Early phagosomes in dendritic cells form a cellular compartment sufficient for cross presentation of exogenous antigens

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Conventionally, MHC class I-restricted antigen (Ag) processing requires the action of the multimolecular peptide-loading complex within the endoplasmic reticulum (ER). Here we show that early phagosomes from human dendritic cells (DCs) contain the peptide-loading complex, incorporating MHC class I, β2 microglobulin, transporter associated with Ag processing (TAP), calreticulin, tapasin, and ERp57. Antigenic peptides could be translocated into purified phagosomes by TAP and loaded onto cognate class I molecules, inducing their specific dissociation from the loading complex. Endoglycosidase H-sensitive class I molecules were detected at the DC cell surface, suggesting that these molecules traffic there directly from phagosomes. Macropinocytosis also allowed internalized soluble Ags access to an ER-like compartment containing the class I loading complex. Blockade of TAP by endoH of a soluble derivative of human cytomegalovirus protein US6 confirmed that, although retrotranslocation into the cytosol is critical for processing, efficient association of class I molecules with peptides derived from exogenous Ags occurs within a compartment directly accessible to internalized proteins. Together, this evidence suggests that early phagosomes and pinosomes facilitate cross presentation of exogenous Ags by DCs.

The primary function of MHC class I molecules is to display peptides derived from endogenous proteins to CD8+ T cells. Cytoplasmic and newly synthesized proteins are degraded by the proteasome; the resulting peptides are translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen (Ag) processing (TAP). TAP forms the core of a multimeric peptide loading complex, associating with the glycoprotein tapasin, which binds to nascent MHC class I heavy chain-β2 microglobulin (β2m) dimers (reviewed in ref. 1). The loading complex also contains calreticulin (CRT), a lectin-like chaperone binding the monoglucosylated N-linked glycan on class I heavy chain (2), and ERp57, a thiol oxidoreductase noncovalently associated with CRT and disulfide-linked to tapasin (3). TAP-transported peptides, trimmed by a specific ER aminopeptidase (ERAP) (4, 5), bind TAP-associated MHC class I-β2m dimers, inducing their release. An intact loading complex within the ER is critical for efficient MHC class I-peptide association; cells and mice lacking tapasin or TAP are deficient in MHC class I Ag presentation (6–10).

Although MHC class I normally presents cytosolic Ags, a specialized mechanism facilitates presentation of exogenous Ags to CD8+ T cells. Termed cross presentation, this process may be crucial for inducing immunity to tumors and viral infections. Dendritic cells (DCs), the most potent Ag-presenting cells, are primarily responsible for this phenomenon in vivo (reviewed in ref. 11), and emerging evidence suggests that MHC class I may be loaded within the endocytic pathway in these cells. MHC class I-β2m dimers are present in immature DC endosomes, potentially allowing their interaction with internalized Ags, and they traffic to the surface rapidly after encountering a maturational signal (12–14). Recently, it was shown that the ER functions as a membrane donor during phagosome formation in macrophages (15). Early phagosomes resemble the ER in composition before their fusion with lysosomes leads to the degradation of ER-derived components (15, 16). These observations raise the possibility that the ER-based loading complex may intersect with exogenous Ags in phagosomes. Similarly, incorporation of the Sec61-based retrotranslocon, which drives misfolded proteins from the ER to the cytosol for proteasomal degradation (17), could deliver endocytoically acquired proteins into the cytosol. This combination of features could create an alternative pathway for MHC class I loading used in cross presentation.

It remains unknown whether DCs, like macrophages, donate ER to nascent phagosomes. In addition, soluble Ags as well as particulate Ags can be cross presented by DCs. If the interaction of internalized Ags with the ER is crucial in cross presentation, additional endocytic processes, including macropinocytosis, may use a similar ER donation mechanism. To address these questions, we examine the mechanisms governing loading of MHC class I molecules with exogenous Ags in primary human DCs and the human dendritic-like cell (DLC) line, KG-1 (14, 18).

Methods

Cells and Peptides. KG-1 cells (18) and KG-1.LKb cells (14) were cultured in Iscove’s modified Dulbecco’s medium with 20% bovine calf serum as previously described. DC cultures were generated from peripheral blood mononuclear cells as described (19). The peptides SIINFEKL [ovalbumin (OVA)257–264], QVPLRP-MTYK (nef7B; HIV-1 nef53–82), FPVTQPLRPTMYK-KAADVLS (nef7X), RRYQNSTEL (TAP transport substrate; glycosylation acceptor sequence underlined), and ICPr471–483 were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Soluble recombinant USP20–146 was prepared as described (20).

Abs. mAbs to HLA-ABC, HLA-DR, and CD34 were obtained from Beckman Coulter. Rabbit antiserum against β2m (R.β2m) and CRT (R.CRT) were obtained from Boehringer Ingelheim and Affinity BioReagents (Neshanic Station, NJ), respectively. An AlexaFluor-647-conjugated mAb specific for SIINFEKL-Kb complexes, 25-D1.16 (21), was provided by Jonathan Yewdell (National Institutes of Health, Bethesda). A rabbit antiserum against L22 was provided by Joan Steitz (Yale University, New Haven, CT). A rabbit antiserum specific for ERAP (R.ALAP) was provided by M. Tsujimoto (RIKEN, Saitama, Japan). The rabbit antiserum R.RING4C (TAP1), R.SINA (tapasin), R.CNX (calnexin), and R.ERp57 (ERp57) were used as described (14, 22). The mAbs 3B10.7 (HLA class I heavy chains), w6/32 (HLA class I-β2m dimers), GAP.A3 (HLA-A3), 4E (HLA-B,C), H5C6

Abbreviations: Ag, antigen; ER, endoplasmic reticulum; DC, dendritic cell; TAP, transporter associated with Ag processing; ERAP, ER aminopeptidase; CRT, calreticulin; β2m, β2 microglobulin; DLC, dendritic-like cell; OVA, ovalbumin; endoH, endoglycosidase H; R., rabbit antiserum.

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surface proteins were biotinylated with sulfo-N-hydroxysuccinimide-SS-biotin (Pierce) as described (14). Western Blots for TAP-Associated Proteins. Purified phagosomes were lysed in 1% digitonin in TBS with protease inhibitors. The HLA class I-loading complex was immunoprecipitated by using 148,3 or R.RING4C. Molecules unassociated with TAP were immunoprecipitated from the supernatants with the specified Abs. Immunoprecipitated proteins were subjected to Western blotting as described (3). When indicated, cells were pretreated with 20 mM N-ethylmaleimide before phagosome purification (3).

Translocation Assay. Translocation assays were performed on purified phagosomes as described for permeabilized cells (24). When indicated, the addition of 30 units/ml apyrase or 15 μM ICP47 demonstrated the ATP and TAP dependence of translocation, respectively.

Peptide-Mediated Dissociation of Kb from TAP. [35S]Methionine-labeled KG-1.Kb cells were suspended in chase medium and allowed to internalize deep blue-dyed 1.0-μm latex beads (Polysciences) at a final concentration of 1% for 10 min. Isothermal phagosomes were subjected to translocation reactions with 10 μM SiNF4KL or control peptide before solubilization, detergent extraction, TAP immunoprecipitation, and SDS/PAGE.

Cross Presentation. The formation of Kb-SiNF4KL complexes by KG-1.Kb cells from exogenously added OVA was analyzed by flow cytometry by using Alexa 647-conjugated 25-D1.16 mAb or by using the T cell hybridoma B3Z (25), as described (14). IL-2 secretion is shown as the average of three independent experiments.

PaStal Internalization. PaStal (100 μg/ml) mAbs, GAP.A3 (100 μg/ml) mAbs, or PaStal prebound to protein G-coupled latex beads were incubated with labeled KG-1 cells for 30 min. The cells were lysed in 1% digitonin in TBS in the presence of a 10-fold excess of unlabelled KG-1 lysate to inhibit post-solubilization exchange. Immune complexes were immunoprecipitated by adding protein G-Sepharose beads or isolated directly for samples incubated with PaStal beads. MHC class I heavy chains were reprecipitated from SDS eluates with 3B10.7 and subjected to endoH digestion and SDS/PAGE.

Immunofluorescence Microscopy. KG-1 cells were allowed to internalize 100 μg/ml soluble PaStal mAb, fluorescein-conjugated OVA (Molecular Probes), or soluble US6 for 10 min, fixed immediately or washed and then fixed after an additional 30 min before analysis by indirect immunofluorescence (26).

Metabolic Labeling. Cells were starved and labeled with [35S]methionine (0.25 mCi/10^6 cells; ICN) for 15 min at 37°C as described (14). After the pulse, cells were washed in PBS before chasing at 37°C with a 10-fold excess of unlabelled methionine and cytochrome.

Immunoprecipitation. Latex bead-containing phagosomes were purified from ~80–100 million cells as described (15, 23). Cell pellets and purified phagosomes were extracted in 0.15 M NaCl and 0.01 M Tris (TBS), pH 7.4, containing either 1% Triton X-100 (Sigma) or 1% digitonin (Calbiochem) with protease inhibitors. Postnuclear supernatants were subjected to immunoprecipitation and, where indicated, endoglycosidase H (endoH) digestion and separated by SDS/PAGE as described (14). Cell surface proteins were biotinylated with sulfo-N-hydroxysuccinimide-SS-biotin (Pierce) as described (14).

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Processing of nef7X Peptide by Human DCs. Radioiodinated nef7X peptide was incubated at 37°C with immature DCs from HLA-A3+ donors or the HLA-A3+ Epstein–Barr virus-transformed B cell line PeCr2 in Iscove’s modified Dulbecco’s medium with 5% bovine calf serum. After lysis in 1% Triton X-100 in TBS, HLA-A3 complexes and HLA-B and -C complexes were immunoprecipitated and counted in a γ-counter (LKB Compugamma CS).

Results

Phagosomes from DCs and KG-1 DLCs Contain the Peptide-Loading Complex. Preliminary experiments revealed that MHC class I heavy chain, β2m, CRT, ERp57, tapasin, and TAP1/2 as well as calnexin were present in phagosomes from both primary human DCs and KG-1 DLCs (data not shown). ERAP, the aminopeptidase associated with antigenic peptide trimming in the ER, was also detected, whereas a control ribosomal protein, L22, absent from phagosomes (15), was not (data not shown). To determine whether the loading complex is properly assembled, phagosomes were purified from immature DCs (Fig. 1) and KG-1 DLCs (data not shown) after a 10-min internalization of latex beads and solubilized in digitonin. After anti-TAP immunoprecipitation, TAP-associated proteins were eluted in SDS and analyzed by immunoblotting. HLA class I heavy chains were clearly associated with TAP (Fig. 1A) immunoprecipitates from phagosome lysates (TAP), or supernatants from the TAP immunoprecipitates (NTA), were separated by SDS/PAGE and immunoblotted for HLA class I heavy chains (A), CRT (B), TAP (C), ERAP (D), L22 ribosomal protein (E), tapasin (F), and ERp57 (G). (A) Samples were treated where indicated with endoH. (E and F) Cells were pretreated with N-ethylmaleimide before lysis or phagosome preparation, and the samples were reduced where indicated with DTT. Cont, isotype control immunoprecipitates. (C) A second TAP immunoprecipitate of the phagosomal lysate (2nd TAP) confirms TAP depletion. M, markers are indicated on the left.

Fig. 1. 1. DC phagosomes contain a properly assembled MHC class I loading complex. Digitonin lysates of whole cells (TCL), total phagosomes (TP), TAP immunoprecipitates from phagosome lysates (TAP), or supernatants from the TAP immunoprecipitates (NTA), were separated by SDS/PAGE and immunoblotted for MHC class I heavy chains (A), CRT (B), TAP (C), ERAP (D), L22 ribosomal protein (E), tapasin (F), and ERp57 (G). (A) Samples were treated where indicated with endoH. (E and F) Cells were pretreated with N-ethylmaleimide before lysis or phagosome preparation, and the samples were reduced where indicated with DTT. Cont, isotype control immunoprecipitates. (C) A second TAP immunoprecipitate of the phagosomal lysate (2nd TAP) confirms TAP depletion. M, markers are indicated on the left.

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Phagosomal TAP is Functional for Peptide Translocation. For these experiments, we used a radioiodinated glycosylation acceptor peptide (27). KG-1 cells were allowed to internalize latex beads for 10 min before purification of phagosomes. After incubating phagosomes with labeled peptide, glycosylated peptides were isolated by using Con A-Sepharose beads. The presence of a glycan indicates transport and glycosylation by ER enzymes present in the phagosome (Fig. 2A). Inhibition by arsphenamine indicated that transport was ATP-dependent. Translocation was also abrogated by the peptide ICP47-35, a portion of the Herpes simplex ICP47 protein sufficient for TAP inhibition (28, 29).

To determine whether peptides translocated into the phagosome can bind MHC class I, we purified phagosomes from immature HLA-A3+ DCs. A radioiodinated A3-binding peptide (net7B) was added followed by a variable translocation period at 37°C. HLA-A3 complexes were isolated by using an HLA-A3-specific mAb (GAP.A3). Significant specific HLA-A3-specific peptide binding was observed (Fig. 2B). Thus, phagosomes from primary DCs are competent for both translocation and loading of peptides onto MHC class I molecules.

MHC Class I Molecules Dissociate from the Loading Complex After Peptide Binding. TAP-mediated peptide transport initiates the ER induces specific dissociation of MHC class I-β2m dimers from the loading complex (30). To determine whether this occurs in phagosomes, purified phagosomes from metabolically labeled KG-1.Kb cells were incubated with either a Kβ-binding (SIIFNFK) or a control nonspecific peptide and lysed in digitonin, and TAP immunoprecipitates were subjected to SDS/PAGE (Fig. 3A). Kβ runs with a lower mobility than human MHC class I because of an additional glycan, Kβ, but not HLA molecules, dissociated from TAP only on addition of SIIFNFK peptide.

For functional cross presentation to T cells, phagosomal class I-peptide complexes must be expressed on the cell surface. This could involve return to the ER or direct transport from phagosomes. Phagosomal TAP-associated MHC class I molecules are sensitive to endoH; their appearance on the cell surface might reflect direct transport. Labeled KG-1 DLCS were incubated with or without latex beads, and cell surface proteins were biotinylated and isolated from detergent extracts by using streptavidin beads. After elution in SDS, class I heavy chains and tapasin molecules were endoH-sensitive regardless of the exposure of the cells to latex beads (Fig. 3B). Under no circumstances, however, could we find tapasin molecules at the cell surface.

Macropinocytosis by DCs Allows Access to the Peptide-Loading Complex. To determine whether proteins internalized by macropinocytosis can also access the MHC class I loading machinery, we incubated KG-1 cells with the soluble anti-tapasin mAb, PaSta1, or a control mAb. The cells were then solubilized in Triton X-100 and protein G-Sepharose beads were added to isolate putative tapasin-PaSta1 complexes. After elution in SDS, tapasin-associated class I heavy chains could be specifically isolated from KG-1 cells incubated with either soluble or latex bead-bound anti-tapasin mAb (Fig. 4A). Most were endoH-sensitive, confirming their premedial Golgi origin. We also examined the localization of soluble internalized proteins by immunofluorescence microscopy. Ten minutes after endocytosis, internalized soluble OVA and PaSta1 appeared in similar peripheral vesicles. A subpopulation of these vesicles contained the ER marker calnexin (Fig. 4B Top and Middle). Although calnexin is incorporated into nascent phagosomes (15), its association with early pinocytic vesicles has not been previously described. Exogenously added proteins were never observed within the ER. Colocalization with calnexin became infrequent 30 min after protein internalization (data not shown), but the number of vesicles containing both internalized protein and the lysosomal marker CD63 increased (Fig. 4C). With time, the internalized proteins became nearly undetectable, likely because of degradation. It appears that proteins internalized by macropinocytosis can initially enter a peripheral ER-like compartment.

Peptide Translocation into an ER-Like Compartment Is Essential for Cross Presentation. Proteins internalized by phagocytosis, and perhaps macropinocytosis, enter a compartment competent for TAP-mediated translocation of peptides and generation of class I-peptide complexes. Internalized proteins may be retrotranslocated from this compartment to the cytosol and proteolytic fragments then transported back via TAP for binding to class I.

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molecules. To test this idea, we sought a TAP inhibitor that could be internalized by macropinocytosis. US6, a transmembrane protein derived from human cytomegalovirus, blocks TAP-mediated peptide transport by interacting with the luminal region of the TAP heterodimer (31,33). Truncated soluble US6 (amino acids 20–146) retains the ability to inhibit TAP transport (20). By immunofluorescence, soluble US6 internalized by KG-1 DLCs behaved similarly to OVA, colocalizing first with ER components (Fig. 4B Bottom) and then with lysosomal proteins (data not shown). As with OVA, US6 could not be observed within the perinuclear ER.

To determine whether exogenously supplied US6 could interact with TAP, immature DCs were incubated with the protein for 10 min and extracted in digitonin, and internalized US6 was immunoprecipitated with an affinity-purified rabbit antiserum (R.US6.9218). Associated TAP molecules were visualized by immunoblotting for TAP1 (Fig. 5A). R.US6.9218, but not nonimmune serum, coprecipitated TAP only in the presence of exogenous US6. These results were confirmed by immunoprecipitation of TAP and immunoblotting for associated US6 (Fig. 5B). Thus, US6 is internalized into a TAP-containing compartment, where it associates with the transporter.

When endogenously expressed, US6 down-regulates surface MHC class I expression. The presence or absence of US6 had no effect on cell viability, presentation of exogenous SIINFEKL peptide, or surface levels of Kb, HLA-DR, CD34, or transferrin receptor (data not shown). The results were confirmed by using the B3Z hybridoma, which recognizes Kb complexed with SIINFEKL complexes, addition of US6 inhibited the cross presentation of OVA whereas BSA had no effect (Fig. 6A). To determine whether the compartment accessed by soluble Ags is crucial for their cross presentation, we added soluble OVA to KG-1.Kb cells in the presence of either BSA or soluble US6. As measured by staining with 25D1.16 mAb, which recognizes Kb complexes with SIINFEKL complexes, addition of US6 inhibited the cross presentation of OVA whereas BSA had no effect (Fig. 6A). The presence or absence of US6 had no effect on cell viability, presentation of exogenous SIINFEKL peptide, or surface levels of Kb, HLA-DR, CD34, or transferrin receptor (data not shown). The results were confirmed by using the B3Z hybridoma, which recognizes the same MHC class I-I peptide complex (Fig. 6B).

Cross Presentation Requires Cytoplasmic Processing. The preceding data suggest that retrotranslocation to the cytosol and TAP-mediated peptide translocation is required for cross present-
phagosomes dissociate from TAP. Our data suggest that these complexes may then be transported directly to the cell surface, without trafficking back to the ER or Golgi. Other ER-retained, endoH-sensitive molecules, e.g., tapasin, are not detectable at the cell surface, arguing for a specialized alternative trafficking pathway specific for MHC class I-peptide complexes. These data support existing evidence that DCs regulate the trafficking of MHC class I molecules throughout DC maturation (12–14, 34).

Desjardins and coworkers (15) established that macrophages recruit ER membrane to the nascent phagosome, and we have extended these findings to human DCs. The role of the ER in other forms of endocytosis, however, has not previously been explored. We present evidence that donation of ER membrane to nascent vesicles also functions in DC macropinocytosis (Fig. 4), allowing soluble proteins access to an ER-like compartment competent for MHC class I peptide loading. ER donation of membranes to nascent endocytic vesicles may be a generalized cellular mechanism in DCs, perhaps explaining the efficiency with which DCs can present exogenous Ags internalized by a variety of forms of endocytosis. Further studies will be required to determine whether similar recruitment of ER membrane occurs in other cell types.

Exogenously added soluble US6 severely abrogates cross presentation. Because US6 interacts with the luminal region of TAP, active TAP inhibition must occur in compartments where internalized proteins interact with ER components. The severity of the inhibition of cross presentation (Fig. 6 A–C) implies cytoplasmic processing of exogenous Ags, as does its inhibition by lactacystin (Fig. 6C). Two potential mechanisms can be envisioned. First, US6 may encounter TAP molecules within the ER, resulting in the complete inhibition of cross presentation and the drastic down-regulation of surface MHC class I in DCs (Fig. 5). This would seem to require luminal continuity between the ER and phagosome, allowing the free exchange of components between compartments. Although we could not observe internalized US6 within the ER (Fig. 4B), immuno-fluorescence may not be sensitive enough to detect low concentrations.

Second, in phagosomes and macropinosomes, the retrotranslocation machinery derived from the ER, required to transport Ags into the cytosol, may be coupled to proteasomes and TAP, allowing processed peptides to be delivered back into phagosomes. If the second hypothesis is correct, the drastic down-regulation of MHC class I surface levels in immature DCs after exogenous US6 treatment (Fig. 5) suggests that endocytically loaded MHC class I molecules must constitute a major proportion of the cell surface complexes. Additional studies will be required to substantiate or refute this idea.

Although powerful evidence supports the idea that exogenous Ags must reach the ER to enter the classical MHC class I loading pathway (35, 36), seemingly irreconcilable studies have suggested an endosomal loading mechanism (37, 38). Substantial evidence, using knockout mice and various inhibitors, has demonstrated the role of TAP-mediated transport and proteasomal degradation in cross presentation. Such inhibitors, however, inevitably reduce transport of MHC class I out of the ER, inhibiting its appearance at the cell surface and possibly in endosomal compartments. Thus, it is difficult to separate the effect of these inhibitors on cross presentation from their more general effect on MHC class I trafficking. Our data may reconcile these previously contrasting observations. Phagosomes contain all of the components necessary for classical MHC class I loading, including ERAP, the aminopeptidase implicated in ER trimming of antigenic peptides. Loading in phagosomes is sensitive to classical inhibitors of MHC class I processing, such as lactacystin and US6, indicating that the mechanisms are similar to those in the ER. Thus, phagosomes and macroinosomes could create the same peptide repertoire as the ER-based,
classical MHC class I loading pathway. Fidelity to classical loading is essential for DCs to stimulate effective cytotoxic immune responses against Ags synthesized by cells that are incapable of cross presentation. The separation of cross presentation from classical MHC class I presentation may make immune responses against Ags synthesized by cells that are loading is essential for DCs to stimulate effective cytotoxic immune responses against Ags synthesized by cells that are incapable of cross presentation. The separation of cross presentation from classical MHC class I presentation may make possible the clinical manipulation of immune responses to tissue-specific Ags occurring in human disease.

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