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Permalink
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Journal
Biomolecular Engineering, 21(6)

ISSN
1389-0344

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Publication Date
2005-02-01

Peer reviewed
A human biotin acceptor domain allows site-specific conjugation of an enzyme to an antibody-avidin fusion protein for targeted drug delivery

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Received 13 July 2004; received in revised form 21 October 2004; accepted 22 October 2004

Abstract

We have previously constructed an antibody-avidin (Av) fusion protein, anti-transferrin receptor (TfR) IgG3-Av, which can deliver biotinylated molecules to cells expressing the TfR. We now describe the use of the fusion protein for antibody-directed enzyme prodrug therapy (ADEPT). The 67 amino acid carboxyl-terminal domain (P67) of human propionyl-CoA carboxylase α subunit can be metabolically biotinylated at a fixed lysine residue. We genetically fused P67 to the carboxyl terminus of the yeast enzyme FCU1, a derivative of cytosine deaminase that can convert the non-toxic prodrug 5-fluorocytosine to the cytotoxic agent 5-fluorouracil. When produced in Escherichia coli cells overexpressing a biotin protein ligase, the FCU1-P67 fusion protein was efficiently mono-biotinylated. In the presence of 5-fluorocytosine, the biotinylated fusion protein conjugated to anti-rat TfR IgG3-Av efficiently killed rat Y3-Ag1.2.3 myeloma cells in vitro, while the same protein conjugated to an irrelevant (anti-dansyl) antibody fused to Av showed no cytotoxic effect. Efficient tumor cell killing was also observed when E. coli purine nucleoside phosphorylase was similarly targeted to the tumor cells in the presence of the prodrug 2-fluoro-2'-deoxyadenosine. These results suggest that when combined with P67-based biotinylation, anti-TfR IgG3-Av could serve as a universal delivery vector for targeted chemotherapy of cancer.

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Keywords: ADEPT; Anti-transferrin receptor-avidin fusion protein; FCU1; In vivo mono-biotinylation; Purine nucleoside phosphorylase

1. Introduction

A major limiting factor in cancer chemotherapy is the toxicity of antiproliferative drugs to normal tissues [1]. Attempts to circumvent this problem have led to the development of various tumor-targeting approaches [2]. One of the most promising of these is antibody-directed enzyme prodrug therapy (ADEPT) in which tumor-specific antibody is used to target an enzyme to the tumor [3–5]. ADEPT is a two-step system. First, an antibody/enzyme conjugate is administered intravenously and allowed to bind selectively to tumor antigens. After allowing sufficient time for unbound antibody/enzyme to be cleared from the circulation, a non-toxic prodrug is administered systemically. The prodrug is then cleaved by the tumor-localized enzyme to generate a potent cytotoxic drug. An advantage of ADEPT is that one enzyme can generate multiple molecules of active drug resulting in a high concentration of cytotoxic drug only in tumors, minimizing systemic toxicity. The activated low-molecular weight drug diffuses throughout the tumor mass, killing not only antigen-expressing cells but also neighboring antigen-negative tumor cells (bystander effect). The tumor cells killed as a result of ADEPT can also induce antitumor immunity [6]. Consequently, the antibody/enzyme conjugate does not have to bind to all tumor cells to elicit an effective response. This rational approach, therefore, can theoretically solve many of the problems observed with standard chemotherapy.
As currently practiced, one important limitation of ADEPT is the difficulty of producing large quantities of homogeneous, uniformly active antibody/enzyme conjugates [1–3]. Although a variety of antibodies and enzymes have been chemically linked to produce a range of conjugates, chemical treatments can be detrimental to the activity of the proteins and the resulting conjugates differ in the number and position of the crosslinks, leading to inconsistent targeting and catalytic activity [7]. This reduces the amount of toxic drug activated at tumors and limits the efficiency of ADEPT. As an alternative approach, recombinant DNA technology could be used to prepare homogeneous antibody–enzyme fusion proteins. However, for reasons we have yet to understand, production levels of such fusion proteins are often very low, limiting their use in clinical (or sometimes even animal) studies [7–9]. In addition, this strategy requires that a different fusion protein be produced for every antibody/enzyme combination, which is cumbersome, and there may be a decrease in activity of one or both covalently linked partners. It would, therefore, be desirable to develop a universal delivery system that eliminates the need to make a specific construct for each application.

We previously constructed antibody-avidin (Av) fusion proteins in which Av was genetically linked to IgG3 at the carboxyl terminus of the heavy chain speciﬁc for either the hapten dansyl or the rat transferrin receptor (TfR) (anti-dansyl IgG3-Av and anti-rat TfR IgG3-Av; see Fig. 1A) [10,11]. These fusion proteins are identical, except for their binding speciﬁcity and each molecule of the fusion proteins contains two Av moieties. Since Av forms a tetrameric structure, the IgG3-Av fusion proteins exist as a non-covalent dimer (Fig. 1B) [10,12]. We demonstrated that anti-TfR IgG3-Av can deliver chemically biotinylated enzymes, i.e., glucose oxidase and β-galactosidase, into rat tumor cells overexpressing the TfR [12]. This suggested that anti-TfR IgG3-Av could serve as a universal delivery vehicle for targeted chemotherapy of tumors overexpressing the TfR. However, the limitation remained that a protein usually contains several potential target residues for chemical biotinylation and thus following biotinylation the products differ in the number and location of the attached biotins [13]. The proteins carrying multiple biotins within a single molecule could form high-molecular weight aggregates when mixed with anti-TfR IgG3-Av. In addition, attaching biotin at certain positions in a protein may disrupt its conformation and/or biological function [14]. Therefore, to successfully use anti-TfR IgG3-Av as a universal vehicle for the delivery of biotinylated molecules for targeted cancer chemotherapy, it is essential to develop methods for site-specific mono-biotinylation of the enzymes.

Biotin protein ligase (BPL) is the enzyme responsible for attaching biotin to a specific lysine of biotin-dependent enzymes [15]. Biotinylation is a relatively rare event, with between one and five biotinylated protein species found in different organisms [16]. Thus, this post-translational modification is extraordinarily specific. In Escherichia coli, for example, only a single lysine residue of acetyl-CoA carboxylase is biotinylated by the organism’s BPL, BirA. Interestingly, the functional interaction between BPLs and biotin-dependent enzymes is highly conserved throughout evolution [15]. Biotinylation occurs even when BPL and biotin-dependent enzyme derive from such divergent species as bacteria and humans. The carboxyl-terminal domain of human propionyl-CoA carboxylase α subunit is naturally biotinylated at lysine-669 by human BPL [17]. Importantly, the same lysine residue is biotinylated in vivo when only the carboxyl-terminal domain (67 amino acids) is expressed in E. coli [17]. This indicates that BirA can biotinylate this human biotin acceptor domain (termed P67) and that P67 alone is sufﬁcient for recognition by BirA. Therefore, fusion of P67 to a protein will provide a site for efﬁcient attachment of a single biotin molecule.

In the present studies, we genetically fused P67 to the yeast enzyme FCU1 [18] and the E. coli enzyme purine nucleoside phosphorylase (PNP) [19]. FCU1 is a genetically engineered chimeric protein consisting of cytosine deaminase (CD) and uracil phosphoribosyltransferase (UPRT). CD can convert the relatively non-toxic prodrug 5-fluorocytosine (5-FC) to the highly cytotoxic agent 5-fluorouracil (5-FU), and UPRT in turn can use 5-FU to synthesize the toxic metabolite 5-fluorouridine 5'-monophosphate.

Fig. 1. Schematic diagrams of the antibody-Av fusion proteins used in this study and FCU1-P67. Diagrams are not drawn to scale. (A) The structure of anti-rat TfR IgG3-Av and anti-dansyl IgG3-Av. Each oval represents a single domain of the heavy (dark gray) and light (light gray) chains of the antibody. Avidin (black circle) is fused to the carboxyl terminus of each heavy chain. (B) The dimeric structure of anti-rat TfR IgG3-Av and anti-dansyl IgG3-Av. (C) The structure of FCU1-P67. An S-tag (white square) is fused to the amino terminus of cytosine deaminase (CD, dark gray circle) and a P67 domain (black oval) is attached, via a linker (wavy line), to the carboxyl terminus of uracil phosphoribosyltransferase (UPRT, light gray circle). (D) A model of the antibody/FCU1 complex that is presumed to form when an antibody-Av fusion protein and FCU1-P67-b are mixed at a molar ratio of 1:2.
sphate (5-FUMP). PNP cleaves the relatively non-toxic prodrug 2-fluoro-2-deoxyadenosine (F-dAdo) to produce the highly cytotoxic drug 2-fluoroadenosine (F-Ade) [20]. In gene therapy models, FCU1 and PNP are more effective in converting prodrugs into cytotoxic agents to kill tumor cells than the commonly used CD and herpes simplex virus type-1 thymidine kinase [18,21]. We have now found that FCU1-P67 produced in E. coli cells overexpressing BirA is metabolically mono-biotinylated at the defined site in the P67 domain with high efficiency. We also found that biotinylated FCU1-P67 conjugated to anti-rat TIR IgG3-Av can efficiently kill rat Y3-Agi1.2.3 myeloma cells [22] in vitro only in the presence of 5-FC. A similar cytotoxic effect was observed when PNP was produced as a P67 fusion protein and the biotinylated PNP-P67 was targeted by anti-rat TIR IgG3-Av to the tumor cells in the presence of F-dAdo. These results suggest that when combined with the P67-based site-specific conjugation technique, anti-TIR IgG3-Av can serve in vivo as a universal vector for targeted chemotherapy of cancer.

2. Materials and methods

2.1. Plasmid construction

The FCU1 gene [18] was constructed using nine overlapping primers with the sequence optimized for mammalian expression vectors. The resulting plasmid was digested with NcoI and SalI, and inserted into the pET9817, replacing the birA gene was inserted into the T7 promoter-based E. coli expression vector pET16b-BS between the BamHI and SalI sites, generating pET9816. [pET16b-BS was constructed by modifying the multiple cloning site of pET16b (Novagen). It has a short DNA fragment carrying the BamHI and SalI sites within the multiple cloning site immediately downstream from the NdeI site and does not contain the original BamHI site.]

A DNA fragment carrying the birA gene was amplified from E. coli genomic DNA using the following PCR primers: 5'-GAGAGGTACCATGGTGA-GGCCAGAATCTATGAAGGATAACACCGTGCCACTG-3' and 5'-CCAAAGGCCT-CTCTGCGGCCGAACAGGC-3'. The amplified fragment was digested with KpnI and EcoRI, and inserted into the multiple cloning site of the T7 promoter-based E. coli expression vector pET29c (+) (Novagen, Madison, WI). This resulted in the introduction of a short DNA sequence encoding the 15 amino acid affinity tag (S-tag) at the 5' end of the FCU1 gene. The plasmid expressing the S-tagged FCU1 protein (denoted just as FCU1) was named pET9811.

A DNA fragment carrying the S67 sequence was amplified by RT-PCR using the polyA RNA isolated from 293T cells using the following primers: 5'-CGTGGATCCCTCGTTCCTCCGATGCCCGGAG-3' and 5'-CTCTGCGGCCGCCTTCCCCAGCTCCCAGCAGCGG-3'. The fragment was digested with BamHI and EcoRI, and inserted into pUC3541 immediately downstream from the flexible (Gly-x-Ser) linker sequence [23]. [pUC3541 is a derivative of pUC19 carrying the linker sequence (an StuI-BamHI fragment) within the multiple cloning site.] The resulting plasmid was digested with StuI and EcoRI to obtain a DNA fragment containing the linker-P67 fusion sequence. The fragment was then inserted into pET9811 immediately downstream from the FCU1 gene.

The plasmid expressing S-tag-FCU1-linker-P67 (called FCU1-P67) was named pET9817.

The K669R mutation was introduced into the P67 gene using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with two complementary oligonucleotides where the sequence of the sense strand is: 5'-GGTGTAGTTAGCCATGGCTATGAGATAGTTACAGC-3'.

A DNA fragment carrying the deoD gene encoding PNP was amplified from E. coli genomic DNA using the following PCR primers: 5'-GAGAGGTACCATGGTGA-GGCCAGAATCTATGAAGGATAACACCGTGCCACTG-3' and 5'-CCAAAGGCCT-CTCTGCGGCCGAACAGGC-3'. The amplified fragment was digested with NcoI and SalI, and inserted into pET9817, replacing the FCU1 gene. The plasmid expressing S-tag-PNP-linker-P67 (called PNP-P67) was named pET9821.

A DNA fragment carrying the birA gene was amplified from E. coli genomic DNA using the following PCR primers: 5'-GCGGATATCTATGGTGA-GGCCAGAATCTATGAAGGATAACACCGTGCCACTG-3' and 5'-CCAAAGGCCT-CTCTGCGGCCGAACAGGC-3'. The amplified fragment was digested with BglII and SacII, and inserted into the multiple cloning site of pDisplay (Invitrogen, Carlsbad, CA). The resulting plasmid was digested with BglII and SalI, and the fragment containing the birA gene was inserted into the T7 promoter-based E. coli expression vector pET16b-BS between the BamHI and SalI sites, generating pET9816. [pET16b-BS was constructed by modifying the multiple cloning site of pET16b (Novagen). It has a short DNA fragment carrying the BamHI and SalI sites within the multiple cloning site immediately downstream from the NdeI site and does not contain the original BamHI site.]

Restriction enzymes and primers were purchased from New England Biolabs (Beverly, MA) and Invitrogen (Carlsbad, CA), respectively.

2.2. Expression and purification of fusion proteins

Anti-dansyl IgG3-Av and anti-rat TIR IgG3-Av were expressed in mouse myeloma cells and affinity-purified from culture supernatants as described previously [10–12]. To produce enzymes, the E. coli strain BL21(DE3) (Novagen) transformed with pET9811, pET9817, or pET9821 was inoculated into LB broth containing 50 μg/ml kanamycin to give an initial optical density of 0.05 OD 600. Ampicillin (100 μg/ml) and biotin (10 μg/ml) were also added when the cells were cotransformed with pACYC9834. Cultures were grown at 37 °C to an OD 600 of 0.9, at which time isopropyl-β-D-thiogalactopyranoside was added to a concentration of 1 mM and the growth temperature was lowered to 30 °C. Four hours later, cells were harvested by centrifugation and stored at −20 °C. Frozen cells were thawed at 37 °C, resuspended in 1/35 of the original culture volume of ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5% glycerol, and 0.1 mM phenylmethylsul-
fonyl fluoride) containing 1 mM DTT and 5 mM EDTA, and incubated on ice for 10 min with lysozyme (1 mg/ml). The cells were further incubated on ice with 0.1% Triton X-100 for 10 min, followed by a 15-min incubation with deoxyribonuclease I (50 μg/ml), ribonuclease A (50 μg/ml), and MgCl₂ (10 mM). The lysate was clarified by centrifugation, added to 0.5–1 ml 50% agarose bead slurry carrying immobilized S-protein (Novagen), and incubated overnight at 4 °C with gentle agitation to capture S-tagged proteins. The beads were then sedimented by a brief centrifugation and washed thoroughly with lysis buffer containing 1 mM DTT until the A₂₈₀ reached <0.01. To elute captured proteins, washed beads were resuspended in 1.5× resin volumes of lysis buffer containing 10 mM DTT and 3 M MgCl₂ and incubated at 4 °C for 30 min with gentle agitation. The beads were then sedimented by a brief centrifugation and the supernatant was saved. The elution process was repeated two more times and the pooled supernatants were desalted by ultrafiltration against phosphate-buffered saline (PBS, pH 7.2) containing 10 mM DTT (and 10 μM ZnCl₂ for FCU1 and FCU1-P67) until the magnesium concentration reached <100 mM. The sample was then desalted against PBS until the magnesium concentration reached <5 mM and concentrated. The concentration and purity of the purified proteins were determined immediately and the proteins were used for various applications without delay. The yields were ~1.5 mg FCU1 or FCU1-P67 per liter and ~30 mg PNP-P67 per liter. The coexpression of BirA did not significantly affect the expression levels of FCU1-P67 and PNP-P67. The purified proteins had the expected sizes (FCU1, 47.0 kDa; FCU1-P67, 52.4 kDa; PNP-P67, 36.4 kDa) and were >98% pure as assessed by Coomassie blue-stained sodium dodecyl sulphate polyacrylamide gel (data not shown).

2.3. Enzyme assays

CD and UPRT activities were determined as described by Erbs et al. [24] and Jensen et al. [25], respectively. PNP activity was determined using EnzChek Phosphate Assay Kit (Molecular Probes, Eugene, OR), as instructed by the manufacturer.

2.4. Determination of the efficiency of in vivo biotinylation

An aliquot of purified protein (~5 μg) was added to 100 μl of 4% agarose bead slurry in PBS carrying immobilized monomeric avidin (Sigma). After incubation on ice for 4 h with occasional gentle agitation, the beads were sedimented by a brief centrifugation, washed four times with 1 ml PBS, and resuspended in 200 μl of PBS. An aliquot of the suspension was used to determine the precipitated CD activity and the result was compared with the CD activity of the equivalent amount of the original (unprecipitated) protein. For a control experiment, the beads were incubated overnight at 4 °C with 100 μl of biotin solution (1 mg/ml), washed with PBS, and mixed with a purified protein.

2.5. Molecular mass estimation by gel filtration chromatography

Chromatography of affinity purified proteins and their complexes was performed at 4 °C on a Superose 6 10/300 GL column (Amersham Biosciences, Piscataway, NJ) at a flow rate of 0.25 ml/min with PBS containing 0.05% sodium azide. The total amount of protein applied to the column was ~50 μg and the sample volume was ~200 μl. Proteins and complexes were detected by absorbance at 280 nm. The column was calibrated with aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) (Amersham Biosciences). The partition coefficients of these standard proteins were plotted against the logarithm of the corresponding molecular mass as described [26,27] and the molecular masses of the proteins and complexes used in this study were calculated from the standard curve.

2.6. Affinity pull-down analysis of antibody/FCU1 complexes

FCU1-P67-b (5 μg) was mixed with 9.5 μg of anti-dansyl (5-dimethylamino naphthalene-1-sulfonyl chloride) IgG3-Av [10] or anti-rat TIR IgG3-Av and incubated overnight at 4 °C. The mixture was added to 50 μl of dansylated BSA coupled to Sepharose beads [28] that had been pre-equilibrated with PBS and incubated at 4 °C for 4 h with occasional gentle agitation. The beads were sedimented by a brief centrifugation, washed four times with 1 ml PBS, and resuspended in 300 μl of PBS. Aliquots of the suspension were used to determine CD and UPRT activities and the results were compared with the CD and UPRT activities of the equivalent amount of the original (untreated) protein. For a control experiment, anti-dansyl IgG3-Av was incubated overnight at 4 °C with 10 μl of biotin solution (1 mg/ml) and mixed with FCU1-P67-b.

The amount of FCU1-P67 in the precipitated complex was determined using FRETWorks S-tag Assay Kit (Novagen). S-tag is a 15 amino acid peptide carrying the amino-terminal sequence of bovine RNase A that binds, with high affinity (K_D ~ 10⁻⁹ M), to S-protein that consists of residues 21–124 of the same RNase [29]. The reconstituted S-tag/S-protein possesses RNase activity. Since FCU1-P67 contains an S-tag at its amino terminus, the amount of the protein can be quantitatively determined by mixing the protein with an excess amount of S-protein and measuring the reconstituted RNase activity. We mixed an aliquot of the above Sepharose bead suspension containing the antibody/FCU1 complexes with an excess amount of S-protein and measured the reconstituted RNase activity using the ArUA substrate that consists of a short, mixed ribo/deoxyribo oligonucleotide having a fluorophore.
on the 5’ end and a quencher on the 3’ end. Fluorescence from the cleaved substrates was detected by Synergy HT (BIO-TEK Instruments, Winooski, VT).

2.7. In vitro cytotoxicity assay

FCU1-P67-b (1.1 μg) was mixed with 2 μg of anti-rat TIR IgG3-Av or anti-dansyl IgG3-Av and incubated overnight at 4 °C. An aliquot of the mixture was used to determine the efficiency of complex formation as described above. Based on this information, the concentration of each conjugate was calculated. The rest of the mixture was used for a serial dilution with Iscove’s Modified Dulbecco’s Medium (IMDM; Irvine Scientific Inc., Irvine, CA) supplemented with 20% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO). The diluted conjugates (50 μl each) were mixed with Y3-Ag1.2.3 cells (1.6 × 10^5 cells in 50 μl IMDM supplemented with 20% FBS) and incubated at 37 °C for 90 min. The cells were washed six times with 3 ml of IMDM supplemented with 20% FBS to remove unbound conjugates, resuspended in IMDM supplemented with 10% FBS, and plated into microtiter plates (5 × 10^5 cells/well).

5-FC (Sigma) dissolved in IMDM supplemented with 10% FBS was then added at varying concentrations and after 3 days cell viability was determined by the MTS assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI).

To remove surface-bound conjugates, Y3-Ag 1.2.3 cells incubated at 37 °C for 90 min with anti-rat TIR IgG3-Av/FCU1-P67-b (1 nM) were treated with a mixture of proteinase K and chymotrypsin (50 μg/ml each in IMDM supplemented with 10% FBS) as described previously [12]. The cells were then washed twice with 3 ml of IMDM supplemented with 20% FBS and processed as described above.

The in vitro cytotoxicity assay with PNP-P67 was carried out as described for FCU1-P67 except that PNP-P67-b (30 μg) was mixed with 41 μg of anti-rat TIR IgG3-Av or anti-dansyl IgG3-Av to form complexes. The produg F-dAdo was purchased from Berry & Associates, Inc. (Dexter, MI).

3. Results

3.1. Enzymatic activities of FCU1 fusion proteins

FCU1 was expressed in E. coli cells as a fusion protein in which a small affinity tag (S-tag) is connected to the amino terminus. The purified fusion protein (hereafter denoted just as FCU1) possessed both CD and UPRT activities (Fig. 2, lane 1 of each panel). The enzyme activities were not significantly affected (Fig. 2, lane 2 of each panel) when P67 was connected to the carboxyl terminus of FCU1 via a flexible linker to produce FCU1-P67 (Fig. 1C). To efficiently mono-biotinylate P67 in vivo (see below), BirA was coexpressed with FCU1-P67. FCU1-P67 purified from E. coli cells overexpressing BirA (called FCU1-P67-b) exhibited CD and UPRT activities similar to those of FCU1 and FCU1-P67 (Fig. 2, lane 3 of each panel).

3.2. Efficiency of in vivo mono-biotinvlation

To estimate the efficiency of in vivo biotinylation, FCU1-P67-b was incubated with an excess of avidin immobilized on agarose beads and the CD activity precipitated by the beads determined. Precipitation of biotinylated proteins with avidin (or streptavidin) immobilized on a solid support is very efficient and has been successfully used to estimate the efficiency of in vivo biotinylation [30,31]. For a control, FCU1 was treated similarly. As shown in Fig. 3, CD activity was not coprecipitated when FCU1 was incubated with the beads (lane 1). In contrast, when FCU1-P67-b was mixed with the beads, >95% of the original CD activity was

Fig. 2. The CD and UPRT activities of FCU1 and FCU1-P67. CD activity was determined by measuring the conversion of cytosine to uracil [24] and expressed as micromoles of cytosine deaminated/min/mg of protein. UPRT activity was determined by measuring the synthesis of uridine 5’-monophosphate from uracil and 5-phosphoribosyl-1-pyrophosphate [25] and expressed as micromoles of uracil phosphorylated/min/mg of protein. Each bar represents the average value (± standard deviation (S.D.)) of three independent assays: 1, FCU1; 2, FCU1-P67; 3, FCU1-P67-b.

Fig. 3. The efficiency of in vivo biotinylation of FCU1-P67. The CD activity of each protein precipitated by monomeric avidin immobilized on agarose beads was determined as described in Section 2. Each bar represents the average CD activity (±S.D.) obtained from three independent assays expressed as the percent of the control value. (Controls are the CD activity of each protein measured without precipitation.) 1, FCU1; 2, FCU1-P67-b; 3, FCU1-P67-b mixed with the avidin-agarose beads preincubated with an excess amount of biotin; 4, FCU1-P67(K669R) purified from E. coli cells overexpressing BirA; 5, FCU1-P67 purified from E. coli cells that do not overexpress BirA.
recovered (lane 2). Coprecipitation of the enzyme activity was almost completely eliminated when the beads were preincubated with an excess amount of biotin before being mixed with FCU1-P67-b (lane 3). Similarly, the enzyme activity was not precipitated when a mutant FCU1-P67 protein [FCU1-P67(K669R)], in which the lysine-669 residue (the single biotinylation site) of P67 was replaced with arginine [17], was purified from E. coli cells overexpressing BirA and mixed with the beads (lane 4). Interestingly, when the same experiment was carried out with FCU1-P67 purified from E. coli cells that do not overexpress BirA, ~50% of the original CD activity was coprecipitated with the beads (lane 5). This suggests that the endogenous BirA proteins expressed by E. coli were sufficient to biotinylate ~50% of the overproduced FCU1-P67 proteins. Taken together, these results strongly suggest that FCU1-P67 is uniformly mono-biotinylated at the lysine-669 residue of P67 and that the efficiency of in vivo biotinylation is >95% in E. coli cells overexpressing BirA.

3.3. Formation of antibody/FCU1 complexes

To determine whether biotinylated FCU1-P67 can form a complex with an antibody-Av fusion protein, we mixed anti-dansyl IgG3-Av and FCU1-P67-b at a molar ratio of 1:2 and pulled down the antibody using dansyl-BSA immobilized on Sepharose beads. The efficiency of complex formation was then determined by measuring the coprecipitated CD and UPRT activities. This assay protocol mimics the molecular events that occur during ADEPT and thus would indicate the efficiency of antibody-dependent enzyme delivery. As shown in Fig. 4A, ~80% of the original CD and UPRT activities were coprecipitated with anti-dansyl IgG3-Av. Coprecipitation of the enzyme activities was barely detectable when the same experiment was carried out with anti-rat TfR IgG3-Av instead of anti-dansyl IgG3-Av, or when anti-dansyl IgG3-Av was preincubated with an excess amount of biotin before being mixed with FCU1-P67-b. These results suggest that biotinylated P67 can bind to the Av moiety of anti-dansyl IgG3-Av. Importantly, the results also indicate that antibody-conjugated FCU1 is enzymatically active. To examine whether the formation of the antibody/FCU1 complex affects the catalytic activity of the enzyme, we determined the amount of FCU1-P67 in the precipitated complexes using the commercially available S-tag assay system (see Materials and methods). The result also indicated that ~80% of FCU1-P67-b mixed with anti-dansyl IgG3-Av was present in the complexes (data not shown). This implies that FCU1-P67 conjugated to anti-dansyl IgG3-Av does not significantly lose its enzymatic activities. When anti-rat TfR IgG3-Av was mixed with FCU1-P67-b and immuno-precipitated with anti-light chain antibodies, CD and UPRT activities were also coprecipitated with similar efficiency (data not shown). Addition of excess FCU1-P67-b did not significantly increase the amount of activity precipitated (Fig. 4B). Since >95% of FCU1-P67 is biotinylated (Fig. 3), the result may indicate either that ~20% of the anti-dansyl IgG3-Av molecules are unable to form the Av/biotin complex possibly because biotin from the growth medium had bound to the avidin or that less antibody was present than originally estimated. It is also possible that the efficiency of this affinity pull-down assay was not as high as that of the experiment described in Fig. 3.

To assess the antigen-binding ability of anti-dansyl IgG3-Av conjugated to FCU1-P67, ELISA assays were performed as described in Fig. 4C. The result indicates that the antigen
binding ability of anti-dansyl IgG3-Av incubated with FCU1-P67-b is similar to that of the same antibody-Av fusion protein incubated with PBS. Since ~80% of the anti-dansyl IgG3-Av molecules incubated with FCU1-P67-b form the antibody/enzyme complex, the result suggests that the binding of FCU1-P67 to the carboxyl terminus of anti-dansyl IgG3-Av through the Av-biotin interaction does not significantly affect the antigen-binding ability of anti-dansyl IgG3-Av.

We then used gel filtration chromatography to determine the molecular form of the complex of biotinylated FCU1-P67 with an antibody-Av fusion protein. Anti-dansyl IgG3-Av, which has a monomer molecular mass of 200 kDa [10], eluted in a peak corresponding to a molecular mass of ~400 kDa (Fig. 5A), confirming our previous observations that both anti-dansyl and anti-rat TIR antibody-Av fusion proteins exist as a non-covalent dimer [10,12] (see Fig. 1B). The higher order structure of FCU1 has not been elucidated. However, under the conditions used in this gel filtration, FCU1-P67-b eluted in a peak corresponding to a molecular mass of ~200 kDa (Fig. 5B). Since the molecular mass of the FCU1-P67 monomer is 52 kDa, the result suggests that FCU1-P67-b exists as a tetramer. Based on these observations, we generated antibody/FCU1 complexes by mixing anti-dansyl IgG3-Av and FCU1-P67-b at a molar ratio of 1:2. Since there are two avidin moieties on each IgG3-Av monomer, the molar ratio between Av and P67 would be 1:1.

We hypothesized that four FCU1-P67-b monomers would bind to the four Av molecules present at the carboxyl terminus of the anti-dansyl IgG3-Av dimer, generating an antibody/FCU1 complex with a molecular mass of ~600 kDa (see Fig. 1D). Consistent with this hypothesis, when the mixture of anti-dansyl IgG3-Av and FCU1-P67-b was analyzed by gel filtration chromatography, the majority of the proteins eluted in a peak corresponding to a molecular mass of ~600 kDa (Fig. 5C).

**3.4. In vitro cytotoxicity of FCU1-P67 conjugated to anti-rat TIR IgG3-Av**

Anti-rat TIR IgG3-Av is identical to anti-dansyl IgG3-Av except for its binding specificity [10–12]. Therefore, anti-rat TIR IgG3-Av and FCU1-P67 are expected to form a functionally active complex that is similar to the anti-dansyl IgG3-Av/FCU1-P67 complex described above. To confirm this, the antigen binding activity of anti-rat TIR IgG3-Av conjugated to FCU1-P67-b and the effects of the conjugate on the cytotoxicity of 5-FC were assessed in vitro using rat Y3-Ag 1.2.3 myeloma cells overexpressing the TIR. Based on the observations described above, anti-rat TIR IgG3-Av and FCU1-P67-b were mixed at a molar ratio of 1:2. Fig. 6A shows that in the absence of the conjugate, 5-FC was cytotoxic to Y3-Ag 1.2.3 cells only at high concentrations, with an IC50 of ~1.5 mM. The conjugate showed a dose-dependent cytotoxic effect and when the cells were treated with 3.3 nM of the conjugate for 90 min and then extensively washed, the cytotoxicity of 5-FC was increased by ~50-fold (IC50 ~0.03 mM). We previously showed that ~50% of Y3-Ag 1.2.3 cells were killed by 3.3 nM of anti-rat TIR IgG3-Av in the absence of 5-FC when the cells were incubated with the fusion protein continuously for 48 h [12]. However, no cytotoxic effect was observed in this study when the cells were incubated, in the absence of 5-FC, with 3.3 nM of anti-rat TIR IgG3-Av or the anti-rat TIR IgG3-Av/FCU1-P67-b conjugate for only 90 min and then extensively washed (data not shown). In addition, 5-FC exhibited the same cytotoxicity to Y3-Ag 1.2.3 cells treated for 90 min with 3.3 nM of anti-rat TIR IgG3-Av alone as was seen with untreated cells (IC50 ~1.5 mM; data not shown). Furthermore, there was no cytotoxic effect in the presence of 5-FC when cells were treated with 3.3 nM of the anti-dansyl IgG3-Av/FCU1-P67-b conjugate (Fig. 6A). These results indicate that anti-rat TIR IgG3-Av retains its antigen-binding activity after being complexed with FCU1-P67-b and can specifically deliver FCU1 to the tumor cells where the enzyme generates the cytotoxic agent from the prodrug 5-FC.

To determine whether surface-associated or internalized FCU1 was responsible for the cytotoxic effect of anti-rat TIR IgG3-Av/FCU1-P67-b, Y3-Ag 1.2.3 cells incubated with the conjugate were treated with proteases to remove surface-bound conjugates [12] and then mixed with 5-FC. For a control, half of the cells incubated with the conjugate were treated only with the medium used to dissolve the proteases. Consistent with the results shown in Fig. 6A, ~80% of the cells treated with medium alone were killed in the presence of 5-FC (Fig. 6B). In contrast, cell death was almost completely eliminated when the cells were treated with proteases. Therefore, the cytotoxic effect observed with...
protein. When produced in outside of the cell. To examine whether the P67-based 
Thus, PNP can exhibit its cytotoxic effect both inside and 
and biotinylated PNP-P67 (PNP-P67-b) was enzymatically 
8
substrate of PNP, F-dAdo and the cleavage product F-Ade 
differences in the IC50 values obtained by these data are statistically 
* value is the average of three assays. The largest S.D. was 
treated with the medium alone and incubated in the absence of 5-FC.) Each 
activity (\(\text{Control cells were treated only with the} \)) 
37
was caused mostly by surface-bound conjugates. 
anti-rat TfR IgG3-Av/FCU1-P67-b (1 nM) were 
C for 90 min with anti-rat TfR IgG3-Av/FCU1-P67-b (1 nM) were 
fi
antibody/PNP complexes generated at this molar ratio were 
7.5%. The differences in the IC50 values obtained by these data are statistically signi 
Student’s t-test, \(p < 0.05\). (B) Y3-Ag1.2.3 cells incubated at 
37 °C for 90 min with anti-rat TfR IgG3-Av/FCU1-P67-b (1 nM) were 
processed as described above. Each bar represents the average enzymatic 
activity (±S.D.) obtained from three independent assays expressed as the % of the activity of control cells. (Control cells were treated only with the 
medium and incubated in the absence of 5-FC.).

3.5. In vitro cytotoxicity of PNP-P67 conjugated to anti-rat TfR IgG3-Av

E. coli PNP is a homohexameric enzyme with each monomer having a molecular mass of 26.0 kDa [19]. The 
substrate of PNP, F-dAdo and the cleavage product F-Ade 
are both freely diffusible across cell membranes [20,32]. 
Thus, PNP can exhibit its cytotoxic effect both inside and 
outside of the cell. To examine whether the P67-based 
biotinylation can be used for anti-TfR IgG3-Av to deliver a 
different enzyme, PNP was expressed as a P67 fusion 
protein. When produced in E. coli cells overexpressing 
BirA, PNP-P67 was biotinylated as efficiently as FCU1-P67 
and biotinylated PNP-P67 (PNP-P67-b) was enzymatically 
active (data not shown). To determine whether PNP-P67-b 
can also form a hexameric structure, purified PNP-P67-b 
was applied to a gel filtration column. The result showed that most of the protein eluted in a peak corresponding to a 
molecular mass of \(~200 \text{ kDa} \) (Fig. 5D). Since the molecular 
mass of the PNP-P67 monomer is 36 kDa, the result suggests that PNP-P67-b exists mainly as a hexamer.

To estimate the antibody: enzyme molar ratio that can 
deliver the highest number of PNP to tumor cells, anti-dansyl 
IgG3-Av and PNP-P67-b were mixed at varying molar ratios 
and the efficiency of coprecipitation was determined as 
described in Fig. 4B. The result indicated that when anti-
dansyl IgG3-Av and PNP-P67-b were mixed at a molar ratio of 
1:4, 70–80% of the original PNP activity was coprecipitated 
with the antibody and that the use of larger amounts of PNP-
P67-b did not significantly increase the amount of enzyme 
associated with anti-dansyl IgG3-Av (data not shown). The 
antibody/PNP complexes generated at this molar ratio were 
then analyzed by gel filtration chromatography. As shown in 
Fig. 5E, most of the applied proteins eluted in a peak 
corresponding to a molecular mass of \(~600 \text{ kDa} \). The two 
minor peaks corresponding to molecular masses of \(~400 \) and 
\(~200 \text{ kDa} \) are likely to represent unconjugated anti-dansyl 
IgG3-Av dimers and PNP-P67-b hexamers, respectively. 
Although a slight shoulder on the left side of the major peak 
may indicate the existence of a small amount of high-
molecular weight complexes or aggregates, the chromatogram 
suggests that under the conditions used for this gel filtration, 
the majority of the proteins form a uniform complex in which 
one hexamer of PNP-P67-b is bound by one dimer of anti-
dansyl IgG3-Av.

Based on these observations, anti-rat TfR IgG3-Av/ 
PNP-P67-b and anti-dansyl IgG3-Av/PNP-P67-b complexes 
generated at a molar ratio of 1:4 were examined for their 
ability to specifically target enzyme to cells and generate 
cytotoxicity in vitro by prodrg conversion as described 
above. Y3-Ag1.2.3 cells are more susceptible to F-dAdo 
than 5-FC (IC50 \(~10 \mu \text{M} \), Fig. 7). Anti-rat TfR IgG3-Av/
PNP-P67-b showed a dose-dependent cytotoxic effect and when the cells were treated with 780 nM of the conjugate for 90 min, the cytotoxicity of F-dAdo was increased by ~50-fold (IC₅₀ ~0.2 μM). No cytotoxicity was observed when the cells were treated for 90 min with 780 nM of anti-rat TfR IgG3-Av or anti-rat TfR IgG3-Av/PNP-P67-b in the absence of F-dAdo (data not shown) or anti-dansyl IgG3-Av/PNP-P67-b in the presence of F-dAdo (Fig. 7). These results indicate that the P67-based biotinylation can also be used to associate functionally active PNP with anti-rat TfR IgG3-Av and that the complex can be delivered to TfR-bearing cells where the enzyme will convert the prodrug into a cytotoxic agent.

4. Discussion

The management of minimal residual disease is a central problem in the treatment of solid tumors [33,34]. Conventional chemotherapeutic strategies are necessarily limited by various toxicities. ADEPT shows potential for effective and non-toxic chemotherapy but the current protocol used for ADEPT needs to be improved [1–3]. One of the major obstacles is the difficulty of producing homogeneous, reproducible, and active antibody/enzyme conjugates.

Although genetically fused antibody/enzyme conjugates can be produced, we, and others, have observed that the production levels of these conjugates are frequently very low [7–9]. In fact, our initial attempt to produce an antibody–FCU1 fusion protein in myeloma cells was unsuccessful because of low production levels (unpublished data). Although chemical conjugation of an antibody to an enzyme can circumvent this problem, chemical conjugation usually generates heterogeneous products that often have impaired function [7].

The P67-based site-specific attachment of biotin has several advantages over the existing conjugation methods. For example, using the P67-based technique, large quantities of highly purified antibodies and enzymes can be readily obtained by independently optimizing their production protocols (see Section 2.2). Importantly, P67-based biotinylation reproducibly produces a homogeneous product with the enzyme-P67 fusion protein metabolically monobiotinylated at a fixed site within the P67 domain. Lysine-669 in P67 is the only site of biotinylation in the fusion protein since the fusion protein with the K669R mutation fails to be bound by the antibody-Av fusion protein (Fig. 3).

The enzyme is linked to the carboxyl terminus of the antibody where it does not significantly hinder the binding of the antibody to its target (Fig. 4C). In contrast to the genetic fusion strategy, the P67-based biotinylation eliminates the need to make a different antibody/enzyme fusion protein for every antibody/enzyme combination. The antibody/enzyme conjugates created by the P67-based biotinylation should be stable since the interaction between avidin and biotin exhibits extraordinarily high affinity (Kₐ = 10¹⁵ M) and an extremely slow dissociation rate [35]. Importantly, when BirA is overexpressed in Escherichia coli cells producing the enzyme-P67 fusion protein, the efficiency of biotinylation is extremely high (Fig. 3) [31,36]. Additionally, in vivo biotinylation can also be carried out with high efficiency in mammalian cells [37,38] (our unpublished data), suggesting that this approach could also be used for enzymes that lose activity when expressed in E. coli.

The primary function of serum transferrin (Tf) is to bind iron and transport it through the blood [39,40]. After binding to its receptor (TfR, also known as CD71) on the cell surface, Tf is internalized into an acidic compartment where iron dissociates and the apo-Tf is returned to the cell surface where ligand-receptor dissociation occurs [39]. We have previously demonstrated that anti-rat TfR IgG3-Av can be directly cytotoxic to Y3-Ag1.2.3 cells when it is used to treat them for long periods of time (48 h) [12]. However, under the conditions of the present assay in which cells were incubated with anti-rat TfR IgG3-Av for only 90 min, no cytotoxicity was observed.

Anti-rat TfR IgG3-Av can deliver biotinylated (β-galactosidase to the inside of Y3-Ag1.2.3 cells through receptor-mediated endocytosis and that the enzyme remains active after internalization, suggesting that at least a fraction escaped lysosomal degradation [12]. Furthermore, by labeling biotinylated glucose oxidase (b-GOX) with FITC, we showed that ~60% of the cell-associated anti-rat TfR IgG3-Av/b-GOX-FITC complex was internalized after 45 min at 37 °C [12]. Therefore, it was surprising that the cytotoxic effect of anti-rat TfR IgG3-Av/FCU1-P67-b mainly resulted from conjugates that were located outside of Y3-Ag1.2.3 cells after the 90 min incubation (Fig. 6B). Since chemically biotinylated enzymes were delivered by the same antibody-Av fusion protein into the same cells, it is possible that the presence of P67 in the conjugate may block receptor-mediated endocytosis. Alternatively, P67 may block the escape of the endocytosed FCU1 in the endosome or facilitate its delivery to the lysosome. FCU1 is a newly engineered enzyme and it is not known whether it will remain functional at the low pH of the endosome. The enzymes [GOX (186 kDa) and ((-galactosidase (464 kDa)) delivered by anti-rat TfR IgG3-Av into the cells are comparable to or much larger than the FCU1-P67 tetramer (210 kDa). Therefore, it seems unlikely that the size of the anti-rat TfR IgG3-Av/FCU1-P67-b complex was responsible for the decrease in intracellular enzymatic activity.

In this study, we have used the enzymes (FCU1 and PNP) that have not previously been used for ADEPT. FCU1 is a bifunctional enzyme consisting of CD and UPRT [18]. CD can convert 5-FU to membrane-permeable 5-FU. In contrast, UPRT synthesizes, from 5-FU, 5-FUMP that would not readily diffuse across cell membranes and its activity located outside of the cell could interfere with the cytotoxic effect of 5-FC. However, for unknown reasons, the CD activity of FCU1 is 100-fold higher than that of the wild-type CD, while
the UPRT activity of FCU1 is equivalent to that of the wild-
type UPRT [18]. Consequently, when incubated with 5-FC,
FCU1 produces large amounts of 5-FU. Thus, FCU1 both
inside and outside of the cell can effectively convert the
prodrug to a cytotoxic agent. As described above, F-Ade
generated by PNP from F-dAdo can also diffuse across cell
membranes [20,32]. Since 5-FU and F-Ade are highly toxic
to both dividing and non-dividing cells [32,41], FCU1 and
PNP would be ideal for eradicating solid tumors with low
growth fractions [18,20].

Tf is considered to be an autocrine regulator of cell
proliferation in malignant tumor cells [39,40]. The elevated
levels of TfR in tumor cells compared to normal cells
suggest that the TfR should be a suitable target for the
delivery of cytotoxic drugs. A major concern is that the anti-
TfR antibody/enzyme conjugates may be cytotoxic to the
normal cells. However, previous preclinical and clinical
studies using toxins chemically conjugated to Tf have shown
that the cytotoxicity was mainly directed to the tumor cells
and that side effects of the treatment were minor or absent
when the conjugate was administrated systematically
[42,43]. ADEPT is a promising therapeutic approach to
the treatment of minimal residual disease in solid tumors.
However, it is currently limited by the difficulty in producing
well-defined, functionally active antibody/enzyme conju-
gates. Our approach addresses the problem and should
significantly expand the use of ADEPT for the treatment of
malignancy.

Acknowledgements

This work was supported by National Institutes of Health
Grants CA87990 and CA86915, the UCLA Jonsson
Comprehensive Cancer Center Interdisciplinary Grant,
and Cancer Center Core Grant CA-16042 (UCLA). PPN
was supported by U.S. Public Health Service National
Research Service Award GM07185.

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