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Research paper

Assembly of targeting complexes driven by a single-chain antibody

Marina V. Backer*, Jennifer Elliot, Timur I. Gaynutdinov, Joseph M. Backer

SibTech, Inc., 705 North Mountain Road, Newington, CT 06111, USA

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Abstract

Rapid development in design and production of recombinant antibodies and antibody fragments specific for cell surface markers opens new opportunities for targeted delivery of therapeutic or imaging agents. However, the progress in this field is slowed by inactivation of many antibodies by chemical conjugation of payloads and by lack of internalization of complexes formed on the cell surface. Here, we describe conversion of a non-internalizing single chain Fv (scFv) antibody P4G7 specific for vascular endothelial growth factor receptor 2 (VEGFR-2) into a targeting protein (Hu-P4G7) for assembly of a novel type of targeting complexes. Hu-P4G7 contains an N-terminal "docking" Hu-tag, a 15-aa fragment of human RNase I, capable of high affinity binding of S-protein fragment of human RNase I or bovine RNase A. Purified Hu-P4G7 and complexes of Hu-P4G7 with S-protein bind both soluble and full-length cellular VEGFR-2. To assemble targeted DNA delivery complexes, S-protein modified with a DNA condensing agent was "docked" to Hu-P4G7, and then loaded with luciferase plasmid DNA. As expected for a non-internalizing targeting protein, Hu-P4G7-based complexes did not deliver DNA in VEGFR-2 expressing cells. However, in the presence of vascular endothelial growth factor (VEGF), these complexes selectively delivered DNA into the cells overexpressing VEGFR-2 suggesting that even a non-internalizing scFv antibody can be used for targeted intracellular drug delivery.

Keywords: Targeted drug delivery; scFv antibody

1. Introduction

Advances in antibody engineering including direct selection from phage display libraries resulted in development of several approved antibody-based anticancer therapeutics, and many more moving through clinical trials (see, for reviews, Neilsen and Marks, 2000; Wu and Yazaki, 1999). The utility of recombinant antibodies specific for cell surface markers would be increased significantly, if they could be "loaded" with therapeutic or imaging agents through a standardized procedure without damaging their binding epitopes. Furthermore, for many applications, it would be useful if antibodies carrying a "payload" were internalized upon binding to the cell surface targets.

We have recently proposed to link drug payloads to a standardized adapter protein that can bind to a "docking" tag fused to a targeting protein (Backer et al., 2002 and Fig. 1). The first adapter/docking tag system for assembly of targeting complexes was

Abbreviations: scFv, single chain fragment; VEGFR-2, vascular endothelial growth factor receptor 2; VEGF, vascular endothelial growth factor; KDR-Fc, soluble VEGFR-2; SLT, Shiga-like toxin; PEI, polyethylenimine.

* Corresponding author. Tel.: +1-860-953-1164; fax: +1-860-953-1317.

E-mail address: mbacker@sibtech.com (M.V. Backer).

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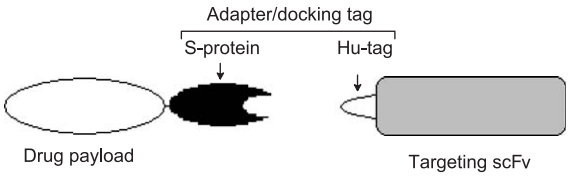


Fig. 1. Assembled targeting complexes for delivery of therapeutic and imaging payloads. Assembly is based on a high affinity interaction between an adapter protein and a docking tag fused to a targeting protein.

based on high affinity interactions between two fragments of bovine RNase A: a 1–15-aa S-peptide (S-tag) and an 18–124-aa fragment S-protein (Backer et al., 2002). Recently, we have established that similar fragments of human RNase I, 1–15-aa Hu-tag and 18–124-aa human S-protein can be used for assembly of targeting complexes for receptor-mediated DNA delivery (Backer et al., 2003). It should be noted that fragments of RNase I (1–15 and 21–124 aa) fused to C-termini of different scFv antibody fragments were successfully used for formation of a bispecific antibody (Dubel, 1999).

To use targeting scFv antibodies in assembled drug delivery complexes (Fig. 1), it is necessary to establish (1) if fusion of a docking tag affects scFv antibody activity, (2) if binding of an adapter affects activity of tagged scFv antibody and (3) if scFv-based assembled targeting complexes can deliver payloads to the targets. For this study, we have chosen P4G7, a non-neutralizing scFv antibody for vascular endothelial growth factor receptor 2 (VEGFR-2) selected from a phage display library (Lu et al., 1999). Here, we report that P4G7 retains functional activity after fusion to N-terminal Hu-tag, after binding of adapter protein and after assembly of DNA-delivery complexes.

VEGFR-2, a major receptor for crucial pro-angiogenic vascular endothelial growth factor (VEGF), is selectively expressed on endothelial cells and over-expressed on growing endothelial cells in tumor vasculature (Brown et al., 1993; Plate et al., 1993; Brown et al., 1995). Targeting this receptor with antibody-based constructs might provide new opportunities for anti-angiogenic cancer therapy. In this study, we demonstrate that in the presence of VEGF, P4G7-driven DNA complexes efficiently delivered DNA into VEGFR-2 expressing cells. These data suggest that some non-internalizing antibodies specific for cell surface receptors might be used for intracellular delivery upon stimulation of receptor-mediated endocytosis with physiological ligands of the receptors.

2. Materials and methods

2.1. Construction and expression of Hu-tagged P4G7

The pCANTAB/p4G7 plasmid encoding P4G7 scFv mouse antibody for human VEGFR-2 was kindly provided by ImClone Systems (New York, USA). The coding sequence for P4G7 including E-tag (GGT GCG CCG GTG CCG TAT CCG GAT CCG CTG GAA CCG CGT encoded by pCANTAB vector, Amersham) was amplified by PCR from the pCANTAB/p4G7 plasmid DNA using primers introducing *Eco*RI restriction sites (underlined): 5′ -CTGC GAATTC CAG GTG AAA CTG CAG GAG TC, sense, and 5′ - CTTC GAATTC CTA TGC GGC ACG CGG TTC, antisense. The gel purified PCR fragment was cloned in *Eco*RI site of the pET29-Hu vector constructed as described (Backer et al., 2003) in-frame with Hu-tag (a 1–15-aa fragment of human RNase I, human S-peptide). Clones with correct p4G7 orientation were selected by PCR using a T7-based sense primer, and confirmed by sequencing. The resulting protein comprised of a 15-aa Hu-tag, a 16-aa peptide linker, 238-aa P4G7 and a 13-aa E-tag was designated Hu-P4G7 and expressed in BL21(DE3) *Escherichia coli* (Novagen, USA). Expression of Hu-P4G7 was induced by the addition of 1 mM of IPTG to the cultures grown to an optical density of 0.5 OU at 600 nm. The induced cultures were grown for 3 h at 37 °C, and then harvested by centrifugation for 15 min at 5000 × *g*. Inclusion bodies were separated using BugBuster reagent (Novagen) according to the manufacturer's protocol, and then extensively washed using a combination of BugBuster, CelLytic B (Sigma, USA), and 1 × HisBind buffer (Novagen) according to manufacturers' instructions. Inclusion bodies were solubilized by sonication in a buffer containing 6 M Gu–HCl, 20 mM Tris–HCl, 100 mM NaCl, pH 8.0, and dialyzed as follows: first, 20 h against 20 volumes of 20 mM Tris–HCl, 2 M urea, 0.5 M arginine, 1 mM glutathione (Novagen), 0.4 mM oxidized glutathione (Novagen), pH 8.0; then 24

h against 100 volumes of 20 mM Tris–HCl, 150 mM NaCl, pH 8.0. Misfolded oligomers were removed from protein preparations by passing them through HiTrap Fast Flow SP Sepharose (1-ml pre-packed column, Amersham). Purity of Hu-P4G7 was estimated by SDS-PAGE followed by SafeBlue staining (Invitrogen, USA), and by RP HPLC on C18 Waters Nova-Pack 5 μm column (150 × 3.9 mm) with elution at 0.75 ml/min with 0.1% TFA (v/v) and a linear gradient of acetonitrile (5–50% over 20 min). The concentration of Hu-P4G7 was calculated using 216-nm integral absorption in HPLC profiles. HP 1090 Series II instrument was used for RP HPLC.

2.2. Tissue culture

HEK293 human transformed embryonic kidney cells (CRL-1573) were obtained from American Type Culture Collection (Rockville, USA). 293/KDR-Fc cells expressing soluble form of VEGFR-2 (Kaplan et al., 1997) and PAE/KDR porcine aortic endothelial cell expressing full-length VEGFR-2 were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY, USA). 293/KDR cells expressing 2.5×10^6 VEGFR-2/cell have been described previously (Backer and Backer, 2001a). All cells were grown in DMEM with 10% FBS, 2 mM L-glutamine and antibiotics at 37 °C, 5% CO_2.

2.3. Ribonuclease activity assay

Human S-protein, expressed and purified as described previously (Backer et al., 2002, 2003), and bovine S-protein (Sigma) were used to test functional activity of Hu-tag in Hu-P4G7 protein. Varying amounts of human or bovine S-proteins were added to reaction mixtures containing 20 mM Tris–HCl, 100 mM NaCl, pH 7.5, 0.1 mg/ml poly(C) and 50 nM Hu-P4G7. After 5-min incubations at room temperature, the reactions were stopped by addition of 1/4 volume of ice-cold 10% trichloroacetic acid, incubated on ice for 5 min, and centrifuged for 10 min at 14,000 × *g* at room temperature. Reconstituted ribonuclease activities were measured by absorbance of the supernatants at 280 nm. One optical unit of acid-soluble material released from poly(C) incubated with reconstituted ribonuclease was defined as one relative unit of ribonuclease activity.

2.4. Binding to soluble VEGFR-2

Soluble VEGFR-2 was purified from 293/KDR-Fc cells conditioned medium by precipitation with protein A sepharose (Amersham) as described (Kaplan et al., 1997). Varying amounts of Hu-P4G7 were mixed with VEGFR-2 collected on protein A sepharose, incubated for 1 h at 4 °C, and precipitated by centrifugation at 1000 × *g* for 3 min. Non-specific proteins were removed from the pellets by washing them with buffer containing 50 mM Tris–HCl pH 8.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS (RIPA buffer), three times for 5 min. Binding of Hu-P4G7 to soluble VEGFR-2 was analyzed by Western blotting using HRP/Anti-E Tag conjugate (anti-E-tag antibody conjugated to horse reddish peroxidase, Amersham) diluted 1:10,000. Immune complexes were visualized by ECL Plus kit (Amersham).

2.5. Immunoprecipitation

293/KDR cells were plated on 6-well plates, $0.5–1 \times 10^6$ cells/well. Twenty hours later, cells were lysed in ice-cold IP buffer (10 mM Tris–HCl pH 7.5 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, protease inhibitors) for 30 min at 4 °C, and clarified by centrifugation at 10,000 × *g* for 5 min. Varying amounts of Hu-P4G7 were mixed with clarified cell lysates, and incubated for 1 h at 4 °C. After binding, Hu-P4G7 was collected from solutions by S-protein agarose (Novagen) for 1 h at 4 °C, and precipitated by centrifugation at 1000 × *g* for 3 min. Non-specific cellular proteins were removed from the S-protein agarose pellets by extensive washing with IP buffer. Binding of cellular VEGFR-2 to Hu-P4G7 was analyzed by Western blotting using Flk-1 (A-3) mouse monoclonal antibody sc-6251 (Santa Cruz, USA) diluted 1:200, followed by anti-mouse IgG/HRP conjugate (Amersham).

2.6. VEGFR-2 autophosphorylation

Recombinant Hu-tagged $VEGF_{121}$ (Hu-$VEGF_{121}$, expressed and purified as described in Backer et al., 2003) was used for induction of VEGFR-2 autophosphorylation performed as described (Backer et al., 2002). Briefly, 293/KDR cells after overnight starvation in 0.5% FBS were stimulated with varying

amounts of Hu-VEGF$_{121}$ alone, or in the presence of 1 μM Hu-P4G7. In another setting, cells were pre-incubated with 1 μM Hu-P4G7 for 45 min at 37 °C, and then stimulated with Hu-VEGF$_{121}$. After 5-min stimulations at 37 °C, cells were analyzed by Western blotting using anti-phosphotyrosine RC20/HRP conjugate (BD Transduction Labs, USA) diluted 1:2500.

2.7. Cytotoxicity assay

Recombinant fusion protein SLT-VEGF containing Shiga-like toxin (SLT) subunit A fused to human VEGF$_{121}$ was expressed as described (Backer and Backer, 2001b). PAE/KDR cells were plated on 96-well plates, 500–1000 cells/well. Twenty hours later, cells in quadruplicate wells were treated with varying amounts of SLT-VEGF alone, or in the presence of 100-fold molar excess of competitors: Hu-VEGF$_{121}$ or Hu-P4G7. Cells were quantitated 72 h later by CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay kit (Promega, USA) according to manufacturer's instructions.

2.8. Assembly of DNA delivery complexes

Bovine S-protein (Sigma) was crosslinked to linear 25-kDa polyethylenimine (PEI, Polysciences, USA) as previously described (Backer et al., 2003). Briefly, PEI was modified with Traut's reagent (Pierce, USA) to the molar ratio of 1:1 according to the manufacturer's protocol. S-protein was modified with a two-fold molar excess of sulfo-MBS (Pierce) in conjugation buffer (0.1 M NaPi, 0.15 M NaCl, pH 7.2) for 30 min at room temperature. Modified PEI and S-protein were mixed at the molar ratio of 1:1 in conjugation buffer and incubated for 4 h at room temperature. PEI/S-protein conjugates were purified by gel-filtration on SE40/100 gel (BioRad Laboratories, USA). Complexes of Hu-P4G7 or Hu-VEGF$_{121}$ with PEI/S-protein conjugates at the S-protein to Hu-tag ratio of 1.5:1 were prepared in 5% glucose. The same amount of glucose was added to a PEI/S-protein conjugate solution to prepare control complexes without a targeted protein. All mixtures were incubated on ice for 20 min, then added separately to the pGL3 plasmid DNA (Promega) to form complexes with the N/P ratio of 3, where N is the concentration of PEI monomers and P is the concentration of DNA phosphate groups. After 20-min incubations on ice, the complexes were added to cells in complete culture medium to final concentrations of 10 nM Hu-tagged protein, 32 nM PEI/S-protein and 1 μg DNA/well.

3. Results

3.1. Functional activity of Hu-tagged P4G7

To arm P4G7 with an N-terminal docking tag, the P4G7 coding sequence including a C-terminal E-tag was cloned into the pET29/Hu bacterial vector for expression of Hu-tagged proteins (constructed as described by Backer et al., 2003). The resulting Hu-P4G7 fusion protein with an N-terminal Hu-tag was expressed in BL21(DE3) *E. coli* and found in the inclusion body fraction (Fig. 2). The protein was solubilized from inclusion bodies, refolded via a two-step dialysis and purified by cation-exchange chromatography with a yield of 12–14 mg/l of more than 95% pure protein. Western blot analysis with HRP/Anti-E Tag antibody revealed a single band with the expected molecular weight of 36 kDa, confirming

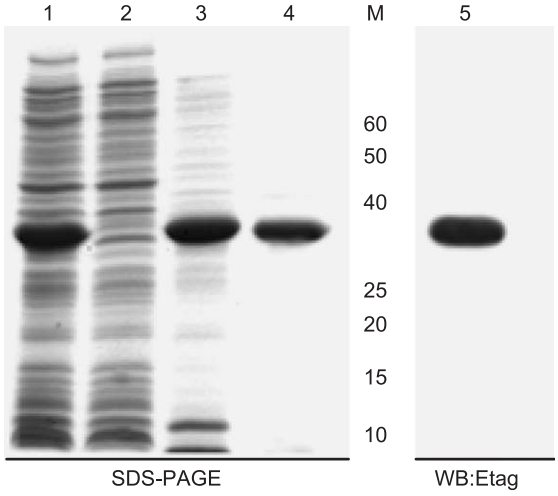


Fig. 2. Hu-P4G7 is purified from inclusion bodies. Samples of total induced bacterial lysate (lane 1), soluble part of bacterial lysate (lane 2), inclusion bodies (lane 3) and purified protein (lane 4) were analyzed by SDS-PAGE on a 17.5% gel followed by staining with SafeBlue (Invitrogen). Molecular weights of protein markers are indicated (lane M). Purified Hu-P4G7 was analyzed by Western blot analysis (lane 5) using HRP/Anti-E Tag conjugate (Amersham) diluted 1:10,000.

the identity of Hu-P4G7 (Fig. 2). To evaluate functional activity of Hu-tagged P4G7, we tested its ability to bind KDR-Fc, soluble VEGFR-2 comprising of extracellular part of VEGFR-2 fused to the Fc portion of human IgG (Kaplan et al., 1997). Hu-P4G7 at final concentrations of 250, 50 and 10 ng/ml was incubated with KDR-Fc pre-absorbed on protein A sepharose beads, then precipitated and analyzed by Western blotting. We found that Hu-P4G7 could be completely depleted by KDR-Fc (Fig. 3) indicating that Hu-tagged P4G7 is functionally active.

3.2. Hu-P4G7 in complex with an adapter protein retains functional activity

Binding of adapter protein to Hu-P4G7 at nanomolar concentrations was readily detected by measuring ribonuclease activity reconstituted upon interaction between Hu-tag and S-protein fragment of human RNase I or highly homologous bovine RNase A (Fig. 4A). These results, as well as similar findings with Hu-VEGF$_{121}$ (Backer et al., 2002, 2003), indicate that an N-terminal Hu-tag is capable of binding adapter S-protein. It should be noted that native bovine S-protein was approximately five-fold more active in this assay than recombinant human S-protein. As we have recently established, it might be due to the presence of significant amounts of inactive human S-protein in our protein preparations (Gaynutdinov et al., 2003). Therefore, further experiments on evaluation of Hu-P4G7/adapter complexes were performed with bovine S-protein.

To test if Hu-P4G7 bound to a derivatized adapter protein still continues to bind VEGFR-2, we used

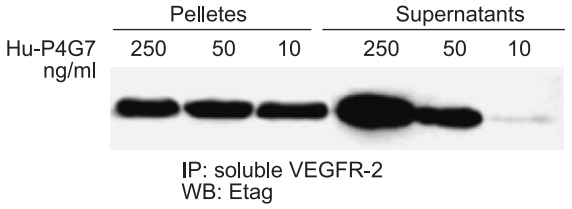


Fig. 3. KDR-Fc binds Hu-P4G7 in a dose-dependent manner. KDR-Fc collected on protein A Sepharose was used to precipitate Hu-P4G7 from solutions containing Hu-P4G7 at concentrations of 250, 50 and 10 ng/ml. The complexes were harvested by centrifugation, and both pellets and supernatants were analyzed by Western blotting with HRP/Anti-E Tag conjugate (Amersham) diluted 1:10,000.

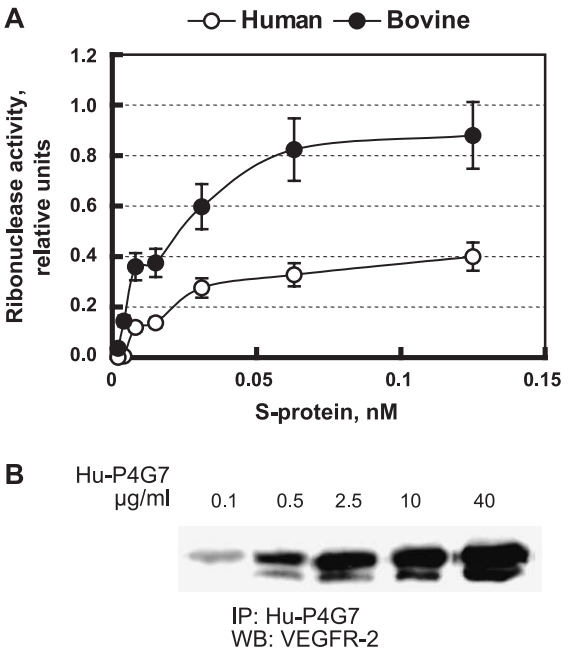


Fig. 4. Human and bovine adapter S-proteins bind Hu-P4G7. (A) Varying amounts of human or bovine S-proteins were mixed separately with Hu-P4G7 taken at a final concentration of 50 nM. Reconstituted ribonuclease activity was measured as described under Section 2. (B) Hu-P4G7 was added to 293/KDR cell lysate to indicated final concentrations. After binding, complexes were precipitated with S-protein agarose (Novagen) and analyzed by Western blotting using VEGFR-2 antibody (Santa Cruz) diluted 1:200.

bovine S-protein cross-linked to agarose. Cells overexpressing VEGFR-2 were lysed, incubated with Hu-P4G7 and immune complexes Hu-P4G7/VEGFR-2 were collected with S-protein agarose. The presence of VEGFR-2 in the immunoprecipitates (Fig. 4B) indicates that binding of a derivatized adapter to Hu-tag is possible and does not prevent P4G7 binding to VEGFR-2.

3.3. Hu-P4G7 does not affect VEGF-induced VEGFR-2 tyrosine autophosphorylation and internalization

The parental scFv P4G7 was reported to be a non-neutralizing antibody that does not inhibit VEGF-induced autophosphorylation of VEGFR-2 in human umbilical vein endothelial cells (Lu et al., 1999). In agreement with these data, we found that Hu-P4G7 added to 293/KDR cells alone or simultaneously with

VEGF did not affect either basal or VEGF-induced VEGFR-2 tyrosine autophosphorylation indicating that Hu-P4G7 does not compete with VEGF (Fig. 5A, compare upper and middle panels). A 45-min pre-incubation of cells with 1 μM Hu-P4G7 also did not affect VEGF-induced VEGFR-2 tyrosine autophosphorylation eliminating the possibility that Hu-P4G7 might stimulate VEGFR-2 endocytosis (Fig. 5A, compare upper and lower panels). To establish if Hu-P4G7 inhibits VEGFR-2-mediated endocytosis, we have tested its effect on cytotoxicity of SLT-VEGF fusion protein comprising of Shiga-like toxin subunit A fused to human $VEGF_{121}$ (described in Backer and Backer, 2001b). Since SLT-VEGF enters cells via VEGFR-2-mediated binding and internalization, it is highly cytotoxic to cells overexpressing VEGFR-2 (Backer and Backer, 2001b). We found that Hu-P4G7 had no effect on cytotoxicity of SLT-VEGF, while Hu-$VEGF_{121}$ used as a positive control effectively rescued PAE/KDR cells (Fig. 5B). Thus, Hu-P4G7 did not affect either formation or internalization of SLT-VEGF/VEGFR-2 complexes.

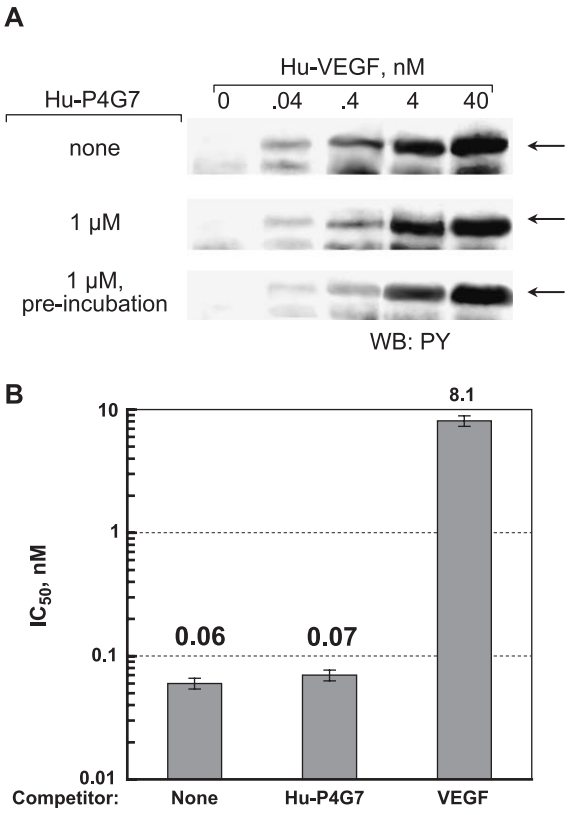
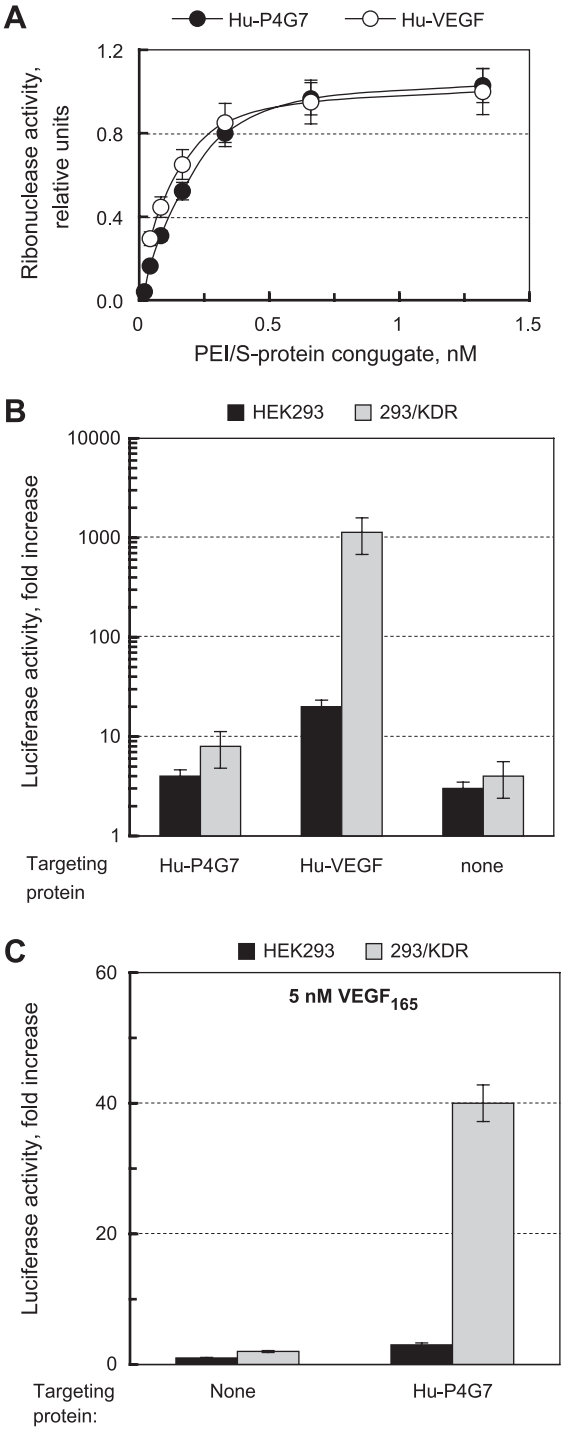


Fig. 5. Hu-P4G7 does not compete with VEGF for binding, activation and internalization of VEGFR-2. (A) Hu-P4G7 does not inhibit Hu-VEGF induced VEGFR-2 tyrosine autophosphorylation in 293/KDR cells. Hu-P4G7 at a final concentration of 1 μM was either premixed with Hu-VEGF (middle panel) or added to cells 45 min prior to Hu-VEGF addition (lower panel). After 5-min incubations with varying amounts of Hu-VEGF, cells were lysed and analyzed by Western blotting using phosphotyrosine RC20/HRP conjugate (Transduction Lab) diluted 1:2500. Arrows indicate positions of VEGFR-2. (B) Hu-P4G7 does not inhibit VEGFR-2 mediated cytotoxicity of SLT-VEGF fusion toxin. PAE/KDR cells expressing $\sim 2 \times 10^5$ VEGFR-2 per cell were plated on 96-well plates (750 cells/well) 20 h before the experiment. Cells in triplicate wells were exposed to varying amounts of SLT-VEGF alone, or in the presence of competitors: 1 μM Hu-P4G7 or 1 μM Hu-VEGF. The IC_{50} values were determined 72 h later by cell quantitation using MTT-based kit (Promega).

3.4. Hu-P4G7-based DNA delivery vehicles do not induce internalization

Since Hu-P4G7 did not induce internalization of VEGFR-2, it appeared to be suitable for targeted delivery of payloads to the cell surface only. However, it is known that increasing the valency of antibodies, as well as scFv fragments, can increase their internalization (Neilsen and Marks, 2000). We have recently demonstrated that Hu-$VEGF_{121}$ driven DNA delivery complexes display higher avidity to VEGFR-2 than free Hu-$VEGF_{121}$, presumably because of the multiplexing of Hu-$VEGF_{121}$ (Backer et al., 2002). Therefore, we tested if multiplexing of Hu-P4G7 molecules in DNA delivery complexes can stimulate VEGFR-2-mediated endocytosis. To create a DNA binding payload module, a DNA condensing agent PEI was conjugated to bovine S-protein at the molar ratio of 1:1, as described (Backer et al., 2002, 2003). PEI/S-protein conjugate was bound to Hu-P4G7, or to Hu-$VEGF_{121}$ serving as a positive control, for targeting of assembled DNA delivery complexes to VEGFR-2 (Backer et al., 2002, 2003). Binding of PEI/S-protein conjugates to Hu-tagged proteins was confirmed by reconstitution of ribonuclease activity upon complex formation (Fig. 6A). In agreement with our previous data (Backer et al., 2002), ribonuclease activity of PEI/S-protein conjugate was about 10-fold lower than that of free S-protein (compare Figs. 4A and 6A) reflecting, most likely, a detrimental effect of random modification of S-protein on its enzymatic activity.



Hu-tagged protein/PEI-S-protein complexes, as well as PEI/S-protein conjugate alone, were loaded with the pGL3 plasmid DNA encoding firefly luciferase at the N/P ratio of 3 (where N is the concentration of PEI monomers and P is the concentration of DNA phosphate groups). Since ~25-kDa PEI contains ~ 600 monomers, and the pGL3 plasmid consists of 5226bp, N/P ratio of 3 is reached at the PEI to plasmid DNA molar ratio of 38:1. Considering that PEI/DNA complexes form supramolecular polyplexes with average size of 200 nm (Backer et al., 2003), and therefore contain several DNA molecules per complex, we can expect multiplexing of Hu-tagged proteins on the surface of the DNA delivery complexes.

Assembled complexes were then added to 293/KDR cells expressing $\sim 2.5 \times 10^6$ VEGFR-2/cell and to HEK293 cells lacking VEGFR-2 receptors and served as a control for non-receptor-mediated uptake. We found that the presence of Hu-P4G7 in DNA delivery complexes did not enhance expression of luciferase relative to the non-targeted PEI/S-protein/DNA complexes (Fig. 6B). In contrast, VEGF-driven DNA delivery resulted in a significant increase over this level (Fig. 6B). Since non-receptor mediated delivery in HEK293 cells was low in both cases, we concluded that cross-linking VEGFR-2 by P4G7 multiplexed on DNA delivery complexes did not induce receptor-mediated endocytosis.

Since P4G7 did not inhibit VEGF-induced tyrosine autophosphorylation and internalization of VEGFR-2 (see Fig. 5), we reasoned that these processes might take place even with the receptors

Fig. 6. Hu-P4G7-based DNA delivery. (A) PEI/S-protein conjugate binds to Hu-tagged proteins. Varying amounts of PEI/S-protein conjugate were mixed separately with 50 nM Hu-tagged proteins. Reconstituted ribonuclease activity was measured as described under Section 2. (B) HEK293 and 293/KDR cells were plated on 24-well plates at 10^4 cells/well 24 h before the experiment. P4G7-driven luciferase DNA delivery complexes were added cells in triplicate wells. Cells were lysed and assayed for luciferase activity (Bright-Glo™ Luciferase Assay System, Promega) 48 h after DNA delivery. Fold increase was calculated over luciferase activity in cells treated with a control PEI/S-protein/DNA complex without a targeted protein. (C) P4G7-driven luciferase DNA delivery complexes were added to cells in triplicates and incubated for 1 h at 37 °C. Recombinant human $VEGF_{165}$ (R&D Technologies, USA) was added then to all wells to a final concentration of 5 nM. Cells were lysed and assayed for luciferase activity 48 h later. All DNA delivery experiments were repeated three times.

being "loaded" with P4G7-driven DNA delivery complexes. Indeed, we found that addition of $VEGF_{165}$ to 293/KDR cells pre-incubated for 1 h with P4G7-driven DNA delivery complexes resulted in a 40-fold increase of luciferase activity as compared to cells pre-incubated with complexes lacking a targeting protein (Fig. 6C).

4. Discussion

This study was undertaken to establish if a scFv antibody can be expressed with an N-terminal Hu-tag for assembly of complexes for targeted delivery of therapeutic or imaging agents (Fig. 1). We have chosen for this study P4G7, a non-neutralizing anti-VEGFR-2 scFv antibody, because of its potential utility for selective targeting of tumor vasculature where VEGFR-2 is overexpressed (Brown et al., 1993; Plate et al., 1993; Brown et al., 1995). We found that Hu-P4G7 refolded and purified from inclusion bodies retains its ability to bind both soluble and cellular human VEGFR-2. On the other hand, as judged by reconstitution of RNase activity, the Hu-tag in Hu-P4G7 retains the ability to bind human and bovine S-protein serving as adapter proteins for loading various cargoes onto targeting proteins (Backer et al., 2002, 2003). Importantly, Hu-P4G7 also binds derivatized S-proteins, such as S-protein agarose, PEI/S-protein or PEI/S-protein-based DNA complexes, indicating that Hu-P4G7 can serve as a targeting protein with a variety of payload modules for delivery of therapeutic or imaging agents. It should be noted, however, that random cross-linking of S-protein to PEI or to liposomes significantly decreases its functional activity (Fig. 6A and Backer et al., 2002). The work is in progress to evaluate an adapter protein with an unpaired cysteine that can be directly modified without interference with activity of assembled targeting complexes (Backer et al., 2004).

Like parental P4G7 scFv antibody, Hu-P4G7 neither activates VEGFR-2 nor competes with VEGF for binding to the receptor. These are very important characteristics for an antibody considered for vascular targeting, since activation of VEGFR-2 might trigger angiogenesis in dormant benign tumors converting them into actively growing ones. Furthermore, Hu-P4G7 does not induce internalization of VEGFR-2 even when it is presented in a multivalent complex for DNA delivery. Importantly, internalization of Hu-P4G7 driven DNA delivery complexes can be stimulated by VEGF, suggesting an approach to intracellular delivery of non-internalizing scFv antibodies.

Taken together, our data suggest that (1) P4G7 fused to a docking tag retains the ability to bind to VEGFR-2, (2) to Hu-P4G7 in complex with an adapter protein still retains its functional activity and (3) P4G7-based assembled targeting complexes can be delivered to the cell surface and into the cell. In this work, we have obtained a proof-of-principle for using Hu-tagged scFv antibodies for non-covalent assembly of targeted drug delivery complexes. We expect that the effects of Hu-tag fusion or a payload module docking might vary and should be established for any particular scFv explored for targeting. On the other hand, we believe that default expression of scFv antibodies as Hu-tagged proteins would provide a standardized approach to test their utility for delivery of therapeutic or imaging agents.

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