ELSEVIER

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

Junbao Yang, Andrés Jaramillo, Ruili Shi, William W. Kwok, and T. Mohanakumar

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 *Bir*A enzyme. However, *in vitro* biotinylation of MHC-BSP (*Bir*A enzyme substrate peptide) fusion protein using *Bir*A enzyme is laborious and is prone to losses of target proteins to unacceptable levels. To circumvent this problem, an *in vivo* biotinylation strategy was developed where the *Bir*A enzyme was coexpressed with target protein, HLA-DR2BSP/MBP, in an insect cell expression system. Bacterial *Bir*A enzyme expressed in *Drosophila melanogaster* 2 (D. Mel-2) cell lines was biologically functional and was able to biotinylate secretary 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. *Human Immunology 65, 692–699 (2004).* © American Society for Histocompatibility and Immunogenetics, 2004. Published by Elsevier Inc.

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

ABBREVIATIONS

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

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 *Bir*A enzyme to biotinylate the lysine residue on an enzyme-specific recognition tag called *Bir*A substrate peptide (BSP) [2] fused at the C-terminus of one of the MHC polypeptides. Biotinylation with *Bir*A 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

From the Departments of Surgery (J.Y., A.J., R.S., T.M.) and Pathology and Immunology (T.M.), Washington University School of Medicine, St. Louis, Missouri; and Benaroya Research Institute (W.W.K.), Seattle, Washington.

Address reprint requests to: Dr. T. Mohanakumar, Department of Surgery, Washington University School of Medicine, Box 8109-3328.CSRB, 660 South Euclid Avenue, St. Louis, MO 63110-1093; Tel: (314) 362-8463; Fax: (314) 747-1560; E-mail: kumart@wustl.edu.

Human Immunology 65, 692–699 (2004)

0198-8859/04/$–see front matter
doi:10.1016/j.humimm.2004.04.001

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 *Bir*A enzyme in the *Escherichia coli* system or by cotransfection of the *Bir*A 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.

*Bir*A 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 biotin carboxyl carrier protein of acetyl-CoA carboxylase is biotinylated by *Bir*A enzyme in all proteins expressed in *E. coli* [11]. The minimum peptide lengths of *Bir*A enzyme recognition site in the biotin carboxyl carrier protein were identified as about 75–105 aa [12]. By use of the library-screening technique, a series of short artificial *Bir*A substrate peptides now known as BSP has also been 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 *Bir*A enzyme. By using an HLA-DRA1*0101-Fos-BSP/MBP-HLA-DRB1*1501-Jun (DR2BSP/MBP in text) fusion protein whose TCR specificity had been determined previously [17] as model protein, we demonstrate here that DR2BSP/MBP could be biotinylated *in vivo*during the expression. Clones that expressed completely biotinylated HLA-DR2BSP/MBP proteins could be isolated by limiting dilution protocol. HLA-DR2BSP/MBP protein isolated by this procedure was ready to be tetramerized with streptavidin without further *in vitro* biotinylation treatment.

MATERIALS AND METHODS

Gene Cloning and Construction of Expression Vectors

Bacterial *Bir*A gene was amplified from the genomic DNA extracted from *E. coli* DH5α strain by PCR that used forward primer 5′-AAA GAA TTC ATG AAG GAT AAC ACC GTG CCA-3′ and reverse primer 5′-AAA GGA TCC TTA TTT TTC TGC ACT TAC GCA-3′ and inserted into the *Eco*RI and *Bam*HI sites of Cu-inducible pRmHa3 insect cell expression vector. This vector was designated as p18BirA vector and expressed a cytosolic form of *Bir*A enzyme. A modified *Bir*A gene with HLA-DR β chain leader sequence (MVCLKLPGGSCMTALTVTLMVLSSPLALSGDTG) encoding region was fused in frame at the N-terminus of *Bir*A enzyme and cloned into pRmHa3 vector. This vector was designated as p21BirA vector expressed secretion form of *Bir*A enzyme in insect cells. An HLA-DR2/mylin basic protein peptide (amino acid 85–99, $MBP_{85–99}$) complex was used as a model molecule for the *in vivo* biotinylation study. The HLA-DR2/$MBP_{85–99}$ expression vectors, pRmHa-DRA1*0101-Fos-Fc and pRmHa3-DRB1*1501-Jun (designated as pDRB/MBP for short), were obtained from Dr. Kai W. Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA). The pRmHa-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, *Bir*A 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 $CuSO_4$ 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 2×10^7 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-l 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 *Bir*A 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 hemoagglutinin 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 *Bir*A gene from *E. coli* K12 strain was cloned into insect cell expression vector under the control of the metallothionein promoter. *In vivo* biotinylation of HLA-DR2BSP/MBP was conducted by cotransfection of pDRA-BSP vector, pDRB/MBP vector, and *Bir*A enzyme expression vector p18BirA into D. Mel-2 cells. HLA-DR2BSP/MBP was biotinylated by cytosolic *Bir*A

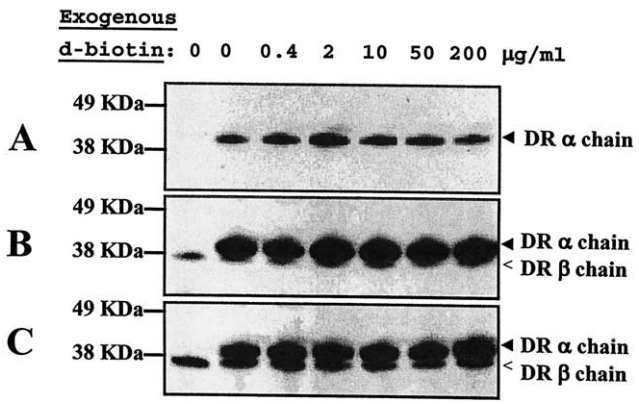


FIGURE 1 Biotinylation of HLA-DR2BSP/MBP *in vivo* by cotransfection of *Bir*A gene. (A) Western blot analysis of expression of biotinylated HLA-DR2BSP/MBP protein. Expression of biotinylated HLA-DR2BSP/MBP protein was detected with SA-HRP. Lane 1, D. Mel-2 cells were cotransfected with 0.5 μg of pDRA-BSP and 0.5 μg of pDRB/MBP expression vectors. Lanes 2–7, D. Mel-2 cells were cotransfected with 0.5 μg of pDRA-BSP, 0.5 μg of pDRB/MBP, and 1 μg of p18BirA expression vectors. Biotin was added in the culture media as indicated. (B) Comparison of biotinylation efficiency of HLA-DRA-BSP by coexpression of cytosolic form *Bir*A enzyme. HLA-DR2BSP/MBP was expressed and biotinylated as in (A) by cotransfection with p18BirA vector. Expression of biotinylated HLA-DR2BSP/MBP protein was detected with SA-HRP. Expression of HLA-DR2/MBP β chain was detected with HRP-conjugated anti-β chain monoclonal antibody. (C) Comparison of biotinylation efficiency of HLA-DRA-BSP by coexpression of secretion form *Bir*A enzyme. HLA-DR2BSP/MBP was expressed and biotinylated as in (A) except p18BirA vector was replaced with p21BirA vector. Expression of biotinylated HLA-DR2BSP/MBP protein was detected with SA-HRP. Expression of HLA-DR/MBP β chain was detected with HRP-conjugated anti-β chain monoclonal antibody.

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 *Bir*A 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 *Bir*A 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 *Bir*A 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 *Bir*A enzyme could biotinylate HLA-DR2BSP/MBP proteins, the cytosolic *Bir*A enzyme had a higher biotinylation efficiency than the secretion form of *Bir*A 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 *Bir*A vs. 5:1 for secretion form *Bir*A). Furthermore, the exogenous d-biotin required for achieving maximal biotinylation for cytosolic form *Bir*A enzyme was 2 μg/ml, whereas for secretion form *Bir*A enzyme it was 200 μg/ml. Therefore, cotransfection with cytosolic *Bir*A enzyme has a higher biotinylation efficiency than secretion form *Bir*A 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 *Bir*A 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-DR2BSP/MBP and *Bir*A enzyme expression vectors at 1:1 (0.2 μg of pDRA-BSP and pDRB/MBP vectors vs. 0.2 μg of p18BirA vector), the maximal production of biotinylated HLA-DR2BSP/MBP was achieved. Further increasing the amount of p18BirA vector reduces the production of biotinylated HLA-DR2BSP/MBP product. Figure 2B shows that the vector ratio of pDRA-BSP and pDR2/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 p18BirA 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: pDRABSP: pDRB/MBP) (Figure 2B).

Efficiency of Biotinylation of HLA-DR2BSP/MBP by Coexpression of *Bir*A Enzyme

One critical issue is the efficiency of biotinylation of target protein being biotinylated by coexpression of *Bir*A 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, pDRABSP, 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

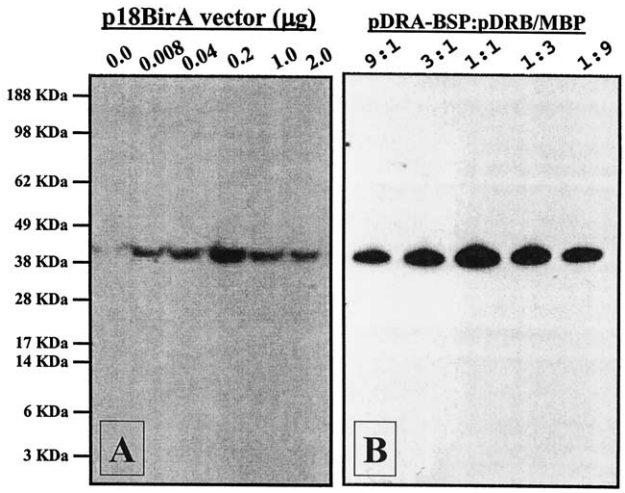


FIGURE 2 Optimizing expression vector ratios to maximizing biotinylation efficiency. (A) Various amount of p18BirA vector (0, 0.008, 0.04, 0.2, 1, and 2 μg vector DNA) along with fixed amount of pDRA-BSP (0.2 μg) and pDRB/MBP (0.2 μg) vectors were cotransfected into D. Mel-2 cells. (B) Fixed amount but at various ratios (1 μg total) of pDRA-BSP and pDRB/MBP vectors along with 1 μg of p18BirA vector were cotransfected into D. Mel-2 cells. Expression of biotinylated HLA-DR2BSP/MBP was detected with SA-HRP.

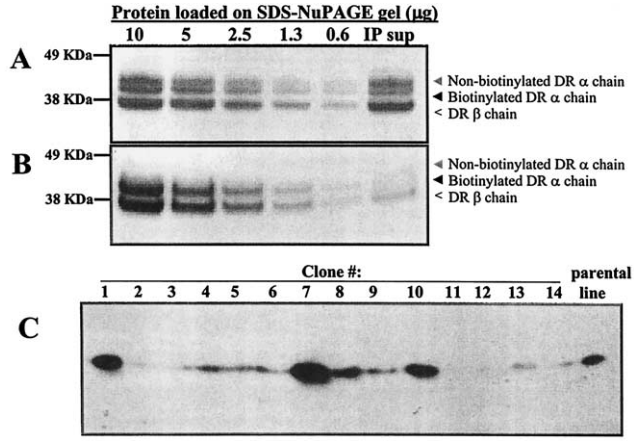


FIGURE 3 (A) Evaluation of biotinylation efficiency of HLA-DR2BSP/MBP expressed by D. Mel-2 cell line. Ten micrograms of purified HLA-DR2BSP/MBP were adsorbed with streptavidin beads and proteins remained in supernatant (lane "IP sup") were compared with the 10 μg and its twofold serial dilutions of HLA-DR2BSP/MBP on SDS-NuPAGE gel. (B) Evaluation of biotinylation efficiency of HLA-DR2BSP/MBP expressed by D. Mel-2 clone 7 from Figure 4C. Ten micrograms of purified HLA-DR2BSP/MBP were adsorbed with streptavidin beads, and proteins that remained in the supernatant were compared with the 10 μg and its twofold serial dilutions of HLA-DR2BSP/MBP on SDS-NuPAGE gel. (C) Isolation and evaluation of D. Mel-2 clones expressing biotinylated HLA-DR2BSP/MBP protein.

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 *Bir*A 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 $CuSO_4$ 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 hemoagglutinin peptide ($HA_{306-318}$) as described previously [14] and tetramerized with PE-SA. T cells from health donor with HLA-DRB1*0103 allele were stimulated with $HA_{306-318}$peptides for 16 days and stained with HLA-DRB1*0103/$HA_{306-318}$tetramer. As shown in Figure 5B, *in vivo* biotinylated HLA-

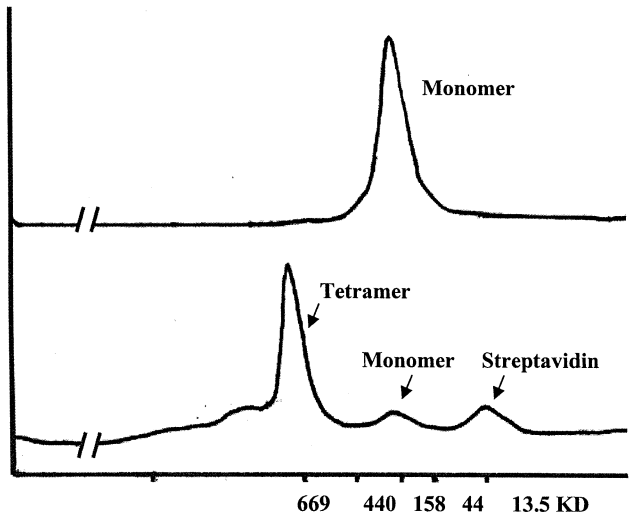


FIGURE 4 Gel filtration analysis of tetramerized HLA-DR2BSP/MBP. 200 μg of HLA-DR2BSP/MBP monomers or tetramers were loaded on Superose 6, 1.6 × 30−cm column. Sample was eluted out with PBS at a flow rate of 0.1 ml/min. The chromatograms were combined in one figure manually.

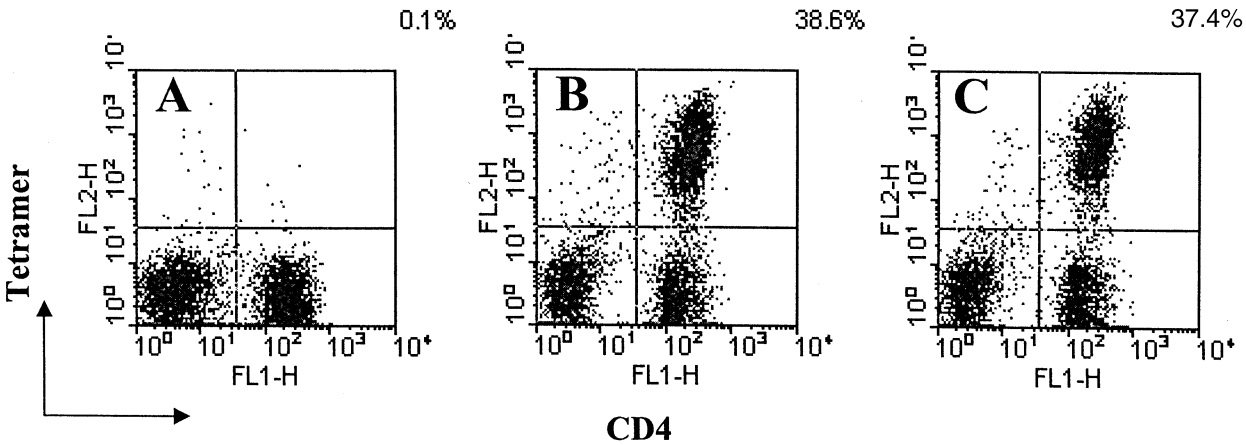
DRB1*0103/$HA_{306-318}$tetramer stained 38.6% of T cells, which is comparable to 37.4% of T cell staining by the *in vitro* biotinylated HLA-DRB1*0103/$HA_{306-318}$ tetramer (Figure 5C). *In vivo* biotinylated tetramer also has a minimum background staining, as shown in Figure 5A, only 0.1% T cells 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 *Bir*A enzyme with MHC class II molecule is sufficient to *in vivo* biotinylate MHC class II molecules for tetramerization and staining antigen-specific T cells.

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.

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 *Bir*A 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 *Bir*A 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 *Bir*A 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 *Bir*A enzyme, but most importantly eliminates long and tedious dialysis and concentration steps for the removal of free biotin. It also avoids contamination of *Bir*A enzyme and may decrease the chance of protein degradation introduced by *Bir*A enzyme.

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



tinylated 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 *Bir*A 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 *Bir*A enzyme in the insect cell [9]. Biotinylation is a posttranslational modification reaction. In the *E. coli* system, *Bir*A 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 excocytosed to the extracellular environment. During this constitutive process, the secretory protein is sequestered from the cytoplasm where the *Bir*A enzyme is located. Therefore, one would speculate that MHC class II-BSP/peptide monomer is only able to be biotinylated when *Bir*A 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 *Bir*A and secretion form of *Bir*A 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 *Bir*A enzyme was coexpressed in cytosol. Therefore we expressed cytoplasmic form *Bir*A 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 *Bir*A 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.

ACKNOWLEDGMENTS

This work was supported by the U.S. National Institutes of Health (NIH grant HL56543). We thank Dr. Kai W.

Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA) for providing the pRmHa-DRA1*0101-Fos-Fc and pRmHa3-MBP-DRB1*1501-Jun vectors.

REFERENCES

1. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM: Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94, 1996.
2. Schatz PJ: Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. BioTech 11:1138, 1993.
3. Bayer EA, Wilchek M: Protein biotinylation. Methods Enzymol 184:138, 1990.
4. Crawford F, Kozono H, White J, Marrack P, Kappler J: Detection of antigen-specific T cells withmultivalent soluble class II MHC covalent peptide complexes. Immunity 8:675, 1998.
5. Kozono H, White J, Clements J, Marrack P, Kappler J: Production of soluble MHC class II proteins with covalently bound single peptides. Nature 369:151, 1994.
6. Scott CA, Garcia KC, Carbone DR, Wilson IA, Teyton L: Role of chain pairing for the production of functional soluble IA major histocompatibility complex class II molecules. J Exp Med 183:2087, 1996.
7. Parrott MB, Barry MA: Metabolic biotinylation of secreted and cell surface proteins from mammalian cells. Biochem Biophys Res Commun 281:993, 2001.
8. Tsao KL, DeBarbieri B, Michel H, Waugh DS: A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in *Escherichia coli*. Gene 169:59, 1996.
9. Chapman-Smith A, Cronan JE: *In vivo* enzymatic protein biotinylation. Biomol Eng 16:119, 1999.
10. Barker DF, Campbell AM: The *birA* gene of *Escherichia coli* encodes a biotin holoenzyme synthetase. J Mol Biol 146: 451, 1981.
11. Chapman-Smith A, Cronan JE: The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. Trends Biochem Sci 24:359, 1999.
12. Beckett D, Kovaleva E, Schatz PJ: A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein Sci 8:921, 1999.
13. Frayser M, Sato AK, Xu L, Stern LJ: Empty and peptide-loaded class II major histocompatibility complex proteins produced by expression in *Escherichia coli* and folding *in vitro*. Protein Expr Purif 15:105, 1999.
14. Novak EJ, Liu AW, Nepom GT, Kwok WW: MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenze A antigen. J Clin Invest 104:R63, 1999.
15. Kotzin BL, Falta MT, Crawford F, Rosloniec EF, Bill J, Marrack P, Kappler J: Use of soluble peptide-DR4 tetramers to detect synovial T cells specific for cartilage antigens in patients with rheumatoid arthritis. Proc Natl Acad Sci USA 97:291, 2000.
16. Kwok WW, Liu AW, Novak EJ, Gebe JA, Ettinger RA, Nepom GT, Reymond SN, Koelle DM: HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2–infected individuals: direct detection of immunodominant antigen-responsive cells. J Immunol 164:4244, 2000.
17. Appel H, Gauthier L, Pyrdol J, Wucherpfennig KW: Kinetics of T cell receptor binding by bivalent HLA-DR peptide complexes that activate antigen-specific human T cells. J Biol Chem 275:312, 2000.
18. Parrott MB, Barry MA: Metabolic biotinylation of secreted and cell surface proteins from mammalian cells. Biochem Biophys Res Comun 281:993, 2001.
19. Parrott MB, Barry MA: Metabolic biotinylation of recombinant proteins in mammalian cells and in mice. Mol Ther 1:96, 2000.
20. Crothers DM, Metzger H: The influence of polyvalency on the binding eoperties of antibodies. Immunochemistry 9:341, 1972.
21. Hornick CL, Karuch F: The role of multivalence. Immunochemistry 9:325, 1972.
22. Duffy S, Tsao KL, Waugh DS: Site-specific, enzymatic biotinylation of recombinant proteins in *Spodoptera frugiperda* cells using biotin acceptor peptides. Anal Biochem 262:122, 1998.
23. Cull MG, Schatz PJ: Biotinylation of proteins *in vivo* and *in vitro* using small peptide tags. Methods Enzymol 326: 430, 2000.
24. Smith PA, Tripp BC, DiBlasio-Smith EA, Lu Z, LaVallie ER, McCoy JM: A plasmid expression system for quantitative *in vivo* biotinylation of thioredoxin fusion proteins in *Escherichia coli*. Nucleic Acids Res 26:1414, 1998.
25. Henrikson KP, Allen SH, Maloy WL: An avidin monomer affinity column for the purification of biotin-containing enzymes. Anal Biochem 94:366, 1979.
26. Guchhait RB, Polakis SE, Dimroth P, Stoll E, Moss J, Lane MD: Acetyl coenzyme A carboxylase system of *Escherichia coli*: purification and properties of the biotin carboxylase, carboxyltransferase, and carboxyl carrier protein components. J Biol Chem 249:6633, 1974.
27. Rothemund DL, Thomas TM, Rylatt DB: Purification of the basic protein avidin using gradiflow technology. Protein Expr Purif 26:149, 2002.
28. Wisniewski D, LoGrasso P, Calaycay J, Marcy A: Assay for IkappaB kinases using an *in vivo* biotinylated IkappaB protein substrate. Anal Biochem 274:220, 1999.