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Targeting to Static Endosome Is Required for Efficient Cross-Presentation of Endoplasmic Reticulum-Resident Oxygen-Regulated Protein 150-Peptide Complexes

Goro Kutomi,*† Yasuaki Tamura,2* Koichi Okuya,*† Takashi Yamamoto,* Yoshihiko Hirohashi,* Kenjiro Kamiguchi,* Jun Oura,* Keita Saito,*† Toshihiko Torigoe,* Satoshi Ogawa,‡ Koichi Hirata,† and Noriyuki Sato*

Heat shock proteins (HSPs) such as Hsp70, Hsp90, and gp96 have been shown to elicit intriguing, efficient CTL responses by cross-presentation via an as yet entirely unknown mechanism. Oxygen-regulated protein 150 (ORP150), also known as grp170, is an endoplasmic reticulum-resident HSP and is up-regulated by hypoxia. It has been demonstrated that ORP150 binds tumor-associated Ag peptides within cancer cells. Immunization with an ORP150-tumor Ag complex has been shown to generate tumor-specific CTLs. Most recently, it has been shown that exogenous ORP150 induces cross-presentation of a chaperoned Ag, thereby stimulating Ag-specific CTLs. However, the mechanism underlying this efficient cross-presentation is still unsolved. In this study, we show that the ORP150-precursor peptide complex can elicit CTL response through cross-presentation as well as the CD4+ T cell response by dendritic cells. Furthermore, we observed that the internalized ORP150-peptide complex, but not OVA protein, which was not cross-presented, was sorted to the Rab5*, EEA1* static early endosome, followed by translocation to a recycling endosome, where the ORP150-chaperoned peptide was processed and bound to MHC class I molecules. Moreover, we observed that immunization of mice with ORP150-peptide complexes elicited strong peptide-specific CTLs and antitumor effects in vivo. Our data indicate that targeting of the Ag to a “static” early endosome by ORP150 is required for the efficient cross-presentation. The Journal of Immunology, 2009, 183: 0000–0000.

It is well known that tumor-derived heat shock proteins (HSPs) such as Hsp70, Hsp90, and gp96 initiate efficient tumor-specific CTL responses and protective immunity (1–5). Although immunized HSPs are exogenous Ags, these HSP-Ag complexes can gain access to the class I Ag presentation pathway, resulting in the stimulation of CD8+ T cells, termed cross-presentation (6–11). The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes to prime CD8+ T cell responses is due to the following: (1) HSPs are able to form stable complexes with antigenic peptides/proteins, (2) HSP-peptide/protein complexes are able to bind surface receptors on APCs, resulting in receptor-dependent endocytosis, and (3) HSP can stimulate an innate immune response, which is not dependent on tumor Ags (12, 13). It is thought that HSPs bind to receptors on APCs, resulting in secretion of proinflammatory cytokines and maturation and activation of dendritic cells (DCs). To date, CD91 (14–16), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (17), CD40 (18), scavenger receptor type A (SR-A) (19, 20), and scavenger receptor expressed by endothelial cells-I (SREC-I) (20, 21) have been demonstrated to be receptors for several kinds of HSPs expressed on APCs.

DCs are the most potent APCs for efficient cross-presentation. Recently, it has been shown that DCs can internalize HSP-peptide complexes by receptor-mediated endocytosis and direct chaperoned peptides into the intracellular pathway for MHC class I-restricted presentation to CD8+ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion (10). However, the underlying mechanism for efficient cross-presentation, in particular how the HSP-Ag complex can enter the MHC class I pathway, is not well understood. Recently, we have demonstrated that extracellular Hsp90-peptide complexes are efficiently cross-presented via the endosome-recycling pathway (22). In this Hsp90-mediated cross-presentation, the receptor-dependent endocytosed Hsp90-peptide complex was transferred to the early endosome in which a cysteine protease such as cathepsin S processed the precursor peptide. The resulting MHC class I epitope was transferred onto recycling MHC class I molecules, thereby expressing an MHC class I-epitope complex on the cell surface. Furthermore, we have shown that immunization with Hsp90-tumor Ag peptide complexes induces Ag-specific CTL responses and strong antitumor immunity in vivo.

Oxygen-regulated protein 150 (ORP150), also known as glucose-regulated protein 170 (grp170), was first described in 1996 by Kuwabara et al. (23). It is an endoplasmic reticulum (ER)-resident Hsp70 superfamily member, and it is induced by stress conditions such as hypoxia, ischemia, glucose deprivation, reductive reagents,
and interference with calcium homeostasis (24). Subjeck’s group has performed extensive research and shown that the ORP150 induces antitumor immunity in vivo (25–28). Vaccination with ORP150 purified from tumors suppresses the same tumor growth in mice and induces tumor-specific CTLs. Furthermore, it has been demonstrated that ORP150 induces efficient cross-presentation of chaperoned Ags, thereby stimulating the Ag-specific CTLs (28). Moreover, the receptor for ORP150 has been shown to be SR-A and SREC-I (20). However, the precise intracellular mechanism for cross-presentation of ORP150-peptide complexes is not well understood.

In this study, we first examined whether an ORP150-precursor peptide complex could elicit a strong peptide-specific CTL response and antitumor immunity through cross-presentation using bone marrow-derived DCs (BMDCs) as APCs. Furthermore, we analyzed the intracellular trafficking pathway of the ORP150-peptide complex for efficient cross-presentation within DCs. Recently, Lakadamyali et al. have shown that early endosomes are comprised of two distinct populations, called “static” early endosome, which is slow maturing, and rapidly maturing “dynamic” early endosome (29). Furthermore, Burgdorf et al. have demonstrated that the mannose receptor introduced OVA specifically into an endosome (29). Furthermore, Burgdorf et al. have demonstrated that targeting of the ORP150-peptide complex to the EEA1⁺, Rab5⁻static early endosomal compartment for subsequent cross-presentation. In contrast, pinocytosis conveyed OVA to lysosome for class II presentation (30). Our observations revealed that targeting of the ORP150-peptide complex to the EEA1⁺, Rab5⁻static early endosome is crucial for cross-presentation. We propose that the static early endosome plays an important role in HSP-mediated cross-presentation by DCs.

Materials and Methods

Mice

Female B6C3F1 (H-2k) mice, C57BL/6 (H-2b) mice, and TAP1⁻/⁻ (H-2b) mice were purchased from The Jackson Laboratory and used at 6 wk of age. Mice were maintained in a specific pathogen-free mouse facility at Sapporo Medical University according to institutional guidelines for animal use and care.

Cells

The B3Z cell is a CD8⁺ T cell hybridoma specific for the OVA257-264 epitope (SL8) in the context of H-2Kb. The KZO cell is a CD4⁺ T cell hybridoma specific for the OVA257-264 (PL19) in the context of I-Ã. These hybridomas synthesize a reporter enzyme when the TCR engages the H-2Kb-SL8 (SIINFEKL) complex or the I-Ak-PL19 (PDEVSGLEQLESI) complex, respectively. The cell lines were obtained from Dr. N. Shastri (University of California at Berkeley, Berkeley, CA). RMA-S-A²402 cells were RMA-S transfected with the gene encoding HLA-A*2402 (provided by Dr. H. Takasu, Dainippon-Sumitomo Pharmaceutical, Osaka, Japan). TG3 cells are a methylcholanthrene-induced fibrosarcoma derived from the HLA-A*2402 transgenic mouse. TG3–2B cells were TG-3 transfected with the gene encoding human survivin-2B (survivin-2B). YAC-1 cells and 293T cells were obtained from the American Type Culture Collection.

Generation of BMDCs

Bone marrow-derived immature DCs were generated from bone marrow cells that were obtained from the femurs and tibiae of female C57BL/6 mice, B6C3F1 mice, and TAP1⁻/⁻ mice. Bone marrow cells (1 × 10⁶/ml) were cultured in complete RPMI 1640 with 10% FCS and 20 ng/ml GM-CSF (Endogen) for 5 days. Medium with GM-CSF was gently replaced on day 2 and day 4.

Construction of secreted form of ORP150 and generation of 293T cells secreting ORP150

Using human ORP150 cDNA as a template, the sense primer (5’GGACGAGAGGGCCTTGGAGGGCCGCCGGC-3’) and the antisense primer (5’GGACAGACAGGGGCTTGGAGGGCCGCCGGC-3’) were used to generate an NDEI-ORP150 cDNA. The PCR product was digested with BamHI/NcoI and ligated into pIRESpuro3-3myc/His vector (Clontech Laboratories). The sequence of the construct was verified by DNA sequencing. 293T cells were transfected with this construct using Lipofectamine 2000 (Invitrogen). Cells were then subcloned with 1 mg/ml puromycin (Invitrogen) and a 293T cell-secreting ORP150, 293T-ORP4, was established.

Purification of secreted ORP150

293T-ORP4 cells were cultured with 10% DMEM for 3 days. Then, the culture supernatant of 293T-ORP4 cells was collected and applied to a Con A-agarose column (Amersham Biosciences). After extensive washing of the column with Con A-BSA (150 mM NaCl, 5 mM Na2EDTA, 1.35 mM MgCl₂, 1.35 mM MgCl₂), ORP150 was eluted off with Con A-BSA containing 10 mM methyl α-6-mannopyranoside (Sigma-Aldrich). The presence of ORP150 in the eluted fraction was examined by SDS-PAGE stained with Coomassie brilliant blue (Bio-Rad) and Western blotting using a mAb against ORP150 (IBL). Typically, fractions 2–5 contained purified ORP150 and these fractions were pooled and concentrated using Microcon YM-100 (Millipore). The concentration of ORP150 was determined using a Micro BCA protein assay reagent kit (Pierce). Quantification of the endotoxin in the purified ORP150 was performed using the endotoxin-specific chromogenic test (ES test; Seikagaku Kogyo). If the endotoxin in the ORP150 preparation was higher than the limit of detection (<5 pg/ml), endotoxin was depleted using Detoxi-Gel endotoxin removing gel (Pierce) and then requantified.

Peptides and proteins

The following peptides were used (underlined sequences represent the precise MHC class I- or class II-binding epitope): survivin-2B, a (AYAC-NTSTL), SL8 (SIINFEKL), SL8C (SIINFEKLTEWTS), PL24 (PDEVSGLEQLESI) and SLC26 (SIINFEKTLTWTSVNEEKIRKYYL). All peptides were synthesized on a solid phase support using F-moc for transient NH₂-terminal protection and were characterized using mass spectrometry. They were purified by HPLC to >99% homogeneity and stored at 2–8°C in distilled H₂O at −80°C. BSA was purchased from Sigma-Aldrich. Chicken OVA was purified by Calbiochem and stored at 20 mg/ml in PBS at −80°C.

Antibodies

Confocal laser microscopy was used to detect organelles with specific Abs: an anti-Rab5 pAb (MBL) and EEA1 (Abcam) for early endosomes, anti-H-lysosome-associated membrane protein 1 (LAMP-1) pAb (Santa Cruz Biotechnology) for late endosomes/lysosomes, anti-Rab11 pAb (Santa Cruz Biotechnology) for recycling endosomes, anti-KDEL mAb (StressGen Biotechnologies) for ER. mAb 25D-1.16 specific for the K/β-OVA257-264 complexes was provided by Dr. R. Germain (National Institutes of Health, Bethesda, MD). The Abs were each conjugated with Alexa Fluor 488 (Molecular Probes) according to the manufacturer’s instructions. Alexa Fluor 594 (Molecular Probes) was used for labeling ORP150.

Generation of ORP150-peptide complex in vitro

Peptide SLC26 (SIINFEKTLTWTSVNEEKIRKYYL) was iodinated with ¹²⁷I using IODO-BEADS (Pierce) according to the manufacturer’s instructions. ORP150 or control protein BSA was mixed with the ¹²⁷I-labeled peptide in a 50:1 peptide-to-protein molar ratio in 0.7 M NaCl containing sodium-phosphate buffer and heated at 45°C for 30 min, then incubated for 30 min at room temperature. The complex was separated from unbound peptide using a Microcon YM-100 or YM-30 (Millipore), Radioactivity of the complex and unbound peptide was measured in a gamma counter for evaluation of the binding efficiency of the ORP-peptide complex. Samples were then analyzed by SDS-PAGE, followed by autoradiography of the stained gel.

In vitro cross-presentation assay

DCs (1 × 10⁶) from C57BL/6 or TAP1⁻/⁻ mice were pulsed with PLC24 (20 μg) alone, different ORP150/peptide dose ratios, or SL8 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. Fixation was stopped by addition of 2 M l-lysolecithin and the cells were washed twice with RPMI 1640 medium and cultured overnight with 1 × 10⁵ B3Z. The B3Z response was measured as β-galactosidase activity induced upon ligand recognition. The β-galactosidase activity was measured by the absorbance at 595 nm of the cleavage product of chlorophenol red-β-D-galactopyranoside (CPRG; purchased from Roche). In other experiments, DCs (1 × 10⁶) from C57BL/6 or TAP1⁻/⁻ mice were pulsed with PLC24 (20 μg) alone, PLC24 (20 μg) alone, a complex of both generated in vitro, a simple mixture of both, OVA (200 μg/ml), or SL8 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, fixed, and then cocultured overnight with 1 × 10⁵ B3Z.
Ag presentation assay

DCs (1 × 10^5) from B6C3F1 mice were pulsed with ORP150 (20 μg) alone, PLC24 (20 μg) alone, a complex of both generated in vitro, a simple mixture of both, OVA (200 μg/ml), or PL19 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. Fixation was stopped by addition of 2 M L-lysine and the cells were washed twice with RPMI 1640 medium and cultured overnight with 1 × 10^5 KZO. The KZO response was measured as β-galactosidase activity using CPRG by the absorbance at 595 nm.

Inhibition studies

DCs (1 × 10^5) from B6C3F1 mice were preincubated with primaquine (ICN Biomedicals). Primaquine was not toxic in our culture systems. Two hours after preincubation, the DCs were pulsed with the ORP150 (20 μg)/PLC24 (20 μg) complex, SL8 (1 μg/ml), or PL19 (1 μM) for 2 h at 37°C in 100 μl of Opti-MEM, then fixed, washed, and cultured overnight with B3Z or KZO. Stained B3Z or KZO were stained with CPRG and red color was measured as absorbance at 595 nm.

Immunocytological localization of ORP150-SL8C complex

ORP150, transferrin (Molecular Probes), and OVA were conjugated with Alexa Fluor 594 (Molecular Probes) according to the manufacturer’s instructions. Immature BMDCs were incubated at 37°C with Alexa Fluor 594-labeled ORP150 (20 μg) complexed with PLC24 (20 μg) for 1 h. Following incubation, cells were washed twice with ice-cold PBS and fixed with ice-cold acetone for 1 min. Organelles were stained with an anti-Rab5 pAb and EEA1 mAb for early endosomes, anti-LAMP-1 pAb for late endosomes and lysosomes, anti-Rab11 pAb for recycling endosomes, or anti-KDEL mAb for ER, followed by Alexa 488-conjugated goat anti-rabbit IgG or anti-mouse IgG and visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system. For detecting the intracellular localization of H-2K^b/SL8 complex using mAb 25D-1.16, the DCs were first incubated with ORP150 complexed with PLC24 for 1 h and fixed with cold acetone. DCs were then incubated with anti-mouse CD16/CD32 Fc-block to block nonspecific staining, followed by costaining with an Alexa Fluor 594-labeled mAB 25D1.16 and anti-organelle Abs conjugated with Alexa Fluor 488. For evaluation of colocalization, single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed.

51Cr-release assay

Each HLA-A*2402/pk transgenic mouse was immunized s.c. at the base of the tail, twice with a 1-wk interval, with the ORP150 (50 μg)-survivin-2B peptide (50 μg) complex. One week after the last immunization, spleenocytes of immunized mice were cultured with irradiated (100 Gy) and survivin-2B peptide-pulsed naive spleen cells for 5 days. Subsequently, the generation of survivin-2B-specific peptide-specific CTLs was evaluated in a 51Cr-release assay. The specificity of CTLs induced was evaluated using TG3–2B cells, RMA-S-A*2402 cells, RMA-S-A*2402 cells pulsed with 1 μg/ml survivin-2B peptide, and YAC-1 cells as targets.

Transplantation of tumor cells and immunotherapy

TG3–2B cells (5 × 10^5) were intradermally transplanted into the right flank in HLA-A*2402/pk transgenic mice on day 0. When average tumor diameter reached 3–4 mm, the mice were then treated with ORP150 (50 μg) alone, the ORP150 (50 μg)-survivin-2B peptide (50 μg) complex, or survivin-2B peptide (50 μg) via s.c. administration at the nape of the neck twice each week for 2 wk (on days 9, 12, 17, and 21). Control groups of mice were immunized with PBS. Tumor growth was recorded twice each week. Average diameters of the two axes were plotted so that therapeutic effects could be compared among the groups. On day 35, tumor rejection rates were compared among the groups. Average tumor diameters on day 35 were statistically analyzed using the Mann-Whitney U test. Statistical analyses for evaluating the survival advantages were performed using log-rank analysis. All of the experiments were performed with 8–10 mice per group.

Immunohistochemical analysis

After treatment of the preestablished TG3–2B tumor with the ORP150-survivin-2B peptide complex or PBS, tumor tissue was excised on day 35. The frozen tissues were stained with an anti-mouse CD4 mAb (Santa Cruz Biotechnology) or an anti-mouse CD8 mAb (Chemicon International) and then incubated with HRP-conjugated goat anti-rat Ig (Dako), followed by hematoxylin counterstaining. The numbers of tumor-infiltrating CD4^+ and CD8^+ T cells were counted in 10 high-power fields (HPF; ×400).

Statistical analysis

All experiments except for the tumor transplantation experiments were independently performed three times in triplicate. Results were given as means SEM. Comparisons between two groups were performed using Student’s t test, whereas comparisons between multiple groups were done using ANOVA test, with a value of p < 0.05 considered to be statistically significant.

Results

Purification of secreted form of ORP150

Secreted ORP150 was purified from the culture supernatant of 293T-ORP4 cells using a Con A-Sepharose column as described in Materials and Methods. Fractions containing homogeneous ORP150 were collected and characterized by gel staining (Fig. 1A) and immunoblotting using a mAb against ORP150. ORP150 and BSA were mixed with a 125I-labeled peptide in a 50:1 peptide-to-protein molar ratio in sodium phosphate buffer. Samples were analyzed by SDS-PAGE and stained using Coomassie brilliant blue, followed by (B) Western blotting using a mAb against ORP150. C, ORP150 and BSA were mixed with a 125I-labeled peptide in a 50:1 peptide-to-protein molar ratio in sodium phosphate buffer. Samples were analyzed by SDS-PAGE and stained using Coomassie brilliant blue, followed by autoradiography of the stained gel.

Generation of ORP150-peptide complex in vitro

We ascertained and quantified the loading of peptides onto ORP150. We employed the iodinated SLC26 peptide as a tracer. As others and as we have already demonstrated, heat shock treatment accelerates the loading of peptides onto the binding sites of ORP150 and other HSPs (22). As shown in Fig. 1C, ORP150 bound labeled peptides efficiently, but control protein BSA bound
only marginal levels. The molar ratio of the bound SLC26 peptide to ORP150 was 3.95 mol of peptide per mole of ORP150 protein.

**ORP150-PLC24 peptide complex is cross-presented by DCs in vitro and vivo**

We first examined whether ORP150 facilitated the cross-presentation of the chaperoned precursor peptide. To determine the optimal ORP150/peptide dose ratio, DCs from C57BL/6 mice were loaded with different ORP150/peptide dose ratios for 2 h, fixed, and then cocultured with B3Z CD8$^+$ T cell hybridoma. As shown in Fig. 2, A and B, we observed that the complex generated using 20 $\mu$g/ml ORP150 and 20 $\mu$g/ml PLC24 peptide yielded sufficiently enough for the cross-presentation. In contrast, the same dose of PLC24 peptide alone was not cross-presented by DCs. Additionally, we examined whether complex formation between ORP150 and peptide was required for enhanced cross-presentation. DCs were pulsed with ORP150 alone, the PLC24 precursor peptide alone, a complex of both generated in vitro, a simple mixture of both, OVA protein, or SL8 peptide (for positive control) for 2 h at 37°C, then fixed, washed, and cultured with B3Z. The B3Z response was analyzed using CPRG. D, B6C3F1 mice were immunized into the footpads with ORP150 alone, SL8C alone, or a complex of both. After 12 h, CD11c$^+$ DCs were isolated from draining popliteal lymph nodes and cocultured with B3Z. The B3Z response was analyzed using CPRG. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

**FIGURE 2.** Cross-presentation of ORP150-chaperoned peptides by BMDCs in vitro and vivo. A and B, DCs ($1 \times 10^5$) from C57BL/6 mice were pulsed with PLC24 (20 $\mu$g) alone, different ORP150/peptide dose ratios, or SL8 (1 $\mu$M) for 2 h at 37°C in 100 $\mu$l of Opti-MEM, and fixed for 1 min with 0.01% glutaraldehyde. C, ORP150 alone, PLC24 alone, a complex of both, a mixture of both, OVA, or SL8 was loaded onto BMDCs and cultured overnight with B3Z. The B3Z response was analyzed using CPRG. D, B6C3F1 mice were immunized into the footpads with ORP150 alone, SL8C alone, or a complex of both. After 12 h, CD11c$^+$ DCs were isolated from draining popliteal lymph nodes and cocultured with B3Z. The B3Z response was analyzed using CPRG. Data are shown as means ± SEM of three independent experiments. *, $p < 0.01$.

As the PLC24 peptide included both the H-2Kb epitope SL8 and I-Ak epitope PL19, we tested whether the ORP150-PLC24 peptide complex was presented through the MHC class II pathway, thereby inducing CD4$^+$ T cell responses. DCs from B6C3F1 mice were pulsed with ORP150 alone, PLC24 peptide alone, a complex of ORP150 and PLC24 peptide, a simple mixture of both, soluble OVA, or PL19 peptide (for positive control) for 2 h at 37°C, then cells were fixed, washed, and cocultured with KZO CD4$^+$ T cell hybridoma. While ORP150 alone and the PLC24 peptide alone were unable to induce CD4$^+$ T cell responses, the ORP150-PLC24 peptide complex induced a robust CD4$^+$ T cell response as well as a CD8$^+$ T cell response (Fig. 3). As expected, soluble OVA was also presented in association with I-Ak. These data indicated that a cross-presentation competent “ORP150-peptide complex” and incompetent “soluble OVA” might be translocated to different intracellular compartments for Ag processing and presentation. Based on our observation, we focused on the intracellular trafficking mechanism responsible for ORP150-mediated cross-presentation compared with soluble OVA.
ORP150-PLC24 peptide complexes are cross-presented by a TAP-independent and recycling pathway

It is generally accepted that exogenous Ags are cross-presented by two distinct pathways in a TAP-dependent and -independent fashion. We examined whether cross-presentation of the ORP150-PLC24 peptide complex depended on TAP transport. DCs from B6C3F1 or TAP−/− mice were pulsed with the ORP150-PLC24 complex for 2 h. DCs from the TAP−/− mouse could process and present the ORP150-chaperoned peptide as efficiently as did DCs from the wild-type mouse (Fig. 4A). This suggested that ORP150-mediated cross-presentation might involve an endosome-recycling pathway. Therefore, we used primaquine, an inhibitor of membrane recycling, in the cross-presentation assay. DCs were preincubated with primaquine and subsequently pulsed with the ORP150-PLC24 complex. Primaquine did, indeed, show the dose-dependent inhibition of cross-presentation of the ORP150-chaperoned peptide (Fig. 4B). These data suggested that cross-presentation of the ORP150-PLC24 complex accessed recycling MHC class I molecules in endocytic compartments.

Immunocytological localization of ORP150-PLC24 complex

To further support all of the above results, we investigated the intracellular routing of ORP150 after uptake of it in DCs, using confocal laser microscopy. DCs were incubated with the Alexa Fluor 594-labeled ORP150-PLC24 peptide complex for 1 h. Following incubation, the cells were fixed and stained with Abs against markers for organelle structures such as EEA1, Rab5, LAMP-1, Rab11, and KDEL. The Alexa Fluor 594-labeled ORP150-peptide complex was detected in EEA1−, Rab5−, early endosomes and Rab11−, recycling endosomes, but not in lysosomes or ER (Fig. 5A). Quantitative analysis of the colocalization between the exogenous ORP150-peptide complex and EEA1,
some recycling pathway (Fig. 5). An exogenous ORP150-peptide complex was delivered to endosomes; Rab5, and Rab11 revealed an average colocalization incidence of 85.6, 74.4, and 58.9%, respectively, further evidencing that the Rab5, and Rab11 early endosome as well as the LAMP-1- late endosome/lysosome, but not to the EEA1 

To quantify the percentage of the colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments.

Rab5, and Rab11 revealed an average colocalization incidence of 85.6, 74.4, and 58.9%, respectively, further evidencing that the exogenous ORP150-peptide complex was delivered to an endosome-recycling pathway (Fig. 5B). Moreover, we examined the dynamics of Alexa Fluor 594-labeled transferrin as a positive control protein for recycling endosomes (Fig. 6). As expected, transferrin localized to EEA1 -static early endosomes and Rab11- recycling endosomes. In contrast, Alexa Fluor 594-labeled soluble OVA localized to the Rab5 early endosome as well as the LAMP-1 late endosome/lysosome, but not to the EEA1 or Rab11 compartment, thus indicating the dynamic endosomal pathway (Fig. 7). These results indicated that the ORP150-peptide complex was sorted into the static endosomal pathway, not the dynamic endosomal pathway. These data suggested that targeting to the static early endosome was required for efficient cross-presentation by DCs.

Early endosomes and recycling endosomes are the compartments where exogenous ORP150-chaperoned precursor peptides are processed and transferred onto recycling MHC class I molecules

To investigate in which compartment the ORP150-PLC24 peptide complex was processed and resulting peptide bound to MHC class I molecule, we used mAb 25D1.16, as this mAb recognized the SL8 peptide-H2Kb complex. We clearly observed that mAb 25D1.16 was detected in early endosome and recycling endosome, and not in the ER (Fig. 8). To confirm the staining specificity of mAb 25D1.16, DCs were incubated with ORP150-irrelevant peptide survivin-2B 

mAb 25D1.16 and each organelle. We did not observe the specific staining of mAb 25D1.16 (supplemental Fig. 1).4 These data indicated that the ORP150-chaperoned precursor peptides were processed and bound to MHC class I within early endosomes and recycling endosomes, suggesting that recycling MHC class I molecules were necessary for efficient cross-presentation of the ORP150-chaperoned peptides.

FIGURE 6. A, DCs were incubated at 37°C with Alexa Fluor 594-labeled transferrin (TF). Organelles were stained with an anti-EEA1 mAb, anti-Rab5 pAb, anti-Rab11 pAb, and anti-LAMP-1 pAb, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Colocalization of internalized transferrin and each organelle is indicated with arrows. B, To quantify the percentage of the colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *p < 0.01.

FIGURE 7. A, DCs were incubated at 37°C with Alexa Fluor 594-labeled OVA. Organelles were stained with an anti-EEA1 mAb, anti-Rab5 pAb, anti-Rab11 pAb, and anti-LAMP-1 pAb, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG or anti-mouse IgG. Colocalization of internalized OVA and each organelle is indicated with arrows. B, To quantify the percentage of the colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 90 cells (30 cells from three independent experiments) were analyzed. Data are shown as means ± SEM of three independent experiments. *p < 0.01.

Consistent with the results in Fig. 6A, DCs did not show the specific staining of mAb 25D1.16 against established tumors. To confirm the staining specificity of mAb 25D1.16 and each organelle. We did not observe the specific staining of mAb 25D1.16 (supplemental Fig. 1).4 These data indicated that the ORP150-chaperoned precursor peptides were processed and bound to MHC class I within early endosomes and recycling endosomes, suggesting that recycling MHC class I molecules were necessary for efficient cross-presentation of the ORP150-chaperoned peptides.

Potential antitumor effect of immunization with ORP150-peptide complex against established tumor

HLA-A*2402/Kb transgenic mice are a well-established model for studying HLA-A*2402-restricted CTL epitopes and vaccine development (31). We previously reported survivin-2B as a universal tumor Ag and identified an HLA-A24-restricted antigenic peptide, survivin-2B90-98 (AYACNTSTL), recognized by CD8+ CTLs (32). We therefore examined the efficacy of ORP150-based immunotherapy using human tumor Ag survivin-2B as a surrogate Ag. HLA-A*2402/Kb transgenic mice were inoculated with methylcholanthrene-induced fibrosarcoma TG3 transfected with survivin-2B cDNA, TG3-2B. When tumor diameter reached 3–4 mm, treatment with s.c. injection of an ORP150-survivin-2B complex, ORP150 alone, survivin-2B peptide alone, or PBS was conducted (Fig. 9A). As shown in Fig. 9B, the growth of established TG3-2B tumors was significantly retarded in the group treated with the ORP150-survivin-2B complex compared with survivin-2B peptide alone, ORP150 alone, and PBS (vs survivin-2B, p = 0.027; vs ORP150, p = 0.016; vs PBS, p = 0.0002). We also

4 The online version of this article contains supplemental material.
mune therapeutic approach for various types of cancer expressing with the ORP150-tumor peptide complexes were an effective im-

set up tumors. These data strongly suggested that immunization in the ORP150-survivin-2B complex-treated group rejected the es-
diameter reached 3–4 mm, mice were given the treat-
ments.

alyzed. Data are shown as means of 90 cells (30 cells from three independent experiments) were ana-

B

B

FIGURE 9. A: The protocol for immuno-

therapy is shown. B, A total of 2 × 10^3 TG3–2B cells were first injected intradermally into HLA-

A*2402/R^2 mice (10 animals/group). When mean tumor diameter reached 3–4 mm, mice were given the treat-
mint with the ORP150 (50 μg)-survivin-2B#0–88 (50 μg) complex, ORP150 (50 μg) alone, survivin-2B#0–88 (50 μg) alone, or PBS twice a week. C, The remaining 8–10 mice in each group were observed for the tumor rejection rate. D, The frozen tissues excised from mice treated with (a) the ORP150-survivin-2B#0–88 peptide complex or (b) PBS control was stained with an anti-
mouse CD8 mAb (×400). E, Immunization with the ORP150-Ag peptide complex induced peptide specific CTLs. After immunization with the ORP150-survivin-

2B#0–88 Peptide complex, splenocytes of the mice were cultured with survivin-2B#0–88 Peptide for 5 days and tested for cytotoxicity. Representative data are shown as means ± SEM of three independent experiments. *p < 0.05 and **p < 0.01.

Discussion

It is well demonstrated that immunization with tumor-derived HSPs or HSPs complexed with an Ag peptide/protein elicits tu-
mor- or Ag-specific CD8^+ T cell responses. Above all, it has been shown that Hsp70- and gp96-Ag complexes facilitate Ag presen-
tation in association with MHC class I molecules (16, 34, 35). However, the ability of HSPs to facilitate the presentation of MHC class II-restricted epitopes and to prime CD4^+ T cells has been relatively unexplored. In recent reports, it has been shown that Hsp70- and gp96-Ag complexes facilitate Ag presentation in association with both MHC class I and class II molecules (34, 36, 37). In this study, we demonstrated that ORP150-Ag complexes also facilitated Ag presentation in association with both MHC class I molecules and MHC class II molecules via an endosome-recycling pathway. In vaccine development, ORP150 has advantages for the induction of specific CTLs due to the simultaneous activation of specific helper T cells, which are required for efficient

evaluated the tumor rejection rates for the ORP150-survivin-2B peptide complex and compared with survivin-2B peptide alone, ORP150 alone, and PBS (vs survivin-2B, p = 0.070; vs ORP150, p = 0.048; vs PBS, p = 0.007) (Fig. 9C). Notably, six of nine mice in the ORP150-survivin-2B complex-treated group rejected the es-
tablished tumors. These data strongly suggested that immunization with the ORP150-tumor peptide complexes were an effective im-
munotherapeutic approach for various types of cancer expressing survivin-2B and HLA-A*2402. Next, we confirmed whether CD8^+ T cells infiltrated within the established tumor mass in re-
ponse to immunization with the ORP150-survivin-2B complex by immunohistological analysis (Fig. 9D). More CD8^+ T cells were observed in the tumor tissues injected with complex (328 cells/10 HPF) than in the PBS control (59 cells/10 HPF). We also ex-

FIGURE 8. A, DCs were first incubated with ORP150 complexed with PLC24 for 1 h and fixed with cold acetone. DCs were then incubated with anti-mouse CD16/CD32 Fc-block to block nonspecific stain-
ing, followed by costaining with an Alexa Fluor 488 conjugated with Alexa Fluor 488. Co-
localization of H-2Kb/SL8 complex and each organelle are indicated with arrows. B, For evaluation of colocalization, a single z-plane of one cell was evaluated. For 25D1.16 and each organelle combination, a total of 90 cells (30 cells from three independent experiments) were ana-

lyzed. Data are shown as means ± SEM of three independent experi-
ments. *p < 0.01.
CTL induction. In other words, when it is required for activation of CD8⁺ T cells as well as CD4⁺ T cells via Ag presentation in the context of both MHC class I and class II molecules, ORP150 can be a potent enhancer in immunotherapy.

Recent studies have revealed that stressful stimuli induce active release of intracellular HSPs into the extracellular milieu. The extracellular HSPs play an important role in initiating immune responses against microbial infection and neoplastic cells (10, 38). Because virus-infected cells and tumor cells are not able to prime naive CD8⁺ T cells due to the lack of costimulatory molecules, priming of CD8⁺ T cells against tumor cells and virus-infected cells requires cross-presentation by APCs. Extracellular HSP-Ag complexes, which are released from damaged tumor cells or virus-infected cells, are considered to be candidate Ag sources for cross-presentation. The pathway for cross-presentation has been shown to be comprised of two distinct intracellular routes, a proteasome-TAP-dependent pathway and an endosome-recycling pathway (22, 39). Recent reports have identified the pathway wherein peptide exchange onto recycling MHC class I molecules occurs within early endosomal compartments (40). We have shown that ORP150-mediated cross-presentation is independent of TAP and sensitive to priming via the early endosome and is processed in the static endosome-recycling pathway. Very recently, Lak-adamyali et al. (29) have shown that early endosomes are comprised of two distinct populations: a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome, and a static population that matures much more slowly. Cargo destined for degradation, including low density lipoproteins, epidermal growth factors, and influenza virus, are internalized and targeted to the Rab5⁺, EEA1⁺-dynamic population of early endosomes, thereafter trafficking to Rab7⁺-late endosomes. In contrast, the recycling ligand transferrin is delivered to Rab5⁺, EEA1⁺-static early endosomes, followed by trans-locating to Rab11⁺-recycling endosomes. Additionally, Burgdorf et al. clearly demonstrated that a mannose receptor introduced OVA specifically into an EEA1⁻, Rab5⁺-stable early endosomal compartment for subsequent cross-presentation (30).

In contrast, pinocytosis conveyed OVA to lysosomes for class II presentation. Of interest, OVA endocytosed by a scavenger receptor did not colocalize with EEA1, but colocalized with LAMP-1 in lysosome, leading to presentation in the context of MHC class II molecules. We showed that the ORP150-peptide complex is targeted into Rab5⁺, EEA1⁺-early endosome after internalization by DCs, suggesting that preferential sorting to the static endosome is necessary for cross-presentation of ORP150-peptide complexes. In contrast, soluble OVA protein, which was not cross-presented, targeted to the EEA1⁻ and LAMP-1⁺-dynamic early endosome-late endosome/lysosome pathway, leading to degradation and presentation in the context of MHC class II molecules. These data suggested that ORP150 shuttled the chaperoned precursor peptide into the static endosome-recycling pathway, preventing further degradation, followed by transferring the peptide onto recycling MHC class I molecules. If the ORP150-peptide complexes were loaded into late endosomes/lysosomes, they might be quickly degraded. Therefore, we consider that it is necessary that the ORP150-chaperoned peptide complex targets the early endosome and is processed in the static endosome-recyc-

ling pathway.

We have shown that immunization with ORP150-peptide complexes elicits strong CTL responses and antitumor effects. In contrast, we have reported that Hsp70-peptide complex, which is a representative member of Hsp70 superfamily, elicits only weak CTL responses (22). This may be because ORP150 is more efficient in binding peptides than is Hsp70. The ability of ORP150 to bind polypeptides better than other Hsp70 family members is largely due to its enlarged C-terminal helical domain (41, 42). Moreover, it may help that peptide binding to ORP150 is independent of ATP, which is in contrast with ATP-dependent substrate binding of other Hsp70 family members (42).

Finally, to date, SR-A and SREC-I have been identified as recep-tors for ORP150 expressed on APCs (20). However, whether SR-A or SREC-I is responsible for the efficient cross-presentation by DCs remains unclear. Such receptors should introduce the ORP150-peptide complex specifically into a static early endosomal compartment for the subsequent cross-presentation. Sorting in the endocytic system is a complex and highly dynamic process in which a wide variety of sorting motifs are recognized by specific sorting machinery to direct the membrane protein such as a recep-tor to its destination. Namely, after the receptor-mediated endocytosis, sorting of the ORP150-Ag complex into the static early endosome may be regulated by specific sorting motifs. The ORP-specific receptor might bear the sorting motifs, which is responsi-ble for the trafficking ORP150 receptor-ORP150 complex to the static early endosomes. To elucidate the mechanism for sorting ORP150 into these compartments, the specific receptors for the ORP150-mediated cross-presentation should be defined. Moreover, it is very important to identify the motifs intrinsic in such receptors that govern static early endosomal sorting. Identification and characterization of the cross-presentation-responsible receptor and its role as an efficient sorter to the static early endosome have important implications in vaccine development strategies. We are currently investigating the ORP150-specific receptors expressed on APCs responsible for the cross-presentation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1

Figure Legends

Specificity of mAb 25D1.16 staining. MAb 25D1.16 did not show the specific staining when pulsed with ORP150- control survivin-2B\textsubscript{80-88} peptide complex.

Materials and Methods

The DCs were first incubated with ORP150 complexed with control survivin-2B\textsubscript{80-88} peptide for 1 hr and fixed with cold acetone. DCs were then incubated with anti-mouse CD16/CD32 Fc-block to block non-specific staining, followed by costaining with an Alexa Fluor 594-labeled mAb 25D1.16 and anti-organelle Abs conjugated with Alexa Fluor 488. They were visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system (Bio-Rad, Richmond, CA).
Supplemental Figure 1