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Covalent coupling of high-affinity ligands to the surface of viral vector particles by protein trans-splicing mediates cell type-specific gene transfer

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ABSTRACT

We have established a novel approach for the covalent coupling of large polypeptides to the surface of fully assembled adeno-associated viral gene transfer vector (AAV) particles via split-intein mediated protein-*trans*-splicing (PTS). This way, we achieved selective gene transfer to distinct cell types. Single-chain variable fragments (scFvs) or designed ankyrin repeat proteins (DARPins), exhibiting high-affinity binding to cell surface receptors selectively expressed on the surface of target cells, were coupled to AAV particles harboring mutations in the capsid proteins which ablate natural receptor usage. Both, the AAV capsid protein VP2 and multiple separately produced targeting ligands recognizing Her2/*neu*, EpCAM, CD133 or CD30 were genetically fused with complementary split-intein domains. Optimized coupling conditions led to an effective conjugation of each targeting ligand to the universal AAV capsid and translated into specific gene transfer into target receptor-positive cell types *in vitro* and *in vivo*. Interestingly, PTS-based AAVs exhibited significantly less gene transfer into target receptor-negative cells than AAVs displaying the same targeting ligand but coupled genetically. Another important consequence of the PTS technology is the possibility to now display scFvs or other antibody-derived domain formats harboring disulfide-bonds in a functionally active form on the surface of AAV particles. Hence, the custom combination of a universal AAV vector particle and targeting ligands of various formats allows for an unprecedented flexibility in the generation of gene transfer vectors targeted to distinct cell types.

1. Introduction

Gene delivery has become more and more important for the development of innovative advanced therapies during the past years. Since certain gene-based therapeutic concepts have brought substantial clinical benefit not only to patients suffering from inherited disease but also to cancer patients, investment by the pharmaceutical industry has considerably increased [1,2]. Vectors for gene delivery are especially important when therapeutic genes are to be administered directly into the patient. This is for example the case for Glybera, the first gene therapy medicinal product that obtained marketing authorization in the Western world for the treatment of lipoprotein lipase (LPL) deficiency [3]. Patients suffering from this inherited disease accumulate life-threatening levels of chylomicrons if they do not adhere to a strict cholesterol diet. Through multiple intramuscular injections of Glybera, an adeno-associated virus (AAV) derived vector encoding active LPL, LPL levels in blood can be restored. AAV vectors represent the currently most effective tool for *in vivo* gene delivery and are non-enveloped particles with a rigid icosahedral capsid containing a single-stranded DNA genome that encodes the gene to be transferred. Moreover, they exhibit an excellent safety record, are robust, can be generated at high titers and large scale, exhibit low immunogenicity, and due to their particularly small size (25 nm diameter) can efficiently penetrate through various types of tissue [4,5]. Tropism and biodistribution of the vector particles are influenced by the AAV serotype used. For example, for gene transfer to the central nervous system AAV9 is often used whereas serotypes 2, 5

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or 8 are preferred for liver-directed gene delivery to treat hemophilia [6].

However, while there is certainly a preference for particular tissues, unmodified AAV vectors are not selective for a particular cell type, which can result in gene transfer into irrelevant cells and thus reduction of the therapeutically active dose as well as adverse events. This is especially relevant after systemic vector injection, where insufficient target cell selectivity often has to be compensated by the injection of higher vector doses. Accordingly, efforts are ongoing to engineer the capsid of AAV vectors in order to achieve more selective gene delivery into therapy-relevant cell types. Strategies include directed evolution approaches as well as rational-based engineering concepts [7–9]. We have established so called receptor-targeted AAV vectors that allow for the selective genetic modification of rare cell types *ex vivo* and *in vivo* with high specificity [10]. This approach is based on ablating natural receptor recognition by mutating residues R585 and R588 in the capsid proteins which mediate binding of heparin sulfate proteoglycans, and by displaying a so called targeting ligand exhibiting high affinity for the intended target receptor on the vector particle surface. As targeting ligands designed ankyrin repeat proteins (DARPins) are genetically fused to the mutated VP2 capsid proteins [11]. This way, Her2/*neu*- and EpCAM-targeted AAVs were generated which targeted therapeutic genes to tumor cells upon intravenous administration of the vector particles [12].

However, even though this technology allows for the generation of versatile targeted vectors, there is still room for improvement. Since AAV particles assemble in the nucleus of producer cells under reducing conditions, targeting ligands requiring oxidizing conditions in order to form disulfide bonds can so far not be used for AAV receptor-targeting. Single-chain variable fragments (scFvs), nanobodies as well as many natural ligands are therefore not applicable, thus restricting the options basically to DARPins. However, (i) available DARPins cover only a very limited repertoire of cell surface receptors, and (ii) the selection of new DARPins specific for a target of choice is tedious and time-consuming. Moreover, the genetic coupling of targeting ligands implies that for each new receptor targeted, a new type of AAV has to be cloned and produced. This approach is not only laborious but also results in high variability between vector types.

As alternative to genetic coupling, adaptor proteins, which simultaneously contact the vector particle and the targeting ligand, have been described for lentiviral and also adenoviral vectors [13–16]. For AAVs, biotinylation of vector particles followed by decoration with avidin-fused targeting ligands [17] or bispecific antibodies bridging the capsid with the target receptor have been suggested [18]. In this case a single capsid modification is sufficient to generate AAVs displaying any type of targeting ligand. However, targeting ligands do not become covalently linked to the AAV particles by this approach. *In vivo* applications are therefore particularly challenging with adaptor systems.

Covalent coupling of targeting ligands to the surface of AAVs, in contrast, would be better compatible with *in vivo* gene delivery. Protein-*trans*-splicing (PTS) mediated by intein domains can covalently link two polypeptides under physiological conditions. Inteins are naturally occurring intervening protein sequences capable to excise themselves from a protein precursor and ligate the flanking (extein) protein sequences [19]. A natural split intein system that is compatible with particularly efficient expression and that exhibits extremely rapid *trans*-splicing is derived from the DNA polymerase III (DnaE) of *Nostoc punctiforme* (Npu) [20]. Further modifications have improved this intein system now having become the method of choice for the affinity-purification of tagless proteins [21],. Here we demonstrate proof-of-principle for the covalent coupling of targeting ligands to the surface of AAV vector particles using the N- and C-terminal fragments of the Npu intein. We show this for in total four different targeting ligands, which, after coupling, directed AAV vector particles to cells expressing their respective targeted receptor. Notably, this strategy worked equally well with DARPins and scFvs, thus substantially broadening the availability of targeting ligands for the generation of cell type specific AAV vectors.

2. Materials and methods

2.1. Cell lines

HEK-293T (ATCC CRL-3216), CHO-K1 (ATCC CCL-61) and SK-OV-3 (ATCC HTB-77) cells were obtained from the American Type Culture Collection (Manassas, VA). The receptor transgenic cell lines CHO-EpCAM H1 and CHO-Her2-k6 were previously described [12,22]. CHO-CD30 cells were established by stable transfection of CHO-K1 cells with pcDNA3.1-V5-His-Topo-CD30 [23], followed by G418 (Invitrogen, Karlsruhe, Germany) selection and single cell cloning via limited dilution assay. For the generation of CHO-CD133 cells, the CD30 sequence in pcDNA3.1-V5-His-Topo-CD30 was exchanged against that of human CD133. Again, CHO-K1 cells were stably transfected, G418-selected and single clones generated. All cells were maintained in DMEM (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% glutamine (Sigma-Aldrich, Taufkirchen, Germany) and kept in a humidified atmosphere containing 5% CO_2 at 37 °C. Cell lines were regularly tested for mycoplasma contamination and found to be negative.

2.2. Plasmid construction

Sequences encoding the split-intein domains Npu-PCC73102 DnaE-n (Npu^N) and DnaE-c (Npu^C) were extracted from the intein database and registry (InBase, www.inteins.com), optimized for human codon usage (Eurofins MWG Operon, Ebersberg, Germany) and assembled *de novo* via PCR using the primers Npu-DnaE-*n*-for-outer, Npu-DnaE-*n*-for-inner, Npu-DnaE-*n*-rev-inner, Npu-DnaE-*n*-rev-outer as well as Npu-DnaE-*c*-for, Npu-DnaE-*c*-rev. In a second PCR the Npu^N gene was amplified and unique restriction sites as well as a C-terminal HA-Tag were added with the assembly PCR as template using the primer pair Sfi-Not-NpuN-for/Not-NpuN-HA-rev. In parallel, an N-terminal His6-tag was cloned in front of the stability optimized CD30-specific single-chain variable fragment (scFv) HRS3opt2#2 in the mammalian expression construct pCR3.1-HRS3opt2#2 [23] by *Nhe*I/*Sfi*I digesting an amplicon generated with the primer pair *Nhe*I-SigPep-for/SigPep-His-Tag-Sfi-rev and pCR3.1-HRS3opt2#2 as template. Subsequently, the NpuN intein amplicon was digested with *Not*I/*Xba*I and inserted behind the HRS3opt2#2 gene of the novel expression construct pCR3.1-His_6- αCD30. The resulting plasmid, pCR3.1-His_6-αCD30-NpuN-HA, encodes for a scFv-Npu^N fusion with N- and C-terminal affinity tags as targeting ligand (TL). To introduce a flexible glycine-serine linker in-between the scFv and the Npu^N domain, the second PCR was also conducted with Not-NpuN-HA-rev together with Not-$(G_4S)_3$-NpuN-for. Subsequent cloning was performed as described above, yielding pCR3.1-His_6-αCD30-$(G_4S)_3$-NpuN-HA. Likewise, the reading frame of the CD133 scFv was assembled based on the sequence published for the W6B3H10 antibody [24] and resulting in pCR3.1-His_6-αCD133-$(G_4S)_3$-NpuN-HA.

In parallel, the Npu^C reading frame was amplified by PCR using the primer pair *Age*I-*Sfi*I-NpuC-Not-$(G_4S)_3$-for/*Age*I-*Sfi*I-NpuC-Not-$(G_4S)_3$-rev and the Npu^C assembly amplicon as template. The Npu^C gene was inserted into pDARPin-VP2 [10] by sticky-end ligation