In Vivo Biotinylation of the Major Histocompatibility Complex (MHC) Class II/Peptide Complex by Coexpression of BirA Enzyme for the Generation of MHC Class II/Tetramers

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ABSTRACT: Success in generation of major histocompatibility complex (MHC) tetramer relies on application of a key technique, biotinylation of MHC molecule specifically on a single lysine residue using the BirA enzyme. However, in vitro biotinylation of MHC-BSP (BirA enzyme substrate peptide) fusion protein using BirA enzyme is laborious and is prone to loss of target proteins to unacceptable levels. To circumvent this problem, an in vivo biotinylation strategy was developed where the BirA enzyme was coexpressed with target protein, HLA-DR2/BSP/MBP, in an insect cell expression system. Bacterial BirA enzyme expressed in Drosophila melanogaster 2 (D. Mel-2) cell lines was biologically functional and was able to biotinylate secretory target protein (on specific lysine residue present on the BSP tag). Biotinylation efficiency was maximized by providing exogenous d-biotin in the culture medium and optimization of the expression vector ratios for cotransfection. By limiting dilution cloning, a clone was identified where the expressed DR2BSP/MBP protein was completely biotinylated. DR2BSP/MBP protein expressed and purified from such a clone was ready to be tetramerized with streptavidin to be used for staining antigen-specific T cells.


KEYWORDS: in vivo biotinylation; BirA enzyme; MHC class II tetramer; expression of MHC class II/peptide complex

INTRODUCTION
The introduction of peptide–major histocompatibility complex (MHC) tetramer technology has revolutionized identification and enumeration of antigen specific T cells [1]. The success of these techniques rests in the increasing avidity of soluble MHC/peptide complex (monomer) and T cell receptor (TCR) by tetramerization of biotinylated MHC/peptide monomer with avidin or streptavidin. The key technique in production of MHC/peptide tetramer relies on the biotinylation of MHC/peptide complex monomer on a single lysine residue. This is normally accomplished by using bacterial BirA enzyme to biotinylate the lysine residue on an enzyme-specific recognition tag called BirA substrate peptide (BSP) [2] fused at the C-terminus of one of the MHC polypeptides. Biotinylation with BirA enzyme at single lysine residue solves several problems associated with traditional chemical-mediated biotinylation method, including random and heterogeneous modification, inactivation of protein biological function, cross-linking, and aggregation after mixing with streptavidin or avidin [3]. This also enables

ABBREVIATIONS

BSP BirA enzyme substrate peptide
HLA human leukocyte antigen
MBP myelin basic protein
MHC major histocompatibility complex
TCR T cell receptor

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the proper orientation of these complexes for binding to the TCRs. Technically, this enzyme-mediated in vitro biotinylation process is effective and efficient [1, 4]. However, it is generally cumbersome, expensive, and very often results in severe loss of proteins during the buffer exchange and protein concentration steps, which are required for enzyme reaction and final removal of free biotin from the reaction (NIAID Tetramer Core Facility Protocols available at: http://www.emory.edu/WHSC/TETRAMER/pdf/Protocols.pdf). Therefore, elimination of this in vitro biotinylation process could greatly simplify the production of MHC tetramers. Unlike production of MHC class I/peptide monomers, MHC class II/peptide complex monomers are usually expressed as biological functional proteins in insect expression system [4–6]. Therefore, it provides opportunity to biotinylate MHC class II/peptide complex monomers in vivo. Previously, a few studies have demonstrated that proteins fused with biotinylation site could be biotinylated in vivo either by endogenous BirA enzyme in the Escherichia coli system or by cotransfection of the BirA enzyme in mammalian cell system [7, 8]. Although the biotinylation efficiency of the expressed fusion protein is low [7] or not determined [8], these studies indicated that our hypothesis is feasible.

BirA enzyme is a component of biotin holoenzyme localized in the E. coli cytoplasm [9]. Part of the function of this enzyme is to mediate the attachment of biotin molecule to lysine residue that resulted in the formation of an amide linkage between the carboxyl group of biotin and the ε-amino group of the lysine residue [10]. This posttranslational modification has exquisite specificity. Only one lysine residue in the birA protein of Streptococcus equi (Sa) is biotinylated by BirA enzyme in all proteins expressed in E. coli [11]. The minimum peptide lengths of BirA enzyme recognition site in the birA protein were identified as about 75–105 aa [12]. By use of the library-screening technique, a series of short artificial BirA substrate peptides now known as BSP has been also created [2, 12]. Some of these BSPs even have a superior biotinylation kinetic than the biotin carboxyl carrier protein in vitro [12]. Because most MHC class II/peptide complexes were expressed in insect cell system [4, 13–16], we postulated that MHC class II/peptide complex attached with BSP tag could be biotinylated in vivo with coexpression of BirA enzyme. By using an HLA-DRA1*0101-Fos-BSP expression vector (designated as p18BirA vector and expressed a cytosolic form of BirA enzyme. A modified BirA gene with HLA-DR β chain leader sequence (MYCLKLPGGSCMTALTVTMLVSSLASPGDGTG) encoding region was fused in frame at the N-terminus of BirA enzyme and cloned into pRmHa3 vector. This vector was designated as p21BirA vector and expressed secretion form of BirA enzyme in insect cells. An HLA-DR2/mylin basic protein peptide (amino acid 85–99, MBP85–99) complex was used as a model molecule for the in vitro biotinylation study. The HLA-DR2/MBP85–99 expression vectors, pRmHa3-DRA1*0101-Fos-Fc and pRmHa3-DRB1*1501-Jun (designated as pDRB3/MBP for short), were obtained from Dr. Kai W. Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA). The pRmHa3-DRA1*0101-Fos-Fc vector was modified as pRmHa3-DRA1*0101-Fos-BSP expression vector (designated as pDRA-BSP) by replacing Fc encoding region with a 15 aa, GLNDIFEAQKIEWHE, BirA substrate peptide (BSP) [2, 12] encoding gene. The sequences of fusion genes were confirmed by DNA sequencing.

Transfection of Cells With Expression Vectors and Establishing Stable Expression Cell Lines

Two million Drosophila melanogaster 2 cells (D. Mel-2; Life Technologies, Grand Island, New York) were seeded in a 12-well plate. Cells were cultured at 28°C overnight and washed with 2 ml Drosophila serum-free medium (SFM; Invitrogen, Carlsbad, CA). The transfection mixture was prepared by mixing 250 μl SFM containing 2 μg DNA of expression vectors and 250 μl SFM containing 8 μl of CELLfectin reagent (Life Technologies). The mixture was allowed to react for 20 minutes at room temperature, then mixed with 0.8 ml SFM and overlaid on washed cells. After incubation at 28°C for 3–4 hours, the transfection mixture was replaced with 2 ml of fresh SFM and incubation was continued. For transient expression, 24 hours after transfection, the cells were induced with 1 μM CuSO4 in culture medium for additional 72
hours. Supernatants were tested for the expression of target protein. To establish stable expression cell lines, 24 hours after transfection the cells were treated with 25 μg/ml of blasticidin (Invitrogen). The blasticidin treatment was continued for 2 weeks with the change of drug selection medium every 4–5 days until stable drug-resistant cell lines were established.

To isolate individual expression clones, stable cell line was diluted with irradiated (20,000 rads) parental cell line as feeder cells (1 × 10^6 cells/ml) and seeded in 96-well plates at 10 cells/well, 3 cells/well, and 1 cell/well, respectively. The cells were grown in SFM containing 25 μg/ml of blasticidin for 1–2 weeks until clones could be spotted in the plates. Individual clones were transferred to a new plate and expanded.

**Expression and Purification of HLA-DR2BSP/MBP**

Stable transfectants were expanded in *Drosophila*-SFM containing 25 μg/ml blasticidin in conical flasks at 27°C 140 rpm in a rotary shaker. The cells were split at ratio of 1 to 5 when the density reached 2 × 10^9 cells/ml. A total of 800 ml of cell culture at density of 10^7/ml were induced with 1 mM CuSO_4 and 2 μg/ml of d-biotin for 4 days in a 2-1 flask. The cells and debris were removed by centrifugation at 10,000 g for 20 minutes. Supernatant was further clarified by filtration through 0.2-μm filter. The clarified medium was passed through a 2.5-ml bed volume of LB3.1 monoclonal antibody (ATCC, Manassas, VA) affinity column at a flow rate of 0.5 ml/min at 4°C. After the column was washed with 10 bed volumes of phosphate-buffered saline (PBS), pH 7.4, the HLA-DR2BSP/MBP was eluted out with 0.1 M glycine, pH 11.5, and immediately neutralized with 1 M Tris-HCl, pH 6.8. The fractions containing proteins were determined by reading absorbance at optical density 280 nm. The fractions with higher absorbance at 280 nm were pooled and buffer-exchanged with PBS by dialyzing. The protein was concentrated to 1–2 mg/ml by centrifugation with a 10-kDa molecular weight cutoff Centricon (Millipore, Billerica, MA).

**Western Blot Analysis**

After induction, 0.2 ml of culture supernatants from each transfection was immunoprecipitated with 20 μl of LB3.1 monoclonal antibody conjugated Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ) for 4 hours. The beads were washed with PBS three times and resuspended in 25 μl of 1× NuPAGE LDS sample buffer (10% glycerol, 141 mM Tris base, 106 mM Tris HCl, 2% LDS, 0.51 mM EDTA, 0.22 mM SERVA Blue G250 and 0.175 mM phenol red, pH 8.5; Invitrogen). The samples were incubated at 70°C for 10 minutes and pelleted by brief centrifugation. Twenty milliliters of supernatant was loaded on a 4%–12% NuPAGE Bis-Tris gel and separated in NuPAGE MES running buffer (50 mM MES, pH8.5, 50 mM Tris base, 0.1% SDS, and 1 mM EDTA) (Invitrogen) by electrophoresis. The separated proteins were transferred onto a PVDF membrane (Millipore) and blocked with 5% skim milk for 2 hours at room temperature. The membrane was incubated in 10 ml 0.2 μg/ml streptavidin conjugated with horseradish peroxidase (SA-HRP; Molecular Probes, Eugene, OR) and washed three times with PBS with 0.05% (v/v) Tween-20. The biotinylation of HLA-DR2BSP/MBP by BirA enzyme was detected by enhanced chemiluminescence (Amersham Biosciences). Expression of HLA-DR β chain was detected with HRP-conjugated anti-β chain monoclonal antibody TDR31.1 (Ancell, Bayport, MN).

**Tetramerization of HLA-DR2BSP/MBP and Gel Filtration Analysis**

Purified biotinylated HLA-DR2BSP/MBP was incubated with one-tenth of its molar amount of streptavidin for 30 minutes. This procedure was repeated 10 times. The reaction mixture was buffer-exchanged with PBS and passed through gel filtration column (Superose 6, 1.6 × 30 cm; Amersham Pharmacia) equilibrated with PBS on GP-250 FPLC instrument (Amersham Biosciences). For T cell staining, purified biotinylated HLA-DR2BSP/MBP was tetramerized with phycoerythrin-conjugated streptavidin (Molecular Probes).

**Development of T Cell Line and Tetramer Staining**

Five million human peripheral blood lymphocytes from a healthy donor (HLA-DRA1*0103, HLA-DRB1*1501) were stimulated with 20 μg/ml of influenza hemagglutinin peptide 306–318 (HA 306–318; Sigma-Aldrich, St. Louis, MO) in RPMI 1640 with 10% human serum and 50 units/ml of human interleukin-2 for 16 days in a 24-well plate. One million T cells was stained with 20 μg of HLA-DRB1*0103/HA 306–318-PE tetramer at 37°C for 90 min and with anti–CD4-FITC at room temperature for 20 minutes, and analyzed by flow cytometry.

**RESULTS**

**Biotinylation of HLA-DR2BSP/MBP Monomer on BSP Tag In Vivo**

In order to biotinylate HLA-DR2BSP/MBP *in vivo*, the BirA gene from *E. coli* K12 strain was cloned into insect cell expression vector under the control of the metallothionine promoter. *In vivo* biotinylation of HLA-DR2BSP/MBP was conducted by cotransfection of pDRA-BSP vector, pDRB/MBP vector, and BirA enzyme expression vector p18BirA into D. Mel-2 cells. HLA-DR2BSP/MBP was biotinylated by cytosolic BirA.
enzyme. Addition of exogenous d-biotin in the culture medium improved the biotinylation efficiency in a dose-dependent manner. The highest efficiency of biotinylation was achieved with the addition of 2 μg/ml of biotin in the medium (Figure 1A). Further increasing d-biotin inhibited the BirA enzyme activity.

Because the HLA-DR2BSP/MBP was a secretion protein (i.e., translation and maturation of the protein compartmentalized in the lumen of endoplasmic reticulum), the location of the expression of the BirA enzyme [9, 11, 18, 19] could influence the level of biotinylation on HLA-DR2BSP/MBP protein. An additional expression vector was constructed: p21BirA vector expressing secretion form of BirA enzyme under the direction of HLA DR β chain leader peptide. HLA-DR2BSP/MBP expression vectors were cotransfected with either p18BirA vector or p21BirA vector into D. Mel-2 cell lines. As shown in Figure 1B,C both forms of BirA enzyme could biotinylate HLA-DR2BSP/MBP proteins, the cytosolic BirA enzyme had a higher biotinylation efficiency than the secretion form of BirA enzyme as judged by the ratios of α chain to β signals by Western blot analysis (densitometry analysis of α chain to β chain signals was 20:1 for cytosolic form BirA vs. 5:1 for secretion form BirA). Furthermore, the exogenous d-biotin required for achieving maximal biotinylation for cytosolic form BirA enzyme was 2 μg/ml, whereas for secretion form BirA enzyme it was 200 μg/ml. Therefore, cotransfection with cytosolic BirA enzyme has a higher biotinylation efficiency than secretion form BirA enzyme does in vivo.

Influence of Expression Vector Ratio on the Biotinylation and Production of HLA-DR2BSP/MBP Monomer

In order to determine the optimal expression vector ratios of cotransfection to maximize the production of target protein while maintaining the best biotinylation efficacy of BirA enzyme, two experiments were performed. (1) Cotransfection of D. Mel-2 cells was performed with fixed total amount of HLA-DR2BSP/MBP expression vectors with various amount of pBirA vector. (2) Cotransfection of D. Mel-2 was performed with fixed amount of pBirA vector and total amount of pDRA-BSP and pDRB/MBP vectors at various ratios. As shown in Figure 2A, when the plasmid DNA ratios of HLA-DRA-BSP/MBP and BirA enzyme expression vectors at 1:1 (0.2 μg of pDRA-BSP and pDRB/MBP vectors vs. 0.2 μg of pBirA vector), the maximal production of biotinylated HLA-DR2BSP/MBP was achieved. Further increasing the amount of pBirA vector reduces the production of biotinylated HLA-DR2BSP/MBP product. Figure 2B shows that the vector ratio of pDRA-BSP and pDRB/MBP for maximal production of HLA-DR2BSP/MBP protein was 1:1 (0.5 μg of vector pDRA-BSP to 0.5 μg of pDRB/MBP vector) when 1 μg of pBirA vector was cotransfected. Because the sizes of the expression vectors were very close (p18BirA, 4.9 kb; pDRA-BSP, 4.7 kb; and pDRB/MBP, 4.7 kb), the best cotransfection vector molar ratio for in vivo biotinylation and production of biotinylated HLA-DR2BSP/MBP was 2:1:1 (p18BirA: pDRA-BSP: pDRB/MBP) (Figure 2B).

Efficiency of Biotinylation of HLA-DR2BSP/MBP by Coexpression of BirA Enzyme

One critical issue is the efficiency of biotinylation of target protein being biotinylated by coexpression of BirA enzyme in vivo. To address this issue, drug-resistant stable transfection cell lines were generated by cotransfection of D. Mel-2 cells with vectors of p18BirA, pDRA-BSP, pDRB/MBP, and pCoBlast at vector ratio of 2:1:1:1, respectively. HLA-DR2BSP/MBP protein was induced and purified from one of such stably transfected cell lines. The biotinylation efficiency of protein was
evaluated by immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. As shown in Figure 3A, about 50% of the protein was depleted by streptavidin-conjugated beads. This result indicated that in vivo biotinylation efficiency of HLA-DR2BSP/MBP by BirA enzyme was about 50%. We reasoned that this could be because stable cell lines contain a mixture of clones with varying biotinylation efficiency. To isolate the clones with higher biotinylation efficiency, individual HLA-DR2BSP/MBP expression clones were isolated from stably transfected cell lines by limiting dilution. As shown in Figure 3C, among the 14 clones tested, 3 had had higher biotinylation efficiency than the parental cell line. One of the clones, clone 7 was scaled up and induced with CuSO4 for expression of the biotinylated HLA-DR2BSP/MBP. The expressed proteins were purified by LB3.1 monoclonal antibody affinity column. Immunoprecipitation followed by SDS-PAGE analysis (Figure 3B) indicated that >90% of HLA-DR2BSP/MBP protein was biotinylated in this clone. This level of biotinylation efficiency is comparable or superior to the level achieved by in vitro biotinylation method [4].

Tetramerization and T Cell Staining Activity of In Vivo Biotinylated HLA-DR2BSP/MBP

To determine whether in vivo biotinylated HLA-DR2BSP/MBP is ready for tetramerization, purified HLA-DR2BSP/MBP was mixed with one-tenth of its molar amount of streptavidin for 30 minutes and repeated 10 times. Gel filtration analysis demonstrated that the biotinylated HLA-DR2BSP/MBP was effectively tetramerized by addition of streptavidin (Figure 4). Interestingly, the apparent molecular weight of HLA-DR2BSP/MBP monomer was ~160 kDa, which was notably different to the calculated molecular weight (68 kDa). We attribute this discrepancy to the presence of leucine-zip domains that contain >30% charged residues that affect the overall mobility of HLA-DR2BSP/MBP on the Superose 6 column. The glycosylation on the HLA-DR2BSP/MBP monomer may also contribute to this disparity.

To determine the TCR binding function of in vivo biotinylated tetramer, we used the optimized biotinylation condition to produce an in vivo biotinylated empty HLA-DRB1*0103 tetramer. This time, the in vivo biotinylation efficiency was ~70% (data not shown). The empty HLA-DRB1*0103 monomer was loaded with influenza hemagglutinin peptide (HA306–318) as described previously [14] and tetramerized with PE-SA. T cells from healthy donor with HLA-DRB1*0103 allele were stimulated with HA306–318 peptides for 16 days and stained with HLA-DRB1*0103/HA306–318 tetramer. As shown in Figure 5B, in vivo biotinylated HLA-DRB1*0103/HA306–318 tetramer stained the T cells from the healthy donor.
DRB1*0103/HA_{306–318} tetramer stained 38.6% of T cells, which is comparable to 37.4% of T cell staining by the *in vitro* biotinylated HLA-DRB1*0103/HA_{306–318} tetramer (Figure 5C). *In vivo* biotinylated tetramer also has a minimum background staining, as shown in Figure 5A, only 0.1% T cells stimulated with HA_{306–318}Peptide were stained with *in vivo* biotinylated HLA-DRB1*0103 tetramer without loading with HA_{306–318}Peptide. Therefore, coexpression of BirA enzyme with MHC class II molecule is sufficient to *in vivo* biotinylate MHC class II molecules for tetramerization and staining antigen-specific T cells.

**DISCUSSION**

Multimerization of molecules is an efficient way to increase the affinity and avidity between the ligand and the receptor. The magnitude of avidity can be increased several orders after a monomer is multimerized [20, 21]. Such a strategy becomes feasible with the application of BirA enzyme, which only biotinylates a single lysine residue in particular peptide sequences [1, 2]. This enables the destined single lysine residue being biotinylated for a given fusion protein. Until now, this procedure has been performed by using BirA enzyme to biotinylate target proteins such as recombinant MHC/peptide complexes *in vitro* [1, 2, 4, 14, 16]. The target protein is buffer exchanged to remove free biotin from the reaction mixture by chromatography or extensive dialysis. Further purification may also be required if BirA enzyme needs to be removed for additional biochemical studies. Such long and complicated *in vitro* processes often result in large protein losses, which get even worse when target proteins are protease liable. We found that each dialysis process results in two- to four-fold loss of the protein and concentration of protein causes another two- to five-fold loss. Biotinylation of macromolecules *in vivo* not only saves the time and eliminates the use for BirA enzyme, but most importantly eliminates long and tedious dialysis and concentration steps for the removal of free biotin. It also avoids contamination of BirA enzyme and may decrease the chance of protein degradation introduced by BirA enzyme.

*In vivo* biotinylation by coexpression of BirA enzyme has been reported [22, 23]. However, the efficiency was not determined in these studies. In this study, we bio-

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**FIGURE 5** Comparison of the staining effects with tetramers biotinylated *in vivo* or *in vitro*. Peripheral blood lymphocytes (PBLs) from DRB1*0103 donor were stimulated with HA_{306–318}Peptide and stained with (A) no peptide-loaded HLA-DRB1*0103 tetramer, (B) *in vivo* biotinylated HLA-DRB1*0103/HA_{306–318} tetramer, and (C) *in vitro* biotinylated HLA-DRB1*0103/HA_{306–318} tetramer.
tynlated MHC class II–BSP fusion proteins in vivo for making tetramers. The major concern is the efficiency of biotinylation. Because nonbiotinylated HLA-DR2BSP/MBP was not only decrease the efficiency of tetramer formation, but could also inhibit the tetramerized HLA-DR2BSP/MBP binding to antigen specific T cells resulting in decreased staining of the specific T cells. In bacterial system, in vivo biotinylation by coexpression of BirA enzyme had limited successes with a biotinylation efficiency of ~30% [24]. One factor that influences the biotinylation efficiency was concentration of d-biotin in the biotinylation reaction [24]. In this eukaryotic expression system, we observed that the biotinylation efficiency could be increased threefold with increased concentration of d-biotin (2000 μg/L) in media. Another factor that may affect the biotinylation efficiency is the location of the expressed BirA enzyme in the insect cell [9]. Biotinylation is a posttranslational modification reaction. In the E. coli system, BirA enzyme is a cytoplasmic protein [9] that catalyzes the biotinylation reaction in the cytosol. Similarly, the activities of its analogs known as holocarboxylase synthetase (EC 6.3.4.10) from mammalian cells were also detected only in the cytosol or mitochondria [9]. MHC class II–BSP/peptide complex was expressed as secretory protein in insect cells, hence the translation of these proteins were initiated on rough endoplasmic reticulum and completed in the endoplasmic reticulum lumen. Then the protein is transported to the Golgi apparatus for the posttranslational modification and exocytosed to the extracellular environment. During this constitutive process, the secretory protein is sequestered from the cytoplasm where the BirA enzyme is located. Therefore, one would speculate that MHC class II–BSP/peptide monomer is only able to be biotinylated when BirA enzyme was also located in the endoplasmic reticulum compartment. Interestingly, this is not the case. The MHC class II–BSP/peptide was biotinylated by both cytoplasmic form of BirA and secretion form of BirA enzyme. We found that the biotinylation efficiency is higher according to the ratio of biotinylated HLA-DR α chain to the HLA-DR β chain when BirA enzyme was coexpressed in cytosol. Therefore we expressed cytoplasmic form BirA enzyme with target protein HLA-DR2BSP/MBP in the rest of our experiments.

We did not expect that target protein HLA-DR2BSP/MBP to be biotinylated in vivo completely because stable expression cell line established by cotransfection of multiple expression vectors will contain a mixture of expression clones. Therefore, every clone may not incorporate sufficient copy numbers of pBirA vector into cellular genome and expressed sufficient enzymatic activity for biotinylation. To overcome this problem, we used monomeric avidin chromatography [25] to isolate biotinylated HLA-DR2BSP/MBP from nonbiotinylated molecules. Biotinylated HLA-DR2BSP/MBBP will bind to the monomeric avidin beads and could be eluted by free d-biotin under mild conditions e.g. at neutral pH and physiological salt concentration [25, 26]. Thus, one should be able to purify the biotinylated protein without significant denaturation of the MHC class II proteins. Unfortunately, this approach did not work. We think that the reasons for this failure are the following: (1) the MHC class II protein in the medium was highly susceptible to the proteases; (2) there were many insect proteins in the culture supernatant that bound to the avidin affinity beads nonspecifically, presumably as a result of the basic property of avidin (pI 10) [27]; and (3) some of the biotinylated insect cell proteins in the supernatant were also copurified. These facts resulted in a very poor recovery of interested MHC class II proteins. However, we were able to overcome this technical difficulty by selection of high biotinylation clones through limiting dilution experiments. Actually, the overall production of biotinylated HLA-DR2BSP/MBP fusion protein from isolated clone is higher than that expressed by parental cell line.

An additional merit of the use of in vivo biotinylation strategy was that this method provided a very easy and sensitive method to detect the efficiency of expression in a given system [28] and to modulate the transfection ratios for multiple vectors. We found that supernatants from D. Mel-2 cell culture transiently transfected with HLA-DR2BSP/MBP and BirA enzyme expression vectors are ready for the detection of biotinylated DR2BSP/MBP proteins by Western blot. The Western blot signal is strongly correlated with the production and feasibility of purification of HLA-DR2BSP/MBP by monoclonal antibody affinity column. Unlike establishing of stable cell lines, transient expression takes a significant less amount of time and working load. Therefore, it provides an easy and quick mean to prescreen and evaluate the expression levels of other HLA-DR-BSP fusion constructs.

In summary, we have developed an in vivo biotinylation method to biotinylate HLA-DR-BSP fusion protein for making MHC class II tetramer. The biotinylation efficiency can achieve >90% for the proteins expressed. This strategy eliminates traditional in vitro biotinylation processes that involve protein concentration, buffer exchange for optimal enzymatic activity, enzymatic biotinylation at room temperature, and buffer exchange for removal of free biotin from enzymatic reaction [1]. The in vivo biotinylation strategy may potentially useful for expression of MHC class II tetramers.

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