Supporting Online Material for

IRAP Identifies an Endosomal Compartment Required for MHC Class I Cross-Presentation

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**METHODS**

**Antibodies.** Antibodies used for immunoblots were rabbit sera specific for: IRAP (a gift from Metabolex Inc.), HLA class I (R5996-4, Dr. N. Tanigaki (1)), murine ERAP1 (a gift from N. Shastri (2)), murine EEA1 (Abcam), murine MHC class I (P8; a gift of H. Ploegh); and mouse monoclonal antibodies recognizing Lamp1 (clone 1D4B, BD Pharmingen) and MHC class II (clone Y3P; a gift from A.M. Lennon-Dumesnil). Peroxidase-labeled goat antibodies against rabbit Ig light chains were used as secondary antibodies in MHC class I immunoblots (Jackson Immunoresearch).

The antibodies for flow cytometry were: anti-mouse CD11c-PE clone HL3, anti-mouse CD4-PE clone GK1.5 and anti-mouse CD8 clone 53-6.7-FITC or –APC, anti-Vbeta5.1/5.2-PE clone MR9.5 (BD Pharmingen); anti-H2-Kb-biotin clone AF6-88.5, anti-B220-PE clone RA3-6B2 (BioLegends); anti TCR-HY clone T3.70-biotin (eBioscience); anti-Gr1-PE clone RB6-8C5 and anti-mouse CD11b-PE clone M1/70.15 (ImmunoTools). Streptavidin-Alexa 647 (Molecular Probes) was the secondary reagent.

Antibodies used for fluorescence microscopy were (all antibodies murine unless indicated otherwise): a previously characterized immunoaffinity purified rabbit antibody specific for the cytosolic IRAP domain (3), anti-human EEA1-FITC clone 14, anti-human GM130 (BD Transduction Lab.), anti-human Lamp1-FITC clone H4A3, anti-mouse Lamp1-FITC clone 1D4B (BD Pharmingen), anti-KDEL receptor clone 10C3 (Stressgen), rabbit anti-STX6 (ProteinTech Group, Chicago), anti-human/mouse STX6 (clone 3D10, Stressgen), anti-H2-Db clone B22.249 (a gift from F. Lemonnier), anti-Kb clone 5F1 (a gift from E. Gatti), goat anti-mouse TAP1 (M-18, Santa Cruz Biotechnologies), anti-human ERAP1 clone 4D2 (4), anti-human TAP2 clone 429.3 (5), anti-HLA A, B, C clone W6/32, anti-HLA-DR clone L243 (ATCC), rat anti-HA clone 3F10 (Roche, Meylan, France), Cell-Tracker™ CM-Dil
FITC-coupled antibodies were amplified with goat anti-FITC IgG-Alexa 488 (Molecular Probes). Secondary antibodies used were: F(ab’)2 goat anti-mouse Alexa 594, F(ab’)2 goat anti-mouse Alexa 488, F(ab’)2 goat anti-rabbit Alexa 594, F(ab’)2 goat anti-rabbit Alexa 488, donkey anti-goat Alexa 594, and donkey anti-goat Alexa 488, donkey anti-rat Alexa 488 (all from Molecular Probes).

Antibodies used for cytokine ELISAs were: anti-IL2 capture clone JES6-1A12, anti-IL-2 detection JES6-5H4, anti-IFN-γ capture R4-6A2, anti-IFN-γ detection clone XMG1.2 (BD Pharmingen). Antibodies used for fusion protein targeting in vivo were anti-mouse TLR2 (clone MCA2266, Serotec), anti-mouse MR (clone MCA2235, Serotec).

**Microsome fractionation and IRAP purification.** Identification of IRAP in crude microsomal fractions tested for aminopeptidase activity from MGAR human B lymphoblastoid cells was performed as previously described for ERAP1/ERAP2 (4). Mass spectrometry analysis of peak 4 resulted in identification of peptide ILEALASSEDVR derived from IRAP. All other peptides identified in peak 4 were derived either from HLA class I heavy chains or from β2m. For IFN-γ induction of aminopeptidase activities, HeLa cells were treated for 48 h with 500 U/ml human IFN-γ (TEBU-BIO). Analysis of IRAP mRNA expression by quantitative PCR was also performed as previously described for ERAP enzymes (4), using the primers: 5’-TACGTTGAAGATAGAGTACT (sense), and 5’-TCAAACTGAGTTGCTGCAA (antisense).

**Digestions.** For digestions of peptide K15I, recombinant human IRAP truncated by the 130 aminoterminal amino acids, preceded by the signal peptide from the baculovirus capsid protein gp64 (6) and extended by six carboxy(C)-terminal histidine residues was expressed in baculovirus-infected Hi5 cells. Soluble IRAP was purified from insect cell supernatant by affinity chromatography using Ni-Nta-agarose beads (Invitrogen). Ten µg of peptide K15I were
digested with 0.5 µg of IRAP for different periods using previously described experimental conditions (4), followed by separation of products by cation exchange chromatography.

Expression cloning. Complementary DNA encoding murine Rab14 was PCR-amplified from BM-DCs and cloned into pEGFP-C1 (Clontech). To produce expression constructs encoding murine IRAP variants, full length or truncated IRAP cDNA was PCR-amplified with appropriate primers that added a C-terminal HA tag as well as a KDEL sequence in the case of the ER-targeted variant. All constructs were sequenced to confirm absence of PCR-induced errors and cloned into pMAX (Lonza, Cologne, Germany). DCs were transfected with expression plasmids using a Nucleofector™ equipment (Lonza) 36 h prior to microscopy analysis.

Fluorescence microscopy. Human myeloid DCs, mouse BM-DCs or splenic CD11c<sup>hi</sup> DCs purified using paramagnetic beads (Miltenyi) were seeded in IBIDI™ (Martinsried, Germany) slides and fed cross-presentable antigens for various periods. To synchronize phagosome maturation, DCs were first incubated for 10 min at 37°C with yeast cells or beads. Then cells were washed, and incubated further at 37°C until the total phagocytosis period was attained. Cells were fixed with PFA 4% and permeabilized with saponine 0.2%, BSA 0.5% in PBS. For staining of the cell membrane, cells were incubated for 10 min at room temperature in PBS containing 1 µM CM-Dil before permeabilization. For staining of cell surface MHC class I, cells were incubated before phagocytosis with antibodies W6/32, B22 or AF6-88.5-Biotin at 4°C for 15 min. Secondary reagents were Alexa 594-labeled streptavidin or goat anti-mouse antibodies. To examine co-localization of total cellular MHC class I molecules with IRAP (Fig. 2C), cells were first stained with anti-MHC class I rabbit serum P8 followed by a secondary antibody. Then cells were blocked for 1 h with non-immune rabbit serum, and finally stained with rabbit anti-IRAP antibodies labeled with Alexa 594 using a Zenon anti-rabbit kit.
Images were acquired on a Leica DMI 6000 microscope equipped with a piezoelectric-driven stage and Optophotonics XF100-2 (FITC), XF102-2 (Texas Red) and XF06 (DAPI) filters, and processed for 3D deconvolution using Metamorph™ 6.3.7. When cells were co-stained using CM-Dil and Alexa 488 labeled secondary antibodies, the filter XF202 (FITC narrow) was used for detection of green fluorescence. Determination of the percentage of IRAP co-localizing with other markers was carried out using correlation maps (7) together with the Metamorph™ 6.3.7 co-localization module. We used a fixed threshold of the local correlation coefficient of 0.4 for the correlation map and a fixed threshold of 10% of the maximum grey levels for the fluorescence to be quantified. The percentage of co-localization for each protein is reported as the proportion of its fluorescence that overlays the correlation map. This percentage, calculated for at least five cells for each co-localization evaluated, was calculated using the co-localization module of MetaMorph™ 6.3.7 software. Fig. S2 shows an example of co-localization quantification.

**DC culture.** Mouse BM-DCs were prepared by growing BM cells for 7 days in IMDM media with 10% FCS and 20% GM-CSF supernatant from J558 cells at 0.5x10^6 cells/ml. Human DCs were grown from monocytes isolated using anti-CD14 magnetic beads (Miltenyi) and cultured for 6 days in RPMI medium supplemented with 10% human serum, 8 ng/ml human recombinant GM-CSF and 16 ng/ml human IL-4 (R&D Systems).

**Immunoprecipitations and immunoblots.** To study association of IRAP and MHC class I, 5x10^7 BM-DCs were lysed in a 0.5% CHAPS buffer. The lysates were pre-cleared for 4 h with glycine-Sepharose beads, and target proteins were precipitated by incubation for 2 h with specific antibodies pre-adsorbed to protein G-Sepharose beads: a mix of antibodies 28-8-6S, B22.249, 28-14-8S and AF6-88.5 for H-2Kb/Db, immunoaffinity-purified polyclonal rabbit antibodies for IRAP, and the rat monoclonal antibody M5/114 for MHC class II. The immunoprecipitated material was eluted by heating to 95°C in SDS loading buffer except MHC.
class II samples that were eluted at 20°C. For HLA class I immunoblots, 200 µl of anion exchange fractions were precipitated and the proteins analyzed by standard procedures. For analysis of phagosomal proteins, latex bead-containing phagosomes were prepared by sucrose gradients according to published procedures (8). Phagosomes were finally lysed in 1% CHAPS and analyzed by immunoblots for protein composition after determination of protein concentrations with the Bradford reagent (BioRad).

**In vitro antigen presentation assays.** Antigen presentation was examined using both murine BM-DCs and freshly purified spleen CD11c^{hi} DCs, with identical results. In direct MHC class I presentation experiments, 10^5 BM-DCs were infected with previously described vaccinia viruses (9) at a multiplicity of infection of 30 (OVA) or 10 (S8L) for 6-7 h, and fixed with 1% formaldehyde before overnight incubation with 10^5 lymph node cells from OT-I Rag ko mice. For HY direct presentation, 200,000 male BM-DCs were incubated with 10^5 lymph node cells from HY Rag ko mice for 24 h. T cell stimulation was assessed by measuring secretion of IL-2 (naïve T cells) or IFN-γ (effectors) by sandwich ELISA.

Antigens for MHC class I cross-presentation assays included OVA-coated latex beads, obtained by overnight incubation with 50 mg/ml OVA and washed three times in cold PBS. Additional experiments were performed with SF9 insect cells infected for 24 h with recombinant baculoviruses expressing novel fusion proteins, lysed by two freeze-thaw cycles and washed extensively. These fusion proteins consisted of (from N- to C-terminal) two or three immunoglobulin-binding protein G domains, an ubiquitin molecule, and complete OVA, or the minimal epitope S8L, or S8L preceded by the sequence Cys-Ser-Cys. Fusion proteins were used both as particulate antigens associated with necrotic cells obtained as described above, and after purification from serum-free insect cell supernatants and pre-incubation with antibodies recognizing DC surface receptors. When targeted to DC surface receptors using specific
antibodies, these fusion proteins prime and stimulate CD8+ T cell responses in vitro and in vivo with an efficacy at least 100-fold superior to that of unmodified OVA.

To study the standard processing pathway for MHC class II restricted presentation of soluble exogenous proteins, we used OT-II transgenic CD4+ T cells (10). BM-DCs were incubated for 7 h with 1 mg/ml soluble OVA or 100 nM peptide OVA323-39 (ISQAVHAAHAINEAGR), washed and fixed before addition of OT-II effector cells. DCs were fixed in 0.002% glutaraldehyde before addition to OT-I or OT-II cells. OT-II effectors were produced by incubating splenocytes from RAG ko OT-II mice with cognate peptide-pulsed splenocytes from SV129 wt mice in a ratio of 1:1. Forty-eight h later, 10% T cell growth factor was added, and renewed every 48 h. Cells were used between day 6 and 11 after antigenic stimulation, and stimulation was assessed by measuring IL-2 secretion.

Cytochalasin D (10µM) or wortmannin (5nM) were added to the BM-DCs for 1 h before antigen addition. Protease inhibitors were used at the following concentrations unless indicated otherwise: epoxomicin, 0.2 µM; chloroquine, 25 µM; bafilomycin A1, 25 nM; MG132, 0.6 µM.

In vivo T cell proliferation assay. For adoptive transfer tests using OT-I T cells, 2x10^6 lymph node cells from RAG ko OT-I or HY mice were labeled with 10 µM CFSE. CFSE-labeled lymph node cells were injected i.v. into recipient mice, followed 24 h later by i.v. injection of antigens. Cross-presented antigens were: 0.5x10^6 β2m ko splenocytes electroporated with OVA as described (11); 10^7 male Balb/c splenocytes (for HY T cell-injected mice); or fusion protein PrG(3x)-Ub-OVA pre-incubated for 2 h at 4°C with targeting antibodies at a molar ratio of 1:1.

The amount of fusion protein per mouse was: 1 µg for targeting to the MR, 0.8 µg for TLR2 targeting. Three days after antigen injection, spleens were removed, and proliferation measured in CD8+ cells identified using Vß- or clonotype-specific antibodies, as described (11). The division index of transferred cells was calculated by dividing the number of observed OT-I
mitoses by the number of precursor cells, assuming that two cells of a given CFSE intensity arose from the single mitosis of a cell possessing a CFSE intensity immediately greater, as described by Angulo and Fulcher (12). For example, for a sample containing 100 cells not having divided, 200 cells having divided once, and 100 cells having divided twice, the number of precursors is calculated to be: $100 + \frac{200}{2} + \frac{100}{4} = 225$, and the number of mitoses to be: $0 + 100 + (25 + 50) = 175$, resulting in a division index of $\frac{175}{225} = 0.78$. Statistical significance was calculated by two-tailed Mann-Whitney or Student t tests (for TLR2-PrG(3x)-Ub-OVA), using GraphPad Prism™ software.

**Cross-priming of endogenous CD8<sup>+</sup> cells.** Thirty million β<sub>2</sub>m ko splenocytes were loaded at 37°C with 5 mg/ml OVA and 0.2 mg/ml poly-IC (Sigma), washed, and injected i.v. Ten days later, splenocytes of immunized mice were stained with anti-CD8 and a K<sub>b</sub>/S8L pentamer (ProImmune) and analyzed by flow cytometry.
Supporting References

1. N. Tanigaki et al., Immunogenetics 40, 192 (1994).
5. P. M. van Endert et al., Immunity 1, 491 (1994).
Supporting Figure 1. **IRAP mRNA expression is not induced by IFN-γ**. HeLa cells were incubated for 48 h with 500 U/ml IFN-γ or not before mRNA extraction and quantification by quantitative PCR. Levels of the housekeeping gene GAPDH mRNA were used for normalization of mRNA amounts. While ERAP1 and ERAP2 mRNAs are strongly induced by IFN-γ, IRAP mRNA levels remain unchanged.
A

**Raw Images**
- ERAP1 green, IRAP red
- H2-Db green, IRAP red
- Rab14 green, IRAP red

**Scatter Plots**
- IRAP vesicle
- ERAP1, H2-Db, Rab14

B

**Merged**
- ERAP1 green, IRAP red
- H2-Db green, IRAP red
- Rab14 green, IRAP red

**Correlation map**
- IRAP vesicle

C

**Bar Chart**
- % IRAP colocal. w/ the marker
- ERAP1: n=5, H2-Db: n=5, Rab14: n=7
Supporting Figure 2. **Quantification of IRAP co-localization with ERAP1, Rab14 and internalized H2-D\textsuperscript{b} molecules.** Examples of fluorescence scatter plots and correlation maps used to estimate co-localization of IRAP (red) with different markers (green): ERAP1 in human DCs, internalized H2-D\textsuperscript{b} in mouse BM-DCs after yeast phagocytosis for 15 min, Rab14-GFP in human DCs.

**A.** Phase contrast and merged fluorescence images are shown in the left hand panels, and scatter plots are shown in the right hand panels for the whole field and for a selected IRAP vesicle (arrow on merged images). The mean correlation coefficients for the entire images or for the selected IRAP vesicles are given in the corresponding scatter plots.

**B.** Merged fluorescence images and correlation maps are shown in the left hand panels, and scatter plots relating the IRAP fluorescence layer (red) to the correlation maps (white) are shown in the right hand panels for the whole field and for a selected IRAP vesicle. White lines on the scatter plots represent the thresholds of IRAP fluorescence and correlation intensity used for quantification of IRAP co-localization in panel C.

**C.** The percentage of IRAP (means + standard deviation) co-localizing with ERAP1, H2-D\textsuperscript{b} and Rab14 was calculated as the proportion of IRAP fluorescence coincident with the corresponding correlation maps (7), using the co-localization module of MetaMorph™ 6.3.7 software to compare the two images. We used a fixed threshold of the local correlation coefficient of 0.4 and applied a fixed threshold for IRAP images of 10% of the maximum grey levels. Correlation maps were used to detect the co-localization of the IRAP fluorescence intensity profiles with ERAP1, H2-D\textsuperscript{b} and Rab14 fluorescence using a 6x6 pixel Gaussian window. This method allowed us to use a single threshold for all quantifications presented (7).
A

IRAP wt - HA green

IRAP-KDEL - HA green

$\Delta$79-IRAP - HA green

B

HA green, IRAP red

Phase contrast

IRAP wt - HA green
Supporting Figure 3. Lack of detection of wild-type IRAP in the ER is not related to antibody specificity.

A, BM-DCs from IRAP ko mice were transfected with plasmids encoding different IRAP forms all carrying a HA tag: wt IRAP (wt-HA), IRAP truncated by 154 N-terminal residues and carrying an additional C-terminal KDEL sequence (KDEL-HA), or IRAP truncated by 79 N-terminal residues (Δ79-HA). One day after transfection, the cells were stained with an anti-HA antibody together with antibodies recognizing the indicated markers. In agreement with published reports (13), Δ79 IRAP-HA localizes to the cell membrane. IRAP-KDEL-HA shows co-localization with ER markers (mTAP1 and calnexin), while IRAP wt-HA is exclusively localized in STX6+ endosomes.

B, IRAP ko BM-DCs were transfected with a plasmid encoding HA-tagged wt IRAP, and stained with a rat anti-HA antibody together with previously described immunoaffinity-purified rabbit antibodies against the cytosolic domain of IRAP (3), used to detect untagged IRAP throughout this study. Specificity of the rabbit antibodies is confirmed by exclusive staining of the cell expressing transfected IRAP wt-HA.
Supporting Figure 4. **Analysis of IRAP co-localization in murine DCs.** BM-DCs from SV129 wt mice or IRAP ko (top left panel only) mice were stained with rabbit antibodies against the cytosolic domain of IRAP (3) and with antibodies recognizing the indicated markers. Rab14 localization was visualized by nucleofection of a plasmid encoding a Rab14-GFP fusion protein. Anti-IRAP antibodies do not stain IRAP ko DCs. IRAP showed co-localization in BM-DCs with Rab14, MR, STX6 and MHC class I.
Supporting Figure 5.

**Internalized HLA class I molecules co-localize with IRAP but not with a lysosomal marker.** Human DCs were coated at 4°C with a monoclonal mouse anti-HLA-A, B, C antibody before addition of yeast cells. After incubation at 37°C for different time periods, cells were fixed and stained first with a secondary anti-mouse antibody (red) followed by a second staining for EEA-1, IRAP and Lamp1 (green). IRAP and EEA-1 are recruited to early phagosomes, and IRAP co-localizes with internalized HLA. LAMP-1 is recruited to late phagosomes devoid of detectable HLA class I molecules.
Supporting Figure 6. **Normal maturation of IRAP ko DCs.** BM-DCs from IRAP ko and wt mice were incubated for 24 hours with 2 μg/ml LPS or medium and stained for CD11c together with CD86 co-stimulatory molecules, MHC class I (H2-Kb) or MHC class II (I-Ab). The histograms shown were derived by gating on CD11c+ cells. IRAP deficiency has no effect on LPS-induced up-regulation of the markers used.
Supporting Figure 7. **Normal presentation of exogenous synthetic peptide by IRAP ko DCs.**

BM-DCs from IRAP ko, wt and BALB/c mice were incubated for 2 h with different concentrations of the S8L peptide, washed, fixed and added to OT-I T cells (ratio APC : T cells of 1 : 1). IL-2 secretion was measured by ELISA 16 h later. IRAP deficiency does not affect presentation of exogenous peptide by MHC class I molecules.
Supporting Figure 8. **Normal phagocytosis by IRAP ko DCs.** BM-DCs were incubated with 1 μm TransFluoSphere™ 488-560 microspheres at 37°C for 20 min in the presence or absence of wortmannin, washed and analyzed immediately by flow cytometry. IRAP deficiency does not alter the efficiency of bead phagocytosis by BM-DCs.
A

OVA

OT-I

OT-II

Control

6 µM

12 µM

25 µM

6 nM

12 nM

25 nM

0.15 µM

0.3 µM

0.6 µM

0.06 µM

0.12 µM

0.25 µM

Percent T cell stimulation

Peptide

OD (IL-2)

IRAP ko

WT

B

OT-I + OVA Beads

Fixed

MG132 0.6 µM

Epox. 0.2 µM

BafA1 25nM

Chlor. 25 µM

Control

OD (IL-2)

C

OT-II

Peptide Pulsed

OVA

OD (IL-2)
Supporting Figure 9. **Effect of protease inhibitors on standard and cross-presentation pathways**

**A,** left hand panels: effect of inhibitors on MHC class I and class II-restricted presentation of OVA protein. Wt BM-DCs were infected with vaccinia-OVA (open bars), or incubated with 1 mg/ml of soluble OVA for 7 h (filled bars), in the presence of the inhibitors shown. Cells were washed and fixed before the addition of OT-I or OT-II cells in a ratio APC : T cells of 1 : 1. T cell activation was measured by IL-2 ELISA 16 h later. IL-2 secretion without inhibitors was set at 100, and secretion in the presence of inhibitors expressed as percent of that signal. Peptide controls are shown in the right hand panels. For OT-II experiments, 10^{-7} M synthetic peptide OVA_{323-39} was used as control, while infection with a vaccinia virus encoding the pre-processed epitope S8L was used as control for OT-I experiments. 0.2 \mu M epoxomicin, and 0.6 \mu M MG132 inhibit endogenous OVA processing for MHC class I presentation without affecting MHC class II-restricted presentation, while 25 nM bafilomycin A1 and 25 \mu M chloroquine inhibit OVA processing for MHC class II presentation without affecting endogenous MHC class I-restricted presentation.

**B,** BM-DCs from IRAP ko or SV129 wt mice were incubated with OVA-coated latex beads, washed, fixed and added to OT-I cells. Proteasome inhibitors reduce cross-presentation of OVA-coated beads, while inhibitors of lysosomal proteases do not affect (chloroquine) or increase (bafilomycin) cross-presentation, consistent with published reports (14).

**C,** BM-DCs from IRAP ko and SV129 wt mice were incubated with 1 mg/ml soluble OVA or 10^{-7} M peptide OVA_{323-39} for 7 h, washed, fixed and added to OT-II cells. IRAP deficiency does not affect presentation of soluble OVA by MHC class II molecules.
Supporting Figure 10. **IRAP deficiency does not affect circulation and recovery of adoptively transferred CD8⁺ T cells.** Recipient mice were injected i.v. with 2 x 10⁶ CFSE-labeled OT-I cells, and 4 days later splenocytes and lymph node cells were stained with anti-CD8 antibodies. As seen in A, wt and ko mice have equivalent numbers of CD8⁺ T cells in both organs. Moreover, the percentage of transferred OT-I cells among CD8⁺ cells is virtually identical (B). Numbers in the plots correspond to the mean percentages ± SD of 3 mice.
Supporting Figure 11. **Proliferation of adoptively transferred, CFSE-labeled OT-I T cells.**

Mice were injected i.v. with CFSE-labeled T cells followed 24 h later with (from top to bottom) PBS, 2 x 10^6 wt DCs pulsed for 2 h with 10^{-7} M peptide S8L, 5 x 10^5 OVA-loaded β2m deficient spleen cells, or 0.8 μg of TLR2-targeted fusion protein.
Supporting Figure 12. **Model of cross presentation pathways.** In DCs, most recycling MHC class I molecules are transferred from phagosomes to IRAP⁺Rab14⁺ endosomes. Antigens are exported into the cytosol and degraded by the proteasome. Some peptides are transported into endosomes by TAP recruited upon phagocytosis, for trimming by IRAP and assembly with recycling MHC class I molecules, others into the ER, for trimming by ERAP. A proteasome-independent vacuolar pathway complements the two TAP- and proteasome dependent pathways.