Self-Assembled "Dock and Lock" System for Linking Payloads to Targeting Proteins

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Random conjugation of therapeutic or diagnostic payloads to targeting proteins generates functionally heterogeneous products. Conjugation of payloads to an adapter that binds to a peptide tag engineered into a targeting protein provides an alternative strategy. To progress into clinical development, an adapter/docking tag system should include humanized components and be stable in circulation. We describe here an adapter/docking tag system based on mutated fragments of human RNase I that spontaneously bind to each other and form a conjugate with a disulfide bond between complimentary cysteine residues. This self-assembled "dock and lock" system utilizes the previously described fusion C-tag, a 1−15 aa fragment of human RNase I with the R4C amino acid substitution, and a newly engineered adapter protein (Ad-C), a 21−127-aa fragment of human RNase I with the V118C substitution. Two vastly different C-tagged recombinant proteins, human vascular endothelial growth factor (VEGF) and a 254-aa long N-terminal fragment of anthrax lethal factor (LFn), retain functional activities after spontaneous conjugation of Ad-C to N-terminal or C-terminal C-tag, respectively. Ad-C modified with pegylated phospolipid and inserted into the lipid membrane of drug-loaded liposomes (Doxil) retained the ability to conjugate C-tagged proteins, yielding targeted liposomes decorated with functionally active proteins. To further optimize the system, we engineered an adapter with an additional cysteine residue at position 88 for site-specific modification, conjugated it to C-tagged VEGF, and labeled with a near-infrared fluorescent dye Cy5.5, yielding a unique functionally active probe for in vivo molecular imaging. We expect that this self-assembled "dock and lock" system will provide new opportunities for using functionally active proteins for biomedical purposes.

INTRODUCTION

Conjugation of therapeutic or diagnostic "payloads" (e.g. drugs, radionuclides, nanoparticles, liposomes) to targeting proteins encounters a well-recognized problem of functional heterogeneity of the final products (*1*, *2*). The protein−payload conjugates are usually synthesized via random modification of available chemical groups, most often ϵ-amino groups of lysine residues, and are inevitably heterogeneous, both structurally and functionally. One approach to solving this problem is to engineer targeting proteins with an additional cysteine residue that does not interfere with protein folding and can be used for a site-specific conjugation (*3*−*7*). Alternatively, native cysteines dispensable for target recognition can be coupled to a payload, as it has been recently reported for cysteines involved in the interchain disulfide bonds in IgG molecules (*8*, *9*). Although these strategies eliminate or decrease heterogeneity of the conjugates, they do not solve sterical problems associated with coupling of either bulky or highly charged payloads, which might interfere with functional activity of the resulting conjugates. To minimize this interference, payloads can be linked to an adapter protein that can be docked to a "tag" engineered in targeting protein. Currently, streptavidin/biotin is the most popular adapter/docking tag system, although it is hardly suitable for clinical development (*10*−*13*). Another promising but highly specialized approach is the engineering of bispecific recombinant antibodies recognizing one epitope on the cell surface and the other one on a drug carrier (*14*, *15*).

We have recently developed and tested in vitro and in vivo a fully humanized adapter/docking tag system based on high affinity interactions between two fragments of human RNase I,

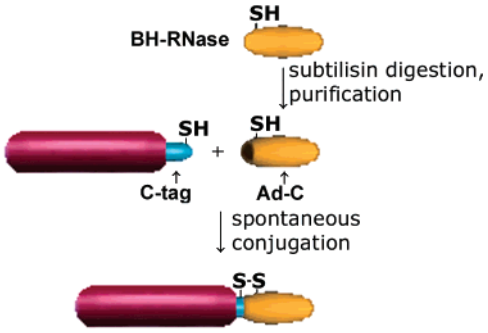

Figure 1. "Dock and lock" system.

a 1−15 aa fragment (Hu-tag) fused to a targeting protein as a docking tag, and a 21−127 aa fragment (HuS) as an adapter (*16*, *17*). However, the utility of this, as well as all other noncovalent adapter/docking tag systems, depends on their stability in a complex environment of the human bloodstream. We reasoned that "locking" of an adapter/docking tag complex with an intra-complex disulfide bond would eliminate a stability problem. We describe here such a "dock and lock" system, which is based on appropriately mutated fragments of human RNase I (Figure 1). Docking tag, named C-tag, contains a cysteine in position 4; it has been recently used for direct site-specific modification of C-tagged VEGF with derivatized dendrimers (*7*). An adapter, named Ad-C, contains cysteine in position 118 (positions are indicated for full-length human RNase I). This choice of cysteine positions was based on an observation that stability of human RNase I (R4C,V118C) double mutant is increased relative to wild-type molecule, suggesting possible formation of an intramolecular C4−C118 disulfide bond (*18*).

We hypothesized that C4 and C118 introduced in C-tag and Ad-C, respectively, would spontaneously form a disulfide bond

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in the adapter/tag complex. To test this hypothesis, we used two proteins: a newly constructed LFn-C, a 254-aa long catalytically inactive N-terminal fragment of anthrax lethal factor with no native cysteines, fused to C-terminal C-tag, and previously described human C-tagged $VEGF_{121}$ (18 native cysteines per dimer), expressed with an N-terminal C-tag (*7*). Here we report that both C-tagged proteins covalently bind Ad-C without compromising their respective activities. We demonstrate that doxorubicin-loaded liposomes can be decorated with Ad-C and then coupled to either C-tagged protein, providing a simple and efficient method for molecular targeting of bulky payloads. Furthermore, an additional cysteine introduced in Ad-C in position 88 allowed for site-specific modification of VEGF-Ad conjugate with a charged near-infrared fluorescent dye, Cy5.5. This approach yielded a functionally active molecular probe for in vivo imaging, while direct conjugation of Cy5.5 resulted in a significant drop of VEGF functional activity.

MATERIALS AND METHODS

Construction and Purification of Chimeric BH-RNase-(V118C) Mutant. Chimeric $^{1-29}B/^{30-127}H$-RNase (BH-RNase) consisting of a 1−29-aa fragment of bovine RNase A and a 30−127-aa fragment of human RNase I has been constructed as described (*19*). The V118C mutation was introduced in the pET29/$^{1-29}B/^{30-127}H$-RNase plasmid DNA by site-directed mutagenesis (Gene-Tailor Site Directed mutagenesis kit, Invitrogen) using primers: 5′-AA GGC TCT CCG TAC GTT CCG TGT CAT TTC gac gcg (sense, the mutation underlined) and 5′-CGG AAC GTA CGG AGA GCC TTC GCA TGC AAC (antisense). BH-RNase(V118C) was used for construction of double mutant BH-RNase(N88C,V118C) by introducing the N88C amino acid substitution as described (*20*). The V118C substitution and double V118C, N88C mutations were confirmed by sequencing. BH-RNase(V118C) and BH-RNase(N88C,-V118C) were expressed in BL21(DE3) *E. coli* (Novagen, Madison WI), refolded from inclusion bodies, and purified by ion-exchange chromatography on HiTrap SP Sepharose Fast Flow (1-mL prepacked columns, Amersham Biosciences, Piscataway, NJ) as described for wild-type BH-RNase (*19*). The yield of BH-RNase(V118C) was 45−50 mg/L, while double mutant BH-RNase(N88C,V118C) yielded 20−25 mg/L. Free cysteines were tested by reaction with *N*-(1-pyrene)maleimide (Molecular Probes, Eugene, OR) followed by RP HPLC analysis as described (*21*) Briefly, *N*-(1-pyrene)maleimide dissolved in DMSO was added to protein at the molar ratio of 10:1, incubated for 1 h at RT, and analyzed by RP HPLC.

Purification of Mutant Adapter Ad-C. To release Ad-C, which is a 21−127-aa fragment of BH-RNase(V118C), BH-RNase(V118C) was digested with subtilisin (Sigma) at the protein to protease w/w ratio of 10:1 for 15−20 min at 4 °C. Subtilisin digestion was stopped by 1% ice-cold TFA. After a 5-min incubation on ice, the reaction mixture was purified by ion-exchange chromatography on SP Sepharose Fast Flow as described (*19*, *20*). Digested BH-RNase(V118C) was purified by affinity chromatography on a Hu-column carrying CA-extended Hu-peptide (CA-KESRAKKFQRQHMDS synthesized by Genemed Synthesis, Inc., South San Francisco, CA) as described (*16*). Protein eluted from Hu-column with 0.1 M citric acid was neutralized with 1 M Na_2HPO_4 and analyzed 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 Ad-C was calculated using 216-nm integral absorption in HPLC profiles with bovine S-protein (Sigma) serving as a standard.

Construction of the pET/$(G_4S)_3$Hu-R4C Vector. The pET/Hu-R4C$(G_4S)_3$ vector for bacterial expression of recombinant proteins with N-terminal C-tag was constructed as described (*7*). To construct a vector for expression of proteins with C-terminal C-tag, a DNA fragment encoding Hu-tag (described in *16*) was cloned in *Nde-Kpn* sites of the pT7Blue vector (Novagen). Then a single-stranded DNA fragment encoding a $(G_4S)_3$ linker 5′- pTAT GGG TGG CGG TGG TAG TGG TGG TGG CGG TTC AGG CGG TGG TCA and a complimentary fragment 5′- pTAT GAC CAC CGC CTG AAC CGC CAC CAC CAC TAC CAC CGC CAC CCA (synthesized by GeneLink, Thornwood, NY) were annealed with reconstitution of *Nde*I sites on both termini and ligated immediately upstream of Hu-tag into *Nde* I site of the pT7Blue-Hu vector. Plasmid DNA with correct orientation of the cloned fragment was selected by sequencing, and the inner *Nde* I site was destroyed by site-directed mutagenesis (Gene-Tailor Site Directed mutagenesis kit, Invitrogen). A DNA fragment encoding $(G_4S)_3$-Hu-tag was amplified from pT7Blue$(G_4S)_3$Hu vector with primers introducing an *Nde*I site to the 5′-terminus, a stop codon and a *Kpn*I site to the 3′-terminus and cloned into *Nde-Kpn* of the pET29a(+) bacterial expression vector (Novagen). The R4C amino acid substitution was introduced in Hu-tag of the pET/$(G_4S)_3$Hu vector by site-directed mutagenesis and confirmed by sequencing. The resulting vector for bacterial expression of proteins fused to C-terminal C-tag via a $(G_4S)_3$ linker was designated pET/$(G_4S)_3$Hu-R4C.

Cloning and Expression of C-Tagged Proteins. Coding sequence for a 1−254-aa fragment of anthrax lethal factor (LFn) was amplified from the pGEX-KG LF254 plasmid (kindly provided by Dr. S. Leppla, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). For construction of LFn-C (C-terminally fused C-tag), primers introduced *Nde* I sits (italicized): 5′- CTACCT *CATATG* GCG GGC GGT CAT GGT GATG (sense) and 5′- CTACCT *CATATG* TAG ATT TAT TTC TTG TTC GTT AAA TTT ATC (antisense). For construction of C-LFn (N-terminally fused C-tag), primers introduced *Nco* I sits, and the fragment was cloned in *Nco* I site of the pET/Hu-R4C$(G_4S)_3$ vector downsream of the $(G_4S)_3$ linker. Clones with correct orientation of LFn fragment were selected by PCR using a T7-promoter-based sense primer and then confirmed by sequencing. Both C-tagged LFn fusion proteins were expressed in BL21(DE3). The expression was induced by 1 mM IPTG at the optical density of 0.5−0.7 unit at 600 nm. After induction, cultures were grown for 2.5−3 h at 37 °C with shaking at 300 rpm, harvested by centrifugation, and ruptured using EmulsiFlex-C5 (Avestin, Ottava, Canada). The soluble part of bacterial lysate was dialyzed against 200 volumes of 20 mM Tris-HCl pH 8.0 for 20 h at 4 °C, clarified by centrifugation (15 000*g* for 20 min), and passed through Sepharose Q FF column. C-LFn-containing fractions were pooled together, dialyzed against 100 volumes of 20 mM NaOAc pH 6.5 for 20 h at 4 °C, and further purified on Heparin HP Sepharose followed by hydrophobic interaction chromatography on Butyl FF Sepharose (1-ml prepacked columns, Amersham). The yield of Hu(R4C)-LFn was 8−10 mg/L, purity >98% by SDS−PAGE followed by SimplyBlue Safe Stain (Invitrogen). Human $VEGF_{121}$ was expressed with N-terminal C-tag and recovered from inclusion bodies as described (*7*). Hu-VEGF, human $VEGF_{121}$ fused to Hu-tag, was cloned and expressed as described (*16*).

Synthesis of VEGF/Ad-Cy5.5 Conjugate. BH-RNase(N88C,-V118C) was deprotected with DTT and digested with subtilisin as described for BH-RNase(V118C). Purified double mutant adapter was mixed with C-tagged VEGF at the molar ratio of 3:1 and incubated for 4 °C for 16 h. Cy5.5-maleimide (GE Healthcare) dissolved in dimethylformamide was added then to the reaction mixture to a final protein-to-Cy molar ratio of 1:10, incubated at RT for 1 h, and purified by RP-HPLC on a

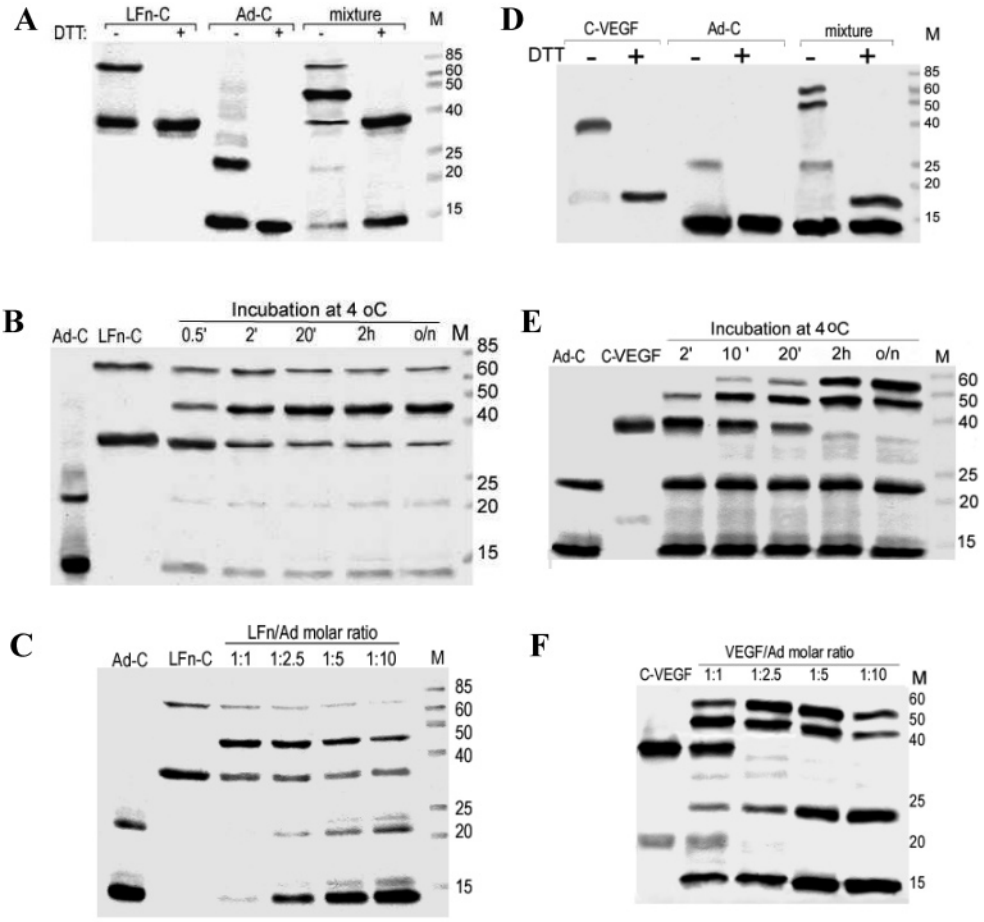

Figure 2. Self-assembly of Ad-C/C-tag conjugates. Ad-C was mixed with LFn-C (panel A) at a molar ratio of 1:1 or C-tagged VEGF (panel D) at a molar ratio of 3:1 and incubated for 16 h at 4 °C. Samples were analyzed by nonreducing (−DTT) and reducing (+DTT) SDS−PAGE on 17.5% gels followed by Bio-Safe Coomassie G-250 staining (Bio-Rad). In panels B and E, nonreducing SDS−PAGE analysis of kinetics of conjugation. Ad-C was mixed with LFn-C at a 1:1 molar ratio (B) or with C-VEGF at a 3:1 ratio (E) and incubated at 4 °C for indicated periods of time. In panels C and F, nonreducing SDS−PAGE analysis of conjugation dose-dependencies: varying amounts of Ad-C were mixed with LFn-C (C) or C-VEGF (F) and incubated at 4 °C for 2 h.

preparative C-4 column (GE Healthcare). The extent of modification was calculated as a ratio of integral peak intensities at 216 nm (for protein) and at 598 nm (for Cy5.5).

Cells. HEK293 human transformed embryonic kidney cells (CRL-1573) and RAW 264.7 mouse monocytes (TIB-71) were from American Type Culture Collection (Rockville, MD). 293/KDR cells expressing 2.5×10^6 VEGFR-2/cell have been developed in SibTech, Inc. (*22*). PAE/KDR porcine aortic endothelial cells expressing 2.5×10^5 VEGFR-2/cell were kindly provided by Dr. B. Terman (Albert Einstein School of Medicine, Bronx, NY). 4T1luc mouse breast carcinoma cells expressing firefly luciferase was developed as described (*23*). All cells were grown in DMEM with 10% FBS, 2 mM L-glutamine and antibiotics at 37 °C, 5% CO_2.

In Vivo Imaging. Female 5−6 weeks old BALB/c mice were from Charles River Laboratories (Wilmington, MA). 4T1luc cells were implanted subcutaneously in the backs (5−10,000 cells/mouse). For imaging, mice with 10−14 days old tumors received 5−7 μg of VEGF/Ad-Cy5.5 per mouse via the tail vein, 5 min later were injected intraperitoneally with aqueous luciferin (0.5 mg/mouse), and anesthetized 5 min later with ketamine/xylazine mixture as described (*7*). Images were obtained on Kodak Image Station 4000. The protocol for the animal studies was approved by the Institutional Animal Care and Use Committee at the University of Connecticut Health Center.

Insertion of Ad-C into Doxorubicin-Loaded Liposomes. Ad-C was modified with 2-fold molar excess Traut's reagent (Sigma) in a buffer containing 50 mM NaPi, 150 mM NaCl, 1 mM EDTA, pH 8.0 for 2 h. Ten-fold molar excess of mPEG-DSPE maleimide (FW 3400, Nectar Technologies) was added to the reaction mixture and incubation continued for 4 h. Equal volume of Doxil (pegylated liposomal doxorubicin, OrthoBiothech) was added to the reaction and incubated at 37 °C for 16 h followed by gel-filtration on Sephadex 4B as described (*20*). The efficiency of Ad-C/DSPE insertion into liposomes was analyzed by RP HPLC, as described elsewhere (*20*). C-Tagged proteins were mixed with Ad-C/Doxil at the C-tag to Ad-C molar ratio of 1:3 and incubated for 16 h at 4 °C followed by purification on Sepharose 4B. C-Tagged protein concentration in purified liposomes was determined by Western blotting.

RESULTS

Preparation Adapter Protein for "Dock and Lock". To obtain Ad-C, an adapter protein that binds to and forms a disulfide bond with C-tag, we engineered the V118C amino acid substitution in BH-RNase, a source of adapter protein (*19*). BH-RNase comprising a 1−29aa long fragment of bovine RNase A and a 30−127 aa long fragment of human RNase I carries a unique subtilisin cleavage site between S20 and A21, making it possible to release a 21−127aa fragment (adapter protein) after limited subtilisin digestion. Enzymatically active BH-RNase(V118C) was expressed, refolded in glutathione-containing Red-Ox buffer, and purified as described elsewhere (*20*). On the basis of our experience with another BH-RNase mutant containing the N88C amino acid substitution (*20*), we expected

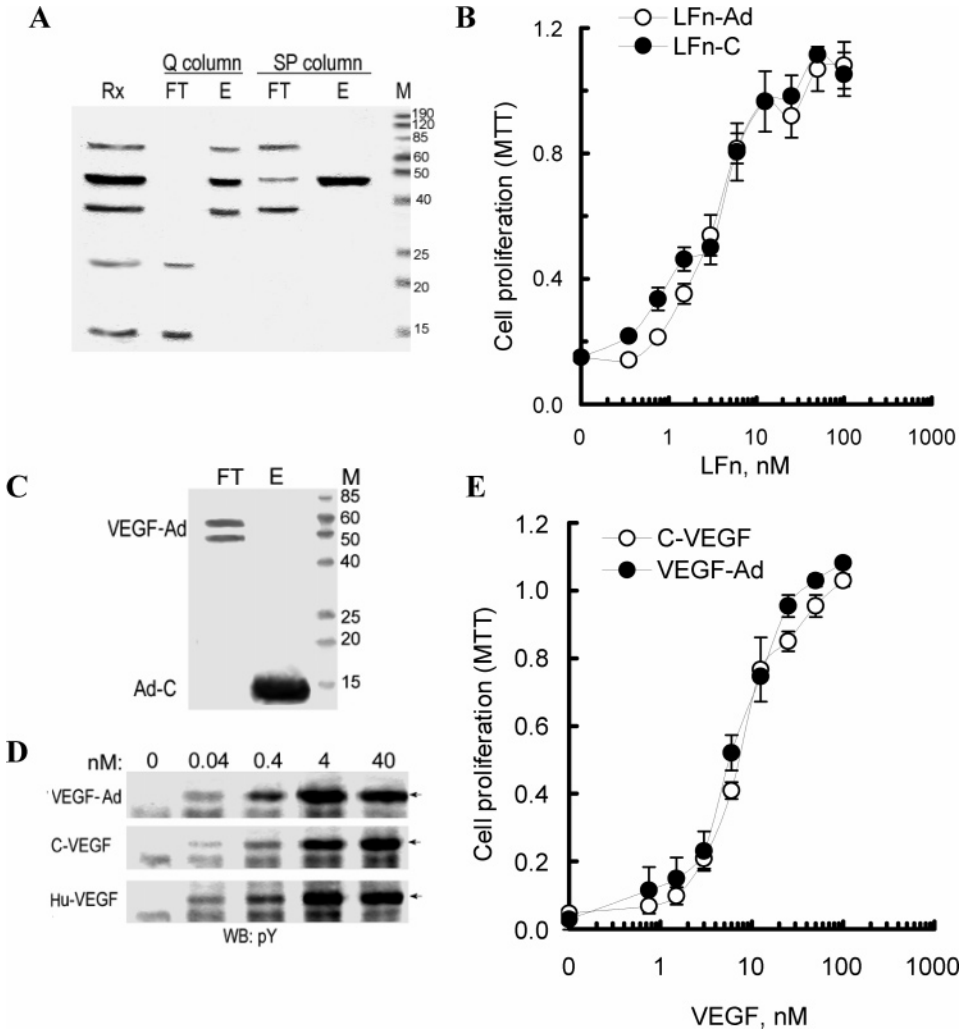

Figure 3. Conjugation to Ad-C does not affect functional activity of C-tagged proteins. In panel A, purification of LFn-Ad by ion-exchange chromatography on a Q-column followed by a SP-column. Rx, reaction mixture; FT, flow-through; E, elution. In panel B, competition of LFn-Ad with LF for binding to RAW cells in the presence of PA. Cells were plated on 96-well plates 15×10^3 cells/well 20 h before the experiment. Varying amounts of LFn-C or LFn-Ad were mixed with LF and PA (List Biological, Campbell, CA) in DMEM complete culture medium and added to cells in triplicate wells to final concentrations of 2 nM PA and 0.2 nM LF. After 2.5 h of incubation at 37 °C in 5% CO_2, viable cell numbers were determined by CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega). In panel C, purification of VEGF-Ad conjugate by affinity chromatography on Hu-peptide column. FT, flow-through fraction; E, elution. In panel D, induction of VEGFR-2 tyrosine autophosphorylation in 293/KDR cells. After overnight starvation (DMEM/0.5% FBS), cells were shifted to serum-free DMEM with 0.5 mM sodium vanadate for 20 min at 37 °C and then stimulated for 10 min at 37 °C, lysed, and analyzed by Western blotting using anti-phosphotyrosine RC20:HRPO conjugate (BD Transduction Labs, San Diego, CA). C-VEGF, C-tagged $VEGF_{121}$; Hu-VEGF, Hu-tagged $VEGF_{121}$. Arrows indicate positions of VEGFR-2. In panel E, competition with SLT-VEGF chimeric toxin. 293/KDR cells were plated on 96-well plates 20 h before the experiment, 1000 cells/well. Varying amounts of VEGF-Ad or C-tagged $VEGF_{121}$ were mixed with SLT-VEGF in complete culture medium, and added to cells in triplicate wells to a final SLT-VEGF concentration of 1 nM. Viable cells were quantitated 96 h later by a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI).

that unpaired C118 in BH-RNase(V118C) would be involved in a mixed disulfide with glutathione. Indeed, modification of BH-RNase(V118C) with a sulfhydryl-directed reagent *N*-(1-pyrene)maleimide varied between 0 and 0.3 mol/mol for different preparations. However, after a mild DTT treatment (1.5-fold molar excess DTT at 4 °C for 16 h), modification of BH-RNase(V118C) reached ~1 mol/mol, suggesting that a single thiol group per protein was "deprotected". BH-RNase-(V118C), with or without deprotection, was digested with subtilisin, and Ad-C was purified by affinity chromatography on Hu-peptide affinity column as described (*20*). The resulting protein was more than 98% pure by SDS−PAGE (Figure 2). Under nonreducing conditions, a part of Ad-C migrated as a dimer that was absent in the presence of DTT reflecting dimerization of Ad-C via unpaired C118 residues.

Self-Assembly of Conjugates between Ad-C and C-Tagged Proteins. We expected that the close proximity of C4 and C118 thiol groups in Ad-C/C-tag complex might lead to formation of a C4−C118 disulfide bond, even when one or both thiol groups were involved in mixed disulfides with glutathione. We first tested this hypothesis using Ad-C and LFn-C, a catalytically inactive fragment of anthrax lethal factor with C-terminal C-tag. LFn-C was bacterially expressed and purified from a soluble fraction through a process that did not require red-ox refolding, and therefore C4 thiol group was available for conjugation. LFn-C, Ad-C, and their 1:1 mixture were incubated at 4 °C for 16 h and then analyzed by SDS−PAGE. As expected, for both LFn-C and Ad-C incubated separately, part of each protein migrated in DTT-sensitive bands corresponding to homodimers of these proteins (Figure 2A). However, in the LFn-C/Ad-C mixture, the majority of protein was found in a DTT-sensitive band corresponding to ~46 kDa LFn-Ad conjugate (Figure 2A). Thus, this conjugation occurs even when the majority of C118 thiol groups in Ad-C were involved in mixed disulfide bonds with glutathione. Importantly, LFn-Ad conjugation was more efficient than homodimer formation, supporting the notion that

the process proceeded in the noncovalent complex between C-tag and Ad-C, rather than via random collisions. We found that conjugation was detectable after 30 s of incubation at 4 °C and reached a plateau by 20 min (Figure 2B). Interestingly, an increase of the Ad-C to LFn-C molar ratio did not enhance the yield of the conjugate (Figure 2C), suggesting that a fraction of LFn-C was proteolytically degraded and thus has a nonfunctional C-tag.

To test the ability of protected C4 thiol group to form a disulfide bond with Ad-C, we used recently described C-tagged human $VEGF_{121}$, a dimeric protein with C-tag fused to the N-terminus of each monomer (*7*). This protein was recovered from inclusion bodies and had both C4 thiol groups in the form of mixed disulfides due to refolding in a glutathione-containing red-ox buffer (*7*). C-Tagged $VEGF_{121}$, Ad-C, and their mixture at a molar ratio of 1:3 were incubated at 4 °C for 16 h and then analyzed by SDS−PAGE. As shown on Figure 2D, under nonreducing conditions, C-tagged $VEGF_{121}$ migrated in a DTT-sensitive band corresponding to a native dimer, while only a part of Ad-C (smaller in this Ad-C preparation, than in the one shown on Figure 2A) migrated as a dimer. However, in the protein mixture, virtually all C-tagged VEGF was found in DTT-sensitive bands corresponding to ~45 kDa and ~57 kDa VEGF-Ad conjugates with either one or two adapter molecules per VEGF dimer, respectively (Figure 2D). Thus, the presence of glutathione on both C4 and C118 did not prevent formation of VEGF-Ad conjugate.

Conjugation of C-tagged VEGF and Ad-C mixed at the molar ratio of 1:3 was detectable after a few minutes of incubation at 4 °C and reached a plateau by 2 h (Figure 2E). Free VEGF disappeared from the reaction mixture at 2.5-fold molar excess of Ad-C (Figure 2F), although complete VEGF modification, e.g. two Ad-C molecules per each VEGF dimer, was not achievable. As with LFn-Ad conjugate, we explain it by possible N-terminal proteolysis during protein purification. Deprotection of C4 and/or C118 by mild DTT treatment did not accelerate conjugate formation (data not shown), suggesting that freeing the thiol groups from the form of mixed disulfide was not a limiting step in this process.

Functional Activities of Adapter-Conjugated C-Tagged Proteins. LFn-Ad conjugate was purified from the reaction mixture by anion-exchange chromatography that removed unconjugated Ad-C, followed by cation-exchange chromatography to remove free LFn-C and its dimers (Figure 3A). Purified LFn-Ad was tested for its ability to rescue RAW 264.7 cells from cytotoxicity of anthrax lethal toxin, a combination of full-length lethal factor (LF) and protective antigen (PA). It was demonstrated previously that LFn completely retained the ability to bind to PA, while it displayed no toxicity for target RAW 264.7 cells (*24*). We found that in this assay, purified LFn-Ad conjugate competed with LF as effectively as free LFn-C, rescuing RAW 264.7 cells with IC_{50} of ~3 nM (Figure 3B), indicating that the conjugation did not affect the ability of LFn to interact with PA.

VEGF-Ad conjugate was purified from unreacted Ad-C by affinity chromatography on a Hu-peptide column that retained free Ad-C (Figure 3C). Purified VEGF-Ad conjugate was found to be as active as free $VEGF_{121}$ in a short-term 10-min assay, induction of VEGF receptor (VEGFR-2) tyrosine autophosphorylation (Figure 3D). In a long-term 3-day assay, VEGF-Ad was tested for the ability to rescue VEGFR-2-expressing cells from the cytotoxicity of SLT-VEGF chimeric toxin (*25*, *26*). In this assay VEGF-Ad was as effective as C-tagged $VEGF_{121}$, rescuing 293/KDR cells with the IC_{50} of ~8 nM (Figure 3E), which was also similar to previously reported IC_{50} value for Hu-$VEGF_{121}$ (*26*). Thus, both assays demonstrated that conjugation did not affect the ability of $VEGF_{121}$ to bind to VEGFR-2.

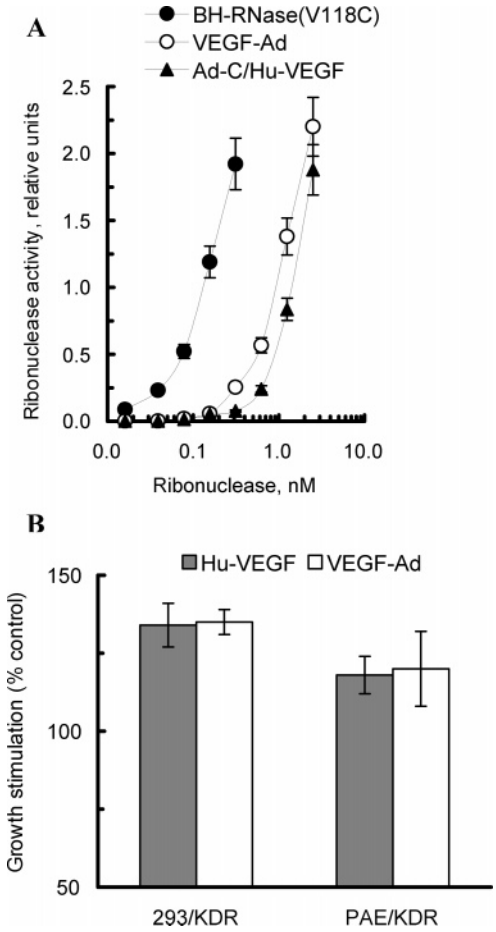

Figure 4. Reconstituted ribonuclease activity of VEGF-Ad conjugate is not cytotoxic. In panel A, BH-RNase(V118C), VEGF-Ad conjugate, or a equimolar mixture of Ad-C and Hu-VEGF was added, separately, to a buffer containing 20 mM Tris-HCl, 100 mM NaCl, pH 7.5, 0.1 mg/mL polyC and incubated for 5 min at RT. Ribonuclease activity was measured by 280-nm absorbance of the reaction mixtures cleared from TCA-precipitated material. One optical unit of TCA-soluble material released from polyC under these conditions was defined as one relative unit of ribonuclease activity (the method is described in details in 16). In panel B, VEGF induced growth stimulation of VEGFR-2 expressing cells. 293/KDR and PAE/KDR cells were plated on 96-well plates at densities of 500−1000 cell/well. Varying amounts of VEGF-Ad, or control Hu-VEGF, were added to cells in triplicate wells 20 h later. Cells were allowed to grow for 72−96 h and then quantitated by a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega).

Since ribonuclease activity is reconstituted in noncovalent complexes of Hu-tagged proteins with Ad (*16*), we tested whether introduction of C4 and C118 affected proper alignment of RNase fragments. We found that both VEGF-Ad and LF-Ad conjugates displayed similar ribonuclease activity (Figure 4A, for VEGF-Ad). Although VEGF-Ad activity was ~10-fold lower than that of BH-RNase(V118C), the conjugate was more active than noncovalent complexes between Ad-C and Hu-VEGF (Figure 4A). To address the issue of potential cytotoxicity of "dock and lock" complexes, we tested the effects of VEGF-Ad conjugate on cell growth. VEGFR-2-expressing cells of different tissue origin were plated at low densities and allowed to grow for 72−96 h in the presence of 10 nM VEGF-Ad conjugate. Both cell lines displayed low but significant growth stimulation in response to either VEGF-Ad or Hu-VEGF served as a control, indicating no toxic effects of VEGF-Ad (Figure 4B).

Self-Assembly of Conjugates between C-Tagged Proteins with Ad-C Decorated Liposomes. To assess self-assembly of "dock and lock" conjugates in the presence of bulky payloads,

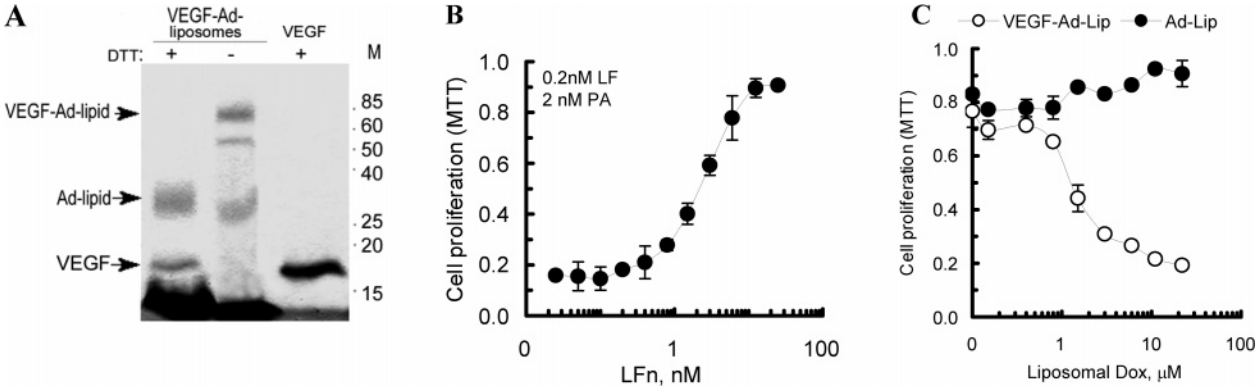

Figure 5. C-Tagged proteins conjugated to liposomes derivatized with Ad-C retain the functional activity. In panel A, VEGF-Ad-liposomes purified on Sepharose 4B were analyzed by SDS−PAGE as described in legend to Figure 2. In panel B, LF-Ad-liposome competition with the full length LF in the presence of PA on Raw cells was done as described in legend to Figure 4. In panel C, 293/KDR cells were plated on 96-well plates, 1000 cell/well. Twenty hours later, varying amounts of VEGF-Ad-liposomes or equivalent amounts of Ad-liposomes were added to cells in triplicate wells. After a 2-h incubation at 37 °C, liposome-containing media were removed, and cells were shifted to complete culture medium and allowed to grow for 96 h under normal culture conditions. Cells were quantitated by a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega).

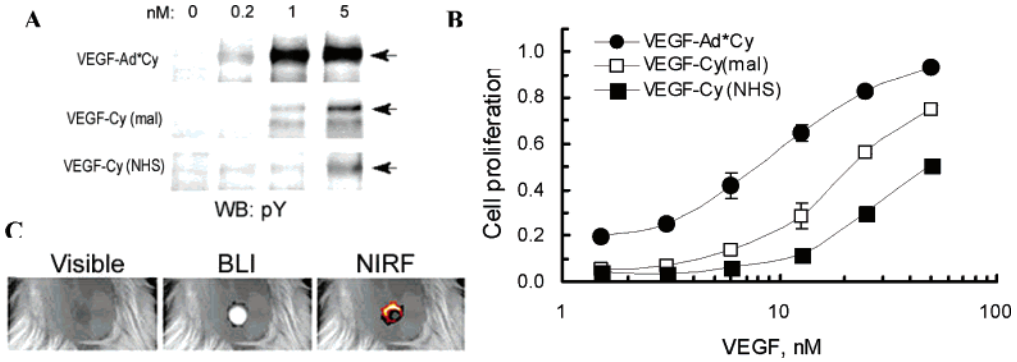

Figure 6. Functionally active Cy5.5-labeled VEGF-Ad* conjugate. VEGF modified with Cy5.5 directly using Cy5.5-maleimide (mal), or Cy5.5-NHS (NHS) or modified via adapter (VEGF-Ad*Cy) was purified and analyzed in VEGFR-2 tyrosine phosphorylation assay (panel A), or SLT-VEGF protection assay (panel B) as described in legend to Figure 3. In panel C, mice bearing 2-week old sc 4T1Luc tumors ($n = 5$) received ip injection of 0.5 mg luciferin and iv injection of 5 μg VEGF-Ad*Cy and were anesthetized and imaged. BLI, bioluminescent image; NIRF, pseudocolored in a "fire" range near-infrared image.

we used commercially available pegylated doxorubicin-loaded liposomes (Doxil). Ad-C with protected C118 was first modified with Trout's reagent to provide one free thiol group per Ad-C molecule and then modified with mPEG-DSPE maleimide, connecting a phospholipid moiety to Ad-C via 3.4 kDa poly-(ethylene glycol) linker. The lipidation mixture was then incubated with Doxil for 16 h at 37 °C and purified by gel-filtration on Sepharose 4B, as described elsewhere (*20*). Purified adapter-decorated liposomes were conjugated to either C-tagged $VEGF_{121}$ or LFn-C for 16 h at 4 °C and purified by gel filtration, and the resulting liposomes were analyzed by SDS−PAGE. We found that all liposome-associated C-tagged $VEGF_{121}$ and LFn were present in the DTT-sensitive bands corresponding to conjugates formed with lipidated adapter (Figure 5A, for C-tagged $VEGF_{121}$). Although a fraction of liposome-associated Ad-C remained free, the conjugation yielded ~25−50 molecules of C-tagged proteins per liposome and was sufficient for in vitro testing.

LFn-decorated liposomes were tested for their ability to protect RAW 264.7 cells from LF/PA toxicity and found to be as active as free LFn or LFn-Ad conjugate at the equal protein concentrations (Figure 5B, compare to Figure 3B). $VEGF_{121}$-decorated liposomes were tested for their ability to deliver cytotoxic doxorubicin into 293/KDR cells via receptor-mediated endocytosis. In these experiments, untargeted liposomes served as a control for nonspecific toxicity. We found that only $VEGF_{121}$-decorated liposomes were highly cytotoxic under selected conditions, indicating that the liposome payload did not prevent binding to VEGFR-2 and receptor-mediated endocytosis (Figure 5C).

Adapter for Covalent Self-Assembly and Site-Specific Labeling of Conjugates. To eliminate heterogeneity in Ad-C labeling, we have engineered Ad*-2C adapter by introducing the N88C substitution that provided a thiol group suitable for site-specific conjugation (*20*). We found that Ad*-2C can be recovered from the corresponding BH-RNase(N88C,V118C) mutant and self-assembled into functionally active conjugates with C-tagged $VEGF_{121}$ and LFn, as readily as Ad-C (data not shown). Ad*-2C was first deprotected by mild DTT treatment and then conjugated to C-tagged $VEGF_{121}$, and the conjugate was labeled with a near-infrared fluorescent dye Cy5.5 via maleimide chemistry to the molar ratio of one Cy5.5 per $VEGF_{121}$ monomer. The functional activities of Cy5.5-labeled VEGF-Ad* conjugate were close to that of C-tagged $VEGF_{121}$ in VEGFR-2 tyrosine phosphorylation assay (Figure 6A) and identical to that in protection from SLT-VEGF cytotoxicity (Figure 6B). In contrast, direct labeling of C-tagged $VEGF_{121}$ with either Cy5.5-maleimide or Cy5.5-NHS to the same ratio of one Cy5.5 per $VEGF_{121}$ monomer resulted in a significant decrease of functional activity, in both assays (Figure 6A,B). For in vivo testing, Cy5.5-labeled VEGF-Ad* conjugate was injected intravenously into mice bearing subcutaneous 4T1luc tumors. Since tumor cells were engineered to express luciferase, 4T1luc tumor could be visualized by bioluminescent imaging after intraperitoneal injection of luciferin (*23*). We found that Cy5.5-labeled VEGF-Ad* conjugate rapidly and selectively

accumulated in the tumor region visualized by bioluminescent imaging (Figure 6C). As expected, the strongest Cy5.5 fluorescence was observed at the growing edges of the tumor, which are enriched with VEGFR-2 overexpressing cells (Figure 6C).

DISCUSSION

We describe here self-assembled "dock and lock" conjugates between C-tagged targeting proteins and Ad-C adapter protein, which are based on a unique intermolecular disulfide bond. Since both C-tag and Ad-C are mutated fragments of human RNase I, reconstitution of ribonuclease activity in Ad-C/C-tag conjugate indicates their proper folding and alignment (*16*). Self-assembly proceeds remarkably rapidly, even when both complimentary cysteines are involved in mixed disulfides with glutathione, suggesting that C4−C118 bonding is facilitated by formation of Ad-C/C-tag complex.

The "dock and lock" system presents a flexible platform for linking payloads to targeting proteins. The human origin of both components makes it suitable for potential clinical development. Combined size of C-tag (15-aa) and adapter (105-aa) is significantly smaller than that of biotin/streptavidin complex and is comparable to that of the adapter arm of recombinant diabodies. Importantly, C-tag can be fused to either N- or C-terminus, making it compatible with various targeting proteins. The "dock and lock" self-assembly appears to be a robust process: two completely different C-tagged proteins readily formed functionally active conjugates with Ad-C even when it was associated with such a bulky payload such as a liposome.

On the basis of our previous work on site-specific modification of RNase I derived adapter (*20*), we further optimized "dock and lock" system by engineering an Ad*-2C adapter protein with the N88C substitution. Not only Ad*-2C allows for site-specific modification eliminating heterogeneity of randomly modified Ad-C, but it also positions a payload as far as possible from the tag, protecting C-tagged proteins from potential interactions with the payload (*20, 27*). By design, C88 and C118 are released from mixed disulfides first, and then deprotected Ad*-2C is conjugated to C-tagged protein, leaving the C88 thiol group available for site-specific modification. This procedure was used to prepare VEGF-Ad* conjugate carrying a near-infrared fluorescent dye Cy5.5 that appeared to be fully active in vitro and in vivo. In contrast, direct conjugation of Cy5.5 to thiol groups in deprotected C-tagged $VEGF_{121}$ results in significant loss of VEGF activity, most likely because of interactions between negatively charged Cy5.5 and VEGF lysine residues involved in receptor binding (*28*). These observations suggest that insertion of Ad*-2C between C-tagged $VEGF_{121}$ and Cy5.5 might prevent such interactions, allowing for full activity of the final conjugate. Indeed, we have recently described functional conjugates of C-tagged $VEGF_{121}$ with 5th generation PAMAM boronated dendrimers derivatized with Cy5.5, where dendrimer was functionally "inserted" between C-tagged $VEGF_{121}$ and Cy5.5 (*7*).

Although we describe here conjugates with adapter-carrying therapeutic or diagnostic payloads, a similar approach might be used for surfaces derivatized with functionally active proteins, which might be useful for various devices, tissue scaffolds, and biosensors. In this strategy, the surface is first derivatized with adapter protein, and then conjugates are formed with "working" C-tagged protein(s). Taken together, our results suggest that self-assembled "dock and lock" conjugates might provide a standardized and versatile approach to using recombinant proteins for biomedical purposes.

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