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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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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.

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55 As currently practiced, one important limitation of
56 ADEPT is the difficulty of producing large quantities of
57 homogeneous, uniformly active antibody/enzyme conju-
58 gates [1–3]. Although a variety of antibodies and enzymes
59 have been chemically linked to produce a range of
60 conjugates, chemical treatments can be detrimental to the
61 activity of the proteins and the resulting conjugates differ in
62 the number and position of the crosslinks, leading to
63 inconsistent targeting and catalytic activity [7]. This reduces
64 the amount of toxic drug activated at tumors and limits the
65 efficiency of ADEPT. As an alternative approach, recombi-
66 nant DNA technology could be used to prepare homo-
67 geneous antibody–enzyme fusion proteins. However, for
68 reasons we have yet to understand, production levels of such
69 fusion proteins are often very low, limiting their use in
70 clinical (or sometimes even animal) studies [7–9]. In
71 addition, this strategy requires that a different fusion protein
72 be produced for every antibody/enzyme combination, which
73 is cumbersome, and there may be a decrease in activity of
74 one or both covalently linked partners. It would, therefore,
75 be desirable to develop a universal delivery system that
76 eliminates the need to make a specific construct for each
77 application.

78 We previously constructed antibody-avidin (Av) fusion
79 proteins in which Av was genetically linked to IgG3 at the
80 carboxyl terminus of the heavy chain specific for either the
81 hapten dansyl or the rat transferrin receptor (TfR) (anti-
82 dansyl IgG3-Av and anti-rat TfR IgG3-Av; see Fig. 1A)
83 [10,11]. These fusion proteins are identical, except for their
84 binding specificity and each molecule of the fusion proteins
85 contains two Av moieties. Since Av forms a tetrameric
86 structure, the IgG3-Av fusion proteins exist as a non-
87 covalent dimer (Fig. 1B) [10,12]. We demonstrated that anti-
88 rat TfR IgG3-Av can deliver chemically biotinylated
89 enzymes, i.e., glucose oxidase and β-galactosidase, into
90 rat tumor cells overexpressing the TfR [12]. This suggested
91 that anti-TfR IgG3-Av could serve as a universal delivery
92 vehicle for targeted chemotherapy of tumors overexpressing
93 the TfR. However, the limitation remained that a protein
94 usually contains several potential target residues for
95 chemical biotinylation and thus following biotinylation
96 the products differ in the number and location of the attached
97 biotins [13]. The proteins carrying multiple biotins within a
98 single molecule could form high-molecular weight aggre-
99 gates when mixed with anti-TfR IgG3-Av. In addition,
100 attaching biotin at certain positions in a protein may disrupt
101 its conformation and/or biological function [14]. Therefore,
102 to successfully use anti-TfR IgG3-Av as a universal vehicle
103 for the delivery of biotinylated molecules for targeted cancer
104 chemotherapy, it is essential to develop methods for site-
105 specific mono-biotinylation of the enzymes.

106 Biotin protein ligase (BPL) is the enzyme responsible for
107 attaching biotin to a specific lysine of biotin-dependent
108 enzymes [15]. Biotinylation is a relatively rare event, with
109 between one and five biotinylated protein species found in
110 different organisms [16]. Thus, this post-translational

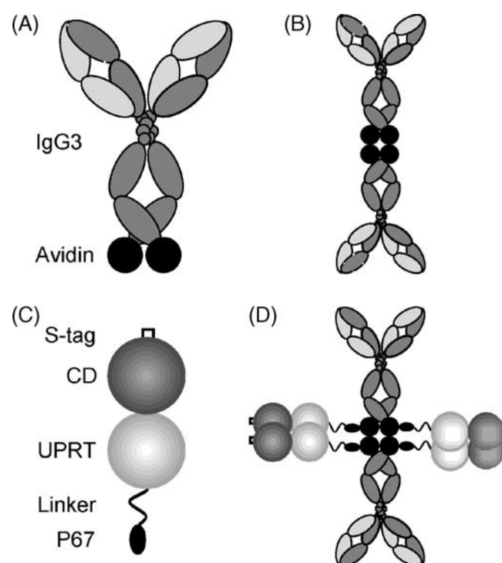

Fig. 1. Schematic diagrams of the antibody-Av fusion proteins used in this study and FCU1-P67. Diagrams are not drawn to scale. (A) The structure of anti-rat TfR IgG3-Av and anti-dansyl IgG3-Av. Each oval represents a single domain of the heavy (dark gray) and light (light gray) chains of the antibody. 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.

111 modification is extraordinarily specific. In *Escherichia coli*,
112 for example, only a single lysine residue of acetyl-CoA
113 carboxylase is biotinylated by the organism's BPL, BirA.
114 Interestingly, the functional interaction between BPLs and
115 biotin-dependent enzymes is highly conserved throughout
116 evolution [15]. Biotinylation occurs even when BPL and
117 biotin-dependent enzyme derive from such divergent species
118 as bacteria and humans. The carboxyl-terminal domain of
119 human propionyl-CoA carboxylase α subunit is naturally
120 biotinylated at lysine-669 by human BPL [17]. Importantly,
121 the same lysine residue is biotinylated in vivo when only the
122 carboxyl-terminal domain (67 amino acids) is expressed in
123 *E. coli* [17]. This indicates that BirA can biotinylate this
124 human biotin acceptor domain (termed P67) and that P67
125 alone is sufficient for recognition by BirA. Therefore, fusion
126 of P67 to a protein will provide a site for efficient attachment
127 of a single biotin molecule.

128 In the present studies, we genetically fused P67 to the
129 yeast enzyme FCU1 [18] and the *E. coli* enzyme purine
130 nucleoside phosphorylase (PNP) [19]. FCU1 is a genetically
131 engineered chimeric protein consisting of cytosine deami-
132 nase (CD) and uracil phosphoribosyltransferase (UPRT).
133 CD can convert the relatively non-toxic prodrug 5-
134 fluorocytosine (5-FC) to the highly cytotoxic agent 5-
135 fluorouracil (5-FU), and UPRT in turn can use 5-FU to
136 synthesize the toxic metabolite 5-fluorouridine 5′-monopho-

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sphate (5-FUMP). PNP cleaves the relatively non-toxic prodrug 2-fluoro-2′-deoxyadenosine (F-dAdo) to produce the highly cytotoxic drug 2-fluoroadenine (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 TfR IgG3-Av can efficiently kill rat Y3-Ag1.2.3 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 TfR 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-TfR 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 codons. The resulting *FCU1* gene was amplified by PCR using the following primers: 5′-GAGAGGTACCATGGTGACAGGGGGAATGGCAAGC-3′ and 5′-CCAAAGGCCTGAACACAGTAGTATCTGTCACC-3′. The amplified fragment was digested with *Kpn*I and *Eco*RI, 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 *P67* sequence was amplified by RT-PCR with the polyA RNA isolated from 293T cells using the following primers: 5′-CGTTGGATCCCTGCGTTCCCCGATGCCCGGAG-3′ and 5′-CTCTGCGGCCGCTCATTCCAGCTCCACGAGCAG-3′. The fragment was digested with *Bam*HI and *Eco*RI, and inserted into pUC3541 immediately downstream from the flexible $(Gly_4\text{-}Ser)_3$ linker sequence [23]. [pUC3541 is a derivative of pUC19 carrying the linker sequence (an *Stu*I–*Bam*HI fragment) within the multiple cloning site.] The resulting plasmid was digested with *Stu*I and *Eco*RI 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′-GTGTGATTGAAGCCATGCGTATGCAGAATAGTATGACAGC-3′.

A DNA fragment carrying the *deoD* gene encoding PNP was amplified from *E. coli* genomic DNA using the following PCR primers: 5′-GAGAGGTACCATGGCTACCCCACACATTAATGC-3′ and 5′-CCTTAGGCCTGCTCTTTATCGCCCAGCAGAACG-3′. The amplified fragment was digested with *Nco*I and *Stu*I, 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′-GGCCAGATCTATGAAGGATAACACCGTGCCACTG-3′ and 5′-GGTCCGCGGTTATTTTTCTGCACTACGCAGGGATATTTC-3′. The amplified fragment was digested with *Bgl*II and *Sac*II, and inserted into the multiple cloning site of pDisplay (Invitrogen, Carlsbad, CA). The resulting plasmid was digested with *Bgl*II and *Sal*I, and the fragment containing the *birA* gene was inserted into the T7 promoter-based *E. coli* expression vector pET16b-BS between the *Bam*HI and *Sal*I sites, generating pET9816. [pET16b-BS was constructed by modifying the multiple cloning site of pET16b (Novagen). It has a short DNA fragment carrying the *Bam*HI and *Sal*I sites within the multiple cloning site immediately downstream from the *Nde*I site and does not contain the original *Bam*HI site.] pET9816 was digested with *Bgl*II and *Cla*I to prepare the *birA* gene carrying the T7 transcription unit. To obtain a BirA expression plasmid that is compatible with pET29, the *Bgl*II–*Cla*I fragment was ligated to the *Bam*HI–*Cla*I fragment of pA CYC177 carrying the replication origin and the *bla* gene, generating pACYC9834.

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 TfR 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-

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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 $MgCl_2$ (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 A_{280} 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 $MgCl_2$ 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 $ZnCl_2$ 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 TfR 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 ($K_D \sim 10^{-9}$ 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 ArUAA substrate that consists of a short, mixed ribo/deoxyribo oligonucleotide having a fluorophore

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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 TfR 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 (5×10^3 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 TfR 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 TfR IgG3-Av or anti-dansyl IgG3-Av to form complexes. The prodrug 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

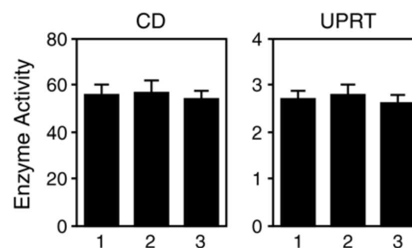

Fig. 2. The CD and UPRT activities of FCU1 and FCU1-P67. CD activity was determined by measuring the conversion of cytosine to uracil [24] and expressed as micromoles of cytosine deaminated/min/mg of protein. UPRT activity was determined by measuring the synthesis of uridine 5′-monophosphate from uracil 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.

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-biotinylation

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

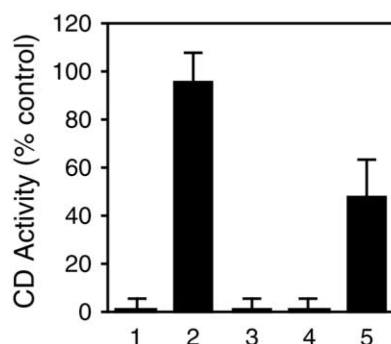

Fig. 3. The efficiency of in vivo biotinylation of FCU1-P67. The CD activity of each protein precipitated by monomeric avidin immobilized on agarose beads was determined as described in Section 2. Each bar represents the average CD activity (±S.D.) obtained from three independent assays expressed as 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.

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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 immunoprecipitated 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

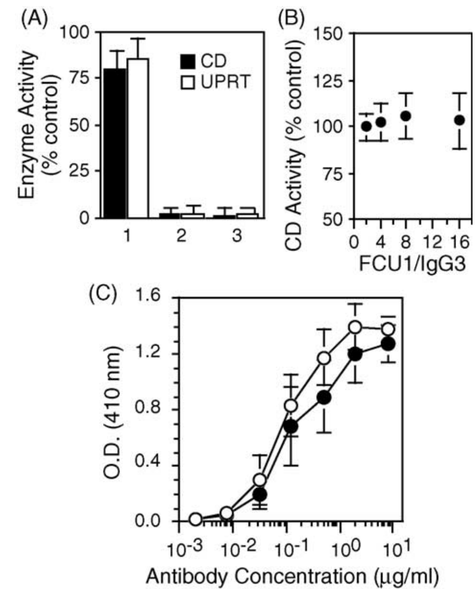

Fig. 4. Analysis of anti-dansyl IgG3-Av/FCU1-P67-b complexes. (A) FCU1-P67-b was mixed with anti-dansyl IgG3-Av (1), anti-rat TfR IgG3-Av (2), or anti-dansyl IgG3-Av preincubated with an excess amount of biotin (3). The molar ratio between an antibody-Av fusion protein and FCU1-P67-b was 1:2. The mixture was incubated overnight at 4 °C and the antibody was precipitated using an excess of dansyl-BSA Sepharose beads. Coprecipitated enzyme activities were determined as described in Section 2. Each bar represents the average enzyme activity (±S.D.) obtained from three independent assays expressed as the percent of the control value. (The control is the CD or UPRT activity of FCU1-P67-b measured without any treatment.) (B) FCU1-P67-b and anti-dansyl IgG3-Av were mixed at the indicated molar ratios (FCU1/IgG3) and incubated overnight at 4 °C. After incubation, the antibody was pulled down as described above and the coprecipitated CD activity was determined. Each symbol represents the average CD activity (±S.D.) obtained from three independent assays expressed as the % of the control value. The control is the average CD activity of the precipitated complex when FCU1-P67-b and anti-dansyl IgG3-Av were mixed at a molar ratio of 2:1. (C) Anti-dansyl IgG3-Av was mixed with PBS (○) or FCU1-P67-b at a molar ratio of 1:2 (●). The mixtures were incubated overnight at 4 °C, diluted with PBS, and added to ELISA plates coated with dansylated BSA. Antigen-bound anti-dansyl IgG3-Av was detected using an anti-human K antibody conjugated to alkaline phosphatase as described previously [10]. Each symbol represents the average absorbance at 410 nm (±S.D.) obtained from four independent assays and is plotted against the concentration of anti-dansyl IgG3-Av in the diluted mixtures. Significant signal was not detected when anti-rat TfR IgG3-Av incubated with FCU1-P67-b at a molar ratio of 1:2 was added to the ELISA plates (not shown).

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

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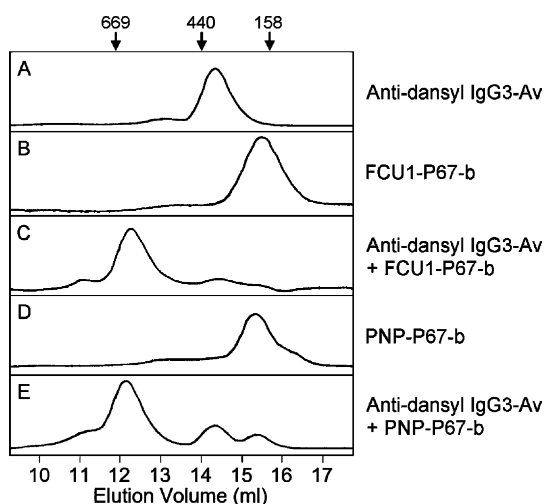

Fig. 5. Analysis of antibody/enzyme complexes by gel filtration chromatography. Elution profiles of Superose 610/300 GL column are shown. Proteins were detected by absorbance at 280 nm. The column was calibrated using aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). The molecular masses of the proteins used in this analysis are as follows: Anti-dansyl IgG3-Av, 200 kDa; FCU1-P67, 52.4 kDa; PNP-P67, 36.4 kDa.

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 TfR 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 TfR IgG3-Av

Anti-rat TfR IgG3-Av is identical to anti-dansyl IgG3-Av except for its binding specificity [10–12]. Therefore, anti-rat TfR 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 TfR 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 TfR. Based on the observations described above, anti-rat TfR 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-Ag1.2.3 cells only at high concentrations, with an IC_{50} 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 (IC_{50} ~ 0.03 mM). We previously showed that ~50% of Y3-Ag 1.2.3 cells were killed by 3.3 nM of anti-rat TfR 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 TfR IgG3-Av or the anti-rat TfR 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 TfR IgG3-Av alone as was seen with untreated cells (IC_{50} ~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 TfR 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 TfR IgG3-Av/FCU1-P67-b, Y3-Ag1.2.3 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

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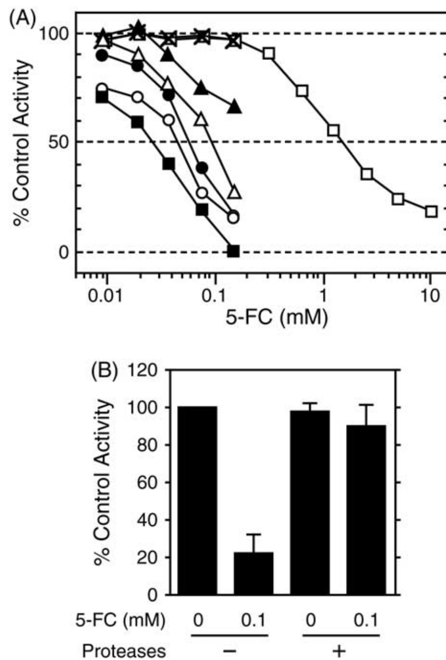

Fig. 6. In vitro cytotoxicity of 5-FC in the presence of the anti-rat TfR IgG3-Av/FCU1-P67-b conjugate. (A) Y3-Ag1.2.3 cells were treated for 90 min with anti-rat TfR IgG3-Av/FCU1-P67-b (■, 3.33 nM; ○, 1.11 nM; ●, 0.37 nM; △, 0.12 nM; ▲, 0.04 nM), anti-dansyl IgG3-Av/FCU1-P67-b (×, 3.33 nM), or medium (□) and processed as described in Materials and methods. The survival of the cells was determined by measuring the dehydrogenase activity using a colorimetric (MTS) assay and expressed as the percent of the enzymatic activity of control cells. (Control cells were treated with the medium alone and incubated in the absence of 5-FC.) Each value is the average of three assays. The largest S.D. was ±7.5%. The differences in the IC_{50} values obtained by these data are statistically significant (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 treated with proteases to remove surface-bound conjugates and then 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.).

anti-rat TfR IgG3-Av/FCU1-P67-b in the presence of 5-FC was caused mostly by surface-bound conjugates.

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 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 kDa. The two minor peaks corresponding to molecular masses of ~400 and ~200 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 prodrug conversion as described above. Y3-Ag1.2.3 cells are more susceptible to F-dAdo than 5-FC (IC_{50} ~10 μM, Fig. 7). Anti-rat TfR IgG3-Av/

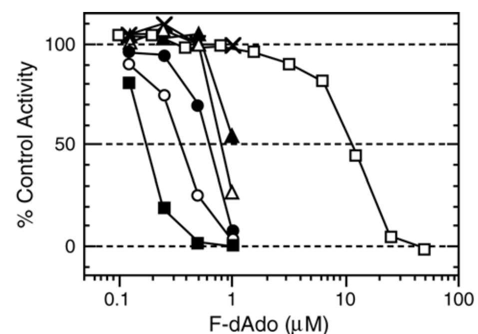

Fig. 7. In vitro cytotoxicity of F-dAdo in the presence of the anti-rat TfR IgG3-Av/PNP-P67-b conjugate. Y3-Ag1.2.3 cells were treated for 90 min with anti-rat TfR IgG3-Av/PNP-P67-b (■, 780.0 nM; ○, 260.0 nM; ●, 78.0 nM; △, 7.8 nM; ▲, 0.8 nM), anti-dansyl IgG3-Av/PNP-P67-b (×, 780.0 nM), or medium (□) and processed as described in Fig. 6A. The largest S.D. in this experiment was ±8.5%. The differences in the IC_{50} values obtained by these data are statistically significant (Student's *t*-test, $p < 0.005$).

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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 (IC_{50} ~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 mono-biotinylated 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 ($K_D = 10^{15}$ M) and an extremely slow dissociation rate [35]. Importantly, when BirA is overexpressed in *E. 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-Ag 1.2.3 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-FC 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

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728 the UPRT activity of FCU1 is equivalent to that of the wild-
729 type UPRT [18]. Consequently, when incubated with 5-FC,
730 FCU1 produces large amounts of 5-FU. Thus, FCU1 both
731 inside and outside of the cell can effectively convert the
732 prodrug to a cytotoxic agent. As described above, F-Ade
733 generated by PNP from F-dAdo can also diffuse across cell
734 membranes [20,32]. Since 5-FU and F-Ade are highly toxic
735 to both dividing and non-dividing cells [32,41], FCU1 and
736 PNP would be ideal for eradicating solid tumors with low
737 growth fractions [18,20].

738 Tf is considered to be an autocrine regulator of cell
739 proliferation in malignant tumor cells [39,40]. The elevated
740 levels of TfR in tumor cells compared to normal cells
741 suggest that the TfR should be a suitable target for the
742 delivery of cytotoxic drugs. A major concern is that the anti-
743 TfR antibody/enzyme conjugates may be cytotoxic to the
744 normal cells. However, previous preclinical and clinical
745 studies using toxins chemically conjugated to Tf have shown
746 that the cytotoxicity was mainly directed to the tumor cells
747 and that side effects of the treatment were minor or absent
748 when the conjugate was administered systematically
749 [42,43]. ADEPT is a promising therapeutic approach to
750 the treatment of minimal residual disease in solid tumors.
751 However, it is currently limited by the difficulty in producing
752 well-defined, functionally active antibody/enzyme conju-
753 gates. Our approach addresses the problem and should
754 significantly expand the use of ADEPT for the treatment of
755 malignancy.

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