Exogenous antigens are processed through the endoplasmic reticulum-associated degradation (ERAD) in cross-presentation by dendritic cells

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Keywords: CHIP, ovalbumin, RNAi, Sec61, VCP

Abstract

Antigen cross-presentation is critical in infectious and tumor immunity where cytotoxic T lymphocytes are induced by dendritic cells specifically equipped with cellular machineries to present exogenous antigens with major histocompatibility complex (MHC) class I molecules. To examine molecular mechanisms of antigen cross-presentation, we employed as a model system a murine dendritic cell line DC2.4 capable of presenting soluble antigens such as ovalbumin (OVA) with MHC class I. Here, we demonstrate that exogenously added OVA is accumulated in the endoplasmic reticulum (ER) and late endosomes followed by retrograde transport to the cytoplasm through the Sec61 transporter complexes, and that CHIP functions as an E3 ubiquitin–ligase for OVA degradation by proteasomes. This mechanism is essentially the same as that known as the ER-associated degradation (ERAD) in the quality control of secretory and membrane proteins.

Introduction

MHC class I molecules are expressed with short peptides generated from endogenous proteins on most nucleated cells and function as immunological self markers (1). Cancer or virally infected cells express cancer- or virus-specific antigenic peptides, respectively, associated with MHC class I molecules as non-self markers (1–3). Cytotoxic T lymphocytes (CTL) attack cancer or virally infected cells by recognizing these non-self markers. CTL derive from naïve CD8⁺ T cells upon appropriate stimulation. However, naïve CD8⁺ T cells, though they express the same T cell receptor repertoire as that of CTL, are unable to respond to CTL-target cells and never differentiate into CTL. This is probably because cancer or infected cells usually express only small amounts of specific antigen-MHC class I complexes and, in addition, lack the co-stimulatory molecules for T cell activation (4). Dendritic cells (DC) function as the unique stimulator for naïve CD8⁺ T cells by presenting cancer or viral antigens along with MHC class I molecules (5,6).

Uptake and processing of exogenous antigens has been well documented for presentation with MHC class II molecules to CD4⁺ T cells, which is called antigen cross-presentation (7–11). A body of evidence has been accumulated that the generation of antigenic peptides for cross-presentation is the ubiquitin-proteasome system- and TAP-dependent (14), as is that for presentation of endogenous antigens with MHC class I molecules. This raised a fundamental question as to how exogenous antigens cross membrane barriers to encounter the ubiquitin–proteasome system. Recently two papers have shown that latex–OVA beads are internalized by phagocytosis, after which OVA and/or hydrolyzed OVA is retrotranslocated to the cytoplasmic surfaces of phagosomes, where it is possibly degraded by the proteasome (15,16). Fusion between phagosomes and ER, which has originally been suggested by proteomics analyses (17,18), seems the key cellular mechanism that allows OVA molecule susceptibility to degradation by proteasomes (13, 17–19). Based on the observation that the Sec61 protein is present in the ER–phagosome compartment, it has been speculated that the Sec61 complex functions in the retrotranslocation of OVA (15,16). More recently, it has been suggested that the loading of peptides from exogenous antigens onto class I molecules take place in endosomes (20) or phagosomes.
Antigen cross-presentation by DC and ERAD

(15,16,21) while the loading of endogenous peptides onto class I takes place in the ER through the conventional pathway for direct antigen presentation. In this study, we investigated molecular mechanisms for intracellular transport and processing of exogenously added soluble OVA in cross presentation.

Methods

Mouse and cell cultures

OT-I (H-2Kb restricted, anti-OVA TCR transgenic) mice were provided by Dr W. R. Heath (The Walter and Eliza Hall Institute, Melbourne, Australia). All experiments with mice were performed in accordance with our Institutional Guidelines. DC2.4, a DC line (22), was provided by Dr K. L. Rock (Dana-Farber Cancer Institute, Boston, MA). Cells were cultured in RPMI-1640 (Sigma, St Louis, MO) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, 100 U/ml penicillin–streptomycin, 55 µM 2-mercaptoethanol, 1 mM CaCl2, and chased for 37°C in 5% CO2 unless otherwise indicated. Bone marrow-derived dendritic cells (BMDC) were induced from bone marrow cells of C57BL/6 mice (SLC, Tokyo, Japan) by 5 ng/ml GM–CSF (MBL, Nagoya, Japan) in RPMI-1640 with 5% FCS. After 5 days culture, CD11c+ cells were purified by magnetic beads and used as BMDC. Polyoxymyxin B (50 µg/ml) was present throughout in cell cultures.

Antibodies and reagents

Anti-BiP (rabbit; MBL), anti-calreticulin (rabbit; Affinity BioReagents, Golden, CA for IP and mouse; Stressgen, Victoria, Canada for WB), anti-caveolin 1 (mouse; BD Biosciences, San Diego, CA), anti-CHIP (rabbit; gift of Dr K. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan for WB and chicken; MBL, Ina, Japan), anti-EEA1 (mouse BD Biosciences), anti-ERK2 (rabbit; Santa Cruz Biotechnology, CA), anti-GM-130 (mouse; BD Biosciences), anti-KDEL (mouse; Stressgen) antibodies, anti-LAMP-1 (rat; BD Biosciences), anti-multi-ubiquitin (mouse; MBL), anti-OVA (rabbit; Polysciences, Warrington, USA), anti-PDI (rabbit; Stressgen), anti-Rab5 (mouse; BD Biosciences), anti-Sec61α (rabbit; Upstate Cell Biotechnology, New York, NY) and β (rabbit; Upstate Biotechnology), anti-TAP1 (goat; Santa Cruz Biotechnology), anti-TAP2 (goat; Santa Cruz Biotechnology), anti-Tfr (mouse; Zymed, South San Francisco, CA) and anti-VCP (rabbit; BD Biosciences for WB and goat; Santa Cruz Biotechnology for IP) were used. As second-staining reagents, streptavidin–peroxidase conjugate (SA–HRP, Vector lab, Burlingame, USA), goat anti-rabbit IgG peroxidase conjugate (Zymed), goat anti-mouse IgG peroxidase conjugate (Zymed), goat anti-Rat IgG peroxidase conjugate (Zymed) and bovine anti-goat IgG peroxidase conjugate (Santa Cruz Biotechnology) were used. OVA was biotinylated using FluoroReporter Biotin–XX protein labelling kit (Molecular Probes, Eugene, USA), bOVA contains 2 mol biotins per 1 mol OVA on average.

Subcellular fractionation and density gradient centrifugation

DC2.4 cells were suspended in 250 mM sucrose in 10 mM Tris–HCl pH 7.4, homogenized by glass beads, and spun at 250 000 g for 30 min. Aliquots were incubated with or without 100 µg/ml trypsin (Sigma) in the presence or absence of 1% Triton X-100 for 30 min at 37°C. Soybean trypsin inhibitor (Sigma) was added at 500 µg/ml before recovery of bOVA. For density gradient centrifugation, DC2.4 cells incubated with bOVA (500 µg/ml) for 3 h were washed twice in PBS, suspended in homogenization medium (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES–NaOH pH 7.4), and then disrupted with 10 strokes of a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 2000 g for 10 min. The post-nuclear supernatant was pelleted at 100 000 g for 45 min, resuspended in homogenization medium and overlaid on a 2.5–30% or 10–30% discontinuous OptiPrep (Gibco/Invitrogen, CA) gradient. Centrifugation was done in Beckman SW 60Ti rotor at 200 000 g for 2.5 h for 2.5–30% gradient or 300 000 g for 3 h for 10–30% gradient at 4°C. Eleven fractions were collected from the top of each centrifuge tube, precipitated with TCA, and analyzed with SDS–PAGE and western blotting.

Recovery and co-purification of biotinylated OVA

DC2.4 cells and BMDC were incubated with bOVA (250 µg/ml), washed twice with PBS and solubilized in TNE (for recovery: 20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5 M EDTA, 1% Nonidet P-40) or Digitonin buffer (for co-purification: 1% digitonin in 20 mM HEPES pH 7.6) with protease inhibitor cocktails (Sigma). Samples were precipitated with streptavidin–agarose beads (SA-beads) (Novagen, San Diego, CA) and anti-goat IgG antibodies for precipitation (Protein G for anti-Sec61α, anti-VCP, anti-BiP, anti-PDI, anti-calreticulin, anti-TAP1 and anti-TAP2) and for adsorption (rabbit anti-IgY column, a gift of Mr S. Seki, MBL for anti-CHIP). Precipitated samples were analyzed by SDS–PAGE and western blotting.

Pulse–chase analysis

2 × 107 DC2.4 cells were labeled with 250 µg/ml of bOVA in medium containing 10 µM MG-132 for 1 h, washed twice with PBS, and chased for various periods at 37°C. The bOVA was recovered and resolved by SDS–PAGE followed by western blotting.

Immunoprecipitation

2 × 107 DC2.4 or BMDC cells were labeled with 250 µg/ml of bOVA in medium containing 10 µM MG-132 for 4 h. Biotin-labeled samples were collected, and solubilized in 1% digitonin in 20 mM HEPES pH 7.6 with protease inhibitors. bOVA (2.5 mg/ml) was post-added to control cell lysates. Samples were pre-cleared with protein G–Sepharose (Amersham Pharmacia Biotech) and incubated with indicated antibodies for precipitation (Protein G for anti-Sec61α, anti-VCP, anti-BiP, anti-PDI, anti-calreticulin, anti-TAP1 and anti-TAP2) and for adsorption (rabbit anti-IgY column, a gift of Mr S. Seki, MBL for anti-CHIP). Precipitated samples were analyzed by SDS–PAGE and western blotting.

Calcium depletion

DC2.4 cells were incubated with bOVA (250 µg/ml) for 1 h, washed twice with 1 mM EGTA in PBS or with PBS alone, and chased for 4 h in medium with or without extra addition of 1 mM CaCl2.
Vector-based RNAi construction

The 1.2 kb of PvuII fragment from pSilencer 1.0-U6 (Ambion, Boston, MA) was inserted into the Smal site of pEGFP-C1 (BD Biosciences) and designated as p60p. p60AC was created by deletion of the 1.3 kb AseI-BglII fragment from p60p. Target siRNA sequences for Sec61, VCP and CHIP were inserted into p60AC (Sec61a-22), p60 (VCP-41) or pSilencer 2.1-U6 hygro vector (Ambion) (CHIP-4) according to the supplier’s protocol, respectively. For Sec61α knockdown, siRNAs against two out of five target Sec61α sequences were significantly effective. Three were not effective. Two out of five target VCP sequences and one out of five target CHIP sequences were significantly effective. The effective target sequences used in this study are Sec61a-22: AATGATCATTACTATCGGT; VCP-41: AATCCTGGTGAATGAGTAGGCT; CHIP-4: CAGTATCGAGGCGC.

Immunofluorescence microscopy

DC2.4 cells and BMDC grown on culture slides (Falcon, Franklin Lakes, USA) were pretreated with MG-132 for 30 min and incubated with 500 μg/ml OVA for 2 h. After washing three times with medium, the cells were fixed with 3.7% formaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and stained with indicated antibodies for 1 h at 37°C in PBS containing 5% BSA/2% FCS. The secondary antibodies were Alexa Fluor 488 goat antimouse IgG F(ab’)2 fragments, Alexa Fluor 488 goat anti-rat IgG and Alexa Fluor 546 goat anti-rabbit IgG F(ab’)2 fragments (Molecular Probes). Images were obtained using an IX70 (Olympus, Tokyo, Japan) inverted microscope equipped with fluorescence optics and Deltavision deconvolution microscopy software (Applied Precision, Washington, DC).

Antigen presentation assay

Parental or knockdown DC2.4 cells were pre-incubated in culture medium supplemented with 50 μg/ml polymyxin B for 30 min in the presence or absence of lactacystin or MG-132, and incubated with OVA (250 μg/ml) or SIINFEKL peptide (10 ng/ml) for 6 h. The cells were washed, fixed with 1% paraformaldehyde, and aliquoted into microtiter plates (5 × 10^4 per well). 5 × 10^6 CD8+ T cells purified from splenocytes of OT-I mice by magnetic beads were added to each well and IL-2 secretion was measured after 24 h incubation by ELISA in triplicate (BD Biosciences).

Results

Proteasome-dependent degradation of accumulated OVA

DC2.4 cells (22) incubated with biotinylated OVA (bOVA) were homogenized and subjected to centrifugation for preparing microsomal fractions. More than 70% of cell-associated bOVA was recovered in microsomal fractions and was trypsin-resistant, indicating that the majority of the incorporated bOVA was accumulated in membranous, subcellular compartments (Fig. 1A). Next, DC2.4 cells were incubated with bOVA for 1 h followed by chase up to 4 h. Cell-associated bOVA was recovered from total cell lysates by streptavidin–agarose and resolved by SDS–PAGE. Cell-associated bOVA decreased as a function of chase time, which was blocked in the presence of lactacystin or MG-132, proteasome inhibitors (Fig. 1B and C). Ammonium chloride did not significantly affect degradation of bOVA (Fig. 1C), suggesting that lysosomal proteases are not involved in the antigen degradation. Essentially the same results were obtained with DC2.4 cells exposed to GST–OVA instead of bOVA and with bone marrow-derived DC (BMDC) exposed to bOVA (data not shown). The majority of cell-associated bOVA had the same molecular mass as intact bOVA (Fig. 1D). In addition, minor but significant fractions of bOVA were found to be poly-ubiquitinated as shown by both anti-poly-ubiquitin antibody and anti-OVA antibody (Fig. 1D). Note that amounts of both total cell-associated bOVA and poly-ubiquitinated bOVA increased in the presence of lactacystin (Fig. 1D). These results suggest that undegraded bOVA was accumulated within membranous organelles and degraded through the ubiquitin–proteasome degradation pathway. Protein degradation with these features is essentially the same as that known as the ER-associated degradation, ERAD (23–25). We provide three lines of evidence that accumulated bOVA in DC2.4 cells is degraded by the ERAD or a quite similar, if not identical, protein degradation mechanism.

Association of accumulated OVA with proteins involved in ERAD

First, we examined interactions of accumulated bOVA with BiP and those involved in the retrograde transport from the ER to the cytosol such as Sec61 (23) and VCP (24,25). To this end,
Sec61 complexes were immunoprecipitated with anti-Sec61β antibody or anti-Sec61α antibody and resolved by SDS–PAGE followed by blotting with streptavidin (SA–HRP). A portion of bOVA was found to be associated with Sec61 in DC2.4 cells that had been incubated with bOVA in the presence or absence of lactacystin (Fig. 2A and B). Immunoprecipitation with anti-VCP antibody from DC2.4 cells incubated with bOVA also demonstrated association of bOVA with VCP (Fig. 2C). Three ER resident proteins, BiP, protein disulfide isomerase (PDI) and calreticulin were also found to be associated with OVA (Fig. 2D–F). Interestingly, it was revealed that a portion of bOVA was associated with CHIP, an E3-ubiquitin ligase (26) (Fig. 2G). Contrary to the above proteins, bOVA was not associated with TAP1 or TAP2 (Fig. 2H and I).

Association of BiP, Sec61α, Sec61β, VCP, calreticulin and CHIP to bOVA was also demonstrated by co-purification of bOVA using streptavidin-beads from cell lysates with these proteins (Fig. 3A). In the purification experiments, we again observed that TAP1/2 were not associated to bOVA. From bone marrow-derived DC (BMDC) that had been incubated with bOVA, Sec61 and VCP were co-immunoprecipitated with bOVA (Fig. 2J and K) and BiP and VCP were co-purified with bOVA (Fig. 3B).

Note that majority of bOVA in DC2.4 cells associated with these molecules including CHIP had the same molecular mass as intact bOVA (Fig. 2A–G). These results suggest that bOVA in unfolded structure was accumulated in the ER or ER-related compartments without degradation, transported to the cytoplasm via the Sec61 transporter, then poly-ubiquitinated at least in part by CHIP, and finally subjected to degradation by proteasomes.

Inhibition of ERAD reduces degradation of accumulated OVA

Secondly, to further confirm involvement of the ERAD, we examined whether accumulation of bOVA is affected by conditions inhibitory for the ERAD such as Ca2+-depletion (27,28) and thapsigargin treatment (29). DC2.4 cells were pulsed with bOVA in the presence of MG-132 followed by washing with PBS or PBS containing 1 mM EGTA and chased for 4 h. As shown in Fig. 4(A), accumulated bOVA did not decrease upon Ca2+-depletion. Readdition of Ca2+ to cells pre-exposed to EGTA restored degradation of bOVA, excluding the possibility that Ca2+-depletion caused irreversible damage to DC2.4 cells (Fig. 4A). Thapsigargin also inhibited the degradation of accumulated bOVA (Fig. 4B). In addition, treatment of DC2.4 cells with tunicamycin (30) also inhibited degradation of bOVA (Fig. 4B). It is likely that tunicamycin treatment resulted in accumulation of N-glycosylation-deficient proteins in ER that are good substrates for the ERAD and, therefore, competed with bOVA for degradation.
Despite this fact, poly-ubiquitination of bOVA was reduced to 34% in CHIP-deficient cells (Fig. 4F). Decrease in the amounts of accumulated bOVA was slowed down in CHIP-deficient cells (Fig. 4D and E). By contrast, knockdown of TAP1 did not affect degradation of bOVA in DC2.4 cells (see Supplementary fig. 1, available at International Immunology Online).

Localization of accumulated OVA in DC2.4 cells

All of the above results strongly support the hypothesis that incorporated bOVA by DC2.4 cells is degraded through the ERAD. However, proteomic analysis and immunoelectron microscopic observations have recently demonstrated that phagosomes where infectious bacteria are taken up consist of constituents of the ER, including those involved in antigen presentation such as MHC class I molecules and TAP1/2 (15, 16). This led us to critically examine the intracellular localization of accumulated OVA in DC2.4 and BMDC by immunofluorescence confocal microscopy. DC2.4 cells were exposed to soluble OVA for 2 h, fixed, and immunofluorescently labeled with anti-OVA antibody. Spots or speckles of OVA distribution were detected in the cells but these OVA distributions appeared not to be superimposed over either early endosomes or caveosomes (Fig. 5A and B). Instead, OVA immunofluorescence was associated at least in part with late endosomes (Fig. 5C) and ER (Fig. 5D). Although ER as labeled with anti-KDEL antibody appeared as meshworks, OVA was not uniformly distributed throughout the ER (Fig. 5D, insets). Similarly, OVA was detected in a restricted area of late endosomes (Fig. 5C). OVA was not associated with the Golgi apparatus (Fig. 5F). Localization of accumulated OVA in ER was confirmed with BMDC (Fig. 5E). These results are consistent with our hypothesis that the incorporated OVA into DC is subjected to ERAD and/or a similar protein degradation pathway in other organelle-containing ER constituents such as phagosomes and late endosomes.

Subcellular fractionation of OVA-containing membranous vesicles by density-gradient centrifugation

We next analyzed distribution of accumulated OVA in vesicular fractions of DC2.4 cells by iodixanol gradient centrifugation. Vesicular fractions were divided into 11 fractions according to their densities. Although OVA was detected throughout the fractions, the peak of OVA was obviously detected in fraction #10 in 2.5–30% density gradient (Fig. 6A) and in fraction #5 in 10–30% density gradient (Fig. 6B). Distribution of Sec61, BiP (KDEL) and Rab5 in the fractions was similar to that of OVA. In contrast, OVA immunofluorescence was superimposed over either early endosomes or caveosomes (Fig. 5A and B). Instead, OVA immunofluorescence was associated at least in part with late endosomes (Fig. 5C) and ER (Fig. 5D). Although ER as labeled with anti-KDEL antibody appeared as meshworks, OVA was not uniformly distributed throughout the ER (Fig. 5D, insets). Similarly, OVA was detected in a restricted area of late endosomes (Fig. 5C). OVA was not associated with the Golgi apparatus (Fig. 5F). These results are consistent with our hypothesis that the incorporated OVA into DC is subjected to ERAD and/or a similar protein degradation pathway in other organelle-containing ER constituents such as phagosomes and late endosomes.

Thirdly, we employed the RNAi method to down-regulate proteins involved in the retrograde transport of substrates in the ERAD pathway (Fig. 4C). In Sec61α-knockdown DC2.4 (S22 and S22-3) cells, accumulated bOVA decreased at slower rates than in parental DC2.4 cells (Fig. 4D and E). Expression of RNA with scrambled sequence of the siRNA for Sec61α knockdown did not affect expression of Sec61α or accumulation of bOVA (data not shown). These results suggest that Sec61α is involved in the transport of bOVA for degradation. Similarly, VCP-deficient DC2.4 (V41 and V41-1) cells retained larger amounts of bOVA than parental DC2.4 cells during chase (Fig. 4D and E), although accumulated bOVA decreased to some extents. We also generated a CHIP-knockdown DC2.4 (C4 and C4F-1) and examined poly-ubiquitination of bOVA under deficiency of CHIP. Uptake of bOVA into CHIP-deficient DC2.4 cells was as low as 70% of that into parental DC2.4 cells (Fig. 4D and F).
recognize a peptide (SIINFEKL) derived from OVA associated with MHC class I, H-2Kb. Production of IL-2 was determined as an indication of T cell stimulation with OVA antigen presented by DC2.4 cells. The octapeptide OVA257–264 antigen was used as a control for demonstrating the ability for direct antigen presentation. As shown in Fig. 7(A), OVA and bOVA were equally antigenic for stimulation of CD8+ OT-I T cells. Proteasome inhibitors suppressed OVA antigen presentation as expected (Fig. 7B). By contrast, NH4Cl did not reduce presentation of the OVA antigen with MHC class I to OT-I CD8+ cells but inhibited presentation with MHC class II to OT-II CD4+ cells (see Supplementary fig. 2). Effect of down-regulation of Sec61, VCP or CHIP was examined using uncloned transfectants (S22, V41 or C4) of DC2.4 with siRNA expression vectors as well as clonal transfectants (S22-3, V41-1 or C4F-1) (Fig. 7C and D). Since Sec61 is involved in both forward and retrograde transport of ER proteins, it is not surprising that down-regulation of Sec61 reduced antigen presentation of exogenously added OVA. Down-regulation of VCP necessary for the retrograde transport of ER proteins also reduced antigen presentation by DC2.4 cells. These results support our hypothesis that exogenously added OVA is processed via the ERAD for cross-presentation. Furthermore, in accord with the effect on accumulation of OVA in DC2.4 cells, down-regulation of CHIP resulted in decrease in the cross-presentation of OVA (Fig. 7C and D), suggesting that CHIP functions as an E3-ligase for ubiquitination of OVA during cross-presentation.

**Discussion**

In the cytoplasm, considerable portions of newly synthesized proteins as well as damaged proteins that are present in situ need to be degraded. The ERAD system is responsible for the intracellular degradation and turnover of proteins.
non-native structure are recognized by the ubiquitination system and degraded by proteasomes (31,32). A portion of generated peptides is transported to the ER through TAP, where these peptides are complexed with MHC class I molecules. This process is well known as direct presentation of endogenous antigens. On the other hand, two non-mutually exclusive models have been proposed for intracellular trans- port and processing of exogenous antigens in the cross-presentation. According to the endosomal model (33), exogenous antigens are accumulated in endosomes, degraded by lysosomal proteases, and loaded onto class I molecules in endo-lysosomal compartments. This process is similar to that for antigenic peptide loading onto class II molecules and is sensitive to NH₄Cl or chloroquine. By contrast, another model, namely the cytosolic pathway model, postulates that incorporated antigenic proteins by endocytosis is delivered to the cytosol where they are poly-ubiquitinated and degraded by proteasomes. The generated peptides are then transported, like those from endogenous antigenic proteins, to the ER via TAP (14). The present results and those previously reported (12) show that cross-presentation of the OVA antigenic peptide by DC2.4 cells is sensitive to proteasome inhibitors, supporting the cytosolic pathway model but not necessarily excluding the endosomal model. However, in this model, it has been enigmatic how exogenous OVA and other antigens cross membrane barriers to encounter proteasomes.

In this report, we provide for the first time direct evidence that exogenously added OVA is accumulated in microsomal fractions, including ER and late endosomes, and retrotrans- ported into the cytosol through the Sec61 complex with the aid of VCP, after which the antigen is poly-ubiquitinated by the CHIP E3-ligase and degraded by proteasomes. This mecha- nism is essentially the same as that for quality control of secretory and membrane proteins in the ER, referred to as ER-associated degradation (ERAD) (24,25,34). The ERAD machinery does not recognize any targeting signal but recognizes non-native conformation of substrate proteins present in ER. Thus, it is reasonable that exogenous antigens cross membrane barriers by utilizing the Sec61 complex together with VCP involved in the ERAD. Discontinuous distribution of OVA in filamentous and/or tubular ER that we observed by immunofluorescence microscopy may represent functionally specialized sites for translocation into the cytosol.

Recent proteomics analyses have raised the intriguing possibility that phagosomes are constructed by fusion of ER to plasmalemma (17–19). More recently, phagosomes have been shown to contain MHC class I heavy chains and their loading machinery consisting of ubiquitinated proteins, proteasome subunits, tapasin, and TAP as well (15,16,21). Thus, it is possible that the ERAD takes place also in phagosome–ER fusion compartments. As soluble OVA was used as an antigen in this study, it was expected that the antigen is incorporated by DC2.4 cells using a cellular mechanism referred to as
endocytosis. However, accumulated OVA was not associated with early endosomes or caveosomes, where albumin receptors are present (35)—even in the early stage of antigen uptake by DC2.4 cells—whereas association of OVA with ER and late endosomes was readily observed. In as much as OVA was not detected in the Golgi complexes, significance of late endosomal OVA in cross-presentation is not clear at the moment. A recent report suggests an intriguing possibility that peptide loading in cross-presentation occurs in late endosomes (20) and/or phagosome (21). Our results shown above are not inconsistent with this report if ERAD occurs also in endosomes that contain ER constituents.

Recent reports have demonstrated that phagocytosis of latex–OVA by phagocytes including DC is driven by fusion of ER with plasma membranes and that such ER-phagosome organelles might be intracellular sites for antigen degradation by proteasomes and peptide loading on MHC class I molecules (15, 16). Thus, the cross-presentation mechanism operating for soluble OVA appears to be different from that for latex–OVA, at least in the initial steps including uptake of antigenic OVA and its transfer to the ER. Nevertheless, it is likely that essential parts of the antigen cross-presentation mechanisms in both systems are common, which have been referred to as ERAD. If cellular systems for substrate accumulation and degradation are tightly coupled to the peptide loading machinery, we would assume that a majority of MHC class I molecule-restricted antigenic peptides in most cells are also produced through the ERAD from secretory and membrane proteins. This hypothesis should be experimentally examined in the near future.

As shown previously, the immune system in higher vertebrates uses degradation products from waste of cytoplasmic proteins for self markers. Similarly, the present finding demonstrates that the protein quality control system in ER is utilized for immune surveillance. Misfolded and damaged proteins both in the cytoplasm and ER are harmful for cells and destined to be degraded (31,32,34). In the cytoplasm, these faulty proteins such as defective ribosomal products (DRIPs) are degraded by the ubiquitin–proteasome system, generating antigenic peptides to be presented with MHC class I molecules (36–39). In ER, the ERAD system recognizes secretary and membrane proteins with defects in folding, assembly and glycosylation, and retrograde-transports them to the cytoplasm for degradation by the ubiquitin–proteasome system (23–25). As shown above, captured OVA by DC is accumulated in ER and degraded through the ERAD, generating the antigenic OVA peptide for cross-presentation.

Supplementary data
Supplementary data are available at International Immunology Online.

Acknowledgements
We thank Satoshi Matsuda for discussion and help in fluorescence microscopy, and Shigeo Murata and Keiji Tanaka for providing antibody. This work was supported in part by Uehara Memorial Foundation, a Grant-in-Aid for Scientific Research on Priority Areas (C) (13226112, 14021110), a National Grant-in-Aid for the Establishment of a High-Tech Research Center in a private University, a Grant for the promotion of the advancement of education and research in graduate schools, and a Scientific Frontier Research Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Abbreviations
BMDC bone marrow-derived DC
CHIP carboxy terminus of Hsc70-interacting protein
DC dendritic cell
DRIPs defective ribosomal products
ERAD endoplasmic reticulum-associated degradation
MHC major histocompatibility complex
OVA ovalbumin
RNAi RNA interference

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