (78).

Chemical modification of AAV vectors has relied on conjugation of moieties to free surface-exposed lysines on the viral capsid, similar to methods used for Ad vectors. We have recently shown that PEGylation of AAV2 vectors confers a modest twofold resistance to NAbs, while retaining wtAAV2 transduction levels (79). Furthermore, careful optimization of the PEG molecular weight and PEG:lysine ratio was critical for the enhanced resistance and retention of high infectivity. Targeting methods for AAV vectors have also been developed through biotinylation of the AAV capsid (80). At levels of 4–5 biotins per viral capsid, streptavidin-EGF fusion proteins mediated 100-fold increases in transduction levels of these biotinylated AAV vectors on cells expressing high levels of EGFR.

In an important initial study employing bispecific adaptors for AAV vectors, Bartlett et al. used a bispecific antibody that recognized both the intact AAV2 capsid and the $\alpha_{IIb}\beta_3$ integrin receptor to retarget AAV2 (81). These vectors demonstrated enhanced binding to the nonpermissive cells and increased transduction by up to 70-fold on these cell lines. While this promising result demonstrated the potential of this approach, the overall transduction levels were 10- to 100-fold below those of wtAAV2 on permissive cells, suggesting that additional gene delivery barriers subsequent to receptor binding may reduce the efficiency of the vectors. An alternate strategy proposed by Ponnazhagan et al. involves the insertion of the protein A ZZ domain after amino acid 587 of the AAV2 capsid to efficiently couple mAbs to the vector, analogous to work with Ad vectors (80). Although several targeting Abs increased targeted transduction on nonpermissive cells, relative to the modified vector without antibody, gene delivery efficiencies were tenfold less than wtAAV2 on these cells and 10,000-fold less than wtAAV2 on permissive cells. Additional refinements in this approach have recently yielded further improvements to the system (48).

For retroviruses and lentiviruses, one critical hurdle for systemic delivery using VSVG-pseudotyped vectors is their susceptibility to neutralization by complement (82). A recent attempt to address this problem showed that PEGylation of VSVG could protect VSVG-pseudotyped HIV vectors from serum-mediated inactivation by 1000-fold (83). Such chemical modifications of the viral surface can also allow targeted gene delivery. For example, tagging of ecotropic MLV vectors with galactose led to specific transduction of a human hepatoma cell line expressing asialoglycoprotein receptors, which bind oligosaccharides with terminal galactose residues (84).

Bispecific linker molecules have commonly been applied to retroviral vector systems. Typically, the linker molecules are fusion proteins composed of a viral receptor, which interacts with the vector particle via the envelope protein, and a targeting ligand, which interacts with its cognate receptor on the target cells (85). Two fusion linkers composed of EGF and either avian leucosis virus A (ALV-A) receptor (TVA) or ALV-B receptor (TVB) mediated efficient ALV-A or ALV-B vector

transduction of cells expressing EGFR (85). Alternatively, an earlier and more complex system involved interactions among three linker molecules. Two biotinylated antibodies, with specificity for the virus envelope and a surface molecule on the target cells, were bridged with streptavidin. The resulting conjugate mediated specific MLV vector transduction of cells expressing major histocompatibility complex class I and class II proteins (86). Such linker-based approaches can be readily extended to other target cell surface molecules.

In another linker approach analogous to Ad and AAV systems, insertion of the ZZ domain into the receptor binding domain of Sindbis virus E2 envelope protein allowed incorporation of targeting antibodies against CD4 or human leukocyte antigen (HLA) into virus particles, ultimately leading to preferential transduction of retroviral vectors into $CD4^+$ and HLA^+ cells, respectively (87). In the Sindbis envelope protein system, where the fusogenic protein E1 functions independently of the receptor-binding protein E2 (87), such insertional mutagenesis for targeting is feasible. Furthermore, insertion of ZZ domain into the E2 protein, in combination with mutations within certain domains of E2 responsible for nonspecific infection, allowed highly specific in vivo transduction of metastatic melanoma in the lung by E2 protein-pseudotyped lentiviral vectors (88).

Although they are highly promising and have enjoyed considerable success, several challenges remain in the development of these adaptor platforms for Ad, AAV, and retroviral vectors. The success of chemical modifications is typically highly dependent upon the reaction conditions, and more controlled site-specific methods will thus greatly enhance the potential of this approach while reducing unwanted side effects, such as reduced transduction levels. Furthermore, the affinity of adaptor:virus complexes in vivo may not be sufficient to prevent dissociation of the adaptor-virus linkage, and endogenous antibodies within a patient's blood may also compete with mAb-based adaptors. Finally, for clinical approval, both the virus and the adaptor must be produced, purified, and fully characterized, adding considerable complexity to vector system production. For these collective reasons, the greatest potential for adaptor systems likely lies in ex vivo therapies, such as for the transduction of a target cell within a heterogeneous cell population prior to reimplantation.

Functional Polypeptide Incorporation into Structural Proteins

Viral capsids or VAPs can be genetically engineered to introduce peptides and in some cases entire protein domains with defined functions, such as cell targeting or affinity purification tags, into well-defined locations on the viral particles (**Figure 2**). Most peptide modification approaches for Ad5 have focused on the fiber, with recent studies exploring the hexon and the structural support protein IX as potential insertion sites. In the initial study, Wickham et al. (89) inserted an RGD ligand, a 7–amino acid polylysine (7K) and other peptides targeting α_v integrins, the laminin receptor, and E-selectins at the C terminus of the Ad fiber. Interestingly, only the RGD and 7K ligand insertions yielded infectious virus that bound to the specified target, highlighting one potential challenge in peptide insertion, that the proper function of the peptide is highly dependent on the local structural environment of the viral protein. Insertion of the 7K peptide, however, enhanced transduction up to 500-fold on some cell types and increased transduction of cardiac smooth muscle cells in vivo. Another study investigated the insertion of peptide epitopes into the HI loop of the Ad fiber. Insertion of an RGD peptide conferred targeted gene delivery to cells expressing high levels of integrins (90) and elevated gene expression in the kidney and lung (91). Further developments in these systems have demonstrated that insertion of both RGD and 7K peptides into the fiber provides an additive effect from both functionalities (92) and that insertion of cell targeting peptides can offset and in some cases restore losses in transduction due

to the ablation of native tropism (93). As further demonstration of the potential of the peptide insertion approach, a novel Ad vector containing an RGD ligand completed animal safety tests and will soon be used clinically to treat ovarian carcinoma (94).

In another approach, Parrott et al. (95) developed a peptide-based platform for general Ad vector engineering. Insertion of a 70-amino acid BAP at the C terminus of the fiber protein resulted in the biotinylation of the Ad vector during production, enabling the attachment of any biotinylated targeting ligand via tetrameric avidin at affinities (10^{-15} M) greatly exceeding those of bispecific antibodies. Peptide modification of Ad vectors within the hypervariable surface loop regions (HVR) of the hexon resulted in variable efficiencies of surface exposure and abilities to mediate targeted gene delivery. Protein IX can also tolerate functional peptide insertions such as single-chain antibodies, although with slight reductions in production levels and thermostability of the vectors (96). Interestingly, several studies have recently demonstrated that imaging ligands, such as fluorescent proteins, inserted into protein IX permit tracking of Ad vectors both in vitro and in vivo (97, 98).

For AAV, recent studies primarily involving AAV2 have identified several sites on the AAV capsid that tolerate the general insertion of peptides. The first study that attempted genetic retargeting of AAV2 involved the fusion of a single-chain antibody against CD34 antigen to the N terminus of VP2 (99). The resulting vector exhibited enhanced transduction of a $CD34^+$ cell line, but the overall transduction levels remained low. It is possible that the incorporation of large polypeptides disrupts the intricate assembly process of the VP1-3 monomers, whereas smaller peptide modifications and different insertion sites may enhance targeting efficiency while retaining the production levels of the wt capsid. To this end, Girod et al. (100) inserted an RGD motif into six new locations, chosen based on the superposition of the AAV2 sequence on the known structure of a related parvovirus. Very importantly, insertion of the RGD motif into one of the six sites, located after amino acid 587 within the surface-exposed loop 4 of the AAV2 capsid, significantly elevated transduction levels relative to wtAAV2 on a nonpermissive cell type, but overall transduction levels were still several orders of magnitude below wtAAV2 expression levels on a permissive cell line. Another study tested two insertion sites identified from sequence comparison of AAV2 and a related parvovirus. Insertion of an NGRAHA peptide, shown to bind the angiogenic vasculature, into loop 4 enhanced AAV transduction levels by up to 20-fold on several tumor cell lines (101). In subsequent work, other ligands were successfully inserted at this site, such as additional RGD peptides (102), a His_6 tag for robust purification (103), and a dynein-binding peptide to facilitate intracellular transport (104). Successful insertion into the analogous region of other AAV serotypes has been reported for a BAP, similar to that used in Ad vectors (105), and a His_6 tag (103). Furthermore, parallel efforts have found that entire proteins can be fused to the N terminus of VP2, including large targeting peptides (>30 amino acids) (106) and a fluorescent protein for viral tracking (107).

Two insertional mutagenesis studies have elucidated insertion locations that disrupt viral assembly and infectivity, as well as highlighted the fact that different peptides experience highly variable success at distinct insertion sites. In one study, sites within VP1 and VP2 were found to tolerate the insertion of a serpin ligand for targeted gene delivery. Interestingly, however, the loop 4 site that has been successful for numerous other peptides (100, 101, 103, 104) did not functionally display the serpin peptide (108). In a separate approach, the combination of several insertion sites along with several sets of possible flanking sequences, which can influence the local environment of the peptide and thus aid in efficient surface presentation, resulted in variable functionality of the inserted peptides (102). The results of these two studies highlight how the optimal insertion site for a given peptide depends not only on the peptide sequence but also on the flanking amino acid context (102, 108).

Various targeting molecules, including short peptides, ligands, and even single-chain antibody fragments (scFv) have been incorporated into retroviral structural proteins. Short targeting peptides inserted into envelope proteins have been shown to mediate targeted gene delivery without severely disrupting the envelope's function. Upon insertion of a single or multiple 13- or 21-amino acid RGD-containing motifs, ecotropic MLV retroviral vectors could transduce A375 human melanoma cell lines. More interestingly, depending on the insertion sites, the natural tropism of MLV vectors was significantly attenuated (109). However, one challenge is that peptide insertion can hinder the intracellular trafficking of envelope proteins during viral production. For example, insertion of a collagen-binding motif at the 24th amino acid of VSVG blocked trafficking to the cell surface at 37°C, thereby severely inhibiting retroviral vector assembly and production (110). Although, at a lower permissive temperature, 30°C, VSVG trafficking to the cell surface was recovered (110).

Because an initial study showed that human low-density lipoprotein receptor (LDLR)-specific single-chain antibodies (scFv) inserted into ecotropic envelope proteins could mediate a specific retroviral transduction of cells expressing LDLR (111), there have been numerous efforts to harness scFvs for targeting. To reduce nonspecific transduction of human cells, ecotropic MLV envelope proteins have been often used as the scaffold for insertion of the scFv-targeting domains (112). These engineered antibody fragments have been primarily inserted into the N-terminal region of envelope proteins through a spacer peptide, thereby likely permitting proper conformations for both the scFv domain and envelope protein. Due to their strong binding affinity and in principle unlimited specificities, scFv molecules have the potential to offer higher specificity than short peptides. However, because of their large size, scFv molecules can disrupt the conformational changes required for envelope proteins to mediate proper membrane fusion. This limitation can be circumvented by coexpressing engineered and wt envelope proteins on virus particles, but with a loss of targeting specificity (111). Alternatively, transduction specificity may be improved by inserting additional cell-specific peptides, such as protease cleavable peptides, between scFv and envelope proteins (113–115).

In addition to peptides and single-chain antibodies, various ligands such as insulin-like growth factor I (IGF-I), EGF, erythropoietin (EPO), and stromal-derived factor-1α (SDF-1α) have been inserted mostly into the N-terminal region or the receptor-binding domain of envelope proteins (116–119). However, in many cases, this ligand insertion strategy can result in low transduction efficiency (116). In particular, virus particles complexed with the targeted receptors were often sequestered on the cell surface or routed to degradative pathways after endocytosis (118, 119). The resulting lack of transduction was due in part to the fact that viral binding to target receptors was not sufficient to trigger conformational changes in the envelope proteins and thereby initiate viral fusion (120). Such ligand-mediated transduction inhibition has led to another targeting strategy, where some ligand domains, such as EGF and CD40, are inserted into amphotropic MLV envelope proteins via a matrix metalloproteinase (MMP)-cleavable linker to block viral entry. Retroviral vectors pseudotyped with these chimeric MLV envelope proteins can be activated by MMP expressed by target cells, which cleave off the blocking domain, thereby resulting in successful transduction (121). Recent improvements in the introduction of targeting ligands into envelope proteins have further enhanced this method (117, 122, 123).

Loss in vector efficiency upon introduction of targeting ligands into envelope proteins can be often reversed by coincorporation of wt envelope proteins into viral vectors (117). An analogous approach incorporated fusogenic HA mutants, which lack the ability to bind receptors, into the vector. MLV packaged with both an engineered MLV envelope protein, containing an N-terminal Flt-3 ligand fusion, as well as a HA mutant, retaining its fusogenic function but unable to bind sialic acid, could transduce cells in a Flt-3 receptor-dependent fashion (122). This strategy can

minimize the undesirable off-target cell binding that accompanies the coexpression of wt envelope proteins to enhance viral fusion. Interestingly, an inserted ligand can enhance retroviral efficiency by altering the target cell's physiological state. For example, when interleukin-2 (IL-2) was fused to the N terminus of a chimeric amphotropic MLV envelope, MLV vectors pseudotyped with both the chimeric and wt envelope proteins could transduce quiescent cells expressing IL-2 receptors 34-fold more efficiently, via activation of the cell cycle, than vectors with only the wt envelope (123).

Although peptide and entire protein domain insertions hold tremendous potential for engineering defined functions into viral vectors, several challenges do remain. Peptide insertion can disrupt multimerization of the capsid monomers, reduce the thermostability of the assembled viral vector, or hinder proper intracellular trafficking (89, 96, 108, 110, 124). Furthermore, the proper function of the inserted peptide is highly dependent on the neighboring amino acids, and thus two peptides inserted in the same location may function with different efficiencies (89, 108, 124). Therefore, high-throughput screening or selection for proper peptide function may enhance the utility of this technique, as well as help explore peptides that help pass gene delivery barriers other than receptor binding.

Rational Point and Domain Mutagenesis

For many gene delivery applications, the native tropism of the virus must be altered or eliminated to reduce infection of off-target cells or modulate biodistribution. Mutagenesis of key amino acids involved in receptor binding can drastically reduce off-target infection, and the addition of a targeting ligand can enable transduction of target cells. Numerous studies have successfully mapped key receptor-binding locations on the capsid or VAP, and initial in vivo experiments emphasize the potential of point mutagenesis for viral vector engineering.

There have been several attempts to alter the biodistribution of the Ad vectors (e.g., reduce liver uptake) by modulating the CAR-binding properties of the fiber protein (125, 126). Mutation of some residues led to 10- to 100-fold reduction in transduction levels in vitro; however, biodistribution studies showed no significant differences compared with wt vectors, except for a slight reduction in gene expression in the heart (126). Recent efforts to engineer an Ad vector with reduced liver transduction have focused on combining the effects of decreasing CAR binding (KO1), integrin binding (PD1), and HSPG binding (S*) (127, 128). These studies confirm that ablation of CAR binding has minimal effects on in vivo gene delivery, but that integrin binding aids in efficient lung, heart, kidney, and liver transduction. The S* phenotype results in a larger than tenfold decrease in transduction in most tissues, including liver, as well as a reduction in the innate immune response to the Ad vector (128). Combining all three mutations generated an Ad vector with a more than 10,000-fold decrease in gene transduction in the liver, and substantial reductions in other tissues (~10–100-fold), thus facilitating the engineering of Ad vectors without a primary liver tropism (127). Further work to restore or exceed wt transduction levels in other tissues using targeting peptides or beneficial compensatory mutations will advance the use of such vectors for in vivo gene delivery.

The solution of the AAV2 crystal structure by Xie et al. (129) has helped to guide several efforts to enhance the knowledge of structure-function relationships for AAV2, as well as aid in viral vector design. Studies by Opie et al. and Kern et al. (130, 131) mutagenized basic residues (i.e., lysine and arginine) in the VP3 region of the capsid and identified five mutations that significantly decreased HSPG binding, in most cases decreased viral infectivity more than tenfold, and altered biodistribution in vivo. In addition, swapping the loop 4 region of AAV2, which contains the key HSPG binding motif, with the analogous region of AAV5, which does not bind HSPG,

Library: a large, diverse pool of related objects, such as proteins or viruses

conferred HSPG binding to AAV5 but also reduced viral infectivity by approximately tenfold. Two additional recent efforts have greatly improved fundamental structure-function knowledge of multiple AAV serotypes. Lochrie et al. (132) analyzed the effects of 127 site-directed mutations on the AAV2 capsid surface to map receptor binding sites and antigenic regions. These results further confirmed the HSPG binding regions and identified a novel dead zone on the viral capsid, which was putatively described as a FGFR-binding region through docking studies. This wealth of information should facilitate the location of analogous receptor-binding regions on other AAV serotypes and aid the design of novel receptor binding sites on AAV capsids through docking studies and mutagenesis. In fact, one study has shown that even single–amino acid changes can alter viral packaging and confer heparin-binding properties to multiple AAV serotypes (133). The database of knowledge amassed from these studies, coupled with the recent crystal structures of AAV2, AAV4, and AAV8 (129, 134, 135), will greatly enhance the rational forward engineering of novel AAV vectors, and future work will hopefully conduct in vivo studies to confirm the altered functionalities.

The protein constituents of retroviral vectors have also been rationally mutagenized to improve existing properties or introduce new functions. For example, substitution of the V3-loop region of the simian immunodeficiency virus (SIV) SU with the corresponding region of a T cell tropic HIV-1 allowed T cell–specific transduction by pseudotyped MLV vectors, even in the presence of anti-HIV-1 sera (136). Another application of rational mutagenesis has addressed the problem that, although heterologous envelope proteins can be robustly incorporated into retroviral and lentiviral vectors, some are selectively excluded from virions. For example, the long C-terminal region of HIV-1 envelope proteins hinders their packaging with MLV core particles during budding, potentially due to steric hindrance (137, 138). To counteract this problem, the cytoplasmic tail of the HIV-1 envelope protein was truncated, leading to efficient packaging of retroviral vectors pseudotyped with HIV-1 envelope proteins (137, 138). As an additional application of site-directed mutagenesis, to minimize random insertional mutagenesis for lentiviral vectors, as well as convert them into transiently expressing vectors, HIV-1 integrase has been altered to reduce its integration ability. Mutations such as RRK to AAH at position 262 (139) and D64V (140) led to transient transgene expression in dividing cells by the nonintegrating HIV vectors carrying the engineered integrases (139), but allowed longer-term gene expression in nondividing cells (139, 140). However, despite the 500-fold drop in integration compared with wt integrase, the residual integration ability of such mutants cannot be ignored (139).

Initial mutagenesis studies have identified key amino acids involved in receptor binding, facilitated the engineering of viral vectors with reduced native tropism, enabled the engineering of vectors with novel tropism, and enhanced vector safety. Future studies involving additional serotypes should enhance basic knowledge of other viral structure-function relationships and aid in the forward engineering of viral vectors through combinations of beneficial point mutations or larger functional domain swappings.

HIGH-THROUGHPUT LIBRARY APPROACHES FOR VECTOR ENGINEERING

Although rational modification techniques have generated viral vectors with novel gene delivery properties, the successful application of these approaches often requires detailed mechanistic knowledge of the viral proteins. However, in the absence of extensive knowledge of the structure-function relationships for the majority of viral proteins, high-throughput library generation and selection schemes can still rapidly generate novel solutions to various gene delivery problems (**Figure 3**). Furthermore, the iterative application of library generation and selection, known as

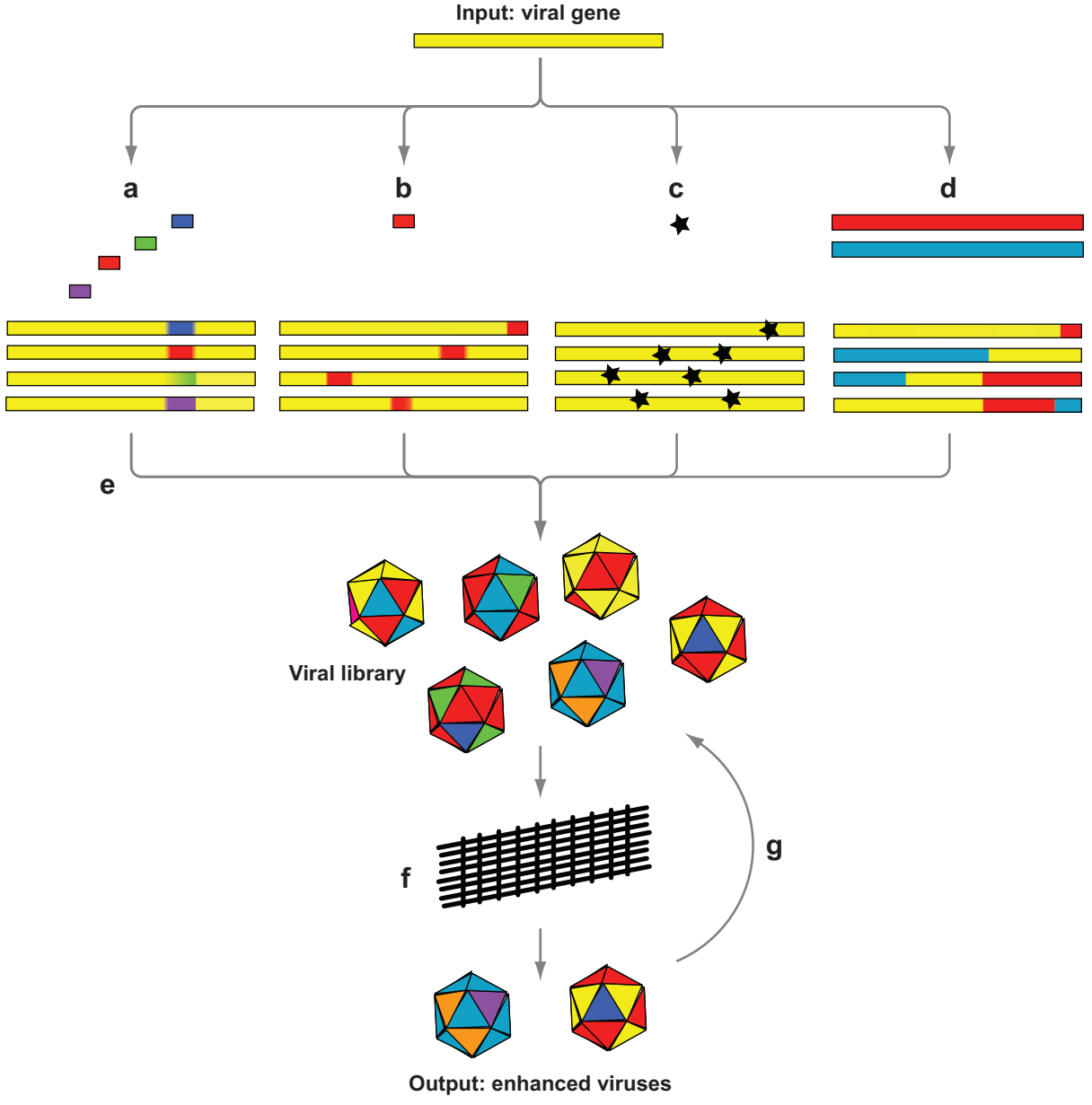

Figure 3

Overview of library protein engineering strategies for viral vectors. Library generation and selection methods include (*a*) display of random peptide in defined location, (*b*) random insertional mutagenesis, (*c*) random point mutagenesis, and (*d*) in vitro recombination. Upon DNA library generation, a highly diverse viral library is produced (*e*). Finally, (*f*) high-throughput selection followed by (*g*) recovery of successful variants and iteration with steps (*a*)–(*d*) and (*e*) employs directed evolution to enhance desired gene delivery properties.

directed evolution, permits the accumulation of beneficial mutations distributed throughout the primary sequence of the protein to engineer viral vectors with novel gene delivery properties in a process that mimics natural evolution.

Viral Peptide Display

As highlighted in Functional Polypeptide Incorporation, above, many peptides that function in one context fail to function properly when inserted into viral capsids or envelope proteins. The insertion may reduce viral stability, disrupt key structural contacts required for viral assembly, alter processing and intracellular trafficking of envelope proteins, compromise membrane fusion, or require proper context to enable functional peptide display. Therefore, methods to select for peptides that function properly in the context of the viral proteins may greatly enhance viral vector engineering.

As mentioned above, the Ad fiber and surface loops of the AAV capsid present attractive locations for the insertion of functional peptides. Pereboev et al. (141) developed a system in which the Ad5 fiber is displayed on the surface of a bacteriophage and showed that peptides incorporated into the fiber are functionally displayed. Therefore, diverse libraries of peptides could be selected for cell surface binding or other properties and likely retain these functions once incorporated into the full Ad vector. This platform has recently been further developed to permit rapid selection of semirandom 14–amino acid peptides within the context of the HI loop of the Ad fiber. Several peptides selected in this fashion enhanced Ad gene delivery by 100-fold relative to wtAd5 on murine fibroblasts (142) or tenfold on mouse myoblasts (143). Similarly inspired by phage display techniques, high-throughput display and selection platforms of peptide-modified AAV2 vectors have also been developed (144, 145). Insertion of a random 7–amino acid peptide into loop 4 of the AAV2 capsid coupled with selection on nonpermissive cell lines, such as endothelial cells, generated AAV vectors with 4- to 40-fold higher transduction levels relative to wtAAV2 and enhanced heart transduction in vivo (144).

Similar high-throughput selection platforms have been developed for retroviral vector envelope proteins. In one study, a random 10–amino acid peptide was substituted into the receptor binding domain of the subgroup A feline leukemia virus (FeLV-A) envelope protein (146, 147). When retroviral libraries displaying the envelope protein mutants were selected on AH927 feline fibroblasts expressing receptors for FeLV-A, -B, and -C, one selected clone exhibited a receptor usage pattern different from that of the parent envelope protein, ultimately conferring novel tropism to canine osteosarcoma cells (146). Selection of a similar library against a *ras*-transformed 143B human cell line yielded one variant with preferential tropism to 143B cells and 293T human embryonic kidney cells, via unknown receptors that were distinct from the FeLV receptor (147).

Random peptide display approaches have been also applied to identify peptides that function as cleavable substrates for proteases specifically expressed on target cells. An early study involved insertion of a random 7–amino acid peptide between a targeting EGF domain and the N terminus of the MLV envelope protein, and the resulting MLV vector library displaying the mutant envelope proteins was selected for the human fibrosarcoma cell line HT1080 that expressed EGFR and Rec-1 receptors. Selected peptides were mostly enriched in basic amino acids, such as arginine, and likely served as substrates for furin, a ubiquitously expressed protease (148). The use of degenerate codons to reduce the generation of furin-sensitive substrates in the library led to isolation of peptides that allowed MMP-2-mediated cleavage and selective retroviral transduction of HT1080 cells. The selected envelope protein variants exhibited not only increased MMP-2 sensitivity but also a high efficiency of incorporation into virus particles (149).

In each of these three vector platforms, peptides were selected for the ability to enhance transduction of a cell line. Although most of the peptides likely mediate receptor binding, it is possible that some may aid in later stages of gene delivery. Further development of these platforms may identify peptides that overcome gene delivery barriers other than cell surface binding, as well as peptide-displaying viral vectors capable of enhanced transduction of additional therapeutically relevant cell types, such as hematopoeitic stem cells.

Random Insertional Mutagenesis

Another approach for engineering viral vectors involves random insertional mutagenesis. Transposases, enzymes that catalyze the efficient random insertion of a small DNA sequence into acceptor DNA ranging from an organismal genome to a plasmid, have aided in insertional mutagenesis studies to efficiently map permissive insertion sites within viral genomes and proteins (150). Recently, we have extended this approach to incorporate a short peptide tag, a His_6, randomly throughout VSVG, thereby permitting efficient viral purification via column chromatography. Three novel insertion sites that did not significantly compromise viral production or infectivity were identified, and analysis of one clone demonstrated that the improved purity resulted in a weaker immune response upon injection into the rat brain (151). Furthermore, the three novel insertion sites have further applications in engineering targeted VSVG variants. Adaptation of this high-throughput method for other novel properties such as enhanced intracellular trafficking has generated retroviral vectors capable of infecting nondividing cells both in vitro and in vivo and thus extended the potential of retroviral vectors (152). Random insertional mutagenesis thus offers the potential to rapidly select for peptides that confer novel functions and/or enhance multiple steps of the gene delivery process.

Directed Evolution

Directed evolution is a promising approach for rapid and efficient viral vector engineering. Random mutagenesis or in vitro recombination techniques can generate large, diverse protein libraries with novel functions, and subsequent high-throughput selections or screens can yield variants with unique and enhanced functions, including enzymes, antibodies, and cytokines (153–155). Importantly, by allowing the iterative accumulation of key, synergistic mutations distributed throughout the primary protein sequence, often outside of previously identified functional regions, evolution can in some cases dramatically alter protein function. Furthermore, in contrast to rational design, this approach does not require substantial mechanistic knowledge of the substrate protein. Therefore, particularly because extensive knowledge of viral structure-function relationships is often lacking, directed evolution is a promising method to engineer viral vectors with novel gene delivery properties whose basis is distributed throughout the primary sequence of the capsid or VAP, such as virus-cell or virus-immune system interactions.

We have recently developed a novel platform for directed evolution of AAV vectors based on PCR-mediated mutagenesis and recombination of the AAV2 *cap* gene (156). Subsequent viral packaging resulted in a large and diverse viral library, in which each AAV capsid contained the *cap* gene encoding that capsid. Selection for altered heparin binding generated AAV2 variants with higher and lower affinities for heparin, and multiple rounds of mutagenesis and selection in the presence of increasing concentrations of anti-AAV2 serum generated a novel AAV2 variant with larger than 100-fold enhanced resistance to preexisting AAV2 antibodies in vivo. A library selection approach involving a single round of mutagenesis and selection against individual human serum samples resulted in variants with 3.3- to 5.5-fold enhanced resistance to the human serum sample (157).

Furthermore, we have recently shown that high-efficiency in vitro recombination of numerous AAV serotypes generates viable and highly chimeric AAV vectors with an array of cellular tropisms distinct from the parental serotypes, as well as enhanced resistance to preexisting antibodies (J.T. Koerber & D.V. Schaffer, unpublished data). When integrated with efficient iterative selections, these new library-generation protocols present a powerful tool for the generation of novel AAV vectors with customized gene delivery properties.

Library generation and selection methods utilizing in vitro recombination have also generated novel retroviral vectors with novel cell tropism and enhanced stabilities. In an important work, Soong et al. (66) employed DNA shuffling of six parental ecotropic MLV envelope genes to construct a diverse chimeric retroviral library. Following five rounds of selection on the nonpermissive Chinese hamster ovary (CHO) cell line, a novel variant capable of transducing CHO cells at high efficiency was isolated. Selection of a similar library for enhanced stability also identified several novel chimeras capable of withstanding ultracentrifugation (158). Analysis of the resulting chimeras in each case demonstrated the cooperative effect of several recombination events or mutations to generate the final viral phenotype, highlighting the potential of this approach to develop novel solutions to gene delivery challenges without a priori knowledge of viral structure-function relationships.

FUTURE OUTLOOK

Numerous advances in viral vector engineering have resulted in vehicles with novel functionality, and future endeavors will extend these approaches in new directions. For example, most studies to date have successfully engineered a single function into the viral vector, focusing on receptor binding on a target cell, the major barrier to efficient delivery. However, other barriers, such as preexisting immunity, intracellular trafficking, and proteasome degradation, also restrict the gene delivery efficiency of these engineered viral vectors and will likely be the focus of future work.

In addition, recent advances in knowledge of viral structure (129, 134, 135, 159, 160) provide a platform for structure-based design of novel receptor-binding regions and other functions into the viral templates. However, to simultaneously contend with multiple challenges and delivery barriers, future work will likely require more high-throughput methods for vector engineering. For example, regardless of viral structure knowledge, library selection and directed evolution methods promise to create vectors with novel gene delivery properties, often resulting in solutions that could not be predicted a priori, as well as yield additional information on basic virology to further aid rational design. Furthermore, techniques such as the generation of chimeric vectors or directed evolution, which combine multiple functions from different parental vectors and in some cases from different viral families, will advance the engineering of more complex viral vectors with multiple designed functionalities, analogous to current synthetic vectors that are generated from blends of polymers or lipids with user-defined chemical properties.

In general, numerous vector engineering methods have shown great promise in vitro, and future in vivo analysis, particularly for targeting approaches and to address concerns over vector immunogenicity, promises to further extend the utility of engineered vehicles. Collectively, a combination of advanced approaches for optimizing the vector and engineering the cargo, such as through transcriptional targeting (161) or regulated expression systems (162), will result in safe and effective viral vectors that will move forward in the clinic.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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