Published in final edited form as:

Biomol Eng. 2007 September ; 24(3): 283–291. doi:10.1016/j.bioeng.2007.02.003.

Metabolic biotinylation of recombinant antibody by biotin ligase retained in the endoplasmic reticulum

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Abstract

Due to its strength and specificity, the interaction between avidin and biotin has been used in a variety of scientific and medical applications ranging from immunohistochemistry to drug targeting. The present study describes two methods for biotinylation of proteins secreted from eukaryotic cells using the *E. coli* biotin protein ligase. In one system the biotin ligase was co-secreted from the cells along with substrate protein enabling extracellular biotinylation of the tagged protein. In the other system, biotin ligase was engineered to be retained in the endoplasmic reticulum (ER) and metabolically biotinylates the secretory protein as it passes through the ER. An engineered antibody fragment, a diabody with specificity for carcinoembryonic antigen (CEA) was fused to the biotin acceptor domain (123 amino acid) of *Propionibacterium shermanii*. Coexpression of the fusion protein with ER retained biotin ligase showed higher biotinylation efficiency than biotinylation by co-secreted ligase. Biotinylation of the anti-CEA diabody tagged with a short (15 amino acid, Biotin AvitagTM) biotin acceptor peptide was also successful. Utilization of ER retained biotin ligase for biotinylation of protein is an attractive alternative for efficiently producing uniformly biotinylated recombinant proteins for a variety of avidin-biotin technologies.

Keywords

Biotinylation; carcinoembryonic antigen; engineered antibodies; diabody; biotin ligase; BirA

1. Introduction

Biotin (vitamin H), an essential coenzyme required by all forms of life, is only synthesized by plants, most bacteria and some fungi. In living cells, a few metabolic enzymes are naturally biotinylated through post-translational modification that is carried out by an intracellular enzyme, biotin protein ligase (BPL), also known as holocarboxylase synthetase [EC 6.3.4.10]. BPL catalyzes the formation of an amide linkage between the carboxyl group of biotin and the ϵ -amino group of a specific lysine residue of the substrate protein in a two step reaction (Cronan, 1990; Wood and Barden, 1997). The non-covalent interaction between avidin/streptavidin and biotin represents one of the strongest and most specific interactions amongst biological molecules ($K_D = 10^{-14}$ - 10^{-15} M). This property has been exploited by researchers who have

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attached biotin 'tags' to proteins for easy detection, labeling, immobilization and purification (Cull and Schatz, 2000; Kumar and Snyder, 2002; de Boer et al., 2003; Kojima et al., 2006; Krepiy et al., 2006). Biotin labeling has also been applied to drug targeting (Ohno et al., 1996; Asai et al., 2005) and viral gene therapy vector-targeting strategies (Smith et al., 1999; Parrott et al., 2003; Campos and Barry, 2004; Arnold et al., 2006).

Biotin labeling of drugs, proteins or virus has traditionally been performed *in vitro* by chemical methods, where an activated biotin derivative is conjugated to protein surface residues (commonly lysines) or carbohydrate moieties (Bayer and Wilchek, 1990; Diamandis and Christopoulos, 1991; Ohno et al., 1996; Smith et al., 1999). However, these methods result in random and heterogeneous modification, which can lead to the inactivation of biological function and cross-linking or aggregation after mixing with streptavidin or avidin. Antibody biotinylation by chemical methods generally leads to the preparation of heterogeneous conjugates. Furthermore, biotinylation of the residues in the binding site of antibodies can alter their binding properties (Saviranta et al., 1998) and result in loss of affinity.

An alternative approach to chemical methods was first demonstrated by Cronan et al. (Cronan, 1990). Fusion of the biotin attachment sites of proteins from four different species to the carboxyl terminus of β -galactosidase enabled biotinylation in *E. coli* by endogenous biotin ligase. The functional interaction between biotin ligases and their protein substrates shows a very high degree of conservation throughout evolution, since biotinylation occurs even with enzymes and substrates from widely divergent species (Chapman-Smith and Cronan, 1999). The most studied endogenous biotinylated protein is the 1.3 S subunit of the transcarboxylase domain of *Propionibacterium shermanii* (PSTCD) which is structurally very similar to that of *E. coli* acetyl-CoA carboxylase (Reddy et al., 1998). By fusing the biotin acceptor peptide domain of PSTCD to the target protein, it was demonstrated that biotinylation could occur in bacterial, yeast, insect and mammalian cells (Smith et al., 1999; Parrott and Barry, 2001; Verhaegen and Christopoulos, 2002). Recent *in vivo* imaging study showed that tumor cells expressing PSTCD tagged surface receptor protein was detected using a variety of imaging agents coupled to streptavidin (Tannous et al., 2006). Biotinylation can occur either by cellular endogenous protein-biotin ligase or by the coexpression of an exogenous biotin ligase, in most cases that of bacterial BirA enzyme (Tsao et al., 1996).

Smaller peptide tags (<23 aa) identified by peptide libraries were also found to be biotinylated *in vitro* with kinetics comparable to those of natural biotin acceptor sequence (Schatz, 1993). A 15 residue peptide (GLNDIFEAQKIEWHE, Biotin AviTag™) (Beckett et al., 1999) with 100% biotinylation efficiency was used for specific biotinylation of fusion protein in *E. coli*, insect and mammalian cells (Smith et al., 1998; Wu et al., 2002; de Boer et al., 2003; Viens et al., 2004; Yang et al., 2004; Warren et al., 2005; Tirat et al., 2006). Utilizing this small peptide *in vivo* biotinylation has also been performed on the surface of yeast (Parthasarathy et al., 2005).

Antibodies can be engineered into a variety of formats that retain binding specificity and exhibit optimal properties for *in vitro* or *in vivo* applications. Single-chain antibody fragments (scFvs), produced by genetically fusing variable light (V_L) and heavy (V_H) chain domains of a parental antibody through a peptide linker, represent the smallest functional unit (25-30 kDa) that still retains the capacity to bind antigen. Production of single-chain antibody scFv dimers (also known as diabodies, 55 kDa) can be forced by shortening the peptide linker, which in turn enhances the binding activity (Holliger et al., 1993). We have previously described an engineered anti-carcinoembryonic antigen (CEA) diabody (Db), constructed from the variable regions of the murine anti-CEA monoclonal antibody T84.66 (Wu et al., 1999). Attempts to biotinylate the anti-CEA diabody by chemical methods resulted in inactivation and finally precipitation of the protein when mixed with streptavidin. To circumvent this problem, we

developed a fusion protein comprised of the anti-CEA diabody and the 123 aa biotin acceptor domain from *P. Shermanii* (referred to here as BD123) to generate Db-BD123. The fusion protein was coexpressed in mammalian cells with BirA, the BPL of *E. coli*. Secreted and ER-retained forms of BPL were developed, and the efficiency of biotinylation of Db-BD123 was higher using ER-retained ligase compared to secreted ligase. BD123 was replaced by a 15 amino acid short biotin acceptor peptide (defined here as BP15) to produce Db-BP15. Biotinylation efficiency of the fusion protein by ER-retained BPL was higher using BP15 compared to BD123. This technology allows metabolic biotinylation of secreted proteins eliminating the need to purify and treat with exogenous reagents. This method is a useful approach for efficiently producing uniformly biotinylated proteins.

2. Materials and methods

2.1. Assembly of genes encoding diabody fusion proteins (Db-BD123 and Db-BP15)

To construct Db-BD123 fusion protein (Fig. 1A), the sequence encoding the 123 amino acid biotin acceptor domain (BD123) of *P. shermanii* was amplified by PCR from pBGLuc-birA (kindly provided by (Verhaegent and Christopoulos, 2002)) using the BD-forward and BD-reverse primers shown in Table 1. The gel purified PCR product was inserted downstream from the anti-CEA Db (Wu et al 1999) in the pEE12 mammalian expression vector (Lonza Biologics, Slough, UK) (Bebbington et al., 1992) using *SpeI* and *EcoRI* sites. The resulting construct Db-BD123 was also inserted into the pcDNA3.1 *neo* vector (Invitrogen, Carlsbad, CA) using *XbaI* and *EcoRI* sites.

Additional three variants of the Db-BP15 (Fig. 1A) were made using the 15 aa sequence, GLNDIFEAQKIEWHE (Biotin AviTag™), BirA substrate peptide (Beckett et al., 1999) at the C-terminus of diabody. One variant contained the anti-CEA Db directly fused to the 15 aa biotinylation tag (Db-BP15). The other two variants contained a 6xHis tag that either preceded (Db-His-BP15) or followed the 15 aa biotinylation tag (Db-BP15-His) (Fig. 1A). These tags were also inserted into pEE12 downstream of the anti-CEA Db as *SpeI-EcoRI* fragments. All constructs included a mammalian leader sequence at their N-terminus for extracellular secretion.

2.2. Design of secreted and ER-retained enzyme biotin ligase (BirA)

The *E. coli* BirA gene was amplified from the pBGLuc-birA vector (Verhaegent and Christopoulos, 2002) by two separate reactions using the primers listed in Table 1. In the first reaction, primers birA10 and birA11/12 were used. The PCR product was gel purified and used as template in the subsequent amplification with birA20 and birA21/22 primers (Table 1). The final PCR products encoding secretory BirA-DYKD and ER retained BirA-DYKDEL (Fig. 1B) were gel purified and cloned into the pEE12 vector using *XbaI* and *EcoRI* sites. The DYKD sequence is the truncated form of the FLAG epitope tag (DYKDDDDK) which allows detection of the recombinant proteins using the M2 anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) and KDEL provides an ER retention signal (Munro and Pelham, 1987; Beerli et al., 1994a; b).

2.3. Mammalian expression, selection, and purification

NS0 murine myeloma cells (Galfre and Milstein, 1981) were transfected and subjected to selection in glutamine deficient medium as described previously (Yazaki et al., 2001; Yazaki and Wu, 2003). Cells transfected with the pEE12/Db-BD123 construct were supplemented with biotin (Sigma-Aldrich) to a final concentration of 100 μ M. For supertransfection, NS0 cells were first transfected with BirA-DYKD and selected in glutamine deficient medium. The expression of BirA-DYKD was detected by Western blot using anti-FLAG M2 peroxidase conjugated antibody (dilution 1:5000). The highest producing clone was supertransfected with

the pcDNA_{neo}/Db-BD123 and double selected using glutamine deficient medium and 1 mg/ml of G418 (Calbiochem, San Diego, CA).

Clones were screened for expression of diabody by ELISA, in which the desired protein was captured by Protein L and detected by alkaline phosphatase (AP)-conjugated goat anti-mouse Fab antibody (Sigma-Aldrich) as described (Olafsen et al., 2004). Supernatants were also analyzed by Western blot using AP-conjugated goat anti-mouse Fab antibody (Sigma-Aldrich, dilution 1:5000) to detect the diabody, mouse monoclonal IgG1 Penta-His antibody (Qiagen, Valencia, CA, dilution 1:1000) to detect the 6xHis tag, and streptavidin horseradish peroxidase conjugate (SA HRP) (GE Healthcare, Piscataway, NJ, dilution 1:2000) to detect biotin. The highest expressing clones were expanded into triple flasks (Nunc, Rochester, NY). Supernatants containing the anti-CEA Db-BD123 fusion protein were loaded onto a Protein L column (Pierce, Rockford, IL). Bound protein was eluted using 0-100% gradient of 0.1 M glycine (pH 2.5) in PBS (pH 7.0). Eluted fractions were collected in the presence of 1/10 volume of 2 M Tris HCl pH 8.0. Hexahistidine-tagged Db-BP15-His fusion protein was purified by Ni-NTA chromatography (Qiagen). Bound proteins were eluted with 250 mM imidazole in presence of PBS (pH 7.0), 300mM NaCl and 0.1% TritonX-100. Eluted fractions containing the desired protein were pooled, dialyzed against PBS and concentrated by Centrprep 30 (Millipore Corp., Bedford, MA). The final concentration of purified proteins was determined by A_{280nm} using an extinction coefficient $\epsilon = 1.5$. The expression level of recombinant diabody in T-flasks was determined by ELISA using anti-CEA diabody as a standard. The protein was captured by Protein L in microtiter plates and detected by AP-conjugated goat anti-mouse Fab antibody (Sigma-Aldrich).

2.4. Characterization of purified biotinylated protein

Purified proteins were analyzed by SDS-PAGE under non-reducing conditions. Samples were also subjected to gel filtration chromatography on a Superdex 75 HR 10/30 column (GE Healthcare) that was run isocratically in 50 mM Na₃PO₄, 0.15 mM NaCl (pH 7.0). Retention times were compared with that of the parental diabody. The Db-BD123 fusion protein was also subjected to gel filtration chromatography on a Superdex 200HR 10/30 column (GE Healthcare) in 50 mM Na₃PO₄, 0.15 mM NaCl (pH 7.0). Retention time was compared with standards of anti-CEA intact chimeric T84.66 antibody, minibody and diabody as described (Kenanova et al., 2005). Proteins were detected by absorbance at 280 nm.

2.5. Binding studies

(A) Binding to Streptavidin Beads—50 μ l of ImmunoPure Streptavidin coated beads (Pierce) were used for 3.5 μ g of purified protein. Binding was done in PBS at room temperature for 1 hour on a rocking platform, followed by 3 washes in PBS. Bound material was eluted by boiling for 5 min in protein sample loading buffer and analyzed by Western blot using SA HRP antibody for detection.

(B) Flow cytometry—LS174T-CEA⁺ human colon carcinoma cells (ATCC, Manassas, VA; #CCL-188), were incubated with purified Db-BD123 or Db-BP15-His fusion protein for 1 hour at 4°C in PBS containing 1% BSA. The presence of biotin in the fusion protein was detected by incubation with Alexa Fluor 488 conjugated streptavidin (Molecular Probes, Inc., Eugene, OR) (dilution 1: 5000 in PBS Tween 20) or Quantum dot 655 streptavidin conjugate (Qdot655SA) (Quantum Dot Corp., Hayward, CA). Antibody binding to CEA was quantified by FACS Calibur flow cytometer (Beckton Dickinson, UK) and data were analyzed by Cell Quest software. FL1 (λ_{em} : 530/30 nm) and FL3 (λ_{em} : 670 nm long pass) were the filters used for Alexa Fluor 488 and Qdot 655 respectively. Biotinylated chimeric T84.66 antibody prepared using an EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce) was used as a positive control.

(C) Confocal microscopy—LS174T-CEA⁺ cells were plated on poly-L lysine coated glass coverslips (BD Biosciences, San Jose, CA) in 12 well-plates in DMEM medium containing 5% Fetal bovine serum (FBS) for 24 hours. The next day, cells were incubated with 2 µg/ml of Db-BD123 or Db-BP15-His fusion protein in PBS/1% FBS on ice for 1 hr. Cells were then fixed with 3.7% paraformaldehyde at 4°C for 30 min. After primary antibody treatment and fixation, cells were incubated in 10 nM Qdot655SA in PBS/1% FBS at room temperature for 1 hour. Coverslips were mounted on glass slides and observed using a Leica TCS-SP inverted confocal microscope equipped with a 100x oil immersion objective lens.

3. RESULTS

3.1. Biotinylation of Db-BD123 fusion protein by co-secretion with biotin ligase

In order to examine the biotinylation of protein secreted from cells, biotin ligase (BirA-DYKD) was co-secreted with the fusion protein of interest, anti-CEA Db-BD123. First, BirA-DYKD was transfected into NS0 cells and clones stably expressing high levels of BirA were identified by Western blot. Fig. 2A shows three representative clones (1D9, 1G6 and 3G7) expressing BirA-DYKD as judged by molecular weight (35.5 kDa) and by anti-FLAG M2 antibody reactivity.

The apparent highest expressing clone, 1G6, was supertransfected with pcDNA_{neo}/Db-BD123 construct. A Western blot probed with anti-Fab antibody shows a single band of 42 kDa, corresponding to combined mass of anti-CEA scFv (27 kDa) and BD123 (15.1 kDa) (Fig. 2B). Staining with SA HRP demonstrated that the Db-BD123 protein can be metabolically biotinylated by the secreted biotin protein ligase (Fig. 2C). The expression level of Db-BD123 (average of 4 clones) ranged from 4 to 6 µg/ml in T-flasks as determined by ELISA.

3.2. Efficient metabolic biotinylation of Db-BD123 by ER-retained biotin ligase

The ER-retained version of biotin ligase (BirA-DYKDEL) was stably cotransfected with the Db-BD123 construct into NS0 myeloma cells. Western blots with anti-FLAG antibody demonstrated stable expression of BirA in cell lysates (Fig. 3A). Furthermore Western blot analysis of supernatants showed the presence of the biotinylated Db-BD123 (Fig. 3B).

To evaluate the efficiency of biotinylation by secreted ligase *versus* ER-retained ligase we compared the extent of biotinylation of two Db-BD123 proteins produced by co-secretion with ligase (5D8) and ER-retained ligase (1G7). Fig. 3C shows that the expression of the diabody moiety is similar for both proteins; whereas the extent of biotinylation differs (the ratio of signals was 1:8 by densitometry). Analysis of additional clones expressing biotinylated Db-BD123 (3A2, 7H5 from Fig. 2B and Fig. 2C) confirmed that ER-retained ligase biotinylated more efficiently than secreted ligase (data not shown).

3.3. Biotinylation of short biotin acceptor peptide

Three versions of diabody fused to a 15 aa biotin acceptor peptide (Db-BP15, Db-His-BD15 and Db-BP15-His) were coexpressed with BirA-DYKDEL. As seen in Fig. 4, diabody fusion protein was expressed in culture supernatants (Fig. 4A, C) and biotinylation was confirmed by probing western blot with SA HRP (Fig. 4B). Hence, the 15 amino acid short biotin acceptor peptide is sufficient for biotinylation. Since there was no obvious difference in the expression and biotinylation of two versions of Db-BP15 containing 6xHis tag, the cell line expressing Db-BP15-His was selected for further study.

3.4. Purification and biochemical characterization of Db-BD123 and Db-BP15-His protein

The expression levels in culture supernatants were 15-20 µg/ml for Db-BD123 and 18-22 µg/ml for Db-BP15-His. A volume of 300 mL culture supernatants containing Db-BD123 and

Db-BP15-His yielded 4.5 mg and 6.12 mg of pure protein respectively, representing 80-90% recovery. Fig. 5A shows that the migration of the fusion proteins was consistent with the predicted molecular weight of a monomer of 30 kDa for Db-BP15-His (lane 1) and 42 kDa for Db-BD123 (lane 2).

Size exclusion chromatography was performed to evaluate the native state of biotinylated fusion proteins. The chromatogram from Superdex 75 HR column (Fig. 5B) shows that the Db-BP15-His elutes at approximately the same time as that of diabody (at 20.70 min) demonstrating that the Stokes' radius of this fusion protein is not significantly changed by the addition of the short 15 aa biotin acceptor peptide and the 6xHis tag. On the other hand, Db-BD123 elutes as a single peak at 18.33 min and earlier than that of the diabody alone (20.69 min) as expected. When Db-BD123 protein was further analyzed on a calibrated Superdex 200 HR 10/30 column (Fig. 5C), main peak eluted at 28.79 min corresponding to an approximate molecular weight of 80-85 kDa.

3.5. Functional Characterization of biotinylated anti-CEA diabody fusion proteins

A critical issue is the efficiency of biotinylation of the target protein by the coexpressed ER-retained ligase. Fusion proteins were immunoprecipitated by streptavidin agarose beads and analyzed by Western blot. Most of the Db-BD123 protein bound to the beads (Fig. 6, lanes 2 and 3) and only about 10% of Db-BP15-His protein remained unbound (Lanes 5 and 6). This demonstrates that the fusions proteins are efficiently biotinylated (>90%) and can be captured by streptavidin agarose beads.

To ensure that the addition of the biotin acceptor peptide and/or biotinylation did not affect the properties of the tagged proteins, binding of biotinylated diabodies was assessed on live LS174T colon carcinoma cells using Alexa Fluor 488 conjugated streptavidin. Flow cytometric analysis showed that *in vivo* biotinylated Db-BP15-His stained better (56%) than the full length Db-BD123 fusion protein (11.7%) (Fig. 7A). In comparison, biotinylated intact anti-CEA chimeric T84.66 antibody showed 65.7% positive staining.

Binding of biotinylated anti-CEA diabodies to LS174T cells was also detected using Qdot655SA. Again Db-BP15-His stained better than Db-BD123 (41% vs. 17.7%), and was comparable to parental chimeric T84.66 (56%).

The binding of biotinylated fusion proteins was also demonstrated by confocal microscopy using Qdot655SA. The immunofluorescence result showed bright surface staining of LS174T cells when the cells were incubated with Db-BD123 (Fig. 8B) and Db-BP15-His (Fig. 8C) followed by Qdot655SA compared to Qdot655SA alone (Fig. 8A).

3.6. Biotin Acceptor Protein is not biotinylated in absence of exogenous biotin ligase

In order to investigate whether proteins containing biotin acceptor sequences could be biotinylated by endogenous mammalian biotin ligases, NS0 cells were transfected solely with the Db-BP15-His construct. Western blots of supernatants probed with anti-mouse Fab (Fig. 9A) and anti-His (Fig. 9B) antibodies demonstrated secretion of the Db-BP15-His fusion protein. Fusion protein was not detected using SA HRP (Fig. 9C) indicating that the protein was not metabolically biotinylated by mammalian cells. This confirms that anti-CEA diabody fusion proteins are biotinylated from the introduced BirA (secreted or ER-retained form) and not by endogenous enzymes already present in the mammalian cells.

4. Discussion

In the present study we have developed a system to metabolically biotinylate the fusion protein in eukaryotic cells. Initial studies showed that the Db-BD123 fusion protein was biotinylated

by both versions of biotin ligase (secreted and ER-retained). In addition three variants of the anti-CEA Db with BP15 was also efficiently biotinylated in presence of ER-retained ligase. Biochemical and biological characterization showed that the fusion proteins assembled into the expected molecular weight fragments and bound antigen effectively. Traditional *in vitro* enzymatic biotinylation is conducted on purified proteins and requires many steps including concentration of protein, buffer exchange for optimal enzymatic activity, enzymatic biotinylation at room temperature, and buffer exchange for removal of free biotin from enzymatic reaction (Altman et al., 1996). In contrast, metabolic biotinylation of protein *in vivo* by biotin ligase shortens and streamlines a lengthy process and reduces the chance of protein degradation during biotinylation by exogenous biotin ligase.

Successful development of biotinylated recombinant antibody fragments required three components: the antibody, the biotin acceptor domain and the linker between the two. The anti-CEA diabody (non-covalent scFv dimer) used in this work was chosen as the minimal, bivalent engineered antibody fragment that is still capable of efficient localization to CEA-expressing tumors *in vivo* (Wu et al., 1999). Furthermore, from the X-ray crystallographic structure of

anti-CEA Db it was found that C-termini of the diabody subunits are almost 70 Å apart and on an alternate face from the antigen combining site (Carmichael et al., 2003). Thus, fusion of an additional partner domain to the C-termini of the diabody should not interfere with the antigen binding. However it was observed that low signal was generated by Db-BD123 in flow cytometry assays using LS174T-CEA⁺ cells (11.7% with Alexa 488 streptavidin as secondary and 17.7% with Qdot655SA as secondary). It may be possible that Db-BD123 binding to CEA sterically blocks the introduced biotin from interacting with streptavidin as replacement of BD123 with BP15 in the Db-BP15-His fusion protein improved the binding activity (Table 2).

Initially the large biotinylation tag (123 aa) derived from the biotin acceptor domain present in naturally biotinylated proteins was used in mammalian cells (Parrott and Barry, 2000; 2001). Small peptide tags obtained by combinatorial libraries were utilized for site specific biotinylation of a wide variety of proteins (Tatsumi et al., 1996; de Boer et al., 2003; Yang et al., 2004; Warren et al., 2005). There are obvious advantages in using smaller artificial tags. First, small tags are much less likely to affect the structure and thus the properties of fusion protein *in vivo*. Second, artificial tags are unlikely to be recognized and biotinylated by endogenous biotin ligases. Indeed we verified that there was no cross-reactivity between the 15 aa tag and endogenous biotin ligase (Fig. 9). When comparing the two fusion proteins (Table 2) we found that, using this small BP15, metabolic biotinylation of fusion protein in eukaryotic cell is a highly efficient process that occurs in presence of ER-retained version of BirA.

Previous studies demonstrated that co-secretion of *E. coli* biotin ligase in mammalian cells resulted in biotinylation of secretory and cell surface proteins (Parrott and Barry, 2001). Here we have expanded this technology to investigate the influence of location of ligase through the production of secreted and ER-retained versions. We found that the protein product directed to the lumen of ER was more efficiently biotinylated than the protein in presence of secreted ligase. For this reason we chose to metabolically biotinylate subsequent fusion proteins in the presence of ER-retained ligase. Recently a similar approach was used to label nonviral, host-derived proteins on the surface of lentiviruses using an ER-retained bacterial biotin ligase (Nesbeth et al., 2006).

More importantly we demonstrated the metabolic biotinylation of protein in eukaryotic cells. A recent report demonstrated biotinylation of an anti-CEA scFv fragment in an *E. coli* strain over-expressing BirA (Warren et al., 2005). The limitation of this approach is that large, complex, glycosylated proteins cannot be produced in *E. coli*, whereas in our system, any protein, antibody or antibody fragments can be biotinylated posttranslationally.

In summary, we have developed an efficient system to metabolically biotinylate secretory fusion proteins in presence of endoplasmic reticulum retained biotin ligase. We demonstrated that the biotinylation efficiency is > 90% for the proteins with the small 15 amino acid biotin acceptor tag at the C-terminus of the diabody. We also generated a stable cell line expressing ER-retained BirA. Supertransfection of the cell line with any other fusion protein containing the 15 aa biotin tag should allow metabolic biotinylation of a variety of proteins, bypassing chemical methods. This *in vivo* metabolic biotinylation strategy is a general approach for production of site-specifically biotinylated proteins for streptavidin-biotin technology.

Acknowledgments

This work was supported by NIH P01 CA 43904, P50 CA 86306 and U54 CA 119367. AMW is a member of the Jonsson Comprehensive Cancer Center (CA 16042). We are grateful to Dr. Theodore K. Christopoulos (University of Patras, Greece) for the pBGLuc-birA plasmid. We thank UCLA Brain Research Institute's Carol Moss Spivak Cell Imaging Facility for confocal microscopy and UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility. We also thank Dr. Tove Olafsen for her help in manuscript preparation.

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Fig. 1.

Schematic presentation of the fusion proteins. (A) Anti-CEA diabody fusion proteins. T84.66 V_L and V_H are joined by an 8 aa linker, to form the diabody. 1-4, Diabody variants with 123 aa biotin acceptor domain (BD123) or 15 aa peptide (BP15) at the C-terminus. A 4aa (GSTS) or a 6 aa (GSTSGS) linker were used between Db and biotin acceptor substrate. (B) Biotin protein ligase (BirA). The amino acids DYKD and DYKDEL at the C-terminus are for secreted and ER-retained biotin ligase respectively.

Fig. 2. Western blots of Db-BD123 fusion protein cosecreted with biotin ligase (BirA-DYKD). (A) Expression of secreted BirA-DYKD was detected with anti-FLAG M2 antibody. (B) Expression of Db-BD123 in different clones was detected with HRP-conjugated anti-mouse Fab. (C) Same Db-BD123 expressing clones detected with SA HRP. The molecular weight standard is indicated. C, control, non transfected NS0 cells.

Fig. 3.

In vivo biotinylation of Db-BD123 fusion protein cotransfecting with ER-retained biotin ligase (BirA-DYKDEL). (A) Western blot of BirA-DYKDEL expression in total cell lysate (5µg per lane). Expression was detected with anti-FLAG M2 antibody. C, total cell lysate from non transfected NS0 cells. (B) Western blots of media supernatants from NS0 cells expressing Db-BD123 using HRP-conjugated anti-mouse Fab and with SA HRP. C, the supernatant from non transfected NS0 cells. Db, diabody (positive control). (C) Biotinylation efficiency of Db-BD123 by secretory (5D8) and ER-retained (1G7) ligase. Equal amount of proteins were loaded in each lane. Diabody was detected with anti-mouse Fab antibody and biotin was detected with SA HRP.

Fig. 4.

Western blots of media supernatants from NS0 cells expressing Db-BP15 using HRP-conjugated anti-mouse Fab and with SA HRP. The presence of 6xHis tag was detected with AP-conjugated anti-His antibody. C, the supernatant from non transfected NS0 cells.

Fig. 5.

Biochemical characterization of purified biotinylated anti-CEA diabody fusion proteins. (A) SDS-PAGE. Lane 1, Db-BP15-His; lane 2, Db-BD123; lane 3, Molecular weight markers. (B) Size-exclusion analysis using Superdex 75 HR column. The major peaks eluted at retention times of 18.33 minutes for Db-BD123 and 20.70 minutes for Db-BP15 consistent with dimers of 84 and 60 kDa respectively. The parental anti-CEA diabody [55 kDa; (Wu et al., 1999)] was used as size standard. (C) Size-exclusion analysis of purified Db-BD123 using a Superdex 200 HR column. The major peak eluted at retention time of 28.79 minutes. Intact chimeric T84.66 IgG [150 kDa; (Neumaier et al., 1990)], minibody [80 kDa; (Hu et al., 1996)] and diabody (55 kDa) with the corresponding retention times of 25.8, 28.5 and 38.2 minutes were used as size standards. Proteins were detected by absorbance at 280 nm.

Fig. 6. Efficiency of biotinylation. Western blot using SA HRP to detect binding of biotinylated fusion proteins to streptavidin agarose. Lanes 1 and 4, starting material; lanes 2 and 5, unbound material and lanes 3 and 6, streptavidin bound material.

Fig. 7.

Flow cytometry of purified fusion proteins binding to live LS174T-CEA⁺ cells. Cells were incubated with no protein (negative control), *in vitro* biotinylated chimeric T84.66 antibody (positive control) and purified biotinylated fusion proteins (Db-BD123 and Db-BP15-His). The cells were stained with (A) Alexa Fluor 488 conjugated streptavidin (FL1, horizontal shift) and (B) Qdot655SA (FL3, vertical shift).

Fig. 8. Fluorescence micrographs of LS174T-CEA⁺ cells following incubation with purified fusion protein (Db-BD123 or Db-BP15-His) and Qdot655SA. Cells were imaged in a confocal microscope. LS174T-CEA⁺ cells treated with (A) no protein (negative control), (B) Db-BD123 and (C) Db-BP15-His. The scale bar corresponds to 20 μ m.

Fig. 9.

Western blots of media supernatants expressing Db-BP15-His in absence of BirA. (A) Expression of diabody was detected with anti-Fab antibody. Lane 1, non transfected NS0 cells (negative control); lanes 2 and 3, cell lines expressing Db-BP15-His in absence of BirA; lane 4, cell line expressing Db-BP15-His in presence of BirA (positive control). (B) Detection of 6xHis tag using AP-conjugated anti-His antibody. Lane 5, non transfected NS0 cells (negative control); lanes 6 and 7, cell lines expressing Db-BP15-His in absence of BirA. (C) Detection of biotin using SA HRP. Lanes 8 and 9, cell lines expressing Db-BP15-His in absence of BirA; lane 10, cell line expressing Db-BP15-His in presence of BirA (positive control).

Table 1PCR primers^a

BD-forward	CGGACTAGTATGAACTGAAGGTAACAGTCAACGGC <i>SpeI</i>
BD-reverse	CCGGAATTTCGCGGCCGCCTATCATTCGATGAGCTCGAGATCCCC <i>EcoRI</i>
birA10	CTATGGGTGCTGCTGCTCTGGGTTCCAGGTTCCACCGGTATGAAGGATAACACCGTGCCAC
birA11(DYKD)	ATCTTTGTAATCTTTTCTGCACTACGCAGGGATATTTC
birA12(DYKDEL)	CAGTTCATCTTTGTAATCTTTTCTGCACTACGCAGGGATATTTC
birA20	GCGGAATTCTCTAGAG CCGCCACC ATGGAGACAGACACACTCCTGCTATGGGTGCTGCTGCTCTGGG <i>XbaI</i>
birA21(DYKD)	CCGGAATTTCGCGGCCGCCTATCAATCTTTGTAATCTTTTCTGCAC <i>EcoRI</i>
birA22(DYKDEL)	CCGGAATTTCGCGGCCGCCTATCACAGTTCATCTTTGTAATCTTTTTC <i>EcoRI</i>

^a Restriction sites are shown in italics, stop codons are underlined and Kozak consensus sequence is in boldface type.

Comparison between two versions of biotinylated anti-CEA diabody

	<u>Db-BD123</u>	<u>Db-BP15-His</u>
Molecular Weight	84 kDa	60 kDa
Concentration in T flask	15-20 µg/ml	18-22 µg/ml
Yield	80-90%	80-90%
Binding with streptavidin agarose	95%	90%
LS174T binding Streptavidin Alexa	11.7%	56%
Qdot Streptavidin	17.7%	41%