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Design and synthesis of a Tat-related gene transporter: A tool for carrying the adenovirus vector into cells

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Abstract—A Tat-related peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide, designed to transport an Adenovirus vector (Ad) into cells, was synthesized. The synthetic peptide was conjugated to Ad, which potentially can act as an efficient carrier of heterologous genes into cells. The Tat-related peptide was synthesized using the solid phase method and then was coupled to the heterofunctional cross-linking reagent, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester. The resulting peptide-succinimidohexanoic acid *N*-hydroxysuccinimide ester was conjugated to Ad containing the luciferase gene. B16BL6 cells infected with the peptide-conjugated Ad luciferase gene construct exhibit a 50-fold greater luciferase activity than B16BL6 cells infected with wild-type Ad containing the luciferase gene.

Gene therapy has attracted much attention as a potential clinical treatment/cure for intractable diseases.[1] A key to the successful implementation of gene therapy protocols is the design of the transgenesis vector. Adenovirus vectors (Ad) are often used as transport agents during gene therapy experiments and trials since they exhibit suitable transduction and gene-expression properties; but for routine clinical procedures, more efficient transfer vectors need to be developed. Previously, we showed that an Arg-Gly-Asp(RGD)-related peptide that binds to integrin functions as an efficient auxiliary transporter of Ad.[2] The RGD-related peptide, when covalently bound to Ad, transports Ad into dendritic cells via interaction with integrins. For this report, a different type of Ad auxiliary transporter was designed, synthesized, and shown to greatly increase the amount of Ad (containing the luciferase gene) transferred into cells.

The human immunodeficiency virus (HIV)-1 protein, Tat, is a transcriptional activator of HIV and can cross both the plasma and nuclear membranes. Tat contains 86 amino acids, but its translocation activity is associated with the peptide sequence, Tat(48–60), (GlyArgLysLysArgArgGlnArgArgArgProProGln: GRKKRRQRRRPPQ).[3] Futaki et al. reported that certain synthetic arginine-rich peptides can readily cross cell membranes and that the optimal number of arginines required for efficient translocation is approximately eight.[4] We designed the peptide, acetyl-Gly-Arg-Arg-Arg-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Cys amide (Ac-GRRRRRQRRRPPQGC-NH_2), to be an efficient auxiliary transporter of Ad. Since Futaki et al.[4] reported that the number of arginine residues correlates with translocation ability, the sequence, Ac-GRRRRRQRRRPPQGC-NH_2, was designed so that the two lysines found in Tat(48–60) were replaced with arginines. A C-terminal cysteine was added so that the peptide could be linked to Ad through the heterofunctional cross-linking reagent 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester (MHS),[5] which reacts with amine and sulfhydryl moieties (Fig. 1). The peptide was synthesized using an Applied Biosystems Peptide Synthesizer 433A-1. 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids [Fmoc-Gly-OH; Fmoc-Pro-OH; N^{α}-Fmoc-N^{G}-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfony-

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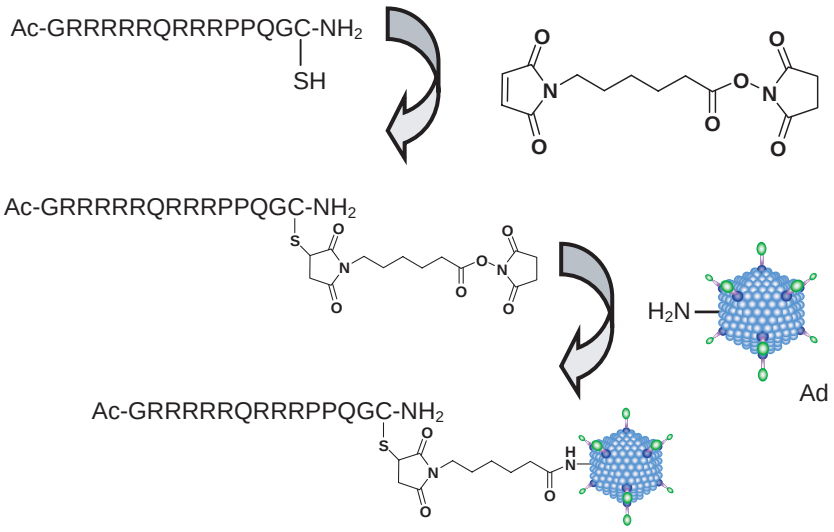

Figure 1. Synthesis of a peptide–Ad conjugate that acts as an efficient heterologous gene transporter.

larginine: Fmoc-Arg(Pbf)-OH; N^{α}-Fmoc-*S*-tritylcysteine: Fmoc-Cys(Trt)-OH; and Fmoc-Gln(Trt)-OH] were coupled in a stepwise manner to Rink amide resin[6] (PE Biosystems. Amino content: 0.67 mequiv/g, 379 mg, 0.25 mmol) using the coupling reagent, 2-(1-*H*-benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU),[7] in *N*-methylpyrrolidone (NMP). After each coupling step, the Fmoc group was removed using 20% piperidine/NMP. The synthetic Fmoc-Gly-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Gln(Trt)-Arg(Pbf)-Arg(Pbf)-Arg(Pbf)-Pro-Pro-Gln(Trt)-Gly-Cys(Trt)-Rink amide resin was treated with 20% piperidine/NMP and then treated with acetic anhydride. The peptide was cleaved from the resin with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (95:2.5:2.5). The resulting crude peptide, (Ac-GRRRRRQRRRPPQGC-NH_2, 410 mg yield), was purified using RP-HPLC.[8] The HPLC profile of the crude peptide mixture is shown in Figure 2 The yield of the purified peptide was 202 mg (28% as calculated from the amino content of the used resin).

The purified peptide (40 mg, 14 μmol), dissolved in PBS (pH 7.2, 500 μL), and the heterofunctional cross-linkage reagent (MHS: 4.3 mg, 14 μmol), dissolved in dimethylsulfoxide (DMSO, 10 μL), were combined and then stirred for 0.5 h. We attempted to purify the product, Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 (SHS: 6-succinimidohexanoic acid *N*-hydroxysuccinimide ester), using HPLC, but could not—the *N*-hydroxysuccinimide ester hydrolyzes easily in water; therefore, the reaction product mixture was frozen immediately and kept at −80 °C until needed. While gently stirring, Ad-Luc, whose chromosome encodes the heterologous luciferase gene, was reacted with Ac-GRRRRRQRRRPPQGC(SHS)-NH_2 at 37 °C for 45 min. To test the relative transduction efficiency of the peptide–Ad conjugate [Tatpep-(Ad-Luc)],

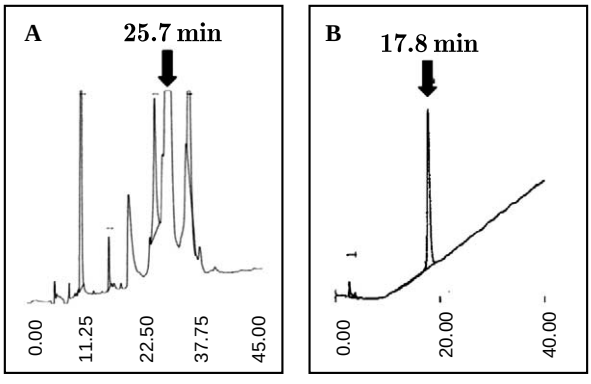

Figure 2. HPLC profile of synthetic crude Ac-GRRRRRQ-RRRPPQGC-NH_2. (A) Preparative HPLC of crude synthetic peptide. Column: DAISOPAK SP-120-5-ODS-B (20 × 250 mm). Flow rate: 10 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 10–70% CH_3CN over the course of 60 min. The absorbance was measured at 220 nm. (B) Analytical HPLC of purified sample. Column: Inertsil ODS-3 (4.6 × 250 mm). Flow rate: 1 mL/min. Eluent: CH_3CN/H_2O containing 0.05% CF_3COOH. Gradient: 5–20% CH_3CN over the course of 40 min. The absorbance was measured at 220 nm.

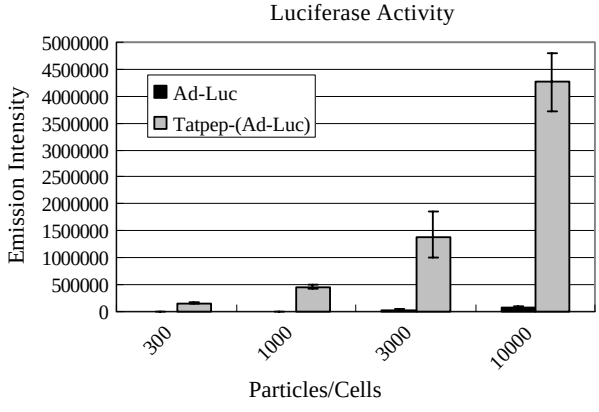

Figure 3. Transduction efficiency of Ad-Luc and Tatpep-(Ad-Luc) into B16BL6 cells Cells (2×10^4) were incubated with 300, 1000, 3000 or 10,000 particles/cell of Ad-Luc (solid bars) or Tatpep-(Ad-Luc) (gray bars). Luciferase activity, which was determined using a Luciferase Assay System Kit (Promega, USA) and a Microlumat Plus LB96 (Perkin-Elmer, USA) after lysing the cells with Luciferase Cell Culture Lysis Reagent (Promega, USA), was measured at the end of a 24 h incubation. The bars report the mean relative unit of light per well ± SD ($n = 3$).

B16BL6 cells were incubated with it or with Ad-Luc for 24 h, at which time luciferase activity was measured (Fig. 3). B16BL6 cells were used because the Coxackie-adenovirus receptor, which transports Ad across the plasma membrane, is nearly absent.[9] At concentrations of 300 and 1000 particles/cell, cells that were exposed to Ad-Luc did not glow, while those exposed to Tatpep-(Ad-Luc) construct clearly did. Ad-Luc infected cells glowed weakly at doses of 3000 and 10,000 particles/cell, while Tatpep-(Ad-Luc) exhibited strong luciferase activity at the same concentrations. The transduction activity of Tatpep-(Ad-Luc) is about 50-fold greater than that of Ad-Luc—a remarkable finding.

In summary, we designed the peptide, Ac-GRRRRRQ-RRRPPQGC-NH_2, to be an efficient auxiliary transporter of Ad into cells. Ad, when covalently bound to this synthetic peptide, exhibits a transduction ability 50-fold greater than does Ad alone. This modified Ad is a promising experimental tool for transduction studies.

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