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ScienceDirect
Protein Expression and Purification 52 (2007) 320–328
Protein Expression & Purification
www.elsevier.com/locate/yprep

A versatile system for site-specific enzymatic biotinylation and regulated expression of proteins in cultured mammalian cells

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Received 25 August 2006, and in revised form 19 September 2006
Available online 29 September 2006

Abstract

We have developed a system for producing biotinylated recombinant proteins in mammalian cells. The expression construct consists of an inducible tetracycline response element (TRE) that drives expression of a bicistronic cassette comprising a biotin acceptor peptide (BioTag) fused to either terminus of the target protein, the gene for *Escherichia coli* biotin ligase (BirA), and an intervening internal ribosome entry site (IRES). By either transient or stable transfection of Chinese hamster ovary (CHO) Tet-On cells, we successfully expressed, detected, and immobilized biotinylated human Itch, a pleiotropic multi-domain ubiquitin–protein ligase, as well as Gla-RTK, a putative vitamin K-dependent receptor tyrosine kinase. The biotinylation of recombinant Itch in transiently transfected CHO Tet-On cells required biotin supplementation and coexpression of BirA, occurred quantitatively and specifically on the lysine residue of the BioTag, and enabled detection of Itch by Western blot in as little as 10 ng of total lysate protein. Stably selected clones were rapidly pre-screened for doxycycline (dox)-inducible BirA expression by ELISA, and subsequently screened for dox-inducible expression of biotinylated Itch. Biotinylated Gla-RTK was detectable in as little as 5 ng of total lysate protein from transiently transfected CHO Tet-On cells, and exhibited pronounced tyrosine phosphorylation. In stable clones however, constitutive phosphorylation was prevented by reducing the expression level of Gla-RTK through the titration of dox. These results demonstrate the utility of this system for the expression of 'difficult' proteins, particularly those that are cytotoxic or those that may require lower expression levels to ensure appropriate post-translational modification.

Keywords: Biotin; Avidin; Streptavidin; Ubiquitin–protein ligase; Receptor tyrosine kinase; Doxycycline-regulated expression

The extraordinarily high affinity of biotin for avidin and streptavidin has been widely exploited in biological research, most commonly for the immunological detection and selective adsorption of proteins. The extremely tight binding of biotin to (strept)avidin ($K_d = 10^{-15}$ M) [1] is driven by an off-rate on the order of days [2], and represents the strongest non-covalent intermolecular interaction known. A wide variety of reactive biotin derivatives are commercially available, allowing the chemical conjugation of biotin and its derivatives to specific types of reactive groups in proteins, carbohydrates and nucleic acids. An array of (strept)avidin derivatives are also available, including conjugates to reporter enzymes, fluorophores, and solid supports such as chromatographic resins, microplate surfaces, magnetic beads, and semiconductor nanoparticles.

Given the widespread use of biotin and its derivatives, the basic biology of biotin is often overlooked. Biotin (vitamin H) is an essential cofactor for metabolic carboxyl transfer reactions, and is utilized by all life forms from archaebacteria to complex metazoans. *Escherichia coli* possesses only a single biotin-dependent enzyme, the acetyl-CoA carboxylase complex, where biotin is found covalently attached its biotin carboxyl carrier protein (BCCP)[1] subunit within a region known as the biotinyl domain [3].

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[1] *Abbreviations used:* BCCP, biotin carboxyl carrier protein; TRE, tetracycline response element; IRES, internal ribosome entry site; MCS, multiple cloning sites; SA, streptavidin; HRP, horseradish peroxidase; RTK, receptor tyrosine kinase.

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doi:10.1016/j.pep.2006.09.011

Biotin is coupled via an amide bond to the ε-amino group of a specific lysine residue within the biotinyl domain by the biotin–protein ligase BirA.

Heterologous expression systems emulating such a 'metabolic' approach offer distinct advantages over chemical biotinylation of target proteins. Recombinant biotinyl domain fusion proteins can be biotinylated in living cells without the use of specialized reagents and without non-specific and heterogeneous biotinylation at sites other than the acceptor lysine of the biotinyl domain. Biotinylated chimeras consisting of proteins fused to a 75 residue bacterial biotinyl domain have been produced in both *E. coli* and yeast, demonstrating cross-species specificity of the bacterial biotinyl domain and the endogenous yeast biotin ligase [4]. The use of this approach was expanded to include higher eukaryotic hosts such as cultured mammalian cells and transgenic mice [5]. However, some features of this approach were less than ideal, including the introduction of a bulky (>75 residue) biotinyl domain [5] and incomplete biotinylation of the target protein [6]. The first of these issues was resolved by the successful screening of random peptide libraries for small peptides capable of accepting biotin from *E. coli* BirA [7], and the subsequent determination of a minimum peptide substrate (BioTag) consisting of only 14 residues [8]. The BioTag can serve as a substrate for biotinylation whether fused to the amino or carboxy terminus of the protein of interest [9].

The BioTag sequence is not biotinylated when expressed in eukaryotic cells, necessitating co-expression of *E. coli* BirA and the target protein from separate vectors [10], from two genes on the same vector [11], or from a single bicistronic expression construct [12]. Of these three strategies, the bicistronic approach offers distinct advantages. First co-expression of both the target protein and BirA is directed by a single plasmid, allowing production of a biotinylated target protein using a single transfection. Second, the coordinated expression of both proteins allows for the rapid pre-screening of stably selected cells by an ELISA designed to detect BirA antigen as a surrogate marker for the protein of interest. This feature is particularly useful when screening a large number of stably selected clones, especially when antibodies directed against the protein of interest are unavailable.

An additional consideration in the design of this expression system was the choice of promoter. Most commercially available mammalian expression vectors utilize viral promoters that direct high-level constitutive expression. However, in some instances they may be unsuitable for proteins that undergo complex post-translational maturation involving potentially rate-limiting processes such as chaperone-mediated folding, compartmentalization, vesicular sorting, limited proteolysis, disulfide formation, glycosylation and a host of other residue-specific modifications. In addition, high-level constitutive expression is incompatible with the generation of stable cell lines that express a cytotoxic target protein, since production of the target protein may cause cell death or senescence during the selection procedure. These issues can often be circumvented by the use of a regulated promoter. On the one hand, the expression of a complex or heavily modified target protein can be adjusted to a level commensurate with the host cell's biosynthetic capacity. On the other, stable selection of cells capable of expressing a cytotoxic target protein can be achieved by performing the selection with the promoter maintained in the silent state [13,14]. We designed our expression system with these considerations in mind, placing a bicistronic expression cassette under control of tetracycline response element (TRE) [15] to allow coordinated expression of both the protein of interest and BirA in response to the tetracycline derivative, doxycycline (dox).

We expressed two different proteins to demonstrate the general applicability of our system. The first of these, Itch, is a cytoplasmic multi-domain ubiquitin–protein ligase. Previous attempts in our laboratory to establish stable cell lines expressing Itch were unsuccessful, suggesting that supraphysiological expression of Itch is cytotoxic. The second target protein expressed in this study, γ-carboxyglutamic acid-containing receptor tyrosine kinase (Gla-RTK), serves as an example of a post-translationally modified integral membrane protein. Gla-RTK was previously identified in the sea squirt, and is predicted to consist of an extracellular vitamin K-dependent γ-carboxyglutamic acid-containing module (the Gla domain), a single-pass transmembrane region, and a cytoplasmic tyrosine kinase domain [16].

Materials and methods

Reagents

Vitamin K_1 was from Abbott Laboratories (Abbott Park, IL). Rabbit anti-Itch polyclonal antibodies were from Abgent (San Diego, CA). ECL Plus kit and PD-10 columns were from Amersham Biosciences (Piscataway, NJ). Immun-Blot polyvinylidene fluoride (PVDF) membrane and Affigel-10 and -15 affinity resins were from Bio-Rad (Hercules, CA). Mouse anti-phosphotyrosine monoclonal antibody P-Tyr-100 was from Cell Signaling Technology (Beverly, MA). Plasmids pTRE2pur, pIRES and pACT2, puromycin, tetracycline-free fetal bovine serum, and a Matchmaker adult human brain cDNA library were from Clontech (Mountain View, CA). Prestained molecular weight markers were from Fermentas (Hanover, MD). Plasmids pcDNA3.1/Zeo(+) and pCR2.1-TOPO, a TOPO TA cloning kit, Lipofectamine Plus reagent, Dulbecco's modified Eagle's medium, OPTI-MEM I, penicillin, streptomycin, G418, 2X SDS–PAGE loading buffer, and Novex 4–20% Tris–glycine pre-cast gels were from Invitrogen (Carlsbad, CA). PolySorp microplates were from Nalge Nunc International (Rochester, NY). Restriction enzymes were from New England Biolabs (Beverly, MA). Immobilized avidin, streptavidin–horseradish peroxidase (SA–HRP), goat anti-mouse IgG-HRP, bicinchoninic acid (BCA) protein