Melanosomal Targeting Sequences from gp100 Are Essential for MHC Class II–Restricted Endogenous Epitope Presentation and Mobilization to Endosomal Compartments

Stéphanie Lepage and Réjean Lapointe

Research Centre, Centre Hospitalier de l'Université de Montréal, Hôpital Notre Dame, Université de Montréal and Institut du Cancer de Montréal, Montréal, Québec, Canada

Abstract

$CD4^+$ T lymphocytes play an important role in $CD8^+$ T cell–mediated responses against tumors. Considering that ~20% of melanomas express MHC class II, it is plausible that concomitant presentation by MHC class I and class II shapes positive (helper T cells) or negative (regulatory T cells) antitumor responses. Interestingly, gp100, a melanoma antigen, can be presented by both MHC class I and class II when expressed endogenously, suggesting that it can reach endosomal/MHC class II compartments (MIIC). Here, we showed that gp100 putative NH_2-terminal signal sequence and the last 70 residues in COOH terminus are essential for MIIC localization and MHC class II presentation. Confocal microscopy analyses confirmed that gp100 was localized in LAMP-1^+/HLA-DR^+ endosomal/MIIC. Gp100 targeting sequences were characterized by deleting different sections in the COOH terminus (last 70 residues). Transfection in 293T cells, expressing MHC class I and class II molecules, revealed that specific deletions in COOH terminus resulted in decreased MHC class II presentation, without effects on class I presentation, suggesting a role in MIIC trafficking for these deleted sections. Then, we used these gp100 targeting sequences to mobilize green fluorescent protein to endosomal compartments and to allow MHC class II and class I presentation of minimal endogenous epitopes. We conclude that these specific sequences are MIIC-targeting motifs, which could be included in expression cassettes for endogenously expressed tumor or viral antigens for MHC class II and class I presentation and optimize *in vivo* T-cell responses or as an *in vitro* tool for characterization of new MHC class II epitopes. (Cancer Res 2006; 66(4): 2423-32)

Introduction

Cancer immunotherapy strategies targeting tumor antigens were mainly developed by eliciting $CD8^+$ CTLs. Over the past decade, growing evidence has emerged from animal studies (1–3) and clinical trials (4, 5), indicating that $CD4^+$ helper T lymphocytes play an important role in initiating and maintaining immune responses against cancer (6, 7) by expanding effective and memory $CD8^+$ T cells (8, 9). Thus, optimal antitumor immunity might require the participation of both $CD4^+$ and $CD8^+$ T lymphocytes to generate a strong and durable response against cancer cells (10, 11). However, a subset of $CD4^+$ T cells, defined as regulatory T lymphocytes (T_{reg}) and representing 1% to 2% of the total $CD4^+$ T-cell population (12), may also play a significant role in human cancer considering their cognate immunoregulatory functions (13, 14). T_{reg} have suppressive activities on effective $CD8^+$ (15, 16) and $CD4^+$ (17, 18) T-cell functions, suggesting that MHC class II–mediated antigen presentation can potentially be either positive or detrimental in antitumor immunity.

Considering that ~20% to 25% of melanomas naturally express MHC class II molecules during the process of malignant transformation (19), and perhaps >50% during inflammation and metastases formation (20, 21), it is plausible that concomitant antigenic presentation by MHC class I and class II shapes antitumor responses mediating both positive and negative mechanisms. Thus, activation of tumor-specific $CD8^+$ and $CD4^+$ T cells may occur at the tumor site. This illustrates the importance of better defining MHC class II antigenic presentation from endogenously expressed proteins.

Interestingly, gp100, a melanoma/melanocyte-shared antigen, can be presented by both MHC class I and class II molecules when expressed endogenously by melanoma and nonmelanoma cells. This implies that gp100 can reach endosomal/MHC class II compartments (MIIC) for antigen processing and presentation by MHC class II. Normally, $CD4^+$ T cells recognize exogenous proteins, which are ingested by antigen-presenting cells (APC) and get degraded into peptides which can be coupled with MHC class II molecules in MIIC, which are lysosome-related organelles (22). These peptide/MHC class II complexes then migrate to the cell surface. Interestingly, an endogenous protein can sometimes reach endosomal/MIIC to be processed similarly to an exogenous protein for MHC class II–mediated presentation. However, endosomal/MIIC internal trafficking leading to MHC class II presentation remains poorly understood.

We previously generated a $CD4^+$ T-cell clone specific to gp100 by using retrovirally transduced dendritic cells (23), indicating plausible MHC class II presentation. We also showed that this gp100-specific $CD4^+$ T-cell clone could recognize melanoma cell lines (24), which evoked potential targeting sequences in gp100 for MHC class II–mediated presentation.

In the current work, we prepared different deletions in the gp100 sequence to assess MHC class I and class II–mediated presentation and to evaluate endosomal protein localization, with the aim of finding sequences, within gp100, involved in these processes. We first confirmed gp100 presentation by MHC class II molecules in gp100-transfected 293T cells and its endosomal mobilization. Our data show that both the COOH-terminal (last 70 residues) and putative NH_2-terminal signal sequences are essential for MHC class II–mediated presentation of endogenous gp100 and other antigens. Our results also suggest that gp100 may transit to the cell surface to be internalized in endosomal/MIIC.

Requests for reprints: Réjean Lapointe, Centre de Recherche, Centre Hospitalier de l'Université de Montréal, Hôpital Notre-Dame, Pavillon J.A. De Sève, Y-5605, 2099 rue Alexandre De Sève, Montréal, Québec, Canada H2L 4M1. Phone: 514-890-8000, ext. 25489, 25504 (lab); Fax: 514-412-7591; E-mail: rejean.lapointe@umontreal.ca.

doi:10.1158/0008-5472.CAN-05-2516

A better understanding of the MHC class II presentation of endogenous antigens may help us to optimize the immune response directed to gp100 or other tumor antigens in cancer immunotherapy.

Materials and Methods

Media and cell culture. Complete medium consisted of AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 5% human AB serum (heat-inactivated; Gemini Bio-Products, Calabasas, CA), 2 mmol/L L-glutamine, 100 units/mL penicillin/streptomycin and 10 μg/mL gentamicin (all from Invitrogen). A gp100-specific $CD4^+$ T-cell clone, HLA-DRβ1*0701 restricted (23), and a $CD8^+$ T-cell clone, HLA-A*0201 restricted (25), were cultured as previously described in complete medium supplemented with 300 IU/mL recombinant human interleukin (IL)-2 (Chiron, Emeryville, CA).

CD40-stimulated B lymphocytes (CD40-B) were cultured as previously described (26) in Iscove's modified Dulbecco's medium [Invitrogen and Wisent (St. Bruno, Quebec, Canada)] supplemented with 10% human serum (heat inactivated, prepared from normal donors), 2 mmol/L L-glutamine, 100 units/mL penicillin/streptomycin, 10 μg/mL gentamicin, 500 ng/mL of a soluble trimeric CD40L (Immunex Corporation, Seattle, WA), and 500 units/mL recombinant human IL-4 (Peprotech, Rocky Hill, NJ).

HEK-293T cells expressing HLA-DRβ1*0701 or DRβ1*0401, kindly provided by Dr. Paul F. Robbins and Dr. Suzanne L. Topalian [National Cancer Institute (NCI)/NIH, Bethesda, MD], and HEK-293T cells expressing HLA-A*0201 were cultured in RPMI 1640 (Invitrogen and Wisent) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen and Wisent), 2 mmol/L L-glutamine, 100 units/mL penicillin/streptomycin, and 10 μg/mL gentamicin.

The melanoma cell line MelFB, which was immunoselected for the absence of gp100 and MART-1, was transducted by retroviral vectors encoding gp100 or green fluorescent protein (GFP) as previously described (23). Melanoma cell lines 1087mel, 624.38mel, 624.38mel-CIITA, 1088mel, 1102mel, 1300mel, 397mel, 553mel, and SK23mel were established at the Surgery Branch (NCI/NIH). Breast tumor cell lines MCF-7 and MDA231 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All tumor cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, 100 units/mL penicillin/streptomycin, and 10 μg/mL gentamicin.

Gp100 mutants and other plasmids. Plasmids encoding HLA-A*0201 and A*0101 (pcDNA-A2 and CLNCx-A1, respectively), kindly supplied by Dr. Paul F. Robbins, were cloned from HLA-typed patients at the NIH. Plasmid encoding gp100 (pcDNA-gp100) also was gifted by Dr. Paul F. Robbins (NCI/NIH).

Plasmids encoding the different versions of gp100, deleted in the COOH terminus or NH_2 terminus (presented in Fig. 3*A, left*), were prepared by PCR from the wild-type sequence, cloned in pcDNA3.1, and their sequences were confirmed by sequencing. PcDNA-gp100ΔTM was internally deleted from residues 594 to 615, which corresponded to the transmembrane domain, and pcDNA-gp100 ΔLL was deleted from residues 650 to the end, which corresponded to a putative dileucine motif. These latter two constructs were prepared by Dr. Paul F. Robbins at the Surgery Branch (NCI/NIH). PcDNA-gp100TM was deleted from residue 615 to the end and pcDNA-gp100NoTM from residue 594 to the end. PcDNA-gp100ΔSS was deleted from residues 1 to 20, which corresponded to the putative NH_2-terminal signal sequence. PcDNA-gp100ΔYV was deleted from residues 616 to 627, which corresponded to a sequence including a tyrosine residue. In pcDNA-gp100CD8, the gp100 transmembrane domain (residues 594-615) was exchanged with the CD8 transmembrane domain (residues 183-204 of CD8 sequence). PcDNA-gp100ΔYVCD8 was similar to pcDNA-gp100CD8 but residues 610 to 627 were also removed.

In the construct pcDNA-gp100/GFP (presented in Fig. 6*A*), the entire GFP sequence, from which the first methionine was changed for a valine, was cloned between the putative NH_2-terminal signal sequence (residues 1-20) and the last 70 residues from gp100 (residue 594 to the end). In pcDNA-gp/GFP+epit (presented in Fig. 6*B*), residues 150 to 225 from gp100, which corresponded to minimal MHC class II and class I epitopes, were inserted after the GFP sequence.

Cell transfection and APC pulsing. The day before transfection, cells were plated at 5×10^5 per well in six-well plates to get 50% to 90% confluence on the day of transfection. The cells were transiently transfected employing Lipofectamine Plus Reagent (Invitrogen) according to the instructions of the manufacturer. Transfected cells were cultured for an additional 24 hours. We routinely reached between 30% and 50% transfection efficiency.

In some experiments, MelFB and CD40-B cells were electroporated in a nucleofection system (Amaxa Biosystems, Gaithersburg, MD) according to the instructions of the manufacturer.

The HLA-DRβ1*0701-binding peptide $gp100_{170\text{-}190}$ (23) and the HLA-DRβ1*0701 control binding peptide $Ig\kappa_{188\text{-}202}$ (27) were synthesized at the Surgery Branch (NCI/NIH). Recombinant gp100 protein was prepared as previously described (28). Recombinant NY-ESO-1 protein (29), another tumor antigen, served as a negative control. Peptide or protein pulsing of CD40-B cells (1×10^5) was carried out in B-cell culture medium for 16 hours in 96-well flat-bottomed plates.

T-cell assays. Gp100-specific T-cell clones were analyzed for their capacity to recognize target cells, such as gp100-transfected 293T cells, melanoma cell lines, or CD40-B pulsed with synthetic peptides or recombinant proteins. Target cells (1×10^5) were cocultured with either a specific $CD4^+$ T-cell clone (2×10^4) or a specific $CD8^+$ T-cell clone (1×10^5) in 200 μL of complete medium in 96-well flat-bottomed plates. Supernatants were harvested after 20-hour incubation and human interferon-γ (IFN-γ) was assayed by ELISA with coupled antibodies (Endogen, Woburn, MA).

In some experiments, chloroquine (Sigma, St. Louis, MO) was added on target cells at 100 μmol/L for 4 hours. Cells were washed once and fixed with 0.5% of formaldehyde for 5 minutes. They were then extensively washed thrice and cocultured with T cells in 200 μL of complete medium in 96-well flat-bottomed plates.

Western blotting. Protein extracts were prepared from gp100-transfected 293T cells at 4°C, for 20 minutes, in lysis buffer [20 mmol/L Tris-HCl (pH 8), 137 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1 mmol/L Na_3VO_4, and 2 mmol/L EDTA] containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 2 μmol/L pepstatin A, 2 μmol/L leupeptin; all from Sigma). Cell debris were sedimented and discarded, and protein concentration was measured by Lowry assay with a DC Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were prepared and loaded (7.5 μg/well) on 10% SDS-polyacrylamide gel in a Mini-PROTEAN 3 system (Bio-Rad) according to the instructions of the manufacturer. Proteins were transferred to Hybond enhanced chemiluminescence (ECL) membranes (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) and revealed by incubation with a goat gp100-specific antibody (1:200; K-18; Santa Cruz, Santa Cruz, CA) or a mouse actin-specific antibody (1:4,000; Chemicon, Temecula, CA) for 1 hour. Membranes were washed and reincubated for 1 hour with secondary peroxidase-conjugated antibodies, chicken anti-goat (1:10,000) or goat anti-mouse (1:40,000; both from Chemicon), before detection with ECL Plus Western blotting (Amersham Pharmacia Biotech).

Confocal microscopy. Cells were plated at 3×10^5 per well on poly-D-lysine (Sigma)–treated coverslips in 12-well plates the day before transfection (when necessary) and cultured for an additional 24 hours. Before intracellular staining, the cells were washed once with PBS (Invitrogen and Wisent) containing 0.5% bovine serum albumin (BSA; Sigma), fixed, and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, Mississauga, ON) directly on coverslips for 20 minutes and washed twice with BD Perm/Wash Solution (BD Biosciences).

Permeabilized cells were stained with a gp100-specific antibody (NK-1; Bio-Design, Saco, ME), a LAMP-1-specific antibody (anti-CD107a; BD Biosciences), or a pan-MHC Class II (HLA-DR, P, Q)–specific antibody (TÜ39; BD Biosciences). After 30 minutes of incubation with the first antibody, the cells were washed and reincubated for 30 minutes with isotype-specific secondary antibodies coupled with Alexa Fluor-488 (green), Alexa Fluor-568 (red), or Alexa Fluor-647 (blue; all from Molecular Probes, Eugene, OR). The cells were then washed and the coverslips were mounted

on microscope slides using Geltol (Immunon, Pittsburgh, PA). After overnight incubation at 4°C, the coverslips were sealed with nail polish.

The cells were observed under a Leica TCS-SP1 confocal microscope (Leica Microsystems, Mannheim, Germany) fitted with a 100× oil immersion objective, analyzed by Leica Confocal Software, and processed by Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA).

Cell surface analyzed by flow cytometry. 293T cells were cotransfected by plasmids coding for GFP and gp100 mutants. Preliminary experiments in transient transfection by our group confirmed that the same cells were cotransfected with two different plasmids, gp100 and GFP, for instance (data not shown). Twenty-four hours later, the cells were harvested with trypsin, distributed at $>1 \times 10^5$ per tube in 5 mL polystyrene round-bottomed tubes, and washed with PBS containing 0.5% BSA. For intracellular staining, the cells were fixed and permeabilized with BD Cytofix/Cytoperm for 20 minutes, then washed twice with BD Perm/Wash Solution (both from BD Biosciences).

Intracellular and cell-surface staining were done using a gp100-specific antibody (NK-1) or an isotype-matched control (IgG2b; BD Biosciences). After 30 minutes of incubation, the cells were washed and reincubated for 30 minutes with a phycoerythrin-conjugated isotype-specific secondary antibody (anti-mouse-R-PE; Molecular Probes). The cells were finally analyzed by flow cytometry on a Fluorescence-Activated Cell Sorter Calibur (Becton Dickinson, Mississauga, ON). Only GFP-positive cells, which were also positive for gp100, were analyzed by WinMDI 2.8 software. Gp100 cell-surface expression was compared with total expression in permeabilized cells.

Results

Exogenous and endogenous gp100 can be presented by MHC class II molecules. We have previously reported the generation of a $CD4^+$ T-cell clone specific for an HLA-DRβ1*0701 epitope from gp100 ($gp100_{170\text{-}190}$) using retrovirally transduced dendritic cells (23). With this $CD4^+$ T-cell clone, classic MHC class II–mediated presentation of exogenous gp100 was first controlled. CD40-stimulated B lymphocytes (CD40-B) served as a source of APC (26). As expected, HLA-DRβ1*0701$^+$ APC pulsed with recombinant gp100, but not the DRβ1*0701$^-$ APC, were recognized by the $CD4^+$ T-cell clone (Fig. 1*A*). APC pulsed with different amounts of gp100 peptide, corresponding to the DRβ1*0701 epitope ($gp100_{170\text{-}190}$), were effectively recognized in a dose-dependent manner but DRβ1*0701$^+$ APC pulsed with either a different recombinant protein or a known DRβ1*0701-binding peptide derived from the immunoglobulin κ light chain ($Ig\kappa_{188-202}$) were not recognized.

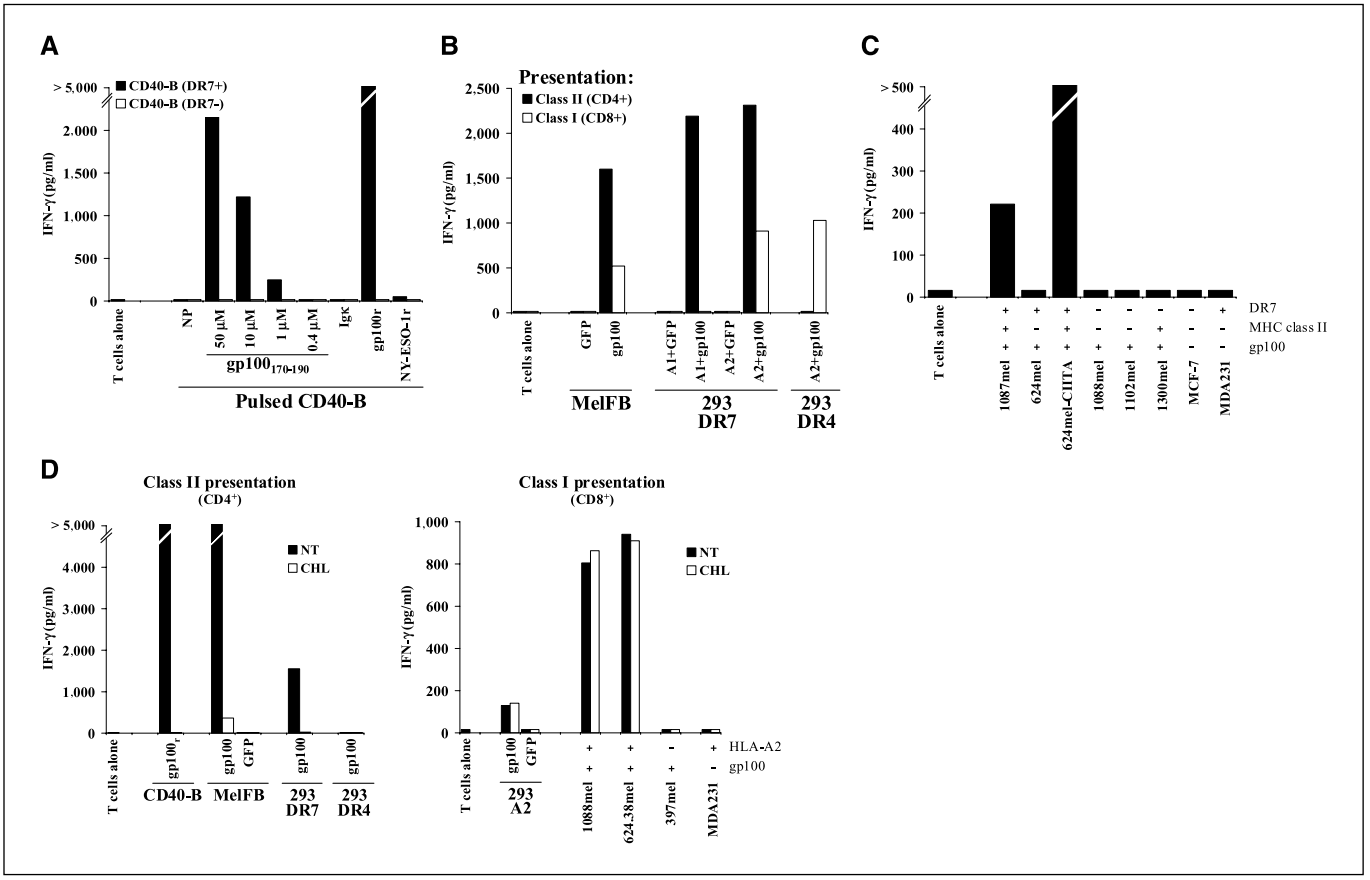

Figure 1. MHC class II–restricted presentation of endogenous and exogenous gp100. *A,* HLA-DRβ1*0701$^+$- or $^-$0701$^-$-stimulated B lymphocytes (CD40-B) were pulsed with recombinant gp100 (*gp100r*), NY-ESO-1 (*NY-ESO-1r*), different amounts of $gp100_{170\text{-}190}$ peptide, or a DRβ1*0701-binding control peptide ($Ig\kappa_{188\text{-}202}$). A gp100-specific $CD4^+$ T-cell clone was cocultured with these target cells. *B,* an HLA-DRβ1*0701$^+$ gp100-deficient melanoma cell line (melFB) was retrovirally transduced using genes encoding gp100 or GFP. 293T cells expressing HLA-DRβ1*0701 or DRβ1*0401 (*DR7* or *DR4*) were cotransfected with plasmids coding for gp100 or GFP and HLA-A*0201 or A*0101 (*A2* or *A1*). Gp100-specific $CD4^+$ and $CD8^+$ T-cell clones were cocultured with these target cells. *C,* melanoma cell lines expressing or not gp100, MHC class II molecules, or HLA-DRβ1*0701 (*DR7*) were cocultured with a gp100-specific $CD4^+$ T-cell clone. *D,* HLA-DRβ1*0701$^+$-stimulated B lymphocytes (CD40-B) pulsed with recombinant gp100 (gp100r), HLA-DRβ1*0701$^+$ gp100-deficient melanoma cell line (MelFB) expressing gp100 or GFP, gp100-transfected 293T cells expressing HLA-DRβ1*0701, DRβ1*0401, or HLA-A*0201 (*DR7, DR4*, or *A2*), and melanoma cell lines expressing or not gp100 or HLA-A*0201 (*HLA-A2*) were treated with 100 μmol/L chloroquine (*CHL*) or left untreated (*NT*) as described in Materials and Methods. Gp100-specific $CD4^+$ and $CD8^+$ T-cell clones were cocultured with these target cells. *A* to *D,* supernatants were harvested after 20 hours of coculture and IFN-γ secretion was determined by ELISA. Representative of ≥5 independent experiments.

We next deployed the same gp100-specific $CD4^+$ T-cell clone to evaluate MHC class II–mediated presentation from endogenously expressed gp100. HLA-DRβ1*0701$^+$ melanoma cells (MelFB), immunoselected for the absence of gp100, and 293T cells were engineered to express gp100 or GFP. As expected, only cells expressing gp100 and HLA-DRβ1*0701 were recognized (Fig. 1*B*). 293T cells expressing a control gene (GFP) or a different MHC class II molecule (HLA-DRβ1*0401) failed to promote IFN-γ secretion from the $CD4^+$ T-cell clone. In all cases, gp100 expression and class I presentation were controlled by cotransfection of an HLA-A*0201 expression plasmid and recognition was monitored by a $CD8^+$ T-cell clone specific to an HLA-A*0201 gp100 epitope ($gp100_{209\text{-}217}$; ref. 25). We have previously confirmed that the amount of IFN-γ secretion by gp100-specific $CD4^+$ (23) or $CD8^+$ T-cell clones correlated with the density of the peptide loaded on APC (data not shown).

In addition, melanoma cells expressing gp100 and the class II transactivator (CIITA), up-regulating invariant chain (Ii), HLA-DM, and HLA-DR molecules, were recognized by the $CD4^+$ T-cell clone (624mel-CIITA, Fig. 1*C*), but not wild-type $CIITA^-$ cells not expressing HLA class II molecules. Melanoma cells naturally expressing HLA class II molecules DRβ1*0701 and gp100 (1087mel) were recognized by the $CD4^+$ T-cell clone. Other melanoma cells expressing HLA class II molecules but not DRβ1*0701 (1300mel) were not recognized, confirming HLA-DRβ1*0701-restricted presentation.

Finally, we evaluated if intracellular antigen processing was required for MHC class II presentation of endogenously expressed gp100. To do so, we treated target cells with chloroquine, which inhibits the processing of exogenous antigen and MHC class II presentation by neutralizing the pH of endosomes. As shown in Fig. 1*D* (*left*), chloroquine treatment resulted in inhibition of MHC class II presentation of exogenous gp100 by HLA-DRβ1*0701$^+$ APC pulsed with recombinant gp100 and endogenous gp100 expressed by the melanoma cell line or 293T cells expressing HLA-DRβ1*0701, indicating that intracellular processing was required for MHC class II presentation. This inhibition was not caused by chloroquine toxicity because similar treatments of tumor cell lines did not inhibit MHC class I presentation of endogenous gp100 (Fig. 1*D, right*) as expected.

Importantly, data revealed that gp100 can be presented by MHC class II from either classic exogenous or endogenous pathways, suggesting protein mobilization to the endosomal compartment and MIIC.

Gp100 localizes to LAMP-1$^+$ endosomal vesicles. Gp100 localization experiments were carried out with laser scanning confocal microscopy. As shown in Fig. 2, gp100 seems to be localized in intracellular vesicles in both melanoma and transfected 293T cells (labeling in green). Double staining was done with anti-LAMP-1, a membrane glycoprotein enriched in the lysosomal membrane and found in endosomes/lysosomes and MIIC (30, 31). Double staining revealed that several gp100$^+$ vesicles were also positive for LAMP-1 (in red), suggesting colocalization in endosomal compartments (yellow vesicles indicated by white arrows). This experiment underscores the importance of the gp100 trafficking to endosomal compartments, which could be linked to its MHC class II presentation.

Mapping of endosomal targeting sequences essential for MHC class II presentation. Endosomal/melanosomal targeting motifs could be involved in gp100 mobilization to MIIC and optimize MHC class II–mediated presentation. We hypothesized that the putative NH_2-terminal signal sequence and the last 70 residues (Fig. 3*C*), which contain a transmembrane domain, a tyrosine residue, and a putative dileucine motif, could be important for gp100 trafficking to endosomal compartments. We prepared different deletions in the gp100 sequence to map potential sequences important for endosomal homing and MHC class II presentation. Plasmids encoding gp100 mutants and HLA-A*0201 were cotransfected in 293T cells expressing HLA-DRβ1*0701. MHC class I presentation was monitored with a $CD8^+$ T-cell clone specific to an HLA-A*0201 epitope from gp100 ($gp100_{209\text{-}217}$). MHC class II presentation was evaluated with a $CD4^+$ T-cell clone specific to an HLA-DRβ1*0701 epitope from gp100 ($gp100_{170\text{-}190}$). The different versions of deleted gp100 are represented in Fig. 3*A* (*left*). Importantly, both MHC class I and class II epitopes remained intact; only the last 70 residues were modified, which is >350 amino acids downstream of the epitopes. As expected, on the basis of IFN-γ secretion, gp100- and HLA-A*0201-transfected 293-DRβ1*0701$^+$ cells were recognized by both $CD4^+$ and $CD8^+$

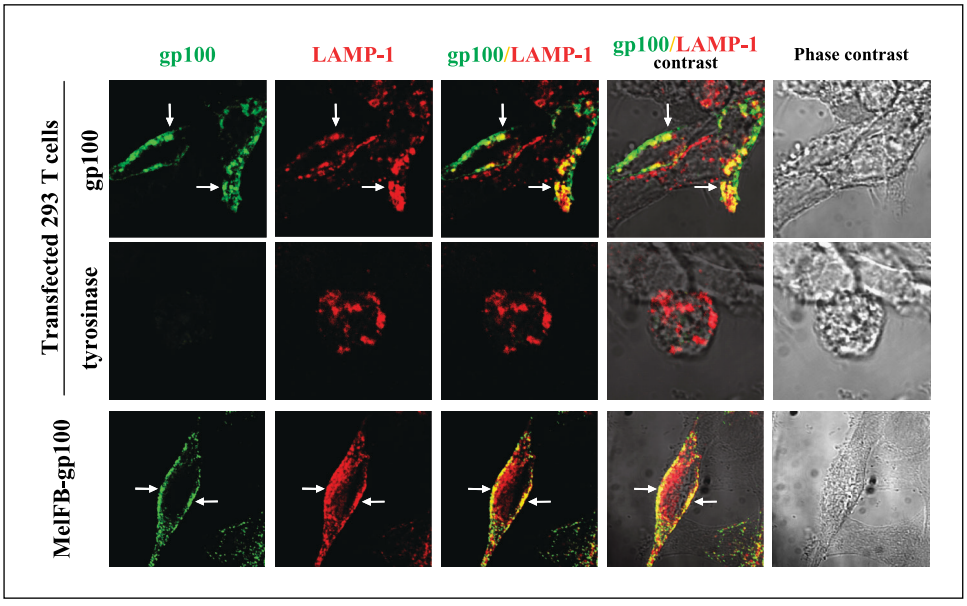

Figure 2. Colocalization of gp100 and LAMP-1. 293T cells transfected with plasmids coding for gp100 or tyrosinase and a melanoma cell line expressing gp100 (MelFB-gp100) were permeabilized and double stained with an anti-gp100 coupled with Alexa Fluor-488 (*green*) and an anti-LAMP-1 coupled with Alexa Fluor-568 (*red*). The cells were analyzed by laser scanning confocal microscopy. Yellow vesicles, in merged images, revealed the colocalization of gp100 and LAMP-1 (*white arrows*). Representative of three independent experiments.

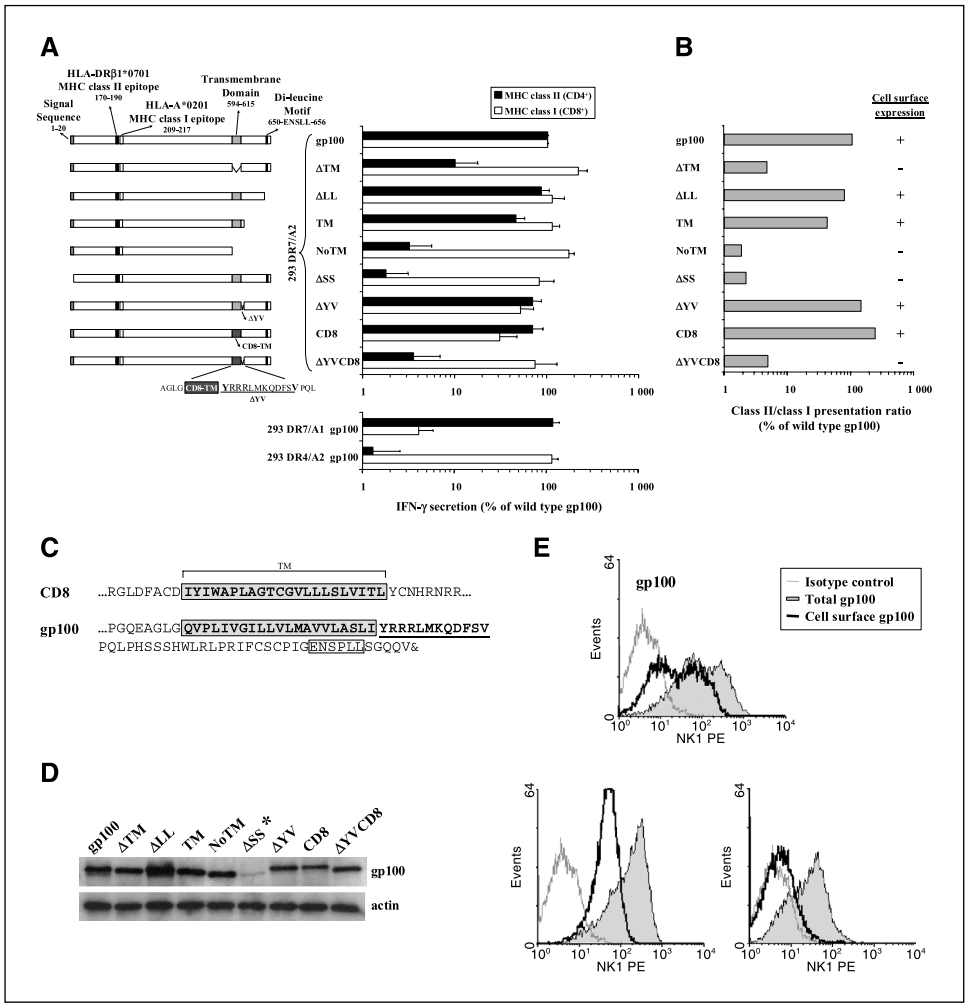

Figure 3. Mapping of gp100-derived targeting sequences essential for MHC class II presentation. *A,* plasmids encoding gp100 mutants and HLA-A*0201 or A*0101 were cotransfected in 293T cells expressing HLA-DRβ1*0701 or DRβ1*0401. Gp100-specific $CD4^+$ and $CD8^+$ T-cell clones were cocultured with transfected cells and peptide presentation was evaluated on the basis of IFN-γ secretion as determined by ELISA. Data from gp100 mutants are presented as a percentage of IFN-γ secretion compared with wild-type gp100, normalized to 100%. Average of 10 independent experiments. *B,* data from (*A*) are presented as a class II/class I antigen presentation efficiency ratio. *C,* amino acid sequences of the transmembrane domain from CD8, the transmembrane domain, and the COOH terminus of gp100. *D,* gp100 expression levels were revealed by Western blot analysis with a gp100-specific antibody. *, the epitope recognized by this antibody is located in the NH_2 terminus; consequently, the expression level of gp100-ΔSS could not be evaluated. *E,* gp100 cell-surface expression was evaluated by flow cytometry (Cell-surface expression). Total gp100 expression was also evaluated by permeabilizing transfected cells before staining (*Total gp100*). Gp100 cell-surface expression from all gp100 mutants is summarized in (*B*). Representative of six independent experiments.

T-cell clones whereas gp100- and HLA-A*0101-transfected 293-DRβ1*0701^+ cells failed to be recognized by the $CD8^+$ T-cell clone. In addition, gp100- and HLA-A*0201-transfected 293-DRβ1*0401^+ cells failed to be recognized by the $CD4^+$ T-cell clone. Although MHC class I–mediated presentation was similar for all gp100 mutants (Fig. 3*A*), deletions in the COOH terminus resulted in decreased MHC class II–restricted presentation. More specifically, deletion of the last 70 residues (gp100-NoTM) or internal deletion of the transmembrane domain (gp100-ΔTM) reduced MHC class II presentation to <10% of wild-type gp100, suggesting an important role for the transmembrane domain. However, as shown by the MHC class II/class I presentation ratio (Fig. 3*B*), MHC class II presentation was not affected by substitution of the transmembrane domain by the one from CD8 (gp100-CD8). Interestingly, deletion of the putative dileucine motif (gp100-ΔLL), a motif previously described as being important for protein trafficking to endosomes (32–35), minimally diminished MHC class II presentation, as illustrated by the MHC class II/class I presentation ratio (Fig. 3*B*). Further deletion in the COOH-terminal sequence downstream of the transmembrane domain (gp100-TM) resulted in 45% IFN-γ secretion by the $CD4^+$ T-cell clone when compared with the full-length sequence (Fig. 3*A*).

Interestingly, deletion of a sequence of 12 residues, including a tyrosine and 3 consecutive arginines, located immediately after the transmembrane domain (gp100-ΔYV), had minimal effect on MHC class II presentation (Fig. 3*A*). However, this deletion combined with CD8 transmembrane substitution (gp100-ΔYVCD8) abrogated MHC class II presentation, which was revealed by the MHC class II/class I presentation ratio (Fig. 3*B*). Finally, as expected, deletion of the NH_2-terminal signal sequence (gp100-ΔSS) resulted in a marked decrease in MHC class II presentation with no change in MHC class I presentation compared with wild-type gp100 (Fig. 3*A*).

These data suggested a similar expression for all mutants considering that MHC class I–mediated presentation was equivalent (Fig. 3*A*). A comparable expression level was further confirmed by analysis of gp100 expression by Western blotting (Fig. 3*D*). The expression level of gp100-ΔSS could not be evaluated because the epitope recognized by the antibody was located in the NH_2 terminus.

Clearly, the data showed that the last 70 residues, including the transmembrane domain and the NH_2-terminal signal sequence, are critical for gp100 MHC class II–mediated presentation. Interestingly, multiple elements within these sequences seemed to be involved in this process.

Gp100 surface expression correlates with MHC class II presentation. Gp100 can possibly reach relevant endosomal compartments by two pathways for proper processing and MHC class II loading: (*a*) directly from the Golgi and (*b*) by transiting to the cell surface with internalization. Interestingly, gp100 modification resulting in decreased MHC class II presentation

was not caused by endoplasmic reticulum/Golgi retention because endoglycosidase H sensitivity patterns were similar for all gp100 mutants (data not shown). Thus, to address the possibility of transition to the cell surface, we evaluated gp100 cell-surface expression by flow cytometry and compared it with total expression in permeabilized cells (Fig. 3*E*). All gated transfected cells were gp100$^+$ and surface expression was detected in 59% of these cells. The assessment of gp100 cell-surface expression determinates for all mutants is summarized in Fig. 3*B*. Interestingly, all cells transfected with plasmid encoding gp100-ΔLL mobilized gp100 at the cell surface (Fig. 3*E*). In contrast, gp100-NoTM, gp100-ΔTM, gp100-ΔSS, and gp100-ΔYVCD8 failed to mobilize to the cell surface. Consequently, as illustrated in Fig. 3*B*, there was a direct correlation between gp100 cell-surface expression and the MHC class II/class I presentation ratio. These results suggest that gp100 could transit to the cell surface to gain access to relevant endosomal compartments and MIIC.

Gp100 cell-surface expression may be due to overexpression in cells without melanosomes. Consequently, we also evaluated gp100 cell-surface and total expression from eight different melanoma cell lines. Gp100 expression was detected in six of seven melanoma cell lines tested (excluding MelFB; Fig. 4) and gp100 cell-surface expression was observed in three of these six melanoma cell lines. We also noted gp100 cell-surface expression in a gp100$^-$ melanoma cell line (MelFB) engineered to express gp100. Surface expression may have clinical implications and needs to be further investigated.

Endosomal localization of gp100 mutants presented by MHC class II. Characterization of the different versions of deleted gp100 was completed by gp100 localization experiments with laser scanning confocal microscopy of transfected 293T cells stained for gp100 and LAMP-1. As expected, gp100 mutants, presented by MHC class II, were located in intracellular vesicles colocalizing with LAMP-1 (yellow vesicles), similar to wild-type gp100 (Fig. 5*A*). In contrast, the other gp100 mutants showed no specific vesicular localization and no colocalization with LAMP-1, showing the importance of these deleted sequences in gp100 trafficking. Interestingly, we found higher gp100 cell-surface expression in gp100-ΔLL-transfected cells than in wild-type gp100-transfected cells (green fluorescence in cell outline), confirming the gp100-ΔLL cell-surface expression already noted by flow cytometry (Fig. 3*E*).

To further confirm that LAMP-1$^+$ endosomes colocalizing with gp100 were MIIC, we stained gp100-transfected 293T cells and melanoma cells with an anti-gp100, an anti-LAMP-1, and an anti-HLA-DR, and laser scanning confocal microscopy was done. As shown in Fig. 5*B*, LAMP-1$^+$ vesicles containing gp100 (yellow vesicles in the left image) were also positive for HLA-DR (white vesicles in the central image), indicating that they were MIIC. LAMP-1$^+$/HLA-DR$^-$ vesicles in MelFB may represent melanosomes.

These latter experiments confirmed that the putative NH_2-terminal signal sequence and the last 70 residues are essential for gp100 mobilization in MIIC.

Gp100 targeting sequences mobilize GFP to endosomes and allow the presentation of minimal class II and class I epitopes. Gp100 targeting sequences were cloned in fusion with GFP, transfected in 293T cells engineered to express HLA class II and accessory molecules, and laser scanning confocal microscopy

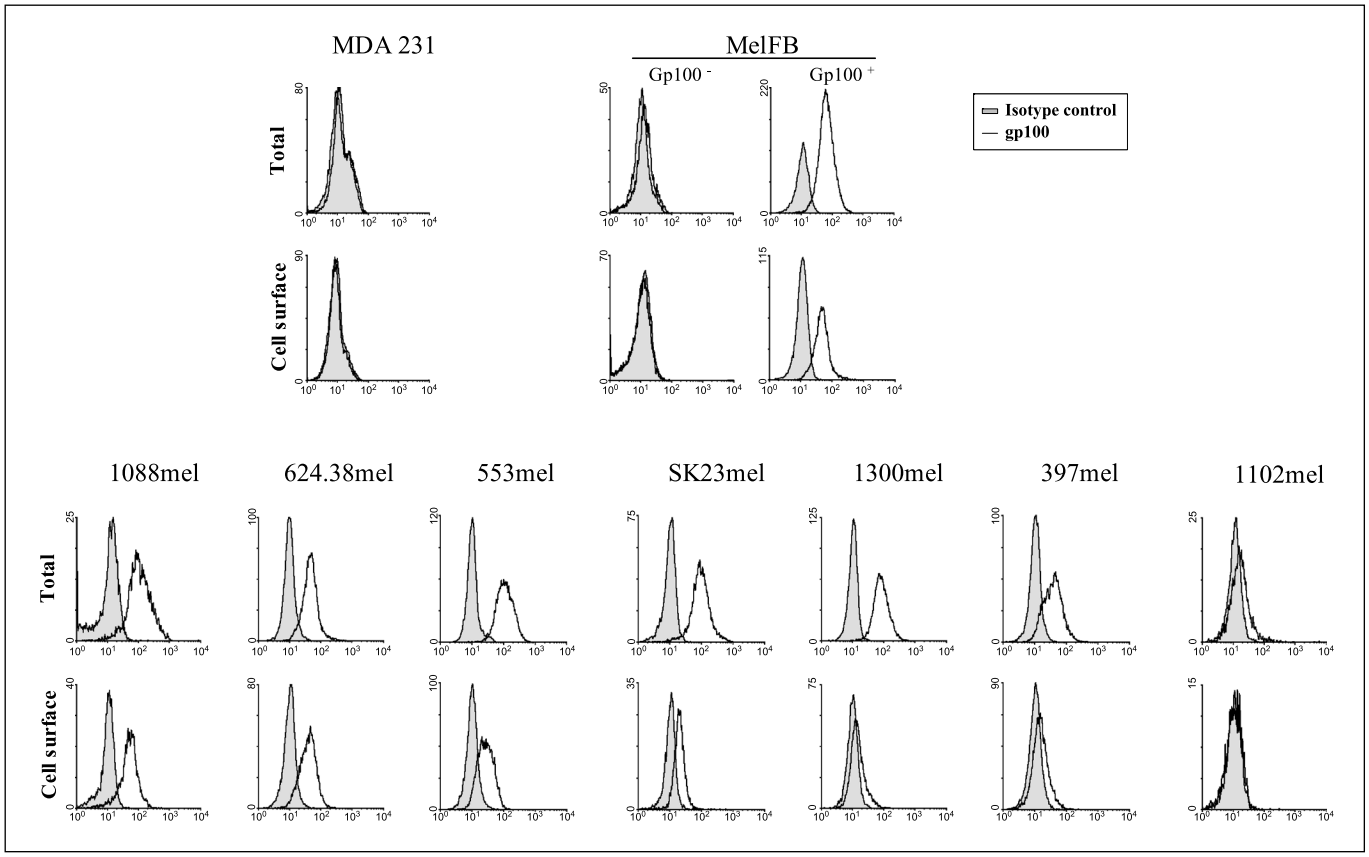

Figure 4. Gp100 cell-surface expression in melanoma cell lines. Gp100 cell-surface expression from different melanoma cell lines was evaluated by flow cytometry (*Cell surface*). Total gp100 expression was also evaluated, by permeabilizing cells before staining (*Total*). Representative of three independent experiments.

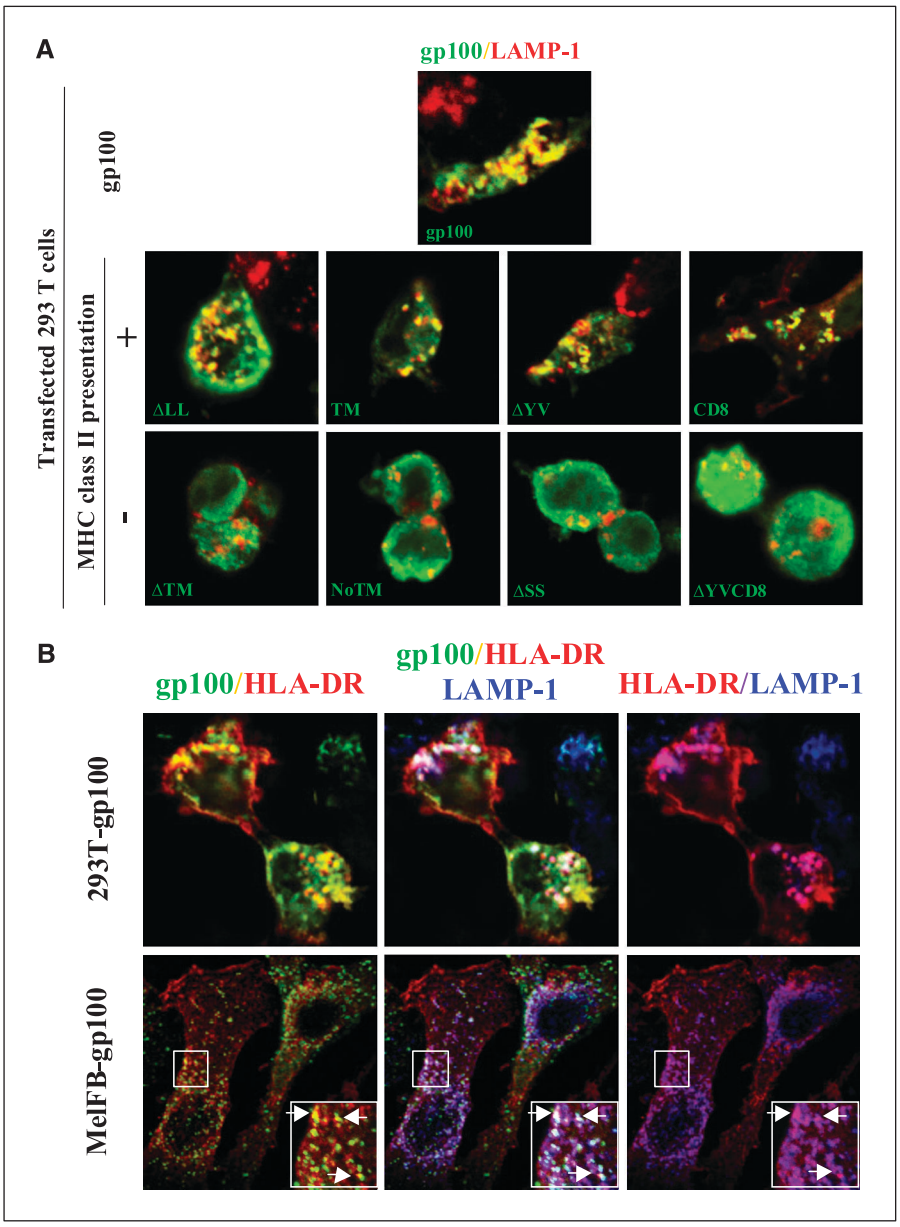

Figure 5. Endosomal localization of gp100 mutant. *A,* 293T cells transfected with plasmid coding for gp100 or gp100 mutants were permeabilized and double stained with an anti-gp100 coupled with Alexa Fluor-488 (*green*) and an anti-LAMP-1 coupled with Alexa Fluor-568 (*red*). The cells were analyzed by laser scanning confocal microscopy. Yellow vesicles, in mutants presented by MHC class II, revealed colocalization of gp100 and LAMP-1. Representative of two independent experiments. *B,* gp100-transfected 293T cells and a melanoma cell line expressing gp100 (MelFB-gp100) were permeabilized and stained with an anti-gp100 coupled with Alexa Fluor-488 (*green*), an anti-HLA-DR coupled with Alexa Fluor-568 (*red*), and an anti-LAMP-1 coupled with Alexa Fluor-647 (*blue*). The cells were analyzed by laser scanning confocal microscopy. White vesicles, in the central image, revealed colocalization of gp100, LAMP-1, and MHC class II molecule HLA-DR. Representative of two independent experiments.

was undertaken. As presented in Fig. 6*A*, wild-type GFP showed no particular mobilization. However, GFP in fusion with the putative signal sequence and the last 70 residues from gp100 (gp100/GFP) colocalized with LAMP-1 (yellow vesicles indicated by white arrows). This confirms the importance of these sequences for protein localization in endosomal compartments and MIIC.

To link endosomal localization to MHC class II–mediated presentation, a short sequence from gp100, corresponding to minimal class II and class I epitopes, was inserted after GFP within the last-mentioned construct. Plasmids encoding this chimeric protein (gp/GFP+epit) and HLA-A*0201 or A*0101 were cotransfected in 293T cells expressing HLA-DRβ1*0701 or DRβ1*0401. GFP expression was confirmed by flow cytometry and vesicular mobilization was studied by fluorescence microscopy (data not shown). As presented in Fig. 6*B*, 293-DRβ1*0701 cells transfected by plasmids encoding gp/GFP+epit or wild-type gp100 were recognized by the $CD4^+$ T-cell clone. As expected, 293-DRβ1*0401 failed to stimulate the $CD4^+$ T-cell clone. Interestingly, gp/GFP+epit and full-length gp100 were efficiently recognized by the $CD8^+$ T-cell clone and the negative control, HLA-A*0101-transfected 293T cells, failed to be recognized. Presentation of gp100-MHC class II epitope was further confirmed in melanoma (MelFB; Fig. 6*C*) and APC (CD40-B).

The latter experiments confirm the importance of these sequences for mobilization to endosomes and for MHC class II–mediated presentation of endogenous gp100.

Discussion

MHC class II antigenic presentation plays a critical role in antitumor immunity. Interestingly, specific endogenous proteins can reach MIIC and get processed for MHC class II presentation, similarly to exogenous proteins. Previous data suggested that endogenous gp100 can be presented by MHC class II molecules and stimulates specific $CD4^+$ T lymphocytes (23, 24). Consequently, we

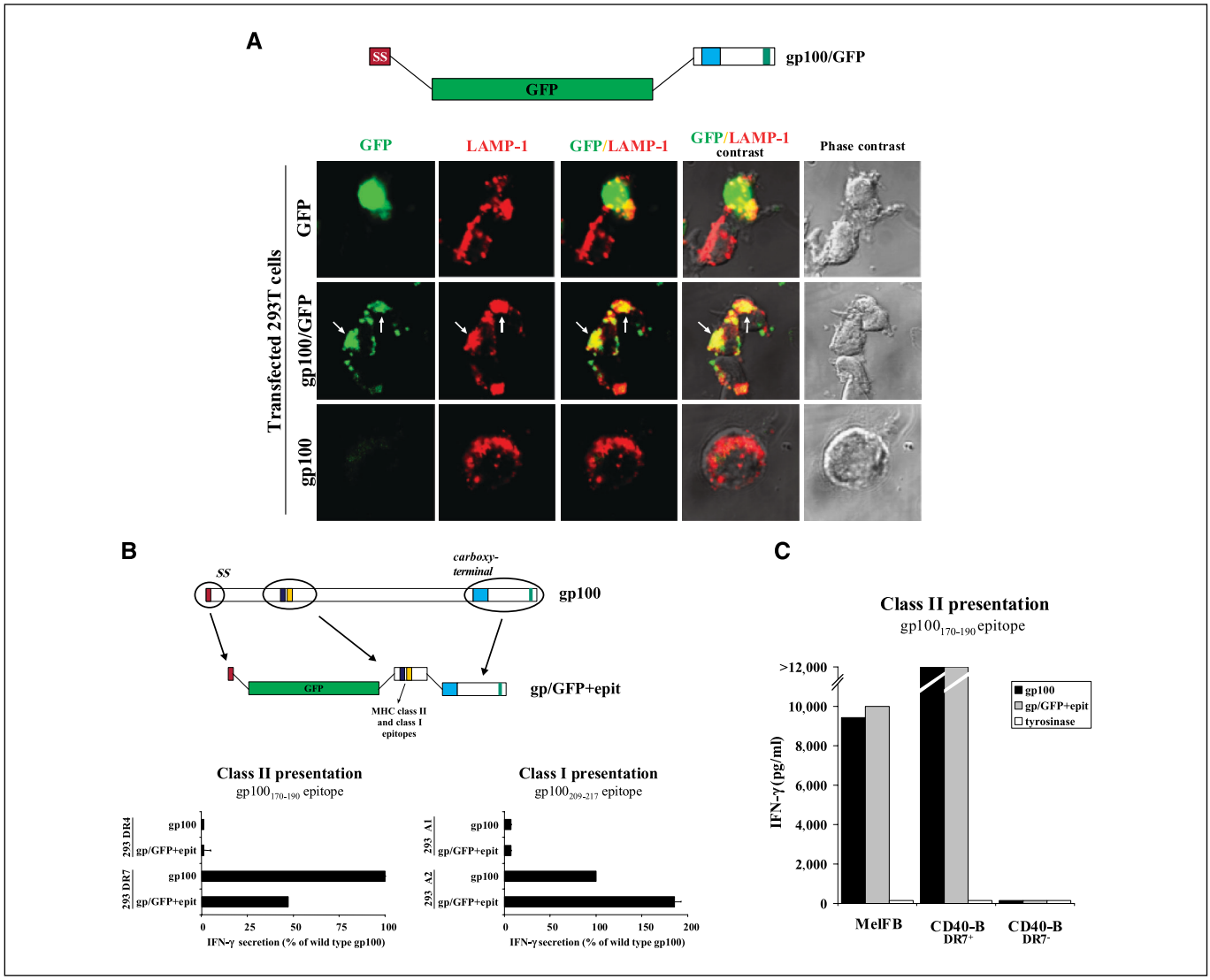

Figure 6. Endosomal mobilization of GFP and presentation of minimal MHC class II and class I epitopes using gp100 targeting sequences. *A,* schematic representation of GFP modified with gp100 targeting sequences (*gp100/GFP*). 293T cells were transfected with plasmids coding for GFP, gp100, or gp100/GFP. The cells were permeabilized, stained with an anti-LAMP-1 coupled with Alexa Fluor-568 (*red*), and analyzed by laser scanning confocal microscopy. Yellow vesicles, in merged image, revealed colocalization of gp100/GFP and LAMP-1 (*white arrows*). Representative of five independent experiments. *B,* schematic representation of GFP modified with gp100 targeting sequences and minimal MHC class II and class I gp100 epitopes (*gp/GFP+epit*). Plasmids encoding gp100 or gp/GFP+epit were transfected in 293T cells expressing HLA-DRβ1*0701 or DRβ1*0401, and a gp100-specific CD4$^+$ T-cell clone was cocultured with these transfected cells (*left*). Plasmids encoding gp100 or gp/GFP+epit and HLA-A*0201 or A*0101 were cotransfected in 293T cells, and a gp100-specific CD8$^+$ T-cell clone was cocultured with these transfected cells (*right*). Supernatants were harvested after 20-hour coculture and IFN-γ secretion was measured by ELISA. Data are presented as a percentage of IFN-γ secretion compared with gp100, normalized to 100%. Average of two independent experiments. *C,* a melanoma cell line (MelFB) and HLA-DRβ1*0701$^+$- or *0701$^-$-stimulated B lymphocytes (CD40-B) were electroporated with plasmids coding for gp100, gp/GFP+epit, or tyrosinase, and a gp100-specific CD4$^+$ T-cell clone was cocultured with these target cells. Representative of two independent experiments.

wanted to further characterize MHC class II–mediated presentation of endogenous proteins and endosomal/MIIC internal trafficking leading to MHC class II presentation. We confirmed that exogenous and endogenous gp100 can be presented by MHC class II. We also showed that gp100 expressed endogenously can reach LAMP-1$^+$/HLA-DR$^+$ endosomal/MIIC, of which processing correlates with presentation by MHC class II. Therefore, we expected to define sequences within gp100 involved in these processes. Our results showed that the putative NH_2-terminal signal sequence and the last 70 residues in the COOH terminus are essential for endosomal localization and MHC class II–mediated presentation of endogenous gp100. Moreover, we showed that gp100 cell-surface expression correlates with MHC class II presentation, suggesting gp100 transition to the cell surface before internalization to relevant endosomal/MIIC.

Mobilization of endogenous proteins to endosomal/MIIC and processing through the MHC class II pathway has already been evoked, especially in melanoma. Some melanosomal proteins might contain epitopes recognized by CD4$^+$ T lymphocytes. Topalian et al. (36) had previously found tyrosinase-specific CD4$^+$ T cells from populations of tumor-infiltrating lymphocytes cultured from a metastatic melanoma lesion. Other groups had also isolated tyrosinase-specific CD4$^+$ T lymphocytes from peripheral blood of melanoma patients (37, 38). It could be suggested that tyrosinase and probably other melanosome membrane proteins, such as gp100, have the capacity to reach MHC class II molecules

transported to endosomal/MIIC. Possibly, melanosomes, endosomes/lysosomes, and MIIC may be compartments with very similar characteristics (39, 40). Because gp100 is known to reach melanosomal compartments via an intracellular pathway (41), it can also possibly reach endosomal/MIIC by a similar pathway.

Two major pathways have been defined by which proteins enter endosomal/MIIC to be processed for MHC class II–restricted presentation and recognition by $CD4^+$ T lymphocytes. The traditional pathway involves the endocytosis of exogenous proteins by APC. In contrast, the second pathway implicates the processing of endogenously synthesized membrane proteins (42). These membrane proteins are believed to enter endosomal compartments by internalization from the cell surface. As we observed a direct correlation between gp100 cell-surface expression and MHC class II–mediated presentation (Fig. 3*B*), it is possible that gp100 also reached endosomal/MIIC by internalization from the cell surface.

Considering previous studies showing that melanosomal glycoproteins were mobilized to melanosomes by a dileucine-based sorting motif (32), we originally predicted that removal of the putative gp100-dileucine motif would impair endosomal/MIIC mobilization and MHC class II presentation. Surprisingly, removal of the gp100 putative dileucine motif (gp100-ΔLL) unchanged mobilization to $LAMP\text{-}1^+$ endosomes (Fig. 5*A*) and presentation by MHC class II (Fig. 3*A*). It is plausible that this putative dileucine motif is not functional even if it fits with the accepted consensus sequence. Actually, functional dileucine motifs from gp75 (43) and tyrosinase (33, 34) are structurally surrounded by basic residues, an acidic spacer, and tyrosine residues, which is not the case for gp100.

However, the gp100 transmembrane domain is clearly important for endosomal/MIIC mobilization and MHC class II–mediated presentation. We showed that substitution of the transmembrane domain by the one from CD8 did not affect gp100 MHC class II–mediated presentation (Fig. 3*A*), indicating that the presence of a transmembrane domain with all other sequences could be sufficient to mediate endosomal/MIIC mobilization of gp100. Interestingly, deletion of a sequence, including a tyrosine residue and three consecutive arginine residues, located immediately after the transmembrane domain and associated with CD8 transmembrane substitution, negatively affected MHC class II presentation whereas deletion of this sequence alone had no effect compared with wild-type gp100 (Fig. 3*A*). This could possibly be caused by an inappropriate folding of the protein within the membrane in the gp100-ΔYVCD8 construct. However, it could also suggest the involvement of important residues in the gp100 Y to V sequence (residues 616-627) that may have a role with the gp100-specific transmembrane domain for endogenous MHC class II presentation, but both are not absolutely required. Interestingly, other membrane glycoproteins, such as LAMP-1 or other lysosomal proteins, contain a lysosomal-trafficking tyrosine residue in a specific position within the cytoplasmic tail (44, 45).

Specifically, the lysosomal targeting signal formed the pattern Y-X-X-hydrophobic residue (46). In the gp100 sequence, the tyrosine residue is followed by three consecutive arginine residues before a leucine, a hydrophobic residue (Y-R-R-R-L). Three arginine residues have already been identified as an endoplasmic reticulum retention signal (RXR; refs. 47, 48), known to play an important role in trafficking of many membrane proteins to the cell surface (49). Here, we observed that deletion of this motif did not affect gp100 cell-surface trafficking (Fig. 3*B*). However, MHC class II presentation was abrogated when this deletion was associated with CD8 transmembrane substitution (Fig. 3*A*).

Interestingly, these findings suggested that more than one sequence have a related role in gp100 MIIC trafficking. Moreover, we confirmed that there are essential sequences present within the gp100 putative NH_2-terminal signal sequence and the last 70 residues in the COOH terminus for endosomal/MIIC localization and MHC class II–mediated presentation of endogenous gp100.

To complete our study, we employed these gp100 targeting sequences to mobilize GFP, as a model endogenous protein, to endosomal compartments (Fig. 6*A*). In addition, we showed that these targeting sequences allow MHC class II and class I presentation of endogenous epitopes linked to GFP in 293T cells, melanoma, and APC (Fig. 6*B* and *C*). Consequently, we believe that these sequences could be exploited as a tool *in vivo* to optimize $CD4^+$ and $CD8^+$ T-cell responses against tumor- and viral-associated antigens. Specifically, MIIC-targeting sequences could be included in expression cassettes for DNA vaccines or for improved expression in APC and optimized MHC class II presentation without apparent negative effect on MHC class I presentation. Thus, these targeting sequences could also be exploited as an *in vitro* tool to characterize new MHC class II epitopes and promote the stimulation of both $CD4^+$ and $CD8^+$ T cells.

Acknowledgments

Received 7/19/2005; revised 11/15/2005; accepted 12/22/2005.

Grant support: Canadian Institutes of Health Research, La Fondation du Centre Hospitalier de l'Université de Montréal, scholarship from Fond pour la recherche en santé du Québec (R. Lapointe), and studentship from the Canderel fund of the Institut du Cancer de Montréal (S. Lepage).

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We thank Ovid Da Silva from the "Bureau d'aide à la recherche" (from Centre Hospitalier de l'Université de Montréal) for text editing, Dr. Jacques Thibodeau for critical review of the manuscript and helpful discussions, and Diane Beauseigle for technical support.

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