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Enhanced in vivo imaging of metabolically biotinylated cell surface reporters

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Abstract

Metabolic biotinylation of intracellular and secreted proteins as well as surface receptors in mammalian cells provides a versatile way for: monitoring gene expression; purifying and targeting of viral vectors; monitoring cell and tumor distribution in real time *in vivo*; labeling cells for isolation; and tagging proteins for purification, localization and trafficking. Here, we show that metabolic biotinylation of proteins fused to the bacterial biotin acceptor peptides (BAP) varies among different mammalian cell types and can be enhanced by over 10-fold upon over-expression of the bacterial biotin ligase directed to the same cellular compartment as the fusion protein. We also show that *in vivo* imaging of metabolically biotinylated cell surface receptors using streptavidin conjugates is significantly enhanced upon co-expression of bacterial biotin ligase in the secretory pathway. These findings have practical applications in designing more efficient targeting and imaging strategies.

Introduction

The strong interaction of biotin with chicken egg white avidin or its bacterial counterpart streptavidin has served as the basis for development of many techniques ranging from protein and DNA detection to imaging and tumor therapy *in vivo*[1–4]. In nature, biotin serves as a coenzyme by binding covalently to carboxylating and decarboxylating enzymes and thereby carrying CO_2 to their substrates[5]. Biotin ligase is the enzyme responsible for the post-translational covalent attachment of a single biotin moiety to a biotin acceptor peptide (BAP) within these enzymes. In general, proteins engineered to contain a bacterial BAP sequence from the 1.3S subunit of *Propionibacterium shermanii* transcarboxylase domain [PSTCD; 129 amino acids (aa)] and expressed in mammalian cells have been shown to be biotinylated to a certain extent by the endogenous biotin ligase, holocarboxylase synthetase (HLCS)[6]. This endogenous metabolic biotinylation of recombinant BAP proteins in combination with streptavidin conjugates has been used in mammalian cells for different purposes such as: purifying proteins and antibodies[7,8]; tracking cells and tumors *in vivo* by expressing a metabolically biotinylated reporter on their surfaces[9,10]; purifying and

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targeting viral vectors to specific cells, such as adenoassociated virus (AAV) and lentivirus[11–13]; and determining the location and molecular structure of triad junctions in skeletal cells by biotinylating receptors, such as the acetylcholine receptor and dihydropyridine calcium channel[14,15].

Mammalian cells have four known endogenously biotinylated proteins. Three of them: pyruvate carboxylase (130 kDa), 3-methylcrotonyl coA carboxylase (75 kDa) and propionyl coA carboxylase (72 kDa) are found in the mitochondria, whereas acetyl coA carboxylase (220 kDa) is located in the cytoplasm[16,17]. In this study, we compared the expression and biotinylation level of these endogenous enzymes as well as the level of holocarboxylase synthetase in different mammalian cells, and show that they vary among different cell types. Further, we show that metabolic biotinylation of proteins fused to the PSTCD BAP have different metabolic biotinylation efficiency in different mammalian cell types and can be enhanced over 10-fold by over-expressing the bacterial biotin ligase which is directed to the same cellular compartment as the fusion protein. We also show that *in vivo* imaging of metabolically biotinylated cell surface reporter is significantly enhanced upon co-expression of bacterial biotin ligase in the secretory pathway.

Experimental Section

Cell lines

Gli36 human glioma cells are a kind gift from Dr. Anthony Capagnoni (UCLA, Los Angeles, CA). 293T human embryonic kidney fibroblasts were obtained from Dr. Maria Calos (Stanford University School of Medicine, Stanford, CA). A549 human lung adenocarcinoma, U251 human glioma as well as U20S human osteosarcoma were obtained from ATCC. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U penicillin and 0.1 mg of streptomycin (Sigma) per ml at 37 °C and 5% CO_2 in a humidified atmosphere. HEI193 human shwanomma cells[18] were maintained in similar growth medium supplemented with 2 μM forskolin and 50 mg/ml gentamicin.

Constructs

All constructs used in this study were initially cloned in the pDisplay vector (Invitrogen, Carlsbad, CA) which tags all genes with an N-terminal epitope of hemagglutinin A (HA). The pBAP-TM construct was generated previously[10]. The cDNA sequence for the 14 BAP was amplified by PCR from pAN-4 (Avidity) and cloned into the BglII/SacII sites of pDisplay vector to generate p14BAP-TM. The cDNA for the single-chain antibody fragment, MR1(scFv), with specific binding to epidermal growth factor receptor-vIII (EGFRvIII)[19] was amplified by PCR and fused in-frame to the 129 aa PSTCD BAP at its 3' terminus in pBAP-TM, replacing TM, to generate pMR1-BAP (60 kDa). The bacterial biotin ligase, codon optimized from mammalian gene expression (hBirA)[20] was amplified by PCR and either cloned in BglII and SacII to generate the secreted form (psshBirA) or cloned in EcoRI and SacII to remove the signal sequence, generating the cytoplasmic version (phBirA). To generate the ER-targeted hBirA (psshBirA-KDEL), a primer set in which the KDEL sequence was introduced to the downstream primer was used to amplify the hBirA and cloned in BglII and SacII sites. The BAP-TM reporter was subcloned into a lentivirus vector together with eGFP separated by an IRES to generate lenti-BAP-TM-IGFP. The secreted biotin ligase sshBirA was subcloned into a lentivirus vector together with mCherry red fluorescent protein separated by an IRES generating lenti-sshBirA-ImCherry[21]. Lentivirus vectors were packaged and tittered as previously described[10].

Western blot analysis

Different types of cells were infected with lenti-BAP-TM-IGFP followed by either lenti-mCherry control or lenti-sshBirA-ImCherry at a multiplicity of infection of 30. Or 293T cells were transfected with different constructs or pDisplay plasmids (as a control) using lipofectamine (Invitrogen). Forty-eight hrs posttransduction, cells were harvested and lysed in 50 µl 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate in 50 mM Tris-HCl, pH 8, with 0.5 µl protease inhibitor (Roche, Indianapolis, IN). The protein concentration of cell lysate was measured using the Bradford reagent (BIO-RAD, Hercules, CA) and 20 µg were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with molecular weight standards, as described[22]. After transferring proteins onto nitrocellulose membranes, blots were stained with Ponceau to verify even protein loading. The endogenously biotinylated proteins as well as the BAP-fusions, BAP-TM (MW 22 kDa), 14BAP-TM (11 kDa), or MR1-BAP (60 kDa) were visualized with streptavidin-horseradish peroxidase conjugate (streptavidin-HRP, Molecular Probes). Blots were also probed with antibodies against either HA epitope (Roche Diagnostics, Indianapolis, IN; to normalize for transduction efficiency), β-tubulin (Sigma, St Louis, MO) or holocarboxylase synthetase (HLCS; Novus Biologicals, Littleton, CO) followed by a secondary antibody-HRP conjugate (Molecular Probes). ECL Reagent (Pierce Biotech, Rockford, IL) was used to develop all blots.

FACS analysis

Cells were transduced with pBAP-TM or p14BAP-TM alone or together with phBirA, psshBirA, or psshBirA-KDEL. A plasmid expressing the enhanced green fluorescence protein under control of CMV promoter was co-transfected with p14BAP-TM construct to normalize for transfection efficiency. Forty-eight hours post-transduction, cells were incubated with anti-biotin antibody conjugated to allophycocyanin (APC; Miltenyi Biotec) diluted 100x in PBS for 5 min at room temperature. Cells were then washed, resuspended in PBS, and analyzed with a FACS-Calibur cytometer (Becton Dickinson, San Jose, CA).

In vivo tumor model. All animal studies were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital and were performed in accordance to their guidelines and regulations. Gli36 human glioma cells were infected with lentivirus vectors carrying the expression cassette for GFP (control), BAP-TM or both BAP-TM and sshBirA. One million of these cells (in 50 µl in PBS) were mixed with same volume of Matrigel and implanted subcutaneously at 3 different sites. Two weeks later, mice (n=5) were intravenously injected with 70 nmol/kg body weight (with respect to the fluorophore) of Streptavidin-Alexa750 conjugate. Twenty-four hours later, coronal fluorescence-mediated tomography (FMT) images were acquired using a continuous wave-type scanner capable of acquiring transillumination, reflectance, and absorption data (VisEn Medical). Prototypes of the device and reconstruction algorithms[23,24] as well as the imaging protocol25 have been described previously.

Results

Expression and biotinylation levels of endogenously biotinylated proteins

To check for the expression level and the biotinylation state of the carboxylases and decarboxylases among different mammalian cell types, SDS-polyacrylamide gel electrophoresis followed by western blot analysis was performed on lysates from different cell lines including a variety of tumor cell lines and fibroblast. Detection with streptavidin-horseradish peroxidase conjugate (streptavidin-HRP) revealed different band intensities for the endogenous biotinylated proteins which could be detected among different cell lines showing that their expression and/or biotinylation varies among different mammalian cell

types (Fig. 1a). To corroborate these results with the expression of the endogenous mammalian biotin ligase in these cells, the same blot was probed with an antibody against HLCS followed by a secondary antibody conjugated to HRP (Molecular Probes), which revealed that HLCS expression also varied among different mammalian cell types (Fig 1a). These findings suggest that metabolic biotinylation of BAP sequences incorporated into different proteins would also vary from cell to cell with different efficiency depending on HLCS expression and the cellular compartment of the protein.

Metabolic biotinylation of proteins incorporating bacterial BAP is not efficient and is enhanced by co-expression of bacterial biotin ligase

Recently, we have described a biotinylated surface reporter which consists of the prokaryotic PSTCD BAP (129 aa) placed between an N-terminal signal sequence followed by an HA-tag and the transmembrane domain (TM) of the platelet derived growth factor receptor (BAP-TM, 22 kDa)[10]. This reporter was shown to be biotinylated by mammalian HLCS, to display biotin on the cell surface, and to be effective in tracking cells expressing it *in vivo* with agents coupled to streptavidin. To determine the efficiency of metabolic biotinylation of the BAP-TM reporter in different mammalian cell types, cells were infected with a lentivirus vector expressing the BAP-TM reporter and eGFP separated by an internal ribosome entry site (IRES) under the control of the cytomegalovirus (CMV) promoter (lenti-BAP-TM)[10]. As a control, cells were infected with similar vector carrying the eGFP expression cassette only. FACS analysis of cells expressing the BAP-TM reporter/eGFP and labeled with anti-biotin antibody conjugated to allophycocyanin (APC) revealed marked labeling of the cells expressing the biotinylated reporter and not the control cells (Fig. 1b–d). Interestingly, the mean fluorescence intensity (MFI) of cells expressing BAP-TM varied among the different mammalian cell lines (Fig. 1b). This supports the hypothesis that levels of metabolic biotinylation of BAP-protein fusions in mammalian cells are cell-type specific and usually not very efficient. In order to enhance the metabolic biotinylation efficiency of BAP-TM, a lentivirus vector was generated containing the codon-optimized bacterial biotin ligase cDNA (hBirA, 35 kDa)[20] preceded by a signal sequence (sshBirA) which directs hBirA to the secretory pathway followed by IRES-mCherry. Different mammalian cells expressing BAP-TM were transduced with this vector or similar vector expressing mCherry (control) and analyzed by western blot and FACS as above. Indeed, over-expression of the hBirA in the secretory pathway enhanced the biotinylation of BAP-TM reporter in all cells, up to 10-fold, as detected by both western blot using streptavidin-HRP and FACS analysis with anti-biotin-APC (Fig. 1a–d). The enhanced biotinylation in the presence of sshBirA is more evident with FACS analysis since some cell lines (e.g. U251, U20S, Gi36) had very high transduction efficiency with lentivirus vectors giving a saturation signal by western blot analysis upon detection with streptavidin-HRP even after 1 second exposure of the membrane. Further, detection of the HA-tag (fused to BAP-TM) using specific antibody, as a marker for transduction efficiency, showed similar band intensities in the presence or absence of sshBirA (Fig. 1a). These results show that although metabolic biotinylation of bacterial BAP-containing proteins in mammalian cells is not efficient, it can be increased by co-expression of bacterial biotin ligase.

Biotinylation of proteins in different cellular compartment is enhanced by directing the bacterial biotin ligase to these compartments

To check for the effect of co-expressing the bacterial biotin ligase in different cellular compartments on the biotinylation efficiency of BAP-containing proteins that are not targeted for the plasma membrane, coding sequences for a single-chain antibody fragment, MR1(scFv), with specific binding to epidermal growth factor receptor-vIII (EGFRvIII)[19] were fused to the 129 aa PSTCD BAP, in-frame, at the 3’ end to generate pMR1-BAP (60 kDa). 293T cells were transfected with pMR1-BAP plasmid alone or together with a

plasmid expressing a biotin ligase with an HA-tag that was directed to either the cytoplasm (phBirA), the endoplasmic reticulum (ER; psshBirA-KDEL) or the secretory pathway (psshBirA). Forty-eight hours post-transfection, cell lysates were analyzed for biotinylation efficiency by western blotting with streptavidin-HRP. As hypothesized, this antibody fusion protein (MR1-BAP, 60 kDa) was not biotinylated efficiently by the mammalian biotin ligase alone (Fig. 2a). When this antibody-BAP was co-expressed with the cytoplasmic hBirA, its biotinylation efficiency increased relatively (2–3 fold). However, consistent with this antibody-BAP being secreted, co-expression of either the secreted or ER-directed ligase increased its biotinylation efficiency dramatically (approximately 10-fold) (Fig. 2a). Interestingly, as observed by western blotting, expression of the bacterial biotin ligase did not have an effect on the biotinylation of endogenous carboxylases and decarboxylases normally biotinylated in mammalian cells, demonstrating that the bacterial biotin ligase did not recognize the mammalian BAP within these enzymes (Fig. 2b)

Co-expression of the bacterial biotin ligase biotinylates small 14 aa BAP efficiently

In order to check for the smallest size of BAP compatible with biotinylation, a new construct was generated in which the 129 aa BAP in the BAP-TM reporter was replaced with a 14 aa BAP (Avidity, 14BAP-TM, 11 kDa) which is known to be biotinylated by bacterial biotin ligase in bacteria and mammalian cells[26]. This new fusion protein was expressed in mammalian cells and, as expected, was not biotinylated by endogeneous HLCS (Fig. 2c). However, when the bacterial biotin ligase directed to the secretory pathway was co-expressed with 14BAP-TM, this fusion protein was efficiently biotinylated, as observed by western blotting analysis with streptavidin-HRP conjugate, proving that a very short BAP sequence can be used for metabolic biotinylation within a cellular compartment in mammalian cells. As an additional conformation, the same 11 kDa band was also detected with a combination of an anti-HA antibody and a secondary antibody-HRP conjugate in both lanes containing lysates from cells transfected with 14BAP-TM or with both 14BAP-TM and sshBirA. Another HA-tagged band of 35 kDa was also observed in the lane containing lysates from sshBirA expressing cells confirming its expression (Fig. 2c).

To check for the effect of co-expressing cytoplasmic, ER-targeted or secreted biotin ligases on the biotinylation of cell surface 14BAP-protein fusions, 293T cells were co-transfected with p14BAP-TM alone or together with phBirA, psshBirA and psshBirA-KDEL and analyzed by FACS after staining with anti-biotin-APC conjugate (Fig. 2d). As expected, co-expressing the cytoplasmic hBirA showed little biotinylation of 14BAP-TM and hence little staining by FACS. When the hBirA was directed to ER (sshBirA-KDEL) or to the secretory pathway (sshBirA), the staining was enhanced greatly (up to 10-fold with no difference between sshBirA and sshBirA-KDEL) suggesting that metabolic biotinylation of secreted or cell-surface BAP-protein fusions is optimized by co-expression of a biotin ligase directed to the same compartment where the fusion protein is being directed.

In vivo imaging of metabolically biotinylated cell surface reporter is enhanced upon co-expression of secreted biotin ligase

We have shown that BAP-TM reporter is useful to track cells expressing it *in vivo*[10]. To corroborate the *in vitro* findings in an *in vivo* model, Gli36 human glioma cells expressing either GFP (control), BAP-TM or both BAP-TM and secreted sshBirA were implanted subcutaneously in nude mice in three different locations. Two-weeks later, mice were intravenously (i.v.) injected with streptavidin-Alexa750 conjugate and fluorescence-mediated tomography (FMT) imaging was performed to quantify the accumulation of the fluorescent conjugate in the different tumor implants. Tumors expressing the BAP-TM showed higher binding/uptake of the fluorophore as compared to control tumors. Moreover, tumors expressing both BAP-TM and sshBirA showed significantly higher accumulation of

the fluorophore as compared to BAP-TM tumors (>5-fold; p=0.001), confirming our *in vitro* findings (Fig. 3). These results reveal that *in vivo* imaging using metabolically biotinylated cell surface receptors is enhanced by co-expression of the bacterial biotin ligase in the secretory pathway.

Discussion

Metabolic biotinylation of BAP-protein fusions have been used in many different fields ranging from protein purification and trafficking to vector purification and targeting as well as non-invasive *in vivo* tumor tracking. The work presented here provides insight into conditions for the optimization of metabolic biotinylation of BAP-protein fusions in mammalian cells. It shows that the expression levels of mammalian biotin ligase, holocarboxylase synthetase as well as the metabolic biotinylation of endogenous carboxylases and decarboxylases and exogenous BAP-protein fusions vary among mammalian cells. Co-expression of the bacterial biotin ligase enhances the biotinylation of BAP-protein fusions in mammalian cells for both 14 aa and 129 aa PSTCD BAPs, especially when directed to the cellular compartment where the fusion protein is targeted. Further, *in vivo* imaging of cells expressing biotinylated surface reporter was significantly enhanced upon co-expression of the bacterial biotin ligase in the secretory pathway. Bacterial biotin ligase did not recognize and hence did not increase the biotinylation of the biotin acceptor sequences within the human carboxylases and decarboxylases normally biotinylated in mammalian cells.

The specificity of the bacterial biotin ligase for the BAP sequences provides a unique way to study cellular biological processes. This methodology could theoretically be applied to any cellular protein irrespective of its cellular compartment and thereby provide an easy and universal means to purify biotinylated protein expressed in mammalian cells with commercially available reagents, such as monomeric avidin beads, and to bring down associated proteins. This strategy can eventually encompass many different proteins in a variety of locations within the cell or secreted from it. The extremely high and stable interaction of biotin with streptavidin makes this system advantageous over genetically encoded tags for antibodies by enabling detection of the label with higher sensitivity and allowing more stringent washing that can increase the specificity and decrease the background interference. This labeling methodology also provides a sensitive means of tracking proteins within living cells during synthesis and processing and under different physiologic conditions, as well as tracking cell movement, proliferation and death *in vivo*.

Recently, a method for labeling surface receptors was developed using exogenous recombinant biotin ligase to biotinylate surface proteins which had been engineered to incorporate BAP sequences[27,28]. In this case, a 14 aa BAP was incorporated into a transmembrane receptor for expression in mammalian cells and then cultured cells were incubated with extracellular recombinant biotin ligase, ATP and free biotin. Although this system has the advantage of using a small 14 aa BAP with great potential for discrete labeling of surface proteins in cultured cells, it would be difficult to use *in vivo* since this BAP is not biotinylated by the endogenous biotin ligase and it would be difficult to administer biotin ligase and ATP to animals/humans. As shown here, co-expressing the bacterial biotin ligase with the 14BAP-TM reporter has the advantage that the cell surface receptors are metabolically biotinylated endogenously during biosynthesis which can be detected in culture as well as *in vivo* after injection of imaging agents coupled to avidin or streptavidin[10].

Optimized metabolic biotinylation in combination with the strong interaction of biotin to avidin or streptavidin provides a unique platform for the development of more efficient

targeting strategies and can be applied to many different fields including vector purification, monitoring of protein fate, investigation of cell biologic events, targeting of therapeutic agents, and monitoring of tumor growth and regression *in vivo*.

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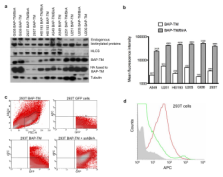

Figure 1. Efficiency of metabolic biotinylation in mammalian cells

Different mammalian cell lines were transduced with lentivirus vectors to express BAP-TM/GFP or both BAP-TM/GFP and sshBirA. (**a**) cells were lysed and analyzed by western blotting using streptavidin-HRP to detect endogenous biotinylated proteins and BAP-TM reporter, as well as anti-HA antibody (detecting HA fused to BAP-TM) and tubulin for normalization. (**b–d**) viable cells were stained with anti-biotin-APC antibody and analyzed by FACS for both APC and GFP. Data presented as mean ± SD (n=3). (**c–d**) Representative FACS analysis results for 293T cells.

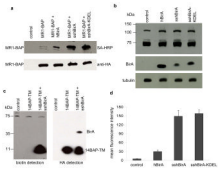

Figure 2. Metabolic biotinylation of secreted and cell surface BAP-protein fusions in mammalian cells

(**a**) 293T cells were transfected with pMR1-BAP with and without hBirA, sshBirA or sshBirA-KDEL. 48 hrs later, cells were lysed and analyzed by western blotting for efficiency of biotinylation with streptavidin-HRP. The same blot was also detected with anti-HA and secondary-HRP to normalize for transfection and loading efficiency. (**b**) Cells were transfected with either phBirA, psshBirA and psshBirA-KDEL and 48 hrs later, cells were lysed and analyzed by western blotting with either streptavidin-HRP for detection of endogenous biotinylated proteins or anti-HA antibody for detection of biotin ligases or anti-β-tubulin antibody for normalization. **(c)** Cells were transfected with either p14BAP-TM or with p14BAP-TM and sshBirA and analyzed by western blotting with streptavidin-HRP for biotin detection (left) and with a combination of anti-HA antibody and a secondary-HRP conjugate (right). (**d**) Cells were transfected with p14BAP-TM alone or together with h either cytoplasmic phBirA, secreted psshBirA or ER-directed psshBirA-KDEL. Live cells were labeled with anti-biotin-APC conjugate and analyzed by FACS for both GFP and APC. Relative mean fluorescence intensity is plotted against the different conditions of transfection. Data presented as mean ± SD (n=3).

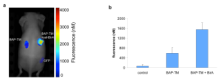

Figure 3. *in vivo* imaging of metabolically biotinylated surface reporter

Gli36 human glioma cells expressing either GFP (control), BAP-TM or both BAP-TM and sshBirA were implanted subcutaneously in nude mice. Two weeks later, mice were i.v. injected with streptavidin-Alexa750 conjugate and imaged using fluorescence-mediated tomography. (**a**) The FMT image is displayed with the fluorescence signals superimposed with the grayscale planar excitation light image of the mouse. (**b**) Quantitation of tumor-associated streptavidin-fluorochrome levels in (a). Data presented as mean ± SD (n=5).